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Rôle du système de transport du phosphate Pst et du régulon Pho dans la virulence de la souche *E. coli* uropathogène CFT073

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Résumé

Les souches d'*Escherichia coli* uropathogènes (UPEC) sont responsables des infections du tractus urinaire (UTI). Les UTI affectent annuellement des millions de personnes et sont causées, à 85 %, par *E. coli*. Malgré une antibiothérapie efficace, des épisodes de résistance aux traitements, de récurrence et de persistance sont de plus en plus communs. Aussi, en raison de l'utilisation massive des antibiotiques, les souches bactériennes y sont de plus en plus résistantes.

L'opéron *pstSCAB-phoU* code pour le système de transport spécifique du phosphate (système Pst) et fait partie du régulon Pho. Ce régulon est contrôlé par le système de régulation à deux composants PhoBR, où PhoR code la protéine senseur membranaire et PhoB la protéine régulatrice cytoplasmique. Donc, en condition de carence en phosphate, PhoBR s'active et induit l'expression des gènes du régulon Pho qui regroupe un ensemble de gènes impliqués dans l'acquisition de diverses sources de phosphate, dont la phosphatase alcaline PhoA et le système Pst. En plus d'être impliqué dans le transport du phosphate, le système Pst régule négativement PhoBR puisque son inactivation l'induit constitutivement. Ainsi, la délétion du système Pst mime un état de carence en phosphate puisque le régulon est constitutivement activé. En plus de son rôle régulationnel et de transport, le système Pst est requis pour la virulence des souches bactériennes pathogènes. Par contre, les mécanismes moléculaires reliant ce système de transport et la virulence ne sont pas encore clairement définis. Précédemment, des analyses de biopuces à ADN chez un mutant *pst* d'une souche d'*E. coli* aviaire ont montré que l'expression des fimbriae de type 1 est réprimée chez ce mutant. Étant donné que ces fimbriae sont essentiels à la virulence des souches UPEC, nous avons donc caractérisé le rôle du système Pst dans la virulence de la souche UPEC CFT073 et plus précisément, son rôle dans la régulation de l'expression des fimbriae de type 1.

Dans un premier temps, nous observons que la délétion du système Pst diminue significativement la capacité du mutant *pst* à coloniser le tractus urinaire murin. De plus, l'atténuation de virulence du mutant *pst* est concomitante avec la répression de l'expression des fimbriae de type 1. Cette répression est la conséquence de l'expression différentielle des régulateurs de ces fimbriae, soit les recombinases *fimB*, *fimE*, *ipuA* et *ipbA*. En exprimant constitutivement les fimbriae de type 1 chez le mutant *pst*, nous observons une restauration de virulence dans le modèle murin d'UTI, montrant ainsi que l'atténuation de virulence observée chez le mutant *pst* est majoritairement causée par la répression des fimbriae de type 1.

Deuxièmement, nous avons voulu identifier les mécanismes moléculaires reliant le système Pst et les fimbriae de type 1. Pour ce faire, une banque de mutants à l'aide du transposon aléatoire *Tn*10 a été construite chez le mutant *pst*. Les mutants obtenus ont par la suite été criblés pour une restauration de l'expression des fimbriae de type 1. Nous avons démontré que YaiC, impliquée dans la biosynthèse du c-di-GMP, relie le système Pst et les fimbriae de type 1. Ainsi, nous observons que *yaiC* est induit chez le mutant *pst* et que cette induction réprime l'expression des recombinases *ipuA* et *ipbA* et par conséquent, réprime l'expression des fimbriae de type 1. De plus, l'inactivation de *yaiC*, chez le mutant *pst*, rétabli la virulence du mutant *pst*. Du point de vue moléculaire, nous observons que *yaiC* est directement et positivement régulé par PhoB, le régulateur du régulon Pho. Finalement, puisque YaiC est

impliqué dans la biosynthèse du c-di-GMP, nous avons déterminé que l'accumulation de cette molécule est concomitante avec la répression des fimbriae de type 1 chez le mutant *pst*.

En dernier lieu, nous avons adapté et développé une méthode simple de complémentation simple-copie dans le chromosome à l'aide du transposon Tn7 afin de prévenir la perte des vecteurs de complémentation *in vivo*, causée par une absence de pression de sélection. À l'aide de cette procédure, nous avons adéquatement complémenté la délétion du système *pst* puisque tous les phénotypes de la souche sauvage sont regagnés chez la souche complémentée. De plus, l'intégration chromosomique ne requiert aucune pression de sélection. Finalement, en plus des souches pathogènes d'*E*. *coli*, cette procédure fonctionne très bien chez *Salmonella enterica* serovars Typhimurium et Typhi, *Klebsiella pneumoniae, Cronobacter sakazakii* et *Citrobacter rodentium*.

Ainsi, dans ce projet de thèse, nous avons déterminé un lien direct entre le système Pst et la virulence. Ainsi, l'atténuation de virulence observée chez le mutant *pst* est causée par la répression des fimbriae de type 1 qui elle, est reliée à l'expression différentielle de ses régulateurs (*fimB*, *fimE*, *ipuA* et *ipbA*), à l'induction de *yaiC* et à l'accumulation du c-di-GMP.

Mots clés:

Régulon Pho, Système Pst, *Escherichia coli* uropathogène, Infection du tractus urinaire, Système de régulation à deux composants, Fimbriae de type 1, Adhésion, Second messager

Abstract

Uropathogenic *E. coli* (UPEC) strains are responsible for urinary tract infections (UTI). They cause up to 85% of all UTI recorded and they annually affect millions of persons. Despite an appropriate antibiotic therapy, episodes of resistance, recurrence and persistence are common. Also, as antibiotics are massively used, pathogenic strains are increasingly resistant.

The *pstSCAB-phoU* operon encodes the Phosphate specific transport system (Pst) and belongs to the Pho regulon, which encodes genes involved in phosphate acquisition and metabolism. Our group, and others, have determined that the Pst system is required for virulence in pathogenic strains. However, the molecular mechanisms connecting the Pst system, the Pho regulon, and virulence are not well defined. We previously identified, by microarray experiments, that inactivation of the *pst* mutant down-regulated type 1 fimbriae in the Avian Pathogenic *E. coli* (APEC) χ7122 *pst* mutant. As these fimbriae are essential for virulence in UPEC strains, we then characterized the role of the Pst system in virulence of the UPEC strain CFT073, especially its role in the regulation of type 1 fimbriae.

Firstly, we observed that inactivation of the *pst* system decreased virulence in the urinary tract infection (UTI) mouse model. Indeed, the *pst* mutant was severely attenuated in its capacity to colonize the bladder and kidneys. Also, this attenuation is concomitant with the down-regulation of type 1 fimbriae. This down-regulation is directly the consequence of the differential expression of *fimB*, *fimE*, *ipuA* and *ipbA*, which regulate expression of type 1 fimbriae. By constitutively activating the expression of type 1 fimbriae in the *pst* mutant, restoration of the virulence in the UTI mouse model was observed, showing that the attenuation of the *pst* mutant is mainly due to the down-regulation of the type 1 fimbriae.

Secondly, we wanted to identify the molecular mechanisms connecting the Pst system, the Pho regulon, and type 1 fimbriae. To address this, a transposon library was constructed in the *pst* mutant and clones were screened for restoration in type 1 fimbriae production. Among them, the diguanylate cyclase encoding gene *yaiC* (*adrA*) connected the Pst system and type 1 fimbriae. Therefore, in the *pst* mutant, *yaiC* was induced and its induction decreased expression of type 1 fimbriae by predominantly altering expression of the *ipuA* and *ipbA* regulators. In the *pst* mutant, inactivation of *yaiC* restored the virulence of the *pst* mutant. Interestingly, expression of *yaiC* was directly and positively regulated by PhoB since transcription of *yaiC* depended on the promoter of *phoA*, a Pho regulon member. As YaiC is involved in c-di-GMP biosynthesis, an increased accumulation of c-di-GMP was observed in the *pst* mutant and was concomitant with the down-regulation of type 1 fimbriae.

Lastly, due to the loss of complementation vectors *in vivo*, in the absence of selective-pressure, we adapted and developed a simple single-copy chromosomal complementation procedure. This approach used the Tn7 transposon which integrates at the chromosomal site-specific *att*Tn7 site located in the transcriptional terminal loop of *glmS*. Using this procedure, we successfully complemented the *pst* mutant as all the phenotypes, i.e. virulence, expression of type 1 fimbriae, adhesion and invasion of bladder cells, etc., of the WT strain were restored in the Δ*pst* complemented strain. Furthermore, as complementation occurs by insertion into the

chromosome, use of antibiotics is not required as Tn7-mediated integration is quite stable. Finally, this approach is also efficient in other *Enterobacteriacaea* species as Tn7 properly integrates at the *att*Tn7 site of pathogenic *E*. *coli* (UPEC CFT073 and 536, APEC χ7122 and EHEC EDL933), *Salmonella enterica* serovars Typhi and Typhimurium, *Klebsiella pneumoniae*, *Cronobacter sakazakii* and *Citrobacter rodentium*.

Thereby, in this project, we determined the connection between the Pst system, the Pho regulon, and the virulence of a UPEC strain. Attenuation observed in the *pst* mutant was mainly due to the down-regulation of type 1 fimbriae, through the differential expression of the recombinase encoding genes *fimB*, *fimE*, *ipuA* and *ipbA*, induction of *yaiC* and accumulation of c-di-GMP.

Keywords:

Pho regulon, Pst system, Uropathogenic *Escherichia coli*, Urinary tract infection, Two-component regulatory system, Second messenger, Type 1 fimbriae, Adhesion

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Discussion

Annexe 1

Liste des sigles et abréviations

ADN :	Acide désoxyribonucléique
A/E :	Lésion de type attachante et effaçante
APEC :	*Escherichia coli* pathogène aviaire
ARN :	Acide ribonucléique
ATP :	Adénosine triphosphate
c-di-GMP :	Di-guanosine monophosphate cyclique
CAM :	Complexe d'attaque membranaire
cGMP :	Guanosine monophosphate cyclique
CNF1 :	Facteur nécrosant cytotoxique 1
CK3 :	Mutant *pst* complémenté de la souche aviaire χ7122
DAEC :	*Escherichia coli* adhésion diffuse
DOC :	Sodium deoxycholate
DFI:	Differential fluorescence induction
DGC:	Diguanylate cyclase
GTP :	Guanosine triphosphate
E. *coli* :	*Escherichia coli*
e.g. :	Par exemple (*exempli gratia* en latin)
EAEC :	*Escherichia coli* entéroaggrégative
EHEC :	*Escherichia coli* entérohémorragique
EIEC :	*Escherichia coli* entéroinvasive
EPEC :	*Escherichia coli* entéropathogène
EPS :	Exopolysaccharides
ETEC :	*Escherichia coli* entérotoxinogène
ExPEC :	*Escherichia coli* pathogène extra-intestinale
Fe :	Fer
GDP :	Guanosine diphosphate
GTP :	Guanosine triphosphate
HlyA :	Hémolysine

HUS : Syndrome hémolytique urémique

IBC : Communauté bactérienne intracellulaire

i.e. : c'est-à-dire (*id* est en latin)

IE : Élément interconvertible

InPEC: *Escherichia coli* pathogène intestinal

IVET: *In vivo* expression technology

K3 : Mutant *pst* de la souche aviaire χ7122

LT: Toxine thermolabile

M: Molaire

ME: Membrane externe

MI: Membrane interne

NMEC: *E*. *coli* causant la méningite néonatale

nt : Nucléotide(s)

PDE : Phosphodiestérase

Pi : Phosphate inorganique

$polyP_i$: Métaphosphate

ppGpp : Guanosine tétraphosphate

PP_i : Pyrophosphate

Pst : Système de transport spécifique du phosphate inorganique

qRT-PCR : Réaction de polymérase en chaîne quantitative en temps réel

RID : Région inversée droite

RIG : Région inversée gauche

RIVET : Recombination-based *in vivo* expression technology

SCOTS: Selective capture of transcribed sequences

SRDC: Système de régulation à deux composants

ST : Toxine thermostable

STEAEC : *Escherichia coli* entéroaggrégative-hémorragique

STM : Signature-tagged mutagenesis

Stx : Toxine de type shiga

T1F : Fimbriae de type 1

T3SS : Système de sécrétion de type 3

T6SS : Système de sécrétion de type 6

µ : micro

µM : Micro Molaire

UPEC : *Escherichia coli* uropathogène

UTI : Infection du tractus urinaire

Vet. Micro. : Veterinary Microbiology

Unité de mesure

g : Gramme

kb : kilobase

K_d : Constante de dissociation

K_M : Constante de Michaelis-Menten

M : Molaire

Mg : Milligramme

nm : Nanomètre

O.D. : Densité optique

µM : Micromolaire (10^{-6} molaire)

Symbole

α : Alpha

β : Beta

χ : Chi

σ : Sigma

> : Plus grand que

< : Plus petit que

Introduction

En plus de faire partie de la flore bactérienne chez l'humain, les bactéries telles que *Escherichia coli* provoquent plusieurs infections. Celles-ci peuvent être de type intestinal ou de type extraintestinal. Les diarrhées sont particulièrement associées aux InPEC (*E. coli* pathogène intestinale) tandis que les infections du tractus urinaire, les méningites et les septicémies sont plus spécifiquement associées aux ExPEC (*E. coli* pathogène extra-intestinale) [1]

En termes de composant cellulaire, le phosphore est le cinquième élément en importance après le carbone, l'oxygène, l'azote et l'hydrogène [2]. Au niveau cellulaire, le phosphore (phosphate) est un élément essentiel puisqu'il entre dans la composition des acides nucléiques, des membranes bactériennes, de la capsule, de l'ATP (énergie) ainsi que dans les voies de signalisation. En raison de son importance, l'homéostasie du phosphate doit donc être extrêmement bien contrôlée. Ce contrôle s'effectue à l'aide du système de régulation à deux composants (SRDC) PhoBR. Celui-ci s'active lorsque les concentrations extracellulaires en phosphate sont inférieures à 4 µM. Cette induction activera l'expression des gènes faisant partie du régulon Pho, qui regroupe un ensemble de gènes codant pour les systèmes d'acquisition et de métabolisme de diverses sources de phosphate, dont le phosphate inorganique (Pi). L'opéron *pstSCAB-phoU* code pour le système spécifique du transport du phosphate (système Pst) et fait partie du régulon Pho. En plus d'agir de transporteur du Pi, le système Pst est un répresseur du SRDC PhoBR puisque l'inactivation de Pst active constitutivement PhoBR, donc le régulon Pho, et ce, indépendamment de la concentration du milieu extracellulaire en phosphate. De cette manière, la mutation du système Pst mime un état de carence en phosphate [3, 4].

En plus de son rôle de transporteur et de régulateur, le système Pst est aussi directement relié à la virulence bactérienne puisque son inactivation atténue la virulence de diverses espèces bactériennes pathogènes [5, 6]. Des études menées par notre groupe ont permis de démontrer que l'atténuation de virulence chez des souches ExPEC d'origine animale est principalement causée par une perturbation de la surface bactérienne et à un effet pléïotrope [7-12]. Malgré ces études, les mécanismes moléculaires reliant l'activation du régulon Pho (carence en phosphate) et la virulence ne sont pas clairement établis. Afin d'identifier ceux-ci, au cours de ma maîtrise

effectuée dans le laboratoire du Dre Josée Harel à l'Université de Montréal, nous avons procédé à des analyses transcriptomiques comparatives chez la souche ExPEC aviaire (APEC) χ7122 et son mutant isogénique *pst* [8]. Ces analyses ont montré qu'en plus d'affecter les voies métaboliques essentielles à la survie et les voies de biosynthèse de la surface bactérienne, la délétion du système *pst* affecte l'expression des gènes impliqués dans la biosynthèse des fimbriae de type 1.

Les fimbriae de type 1 sont des adhésines retrouvées à la surface bactérienne permettant l'adhésion aux tissus de l'hôte. Chez les souches d'*E. coli* uropathogènes (UPEC), les fimbriae de type 1 permettent la colonisation et l'invasion de la vessie. Aussi, ces derniers sont essentiels à l'instauration d'une infection chez les UPEC, donc ils sont un facteur de virulence critique [13, 14]. Étant donné que ces fimbriae sont essentiels pour les UPEC, mais guère impliqués dans la virulence de la souche APEC χ7122, nous avons donc choisi d'investiguer le rôle du système Pst (régulon Pho) dans la virulence de la souche UPEC CFT073.

Les objectifs de cette thèse étaient donc de déterminer le rôle du système Pst dans la virulence de la souche UPEC CFT073. Nous voulions investiguer à quel niveau le système Pst affecte la régulation des fimbriae de type 1. Aussi, nous voulions définir si l'atténuation de virulence d'un mutant Pst chez la souche CFT073 est la conséquence de la répression des fimbriae de type 1, d'une modification de la surface bactérienne ou d'un effet pléïotrope. Aussi, nous désirions déterminer les mécanismes moléculaires reliant le régulon Pho et l'expression des fimbriae de type 1. Finalement, étant donné que les méthodes de complémentation généralement utilisées ne sont pas idéales pour les modèles d'infection *in vivo*, nous avions comme objectif de développer une méthode de complémentation simple-copie dans le chromosome chez des souches faisant partie de la famille des *Enterobacteriaceae*.

REVUE DE LITTÉRATURE

1. *Escherichia coli*

1.1. Caractéristiques générales

La famille des *Enterobacteriaceae* regroupe plusieurs espèces bactériennes dont les *E*. *coli*, les *Salmonella*, les *Shigella* et les *Klebsiella*. L'espèce *E*. *coli* se caractérise par une forme de bacille, une coloration de Gram négative, une respiration de type anaérobie facultative et par la présence de flagelles péritriches [15]. Ces propriétés sont aussi caractéristiques des entérobactéries. Il existe plusieurs souches de *E*. *coli*, qui sont classées sérologiquement selon trois antigènes de surface, à savoir lipopolysaccharidique (antigène O), capsulaire (antigène K) et flagellaire (antigène H). *E*. *coli* est regroupée en deux grandes catégories : les souches commensales et les souches pathogènes. Ainsi, les souches commensales colonisent le tube digestif, sont inoffensives et font partie de la flore intestinale et ce, autant chez les animaux que chez l'homme. Pour sa part, le groupe des pathogènes est divisé en deux sous-catégories, les *E*. *coli* causant des infections de type intestinales (InPEC) et celles causant des infections de type extraintestinales (ExPEC) [1].

Bien que certaines souches soient inoffensives et d'autres hautement pathogènes, il est important de mentionner que toutes les souches d'*E*. *coli* sont génétiquement associées entre elles [1]. Par exemple, 39,2 % des protéines produites par la souche commensale MG1655 sont conservées et également produites par les souches intestinales EDL933 et urinaires CFT073 [16]. Malgré cette similitude, plusieurs distinctions génétiques différencient les souches commensales des souches pathogènes. Ainsi, les souches pathogènes possèdent, en plus de leur noyau de gènes conservés, du matériel génétique supplémentaire et diversifié, absent des souches commensales, localisé soit sur des plasmides ou sur des îlots de pathogénicité. Ces derniers correspondent à des insertions chromosomiques de larges blocs d'ADN codant pour des gènes contribuant à la virulence. De plus, leur ADN chromosomique a subit divers réarrangements, provoqués par des délétions et insertions de transposons et d'ADN phagique [1, 17-19]. Ainsi, ces insertions et réarrangements génétiques permettent aux souches pathogènes d'induire une infection puisque ceux-ci codent, généralement, pour des facteurs de virulence ou de survie. Ces facteurs de virulence peuvent coder, notamment, pour des adhésines, des systèmes d'acquisition du fer, des endo- et exotoxines, une capsule, etc (Fig. 1).

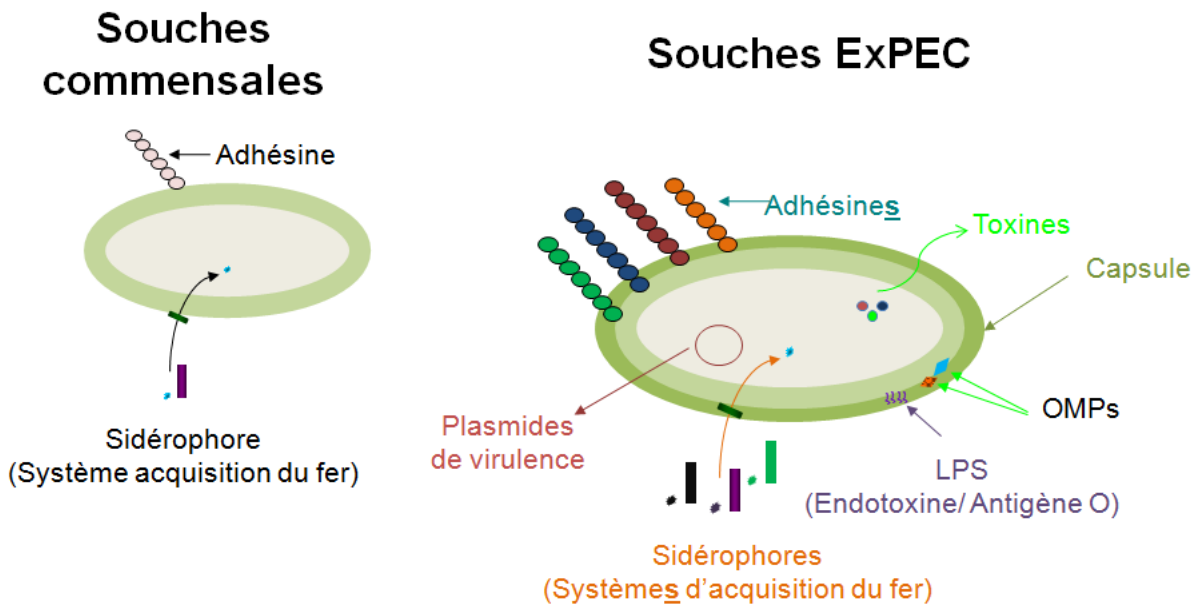

Figure 1. Différences entre les souches commensales et les souches pathogènes (ExPEC). Les souches pathogènes ont acquis du matériel génétique supplémentaire et diversifié. Ce matériel code généralement pour des facteurs de virulence et de survie qui sont absents chez les souches commensales. Les adhésines, toxines, protéines de membrane externe (OMP), les lipopolysaccharides (LPS), les sidérophores et la capsule sont les plus connus. Pour de plus amples détails, voir section 2 sur les facteurs de virulence.

1.2. *E. coli* pathogènes intestinales

Parmi ce groupe, 7 types d'*E. coli* ont été identifiés et caractérisés. De ceux-ci, nous retrouvons les *E. coli* entérotoxinogène (ETEC), entéropathogène (EPEC), entérohémorragique (EHEC), entéroaggrégative (EAEC) et d'adhérence diffuse (DAEC), entéroinvasive (EIEC) et entéroaggregative-hémorragique (STEAEC). L'interrelation entre ces différents pathotypes est que chacun cause des diarrhées. Cependant, le type de diarrhée est caractéristique de chaque souche infectante. Par exemple, les ETEC sont associées aux diarrhées du voyageur ou plus communément appelé « tourista » [20]. Cette diarrhée est causée par les toxines thermolabile (LT) et thermostables (STa et STb) [21]. Pour leur part, les EHEC ont la capacité de former des lésions de type attachant/effaçant (A/E), ce qui a pour effet d'effacer les microvilli intestinaux. Elles produisent les toxines de type Shiga (Stx1 et Stx2) et causent des diarrhées pouvant progresser en fèces sanguinolentes et occasionnellement dégénérer en syndrome hémolytique urémique (HUS), qui se résume en une anémie hémolytique, une thrombocytopénie et une ischémie rénale [1]. Les souches EHEC O157 :H7 sont les plus connues et la plus étudiées. Quant à elles, les souches EAEC, en plus de causer une diarrhée persistante, endommagent la muqueuse intestinale [22]. Elles adhèrent aux cellules

intestinales dans une structure ressemblant à un amoncellement de briques [1, 23]. En 2011, un nouveau type d'*E*. *coli* impliqué dans les infections intestinales a été identifié. Il s'agit d'un hybride entre une souche entéroaggrégative et entérohémorragique. Son identification s'est effectuée à la suite de l'éruption des cas de diarrhées sanguinolentes et de HUS en Allemagne. Il s'agit d'une *E*. *coli* entéroaggrégative-hémorragique (STEAEC) de sérotype O104 :H4 [24, 25]. Comme nous pouvons voir, bien que la caractéristique commune des différentes souches intestinales soit la diarrhée, les types, symptômes et conséquences sont bien différents et propres à chaque souche.

1.3. *E. coli* pathogènes extra-intestinales

Le groupe des *E*. *coli* associé aux infections extraintestinales se nomme ExPEC, pour Extra-intestinal Pathogenic *Escherichia coli*. Comme son nom l'indique, ces souches causent des infections lorsqu'elles se retrouvent à l'extérieur de l'intestin. De ce fait, ces souches peuvent être considérées comme étant des souches commensales intestinales puisqu'elles ne causent aucune infection lorsqu'elles se retrouvent dans cet environnement [26]. Tout comme pour les InPEC, le groupe des ExPEC rassemble plusieurs pathotypes et sont responsables de différentes infections. Les UPEC (*E*. *coli* uropathogène) infectent le tractus urinaire (UTI) et causent des cystites, des pyélonéphrites, des bactériémies et des septicémies. Les NMEC (*E. coli* causant la méningite néonatale) causent des méningites chez les nouveau-nés [1, 26]. Les APEC (*E*. *coli* pathogène aviaire) sont à l'origine de la colibacillose aviaire, qui se manifeste par différentes pathologies dont l'aérosacculite, la péricardite, la périhépatite, la péritonite et la septicémie [27]. Puisque le sujet de cette thèse traite des souches urinaires, au cours des prochaines sections, je m'attarderai plus spécifiquement sur le cycle infectieux et les facteurs de virulence des UPEC.

1.4. Les UPEC et cycle infectieux

Comme mentionné ci-haut, les UTI sont principalement associées aux cystites. Par contre, si la cystite n'est pas adéquatement traitée, l'infection évoluera puisque le système urinaire supérieur sera infecté, ce qui provoquera une pyélonéphrite (infection des reins) pouvant mener ultérieurement à une bactériémie et septicémie [14]. Les UTI sont une des infections bactériennes les plus communes chez les humains et elles affectent, annuellement, près de 150 millions de personnes [28]. Seulement aux États-Unis, les coûts associés aux visites médicales

et aux traitements comptent annuellement pour 4 milliards de dollars [29]. Les UTI causées par *E*. *coli* représentent près de 85 % des infections urinaires [30]. Elles touchent 40 % des femmes et 12 % des hommes. Les jeunes enfants et les personnes âgées sont aussi susceptibles. Malgré une antibiothérapie appropriée, près du quart des femmes développeront une seconde infection dans les six mois suivant l'infection initiale et de celles-ci, 44 % verront une infection récurrente se développer au cours de l'année [31, 32]. Aussi, plus de la moitié des épisodes de récurrence sont causés par la même souche ayant causée l'infection primaire [33, 34].

Infection ascendante

Une UTI est une infection de type ascendante débutant principalement lorsque les UPEC, présentes au niveau de la flore intestinale, s'introduisent dans le méat urinaire. Les UPEC atteignent donc la vessie et adhèrent à l'urothélium à l'aide des fimbriae de type 1. Ces fimbriae reconnaissent des résidus mannosylés, nommés uroplakines, présents à la surface cellulaire de l'urothélium. Suite à l'adhésion, les UPEC se multiplieront, coloniseront et envahiront la vessie (les mécanismes impliqués dans l'invasion de l'urothélium seront traités dans la section suivante). Il y aura donc instauration d'une cystite qui se manifeste par la présence de bactéries (bactériurie) et de leukocytes (pyurie) dans l'urine, une impression d'avoir un besoin urgent et fréquent d'uriner sans en être capable ainsi que la présence de sang dans l'urine (hématurie). Si la cystite n'est pas traitée, l'infection évoluera puisque les bactéries atteindront les reins. L'ascension de la vessie aux reins s'effectue par l'entremise des flagelles. Ceux-ci permettent aux bactéries de « nager » le long des uretères pour atteindre les reins [35]. À destination, les bactéries adhèreront aux cellules rénales par l'entremise des fimbriae P, qui reconnaissent les résidus globosides. La colonisation rénale entraînera l'instauration d'une pyélonéphrite qui se manifestera par une douleur aigüe aux flancs, de la fièvre, des nausées et des vomissements. Tout comme la cystite, si la pyélonéphrite n'est pas traitée, l'infection évoluera puisque les bactéries détruiront les reins, par l'entremise de toxines, et se disséminent dans la circulation sanguine. Cette dissémination se traduira en une bactériémie pouvant évoluer en septicémie (Fig. 2) [1, 14, 36].

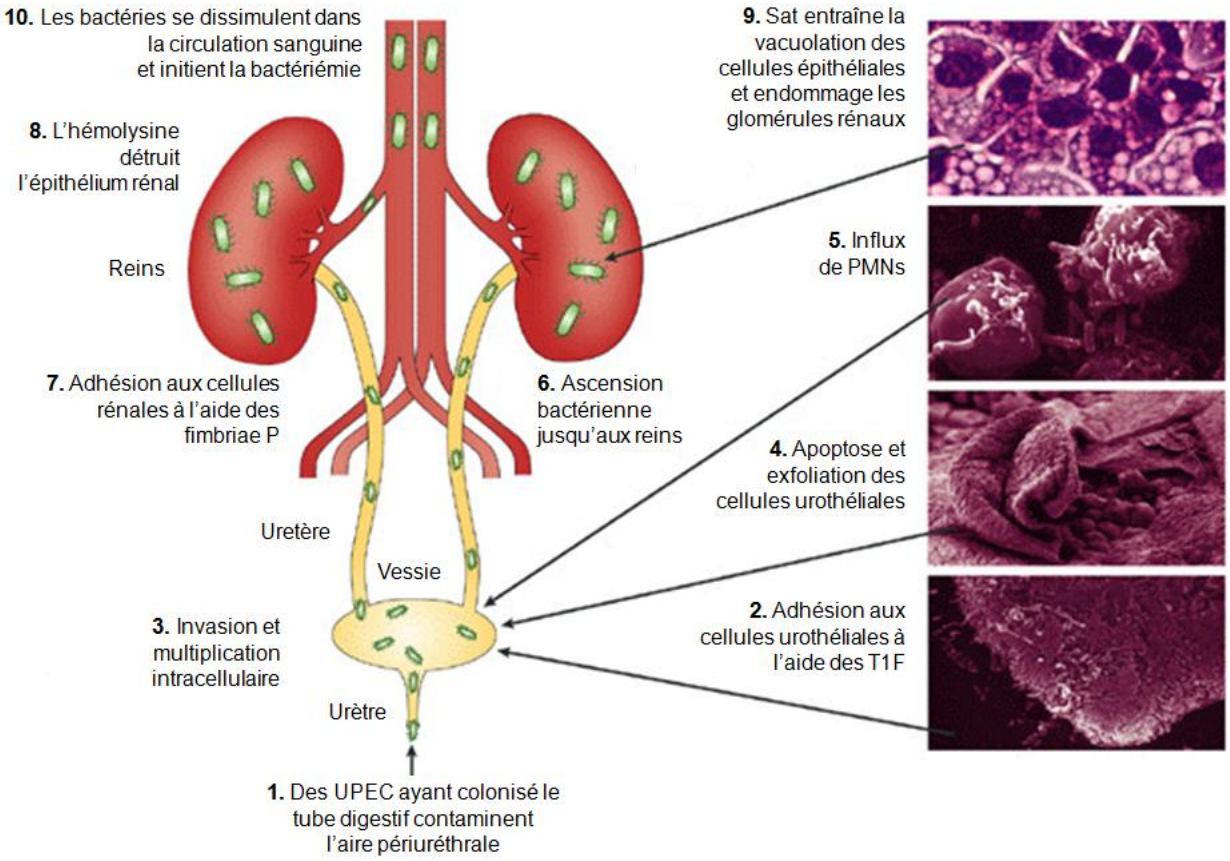

Figure 2. Schématisation de l'infection ascendante du tractus urinaire. Cette figure représente les différentes étapes d'une UTI. Pour plus de détails, se référer au texte à la page précédente. L'hémolysine et l'autotransporteur Sat sont des toxines produites par certaines UPEC. Abréviations : UPEC, *E. coli* uropathogène; T1F, fimbriae de type 1; PMN, leucocytes polymorphonucléaires. Figure adaptée de réf. [1].

Cycle d'infection de la vessie

Longuement considérées comme étant des bactéries extracellulaires, certaines souches d'UPEC ont la capacité d'envahir et de se répliquer à l'intérieur des cellules urothéliales. Ainsi, l'adhésion, à l'aide des fimbriae de type 1, aux uroplakines des cellules urothéliales entraîne un réarrangement de l'actine, activant ainsi des cascades de signalisation / régulation impliquant une multitude de facteurs. Ce réarrangement d'actine formera des protrusions qui envelopperont les bactéries. Celles-ci se retrouveront dans des vacuoles ressemblant aux endosomes tardifs. Par des mécanismes encore inconnus, les bactéries s'évaderont de ces vacuoles et se retrouveront dans le cytoplasme des cellules épithéliales. Cet environnement stimulera leur réplication, entraînant la formation de larges inclusions intracellulaires composées de plusieurs milliers de bactéries. Ces inclusions ressemblent à la structure d'un biofilm et ont été dénommées : Communauté bactérienne intracellulaire (IBC). Dans cette communauté, les

bactéries peuvent entrer en quiescence et perdurer pendant des mois dans le cytoplasme des cellules urothéliales. Suite à des signaux encore inconnus, les bactéries passeront d'une forme coccoïde, forme bactérienne retrouvée dans les IBC, à une forme de bâtonnet motile leur permettant de se détacher de la communauté et éventuellement, sortir de la cellule hôte. Lors de leur sortie, les bactéries se filamenteront et adhèreront aux cellules voisines et démarreront un autre cycle infectieux. Afin de contrer l'invasion, et de surcroît l'infection, les cellules infectées peuvent s'exfolier. Elles se retrouveront donc dans l'urine et seront éliminées lors de la miction. De plus, le processus d'exfoliation active une réponse inflammatoire et immunitaire. Cela aura pour but de contrer l'infection (Fig. 3). Par contre, l'exfoliation cellulaire expose les cellules profondes aux bactéries, les rendant ainsi susceptibles à l'invasion [14, 36-38].

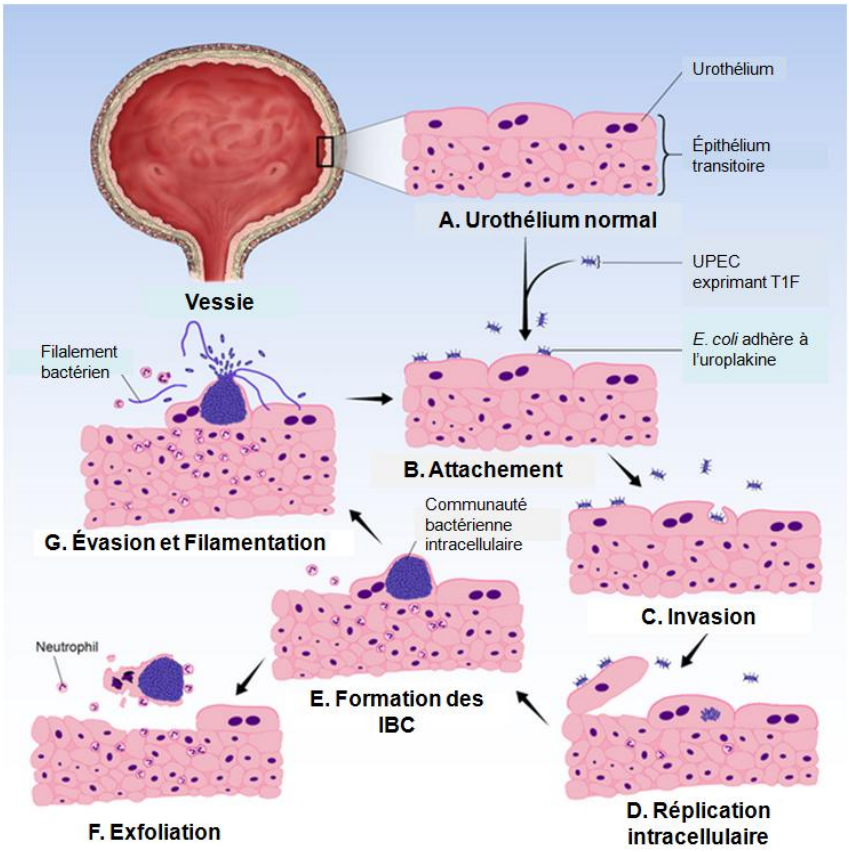

Figure 3. Cycle infectieux dans la vessie. L'urothélium de la vessie (A) se compose d'un épithélium transitoire pseudostratifié. Les cellules superficielles possèdent à leur surface les uroplakines, qui forment, entre autres, la barrière imperméable de la vessie et de récepteurs pour les UPEC. Les bactéries se trouvant dans la vessie adhèreront aux cellules superficielles au niveau des uroplakines, à l'aide des fimbriae de type 1 (B). Suite à l'adhésion, les bactéries envahiront (C) et se répliqueront (D) dans le cytoplasme des cellules. Elles formeront donc une communauté ressemblant aux biofilms (IBC) (E). Ultimement, les bactéries s'échapperont de la niche intracellulaire (G), certaines adopteront une morphologie filamenteuse, où elles adhèreront aux autres cellules et recommenceront un autre cycle infectieux. Au cours de ce processus, les cellules urothéliales infectées peuvent s'exfolier et se retrouver dans l'urine (F). Abréviation : IBC, communauté bactérienne intracellulaire. Figure adaptée de réf. [39].

Étant donné que plus de la moitié des épisodes de récurrence sont causées par la même souche ayant causée l'infection initiale, l'invasion de l'urothélium et la formation des IBC sont associées à la persistance et la récurrence des UTI. De plus, sous la forme d'IBC, les bactéries sont protégées des composants du système immunitaire et des antibiotiques, ce qui peut, en partie, expliquer la forte résistance des UPEC aux traitements [14, 36, 37]. Par contre, il est important de noter que ce ne sont pas toutes les souches UPEC qui sont en mesure de former des IBC. Ainsi, ce processus infectieux a très bien été décrit pour les souches urinaires UTI89 et NU14 tandis qu'il est peu ou pas du tout décrit chez les souches CFT073 et 536.

2. Facteurs de virulence des UPEC

Les facteurs associés à la virulence des UPEC se composent des facteurs d'adhésion (fimbriae de type 1, P, F1C, curli, etc), de flagelles, des polysaccharides de surface (capsule et lipopolysaccharides), de la résistance au sérum, des systèmes d'acquisition du fer, des autotransporteurs, des toxines ainsi que des facteurs dont la fonction est inconnue. En raison du très grand nombre de facteurs de virulence associés aux UPEC, je ne traiterai au cours des prochaines sections que des facteurs d'adhésion, des systèmes d'acquisition du fer, et des toxines.

2.1. Les facteurs d'adhésion

Les fimbriae de type 1

La plupart des souches de *E. coli*, autant commensales que pathogènes, expriment les fimbriae de type 1 [27, 40, 41]. Ces fimbriae se présentent sous la forme d'une tige filamenteuse ressemblant à de la ficelle, possédant un diamètre d'environ 7 nanomètres (nm) et une longueur de 0,1 à 2 micromètres (µM) [42-44]. Ces fimbriae sont codés par l'opéron *fimAICDFGH* qui code pour les protéines impliquées dans la biosynthèse et la structure des fimbriae (Fig. 4). Ces derniers sont composés, d'une part, de la sous-unité majeure FimA qui permet l'ancrage du fimbriae à la surface bactérienne et d'autre part, de l'adhésine FimH, située à l'extrémité distale des fimbriae [43, 45-47]. L'adhésine FimH reconnaît des récepteurs glycoprotéiques contenant des résidus D-mannose retrouvés au niveau des cellules eucaryotes, dont l'épithélium de la vessie (au niveau des uroplakines), de l'intestin, des poumons, des reins ainsi que sur les

cellules inflammatoires [27, 48-50]. De plus, les fimbriae de type 1 permettent la fixation bactérienne au collagène ainsi que sur des surfaces abiotiques [51-53].

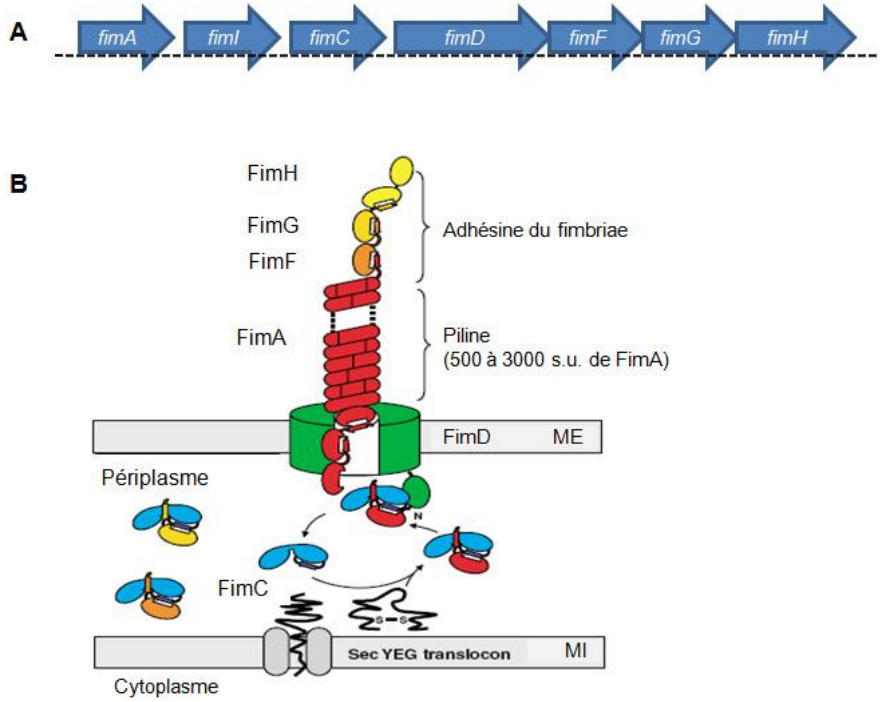

Figure 4. Unité transcriptionnelle et schématisation des fimbriae de type 1. Organisation génétique (A) et structurale (B) des fimbriae de type 1. Le gène *fimA* code pour la sous-unité majeure des fimbriae (nommée fimbrine) et connecte l'extrémité distale (FimFGH) des fimbriae à la membrane externe. Pour sa part, le gène *fimH* code pour l'adhésine et permet l'adhésion des fimbriae aux résidus D-mannose retrouvés au niveau des récepteurs des cellules de l'hôte. Abréviations: s.u., sous-unité; ME, membrane externe; MI, membrane interne. Figure tirée de la réf. [54]

Étant donné que la colonisation de la vessie est dépendante des fimbriae de type 1 et que l'adhésion au tissu hôte est une étape clé dans l'instauration d'une infection, il n'est pas surprenant d'observer que ces fimbriae sont exprimés *in vivo* et sont donc un facteur de virulence essentiel pour les UPEC [50, 55-60]. Comme mentionné à la section 1.4., en plus de permettre l'adhésion aux cellules urothéliales, les fimbriae de type 1 sont aussi impliqués dans l'invasion du tractus urinaire [50, 61] et dans la formation des biofilms [52]. Cela permet donc aux UPEC d'échapper aux mécanismes de défense de l'hôte et de résister à l'antibiothérapie [51].

Régulation de l'expression des fimbriae de type 1

La régulation des fimbriae de type 1 est soumise à la variation de phase et est contrôlée par un promoteur (*fimS*) situé sur un élément interconvertible (IE). Cet IE se trouve en amont de l'opéron *fim* et se compose de 314 nucléotides (nt) bordés de deux régions répétées inversées (RID pour région inversée droite et RIG pour région inversée gauche) [62]. L'IE alterne selon deux orientations, la phase ouverte (ON) et fermée (OFF). Lorsque l'IE est en phase ouverte, les fimbriae sont produits tandis qu'ils sont réprimés lorsque l'IE est en phase fermée puisque le promoteur se trouve en direction inverse de l'opéron *fim*, bloquant ainsi sa transcription (Fig. 5).

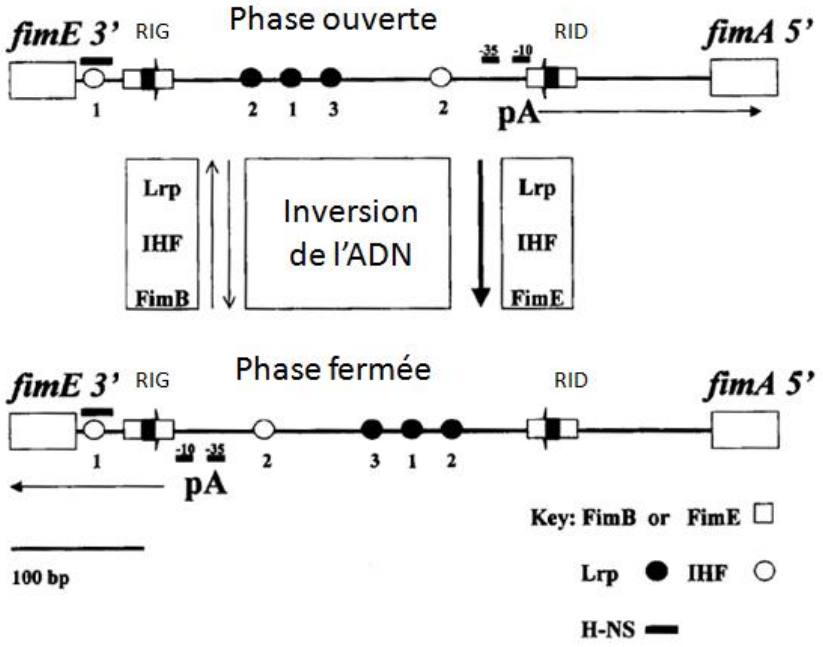

Figure 5. Organisation de l'élément interconvertible (en position ouverte et fermée) ainsi que des sites de fixation des différents cofacteurs impliqués dans la régulation des fimbriae de type 1. Le promoteur des fimbriae de type 1 se trouve sur un élément interconvertible (IE) alternant entre les phases ouverte et fermée. Ce dernier est bordé de régions inversées (RIG et RIG) et contient les sites de fixation des régulateurs des fimbriae de type 1, i.e. FimB, FimE, Lrp et IHF. La fixation de ses régulateurs à leur site de fixation dictera l'expression ou l'inhibition des fimbriae de type 1. Ainsi, la fixation de FimB, Lrp et IHF au niveau de l'IE favorise son positionnement en phase ouverte, menant à l'activation de la transcription de l'opéron *fim* (schéma supérieur). De plus, dans certains cas, la fixation de ce complexe peut mener au positionnement de l'IE en phase fermée et ainsi réprimé l'expression de l'opéron *fim* (schéma inférieure). En contre-partie, la fixation de FimE, Lrp et IHF entraîne le positionnement de l'IE en phase fermée et donc, la répression de l'expression de l'opéron *fim* (schéma du bas). Pour plus de détails, se référer à la section Régulation de l'expression des fimbriae de type 1 de cette revue de littérature. Abbréviations : IE, élément interconvertible; RID, région inversée droite; RIG, région inversée gauche; pA, promoteur de *fimA*, -10 et -35, région -10 et -35 du promoteur de l'opéron *fim*. L'orientation de la flèche associée à pA indique l'orientation de la transcription. Figure adaptée de réf. [63].

Le changement de conformation de l'IE est orchestré par les recombinases *fimB*, *fimE*, *ipuA* et *ipbA* [64-68]. Les recombinases FimB, IpuA et IpbA permettent le changement de conformation de l'IE dans la phase ouverte tandis que FimE entraîne la phase fermée. Ainsi, l'expression de *fimB*, *ipuA* et *ipbA* active l'expression des fimbriae de type 1 tandis que celle de *fimE* la réprime. Afin de coordonner l'expression des fimbriae de type 1, les bactéries inter-convertiront l'IE entre la phase ouverte et fermée. Ainsi, dans la vessie, l'IE est placé en position ON, puis tendra à changer de conformation vers la phase fermée lorsque les bactéries débuteront leur ascension vers les reins, étape durant laquelle les flagelles sont exprimés [35, 60, 69-71].

Afin de permettre la recombinaison de la phase ouverte à fermée et fermée à ouverte, l'expression des recombinases doit être finement régulée. L'expression de *fimB* et *fimE* ainsi que leur activité dépendent de divers régulateurs (Tableau 1) et de diverses conditions environnementales. Étant donné que la régulation de l'expression des fimbriae de type 1 est extrêmement complexe et implique une multitude de facteurs, je ne discuterai ici que des principaux. De plus, puisqu'aucune étude ne traite de la régulation des recombinases IpuA et IpbA, je ne traiterai que de la régulation de *fimB* et *fimE*. Ainsi, l'expression des fimbriae de type 1 est dépendante des cofacteurs IHF (integration host factor), Lrp (leucine-responsive protein), H-NS (histone-like protein) (Fig. 5).

Tableau 1 : Régulateurs influençant l'expression des recombinases *fimB* et *fimE*

		fimB	*fimE*	Références
Régulateurs	CRP-CyaA	-		[72]
	FocB	-		[73]
	H-NS	-	-	[63]
	LrhA		+	[74]
	NagC	+		[75]
	NanR	+		[75]
	PapB	-	+	[76, 77]
	ppGpp	+		[78, 79]
	RcsB	+	-	[80]
	RpoS	+		[78]
	SlyA	+		[81]

Le sigle « + » signifie induction tandis que le sigle « – » signifie répression

L'expression des fimbriae de type 1 dépend de la transcription et de l'activité des recombinases FimB et FimE, qui elles, agissent sur l'orientation de l'IE et permettent soit l'activation soit la répression de la transcription de l'opéron *fim*. Dans un premier temps, il a été mis en évidence que H-NS réprime l'expression de *fimB* et *fimE* [63]. En plus d'être un répresseur transcriptionnel de *fimB* et de *fimE*, H-NS inhibe aussi l'activité de recombinase de FimB [82]. Cette inhibition prend place puisque H-NS se fixe en amont du site de fixation de FimB (RIG) au niveau de l'IE (Fig. 5), causant ainsi un encombrement stérique à ce site. Étant donné que l'expression des recombinases est essentielle à l'inversion de l'IE, le facteur H-NS réprime toute variation de phase, impliquant donc que si l'IE est en phase ouverte, celle-ci restera ouverte tant que H-NS se trouvera fixé au niveau des promoteurs des gènes *fimB* et *fimE*.

Pour leur part, les facteurs Lrp et IHF ne semblent pas être impliqués dans l'expression de *fimB* et *fimE*, mais plutôt dans la variation de phase de l'IE [63, 64, 83]. Ainsi, lorsque l'IE est en phase ouverte, IHF se fixe à un site situé près de la RID tandis que Lrp s'apparie à trois sites localisés près de la RIG [64, 84, 85] (Fig. 5). L'appariement d'IHF et de Lrp au niveau de l'IE joue un rôle dans la régulation de l'activité de recombinase de FimB et FimE, puisque IHF et Lrp introduisent des courbures de l'ADN pouvant atteindre 180°. La fixation d'IHF et de Lrp au niveau de l'IE devrait entraîner la formation d'une boucle en forme d'épingle à cheveux au niveau de l'ADN et la formation de cette boucle a pour effet de juxtaposer les RID et RIG et de ce fait, favoriser la recombinaison par FimB et FimE [64, 83] (Fig. 6).

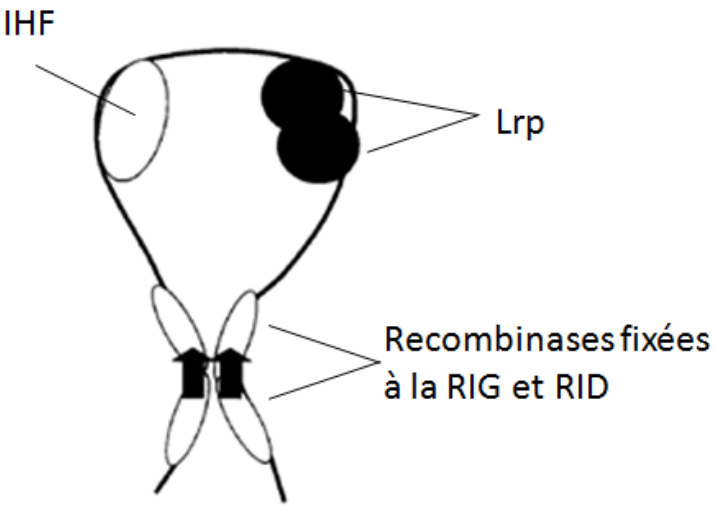

Figure 6. Organisation spatiale de l'élément interconvertible (IE). Organisation spaciale possible de l'IE lorsque FimB ou FimE est fixée au niveau des régions inversée gauche (RIG) et inversée droite (RID) (flèches). La courbure de l'ADN est induite par la fixation d'IHF au site 2 et Lrp aux sites 1 et 2. Cette courbure de l'ADN mène à la juxtaposition des RIG et RID, ce qui permet la recombinaison et la variation de phase. Figure adaptée de réf. [63].

En plus des régulateurs impliqués dans l'expression des fimbriae de type 1, diverses conditions environnementales favorisent l'expression ou la répression de ces fimbriae. Ainsi, il a été mis en évidence que la température, la culture en condition statique, la phase stationnaire de croissance, la présence de certains acides aminés ainsi que le pH et l'osmolarité régulent l'expression des fimbriae de type 1.

Des études contradictoires mettent en relief le rôle de la température dans l'expression des fimbriae de type 1. Dans un premier temps, Dormal *et al*. [86] ont montré que l'expression de *fimA* était réprimée lorsque les souches sont cultivées à 37°C, comparativement à 30°C. Cette observation est surprenante étant donné que les fimbriae de type 1 sont préférentiellement exprimés lorsque les bactéries se retrouvent au niveau de la vessie [59], où la température de l'urine se situe aux environs de 37°C. Cependant, un autre groupe a démontré que l'expression de *fimB* et de l'opéron *fim* est favorisée lorsque les bactéries se trouvent à des températures se situant entre 37 et 41°C et qu'en contrepartie, *fimE* est réprimé à ces températures [87].

Des études contradictoires mettent aussi en évidence le rôle de RpoS dans la régulation de l'expression des fimbriae de type 1. Il a été suggéré que lorsque les bactéries entrent en phase stationnaire de croissance, le facteur sigma (σ) RpoS inhibe l'expression des fimbriae de type 1 en réprimant la transcription de *fimB* [88]. Une fois de plus, ces résultats sont surprenants puisque la condition de culture favorisant l'expression des fimbriae de type 1, *in vitro*, est la croissance statique sur une période de 24 à 48 h [89], condition où les bactéries se trouvent en phase stationnaire de croissance. D'autre part, il a été mis en évidence que l'expression de l'opéron *fim* est maximal lorsque les bactéries entrent en phase stationnaire de croissance [78].

Lrp est impliqué, notamment, dans la régulation de l'opéron *ilvIH*, opéron catalysant les premières étapes de la biosynthèse de l'isoleucine, de la leucine et de la valine [90-92]. Étant donné que la fixation de Lrp au niveau de l'IE est requise dans la variation de phase, il n'est pas étonnant que les acides aminés ayant une chaîne latérale (isoleucine, leucine, valine) et l'alanine inhibent le potentiel de recombinaison de FimB puisque ces derniers inhibent l'appariement de Lrp au niveau de l'ADN (Fig. 5), ce qui a pour effet d'inhiber la variation de phase et de favoriser la positionnement le l'IE en phase fermée [93].

Finalement, il a été mis en évidence que l'expression de *fimE*, donc l'inhibition de l'expression des fimbriae de type 1, est favorisée lorsque les bactéries sont cultivées en condition de haute osmolarité à pH acide (conditions retrouvées dans les reins) [94]. D'autre part, l'induction de *fimB* et de l'opéron *fim* s'effectue en condition de faible osmolarité de pH neutre à moyennement acide (conditions retrouvées dans la vessie) [94]. Ces observations peuvent donc expliquer, en partie, la cascade de régulation des fimbriae de type 1 observée lors d'une infection, i.e. expression des fimbriae de type 1 dans la vessie et leur inhibition dans les reins.

Les fimbriae P

Les fimbriae Pap (fimbriae P) sont codés par un groupe de 11 gènes, les gènes *papIBAHCDJKEFG*. Son nom provient de « pyelonephritis-associated pili ». Les fimbriae P sont associés aux UPEC et, plus précisément, à la pyélonéphrite [95]. Ces fimbriae sont similaires aux fimbriae de type 1, i.e. qu'ils forment une tige protéique de 6,8 nm de largeur, sont constitués d'une sous-unité majeure (PapA) ancrant le fimbriae à la surface bactérienne ainsi que d'une adhésine (PapG) retrouvée à l'extrémité distale des fimbriae [96, 97]. Les récepteurs des fimbriae P sont le globotriasylcéramide (GbO3), le globoside (GbO4) et l'antigène de Forsmann (GbO5). Ceux-ci se retrouvent au niveau des glycolipides membranaires du groupe P des érythrocytes humains ainsi qu'au niveau des cellules uroépithéliales [46, 51, 95, 98, 99].

Contrairement aux fimbriae de type 1, les fimbriae P ne sont pas exprimés au niveau de la vessie, mais plutôt au niveau des reins. Ils sont impliqués dans les cas sévères d'infection urinaire (pyélonéphrite) puisqu'ils permettent l'adhérence bactérienne au niveau des cellules rénales [51, 95].

Curli

La très forte majorité des souches d'*E. coli* produit des curli [27]. Ils se présentent comme étant des appendices ressemblant à des cheveux, minces et en forme de rouleau [100]. Les curli sont codés par deux opérons génétiquement opposés, *csgBAC* et *csgDEF*. Le gène *csgA* code pour la sous-unité fimbriaire tandis que le gène *csgB* code une protéine essentielle à la biosynthèse des curli [101]. De plus, les gènes *csgBAC* sont régulés positivement par le régulateur CsgD. Le rôle des curli dans la virulence bactérienne n'est pas très bien connu. Cependant, il a été mis en évidence qu'ils peuvent adhérer à plusieurs protéines de l'hôte, incluant la fibronectine et la

laminine de la matrice extracellulaire [100-102]. Le rôle des curli dans la virulence des UPEC n'est pas clairement établi. Récemment, il a été mis en évidence qu'en plus de promouvoir la virulence, la production des curli permet aux bactéries d'adhérer aux cellules rénales et de résister au peptide antimicrobien humain LL-37 [103].

Autres types de fimbriae

Les fimbriae F1C sont codés par les gènes *foc* et ressemblent, génétiquement et structuralement, au fimbriae de type 1 [104-106]. Ils permettent l'adhésion aux résidus glycolipidiques β-GalNac-1 et 4β-Gal retrouvés sur les cellules endothéliales de la vessie ainsi que sur les tubules distaux et les tubes collecteurs rénaux [51, 107]. De plus, son adhésion peut s'effectuer sur les récepteurs globotriasylcéramide, qui eux se retrouvent exclusivement dans les reins [108].

Récemment, les fimbriae Yad et Ygi ont été identifiés comme étant impliqués dans la virulence de la souche UPEC CFT073 [109]. Les fimbriae Yad, codés par l'opéron *yadCKLMhtrEecpDyadN* [110], sont impliqués dans la formation des biofilms et dans l'adhésion aux cellules épithéliales de la vessie [109, 110]. Pour sa part, le fimbriae Ygi, codé par l'opéron *ygiLyqiGHI* [110], est impliqué dans la formation des biofilms, dans l'adhésion aux cellules rénales et est important pour la colonisation des reins [109]. De plus, ces deux fimbriae agissent en synergie puisque l'inactivation cumulative de ces derniers atténue la virulence autant dans la vessie que dans les reins [109].

Régulation croisée entre les différentes adhésines

Le séquençage du génome de certaines souches UPEC montre que ces dernières peuvent posséder plus de 10 types de fimbriae. Par exemple, la souche CFT073 possède au moins 12 fimbriae distincts et plusieurs adhésines afimbriaires [16]. De plus, il est suggéré que les bactéries n'expriment qu'une adhésine à la fois [111], comme en fait foi l'étude menée par Snyder *et al*. [59], où les fimbriae de type 1 sont les seuls adhésines exprimées dans la vessie. Étant donné que les fimbriae sont très immunogènes [112], les bactéries n'ont donc pas avantage

à exprimer plusieurs fimbriae à la fois. Ainsi, en plus d'être régulé par les conditions environnementales, une co-régulation existe entre les différents fimbriae dans le but d'exprimer, au moment opportun, les fimbriae appropriés. Cela permettra aux bactéries d'adhérer aux tissus hôtes et de s'adapter aux microenvironnements qu'elles rencontrent au cours de l'infection.

Comme mentionné précédemment, lorsque les UPEC se retrouvent au niveau de la vessie, seuls les fimbriae de type 1 sont exprimés [59], montrant ainsi qu'il y a co-régulation entre les différents fimbriae. Il semble donc que l'expression des fimbriae de type 1 réprime celle des autres fimbriae. De cette manière, il a été montré que l'introduction de mutations au niveau de *fimS*, inhibant la transcription des fimbriae de type 1, active l'expression des fimbriae P. Aussi, la délétion des fimbriae de type 1 et des fimbriae P active l'expression des fimbriae F1C [113]. Il est donc évident que l'expression des fimbriae de type 1 active un ou des facteurs réprimant l'induction des fimbriae P et F1C. Cependant, les mécanismes moléculaires reliant l'expression des fimbriae de type 1 et la répression des fimbriae P et F1C ne sont pas connus.

D'un autre côté, il a été mis en évidence qu'un des régulateurs des fimbriae P, PapB, réprime les fimbriae de type 1. Cette répression passe par l'induction de la transcription de *fimE* [77, 114] et l'inhibition de l'activité de recombinase de FimB [76, 77]. Cette activité de PapB sur *fim* s'effectue par la fixation de PapB en amont et au niveau de l'IE, au niveau de la RIG (site de fixation de FimB permettant la recombinaison de l'IE (Fig. 5)) [77], ce qui a pour effet d'inhiber l'expression de *fim* étant donné que la phase fermée de l'IE est favorisée.

Le régulateur FocB, régulateur des fimbriae F1C, est, lui aussi, impliqué dans la répression de l'expression des fimbriae de type 1 [73]. Il est suggéré que FocB forme un complexe avec PapB. Ce complexe se fixe, comparativement à PapB seul, de manière beaucoup plus ferme au niveau de l'IE. La fixation du complexe PapB-FocB au niveau de l'IE a pour effet d'inhiber l'activité de recombinase de FimB, ce qui réprime l'expression des fimbriae de type 1 puisque le positionnement de l'IE en phase fermée est privilégié.

Conclusion facteurs d'adhésion

Comme nous pouvons le constater, les UPEC possèdent plusieurs adhésines, leur permettant ainsi d'adhérer à différents récepteurs localisés dans différentes niches chez l'hôte. De plus, les flagelles permettent à celles-ci de « nager » d'un site de colonisation à l'autre. Cela leur confère un avantage puisque l'expression séquentielle des facteurs d'adhérence et de motilité permet la colonisation des différentes niches (vessie et reins). Comme discuté à la section 1.4., l'infection urinaire est une infection ascendante pouvant mener à une infection systémique si non traitée. Ainsi, cette expression séquentielle des facteurs d'adhérence et de motilité permet la colonisation de différentes niches et donc, l'établissement d'une infection systémique. En plus de la régulation croisée entre les différents fimbriae, une co-régulation existe entre les fimbriae et les différents composants de la membrane, e.g. entre les fimbriae de type 1 et les flagelles [69], la capsule et les fimbriae de type 1 [115] ainsi que les curli et les flagelles [116]. Étant donné qu'une partie des composants de la membrane externe se retrouvent au niveau du milieu extracellulaire, ceux-ci sont donc très immunogènes. De cette manière, la régulation croisée entre les différents composants a pour effet de diminuer la réponse immunitaire de l'hôte, d'inhiber le possible masquage d'une adhésine par une autre (par exemple, un fimbriae plus grand masque un plus court) et de prévenir un possible effet néfaste d'exportations massives de composants fimbriaires [117].

À chacune des étapes d'une infection, les bactéries orchestrent de façon coordonnée la transcription de leurs gènes afin de s'adapter à chacun des microenvironnements qu'elles rencontrent. Ainsi, les signaux environnementaux rencontrés au niveau de la vessie ne sont pas les mêmes que ceux retrouvés au niveau des reins. De cette manière, les signaux ont pour effet d'activer et de réprimer la transcription des gènes ainsi que l'activité des protéines afin de permettre aux bactéries de survivre.

2.2. Les flagelles

Les flagelles sont impliqués dans la motilité et permettent aux bactéries, chez l'hôte, de « nager » vers les sites de colonisation. Les flagelles sont de longs appendices hélicoïdaux se trouvant au niveau de la surface bactérienne. Ils sont codés par un groupe d'au moins 40 gènes et ils sont composés de sous-unités de flagelline, codée par le gène *fliC*.

Chez la souche UPEC CFT073, il a été montré *in vivo* que l'expression de la flagelline coïncide avec l'ascension de l'uretère et la colonisation des reins [95]. Comme discuté précédemment, les fimbriae de type 1 sont essentiels à la colonisation de la vessie tandis que les fimbriae P permettent celle des reins. Cela suggère que l'expression des fimbriae et des flagelles doit être étroitement régulée puisqu'il semble inapproprié qu'une souche tente de nager et d'adhérer aux tissus de l'hôte en même temps. Ainsi, la régulation réciproque de la synthèse des flagelles et des fimbriae permet à la bactérie de coordonner deux propriétés divergentes, l'adhérence et la motilité. De cette manière, Lane *et al*. [69] ont montré que lorsque les fimbriae de type 1 sont produits, les bactéries expriment moins de flagelles à leur surface, ce qui résulte en une diminution de leur motilité.

2.3. Toxines

Hémolysine

L'hémolysine, où HlyA, codée par l'opéron *hlyCABD*, est une toxine calcium-dépendante de type RTX, i.e. une toxine dont la séquence peptidique est répétée. Elle s'insère et forme des pores de 2 nm dans la membrane plasmique des cellules de l'hôte. Cette insertion mène ainsi à la lyse cellulaire [118], ce qui a pour effet de libérer des nutriments et autres facteurs permettant la croissance bactérienne. En plus de former des pores dans les membranes plasmiques, HlyA stimule la protéolyse des protéines de l'hôte impliquées dans l'adhésion cellulaire, provoquant ainsi l'exfoliation des cellules urothéliales [119], expliquant ainsi les phénotypes associés à HlyA, soit l'induction d'hématuries, de dommages rénaux et l'augmentation des risques de septicémie [120, 121].

Tos

Pour sa part, la toxine putative TosA, elle aussi faisant partie de la famille des toxines RTX, est codée par l'opéron *tosRCBDA*, est uniquement exprimée *in vivo* et est impliquée dans la colonisation de la vessie et des reins dans le modèle murin d'UTI [122]. Plus spécifiquement, TosA est impliquée dans l'adhésion bactérienne aux cellules des voies supérieures du tractus urinaire (urètre et reins) et est importante lors de la bactériémie [123]. Contrairement à HlyA, TosA ne semble pas posséder d'activité cytotoxique et joue donc un rôle différent dans l'établissement d'une UTI. Bien que plusieurs phénotypes soient associés à TosA, la

colonisation du tractus urinaire et lors de l'infection systémique, le rôle précis de cette toxine reste à être déterminé.

Facteur nécrosant cytotoxique 1 (CNF1)

CNF1, toxine de la famille des petites protéines fixant le GTP de la famille Rho. Elle active constitutivement, chez les cellules hôtes, les protéines fixant le GTP de la famille Rho [124]. Ces protéines sont impliquées, notamment, dans l'activation des fibres de stress d'actine, dans la formation de la lamellipodia ou filopodia, dans l'induction du froissement (ruffling) membranaire et dans la modulation des réseaux de signalisation inflammatoire. De plus, l'activation prolongée de ces protéines mène à leur ubiquitination et subséquemment, à leur dégradation par le protéasome [125]. Ainsi, l'effet cytotoxique du CNF1 est causé par l'activation constitutive des protéines Rho. CNF1 est donc impliqué dans l'apoptose des cellules urothéliales, possiblement en stimulant l'exfoliation de celles-ci, dans l'augmentation de l'invasion, causée par le froissement membranaire, et dans l'inhibition de l'activité phagocytaire et chémoattractant des neutrophiles [126-128].

Sat

La toxine Sat (toxine autotransportrice sécrétée) fait partie de la famille des autotransporteurs, ou système de sécrétion de type V. Sat est une sérine protéase causant des dommages rénaux importants puisqu'elle dissout les membranes glomérulaires (couche de la matrice extracellulaire des glomérules rénaux), entraîne la perte des cellules épithéliales tubulaires et provoque la vacuolation du tissu rénal [129, 130]. Étrangement, malgré ces effets néfastes, Sat ne semble pas influencer la capacité de la souche UPEC CFT073 à coloniser le tractus urinaire [129]. Nous pouvons donc émettre l'hypothèse qu'elle est plutôt impliquée dans la sévérité de l'infection et prépare les bactéries à se disséminer dans la circulation sanguine.

2.4. Systèmes d'acquisition du fer par les sidérophores

Le fer est un élément essentiel à la vie de la majorité des organismes. Il est utilisé dans le transport et l'entreposage de l'oxygène, dans la biosynthèse de l'ADN, le transport des électrons (respiration cellulaire) et dans le métabolisme du peroxyde. Les besoins en fer chez les bactéries se situent aux environs de 10^{-7} M. Cependant, chez l'hôte, la concentration en fer du

milieu extracellulaire est d'environ 10^{-24} M puisqu'il est associé à la transferrine, la lactoferrine, l'haptoglobine et l'hémoglobine [131]. En limitant la disponibilité en fer, l'hôte se protège donc d'une possible infection par des pathogènes. Par contre, pour contrer cette carence et subvenir à leurs besoins, les bactéries possèdent des mécanismes de séquestration du fer. Ceux-ci passent, notamment, par les sidérophores et les hémophores. Les sidérophores sont des molécules sécrétées de faibles poids moléculaires ayant une très grande affinité, plus grande que les protéines de l'hôte, pour le fer ferrique (Fe^{3+}). Les bactéries récupèreront les complexes sidérophore-fer à l'aide de récepteurs membranaires où le fer sera libéré au niveau du cytoplasme [118]. Le génome de la souche UPEC CFT073 contient au moins 10 systèmes d'acquisition du fer et plusieurs transporteurs putatifs [16], dont les sidérophores entérobactine, salmochéline, aérobactine, yersiniabactine, le système Chu (système d'acquisition de l'hème) et système Sit (transport fer et manganèse). En raison de la redondance fonctionnelle des systèmes d'acquisition du fer, il a été démontré que le potentiel de virulence ne dépend pas uniquement d'un système [14, 131, 132].

3. Le régulon Pho

Le phosphore, en tant que composant cellulaire, est le cinquième élément en importance après le carbone, l'oxygène, l'azote et l'hydrogène [2]. Le phosphate se retrouve dans la composition d'innombrables molécules telles les lipides membranaires (phospholipides), les sucres complexes (les lipopolysaccharides) et les acides nucléiques. Le phosphate est impliqué dans le métabolisme énergétique et peut aussi être incorporé dans les protéines lors de modifications post-traductionnelles [133]. De plus, le phosphate est impliqué dans plusieurs réactions biochimiques telles la transduction des signaux par les systèmes de régulation à deux composants (SRDC).

E. *coli* (comme plusieurs espèces bactériennes) utilise trois sources différentes de phosphate : le phosphate inorganique (P_i), les organophosphates (esters de phosphate) et les phosphonates. Indépendamment de la source de phosphate, l'incorporation du phosphate suit deux étapes communes. La première consiste en son acquisition et la deuxième repose sur son entrée et son assimilation au niveau du cytoplasme [133].

3.1. Différentes sources de phosphate

La source la plus importante de phosphate est le P_i, qui lui, se retrouve sous trois formes : le P_i, le pyrophosphate (PP_i) et le métaphosphate ($polyP_i$) [4]. Le système impliqué dans l'acquisition du P_i, à partir de l'environnement, est le système Pst (phosphate specific transport system) [4]. Ce système est un membre du régulon Pho (qui sera détaillé plus loin dans ce chapitre) et est sous le contrôle du SRDC PhoBR. Le P_i peut être formé à partir des organophosphates. L'hydrolyse de ces derniers au niveau du périplasme, par une variété d'enzymes dont la phosphatase alcaline (PhoA), libère le P_i, qui lui, est transporté dans le cytosol par le système Pst [4].

3.2. Transport du P_i par le système Pit et Pst

E. *coli* possède deux types de transporteurs du P_i, le système Pit et le système Pst. Le système Pit est un transporteur de faible affinité tandis que le système Pst est un transporteur de haute affinité [133]. En condition d'excès de phosphate, i.e. lorsque la concentration extracellulaire est supérieure à 4 µM, le P_i est transporté par le système Pit. Cependant, en condition de carence en P_i, i.e. lorsque la concentration extracellulaire est inférieure à 4 µM, la production du système Pst est induite plus de 100 fois et le P_i est principalement transporté par ce système [4]. En raison de son induction en condition de carence en phosphate, contrairement au système Pit, le système Pst fait partie du régulon Pho et est sous le contrôle de PhoBR [133].

Le système Pit est composé de deux transporteurs ressemblant à des porines, PitA et PitB. L'énergie nécessaire au transport du P_i par le système Pit est produite à l'aide de la force Proton-motrice (FPM). PitA est constitutivement exprimée tandis que l'expression de PitB s'effectue en condition de forte concentration en phosphate, dû à sa répression par le régulon Pho [134-136]. Pour sa part, l'opéron *pstSCAB-phoU* code pour le système Pst et fait partie de la grande famille des ABC transporteurs (ATP-binding cassette) [133, 137]. Le système Pst est composé d'une protéine périplasmique de haute affinité pour le P_i (PstS), de deux protéines membranaires formant un canal (PstC et PstA), d'une protéine liant l'ATP (PstB) et de PhoU [135, 138]. Les protéines PstSCAB forment l'ABC transporteur tandis que le rôle de PhoU reste à être élucidé. Cependant, il a été démontré que PhoU n'est pas impliqué dans le transport du P_i, mais plutôt dans la régulation de l'activation du régulon Pho et ce, par l'entremise de PhoBR [139, 140]. De plus, la cristallisation de la protéine PhoU, chez *Thermotoga maritima* et *Aquifex*

aeolicus, a montré que PhoU possède une structure STAT (transduction du signal et activateur de la transcription) et des sites de fixation du fer (co-facteur), ce qui renforce l'hypothèse que PhoU est impliqué dans la régulation de l'activation du régulon Pho [139, 141].

La forte spécificité du système Pst pour le P_i est conférée par PstS, qui possède un site unique de fixation pour le P_i (K_d d'environ 1 µM) [142]. Ce site permet aux anions monovalents ($H_2PO_4^-$) et divalents (HPO_4^{-2}) de se fixer directement sur PstS [133]. Suite à la fixation du phosphate sur PstS, le P_i est transloqué du milieu périplasmique au cytoplasme par l'intermédiaire des protéines membranaires PstC et PstA qui forment un canal au niveau de la membrane interne. L'énergie requise pour le transport du P_i est conférée par l'hydrolyse de l'ATP par la protéine PstB, qui se localise au niveau de la face cytoplasmique de la membrane interne (Voir Figure 1 de l'article #1).

3.3. Membres du régulon Pho

En condition de carence en phosphate, lorsque la concentration du milieu extracellulaire est inférieure à 4 µM [133], les cellules bactériennes perçoivent cette condition et activent la transcription des gènes faisant partie du régulon Pho. Chez *E. coli* K-12, le régulon Pho est composé d'au moins 31 gènes (Tableau 2) [133, 143-146] codant, notamment, pour des protéines impliquées dans l'acquisition et le métabolisme de diverses sources de phosphates (P_i, organophosphates et phosphonates). Chez *E. coli*, il a aussi été mis en évidence, par une étude protéomique, que la réponse à une carence en phosphate implique plus de 400 protéines [133]. Cependant, ce n'est pas parce qu'une protéine est produite lors d'une carence en phosphate que celle-ci fait partie du régulon Pho. Les membres du régulon Pho se caractérisent par la présence d'une boîte Pho au niveau de leur promoteur [133, 144]. Une boîte Pho est constituée de deux séquences directes répétées de 7 nucléotides (nt) 5'-CTGTCAT-3' séparées par 4 nt [133]. Cette séquence est reconnue par le régulateur PhoB et l'interaction PhoB-boîte Pho active ou réprime la transcription des gènes (voir Fig. 1 de l'article #1 à la page 48)

Dans le but de prédire les gènes régulés par PhoB, Yuan *et al*. [147] ont effectué une analyse *in silico* des promoteurs des gènes afin d'identifier ceux possédant des boîtes Pho putatives et ce, chez 13 espèces bactériennes. Ils ont construit une matrice à l'aide de 15 séquences de boîte Pho provenant de *Sinorhizobium meliloti* et *E. coli*. À l'aide de cette matrice, l'algorithme

recherche parmi les différents génomes la présence de boîtes Pho au niveau des promoteurs. Un score est alors attribué à chacune des boîtes Pho putative retrouvées dans la région promotrice des gènes. Selon la position des nucléotides, les résultats sont alors filtrés de manière à ce que les membres potentiels du régulon Pho possèdent au moins une boîte Pho putative entre les nucléotides -500 et +100 du codon départ et un score $\geq$ 0,35. De cette manière, chez *S*. *meliloti*, les auteurs ont identifié 96 gènes ou unités transcriptomiques possèdant au moins une boîte Pho putative et de ceux-ci, 34 ont été démontrés comme étant directement régulés par PhoB [147]. De plus, cette analyse a permis de mettre en évidence que le régulon Pho de la souche *E*. *coli* K12 MG1655 possède 107 candidats tandis que la souche entérohémorragique O157 :H7 SAKAI compte 103 candidats, dont sept se retrouvent sur des plasmides. Cependant, des preuves expérimentales sont nécessaires afin de valider cette analyse.

En plus d'être impliqué dans le transport du phosphate, donc dans son homéostasie, le système Pst est aussi impliqué dans la virulence des souches pathogènes puisqu'il a été démontré que la délétion du système Pst atténue la virulence [6]. Dans l'article de revue qui suit, The Pho regulon and the pathogenesis of *Escherichia coli*, nous avons plus spécifiquement traité du rôle du régulon Pho dans la virulence d'*E*. *coli*.

Tableau 2. Gènes composant le régulon Pho

Gène ou opéron	Nombre de boîtes Pho [a]	Fonction	Gène	Description	Références
amn	2		*amn*	AMP nucléosidase	[143, 147]
asr	2		*asr*	Résistance à l'acidité	[146]
iciA	2		*icia*	Initiateur de la réplication du chromosome	[147, 148]
phnCDEFGHIJKLMNOP	2	Transport et metabolisme des phosphonates	*phnC*	Protéine de liaison à l'ATP impliquée dans le transport des phosphonates	[4, 147]
			phnD	Protéine périplasmique fixatrice	
			phnE	Protéine formant un canal membranaire	
			phnF	Régulateur	
			phnGHIJKLM	Composants de la lyase carbone-phosphate	
			phnNP	Protéines accessoires de la lyase carbone-phosphate	
			phnO	Régulateur	
phoA-psiF	2	Hydrolyse des esters de phosphate	*phoA*	Phosphatase alcaline	[4, 147]
		Inconnu	*psiF*		
phoBR	1	Contrôle du régulon Pho	*phoB*	Régulateur	[4, 147]
			phoR	Senseur	
phoE	1	Entrée de polyanions	*phoE*	Porine	[4, 147]
phoH	3	Inconnu	*phoH*	Protéine de liaison à l'ATP	[4, 147]
psiE	1	Inconnu	*psiE*	Protéine hydrophobe	[4, 147]

Gène ou opéron	Nombre de boîtes Pho [a]	Fonction	Gène	Description	Références
pstSCAB-phoU	9	Transport du P_i et contrôle du régulon Pho	*pstS*	Protéine périplasmique fixatrice du P_i de haute affinité	[4, 147]
			pstCA	Protéines formant un canal membranaire	
			pstB	Protéine de liaison à l'ATP	
		Contrôle du régulon Pho	*phoU*	Régulateur	
			ugpC	Protéine de liaison à l'ATP	
			ugpQ	Phosphodiestérase	
yibD	1		*yibD*	Glycosyl transférase putative	[143]
ytfK	1		*ytfK*	Protéine hypothétique	[143]

[a] Le nombre de boîtes Pho identifiées se basent sur l'étude de Yuan *et al*. [147]

3.4. Article #1

The Pho regulon and the pathogenesis of *Escherichia coli*

Sébastien Crépin, Samuel-Mohammed Chekabab, Guillaume Le Bihan, Nicolas Bertrand, Charles M. Dozois and Josée Harel (2011) Veterinary Microbiology 153(1-2): 82-8

A) Contribution de l'étudiant

1- En 2008, notre groupe a publié une revue traitant du rôle du régulon Pho dans la virulence bactérienne [6]. Dans la présente revue, Vet. Micro. 2011, nous avons fait une mise à jour de la revue 2008 et nous avons mis l'emphase sur les souches *E*. *coli* pathogènes. Ainsi, nous introduisons le régulon Pho et son mécanisme d'activation et nous traitons de son rôle dans la virulence des souches ExPEC et InPEC, dans la résistance au stress oxydatif, dans la modification de la surface bactérienne, dans l'adhésion bactérienne aux tissus de l'hôte ainsi que dans l'adaptation à l'environnement.

2- Étant premier auteur de cet article, j'ai écrit près de 80% de l'article. J'ai coordonné l'écriture avec les autres auteurs, corrigé avec l'aide de mes directeurs, assemblé et soumis le manuscrit. Samuel Mohammed Chekabab, Guillaume Le Bihan et Nicolas Bertrand ont écrit les autres sections. L'article de revue a été corrigé par mes directeurs et collaborateurs.

B) Résumé de l'article :

Au cours d'une infection, les bactéries doivent coordonner l'expression de ses gènes afin de s'adapter adéquatement aux stimuli environnementaux. Le régulon phosphate (Pho) est contrôlé par le système de régulation à deux composants (SRDC) PhoBR. Ce SRDC s'active en condition de carence en phosphate et régule les gènes impliqués dans son métabolisme. En plus de son rôle homéostasique, le régulon Pho est impliqué dans la pathogénèse bactérienne puisque son activation module plusieurs processus cellulaires. Ceux-ci inclus une atténuation de virulence par, notamment, une diminution de l'adhérence aux tissus de l'hôte ainsi qu'une sensibilité accrue aux peptides antimicrobiens, à l'acidité et au stress oxydatif. Dans cette revue,

nous traiterons de la relation entre le régulon Pho et la virulence d'*E*. *coli* et montrerons que, en plus de réguler l'homéostasie du phosphate, le régulon Pho joue un rôle majeur dans la virulence et la réponse aux stress.

The Pho regulon and the pathogenesis of *Escherichia coli*

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ABSTRACT

During the course of infection, bacteria must coordinately regulate gene expression in response to environmental stimuli. The phosphate (Pho) regulon is controlled by the two component-regulatory system PhoBR. PhoBR is activated during starvation and regulates genes involved in phosphate homeostasis. Several studies have highlighted the importance of the Pho regulon in bacterial pathogenesis, showing how induction of PhoBR, in addition to regulating genes participating in phosphate metabolism, leads to modulation of many cellular processes. The pleiotropic effects of Pho regulon activation include attenuated virulence and alteration of many virulence traits, including adhesion to host cells and resistance to cationic antimicrobial peptides, acidity and oxidative stresses. This review provides an overview of the relationship between the Pho regulon and virulence in *Escherichia coli* and illustrates that, in addition to regulating phosphate homeostasis, the Pho regulon plays a key role in regulating stress responses and virulence.

Author keywords: Pho regulon, pathogenic *Escherichia coli*, two-component regulatory system, stress response

Introduction

To adapt and survive in different microenvironments, bacteria must sense and respond to extracellular signals. The adaptive response to environmental stimuli can be transduced by two-component regulatory systems [149], which are involved in the regulation of chemotaxis, osmoregulation, metabolism and transport. [150]. A typical two-component regulatory system (TCRS) is composed of an inner-membrane histidine kinase (HK) sensor protein and a response regulator (RR) that acts as a DNA-binding protein, activating or repressing gene expression [151].

Phosphorus, in terms of cellular content, is the third most abundant element. It is found in several molecules, including membrane lipids, complex sugars and nucleic acids. Phosphate is also involved in energy metabolism and in signal transduction, which is mediated by a TCRS [4]. The extracellular concentration of phosphate is sensed by the two-component regulatory system PhoBR, in which PhoR encodes the HK and PhoB the RR. PhoBR responds to phosphate limitation, when the extracellular phosphate concentration falls below 4 µM. In phosphate-limiting conditions, PhoBR induces genes belonging to the Pho (phosphate) regulon, which includes genes involved in acquisition and metabolism of different phosphate groups [4, 6, 152]. The control of the Pho regulon and transmembrane signal transduction by environmental inorganic phosphate (P_i) has been extensively studied in *Escherichia coli* and *Bacillus subtilis.* In *E*. *coli* K-12, the Pho regulon comprises 31 genes [152] and, in addition to being involved in phosphate homeostasis, is also connected to bacterial virulence as its induction results in attenuated pathogens.

Host-pathogen interactions are dynamic processes responding to the diverse environmental conditions encountered by invading pathogens. Survival of a pathogen in different sites in the host requires an adaptive response capable of reacting to different stimuli in its immediate environment. The specialised regulatory systems that control the expression of virulence factors are essential for survival and necessarily complex, with interconnections between regulatory systems at many levels. Although the presence of pathogen-specific genes may dictate the pathogenic lifestyle and virulence potential of pathogenic *E. coli*, products encoded by conserved or “core” genes undoubtedly contribute to functional metabolism, physiology and

adaptation to environmental changes, including host environments and resistance to host defences.

In 2008, Lamarche et al. [6] reviewed the relationship between the Pho regulon, metabolism and pathogenicity. Here, we present a review specifically focusing on the effects on virulence in *Escherichia coli* by the induction of the Pho regulon. First, we briefly overview the induction of the Pho regulon, then discuss the different virulence attributes affected by the induction of the Pho regulon of pathogenic *E. coli*, including resistance to oxidative stress, membrane perturbation, production of adhesins and adaptation to environmental stimuli.

Induction of the Pho regulon

As mentioned above, phosphate starvation is sensed by the TCRS PhoBR. PhoBR is activated when the extracellular phosphate concentration falls below 4 µM, inducing transcription of genes belonging to the Pho regulon. This is enabled by the binding of phosphorylated PhoB to specific DNA sequences, known as Pho boxes, located within Pho-dependent promoter regions. PhoB binding either induces or represses transcription of genes that comprise the Pho regulon, including those involved in phosphate transport and metabolism [4, 152] (Fig. 1). One of these systems, the *pstSCAB-phoU* operon, encodes the phosphate-specific transport (Pst) system. The Pst system encodes an ATP-binding cassette (ABC) transporter involved in the transport of inorganic phosphate (P_i). In addition to acting as a phosphate transporter, the Pst system is required for P_i signal transduction, as mutations in any of the genes of the *pst* operon result in constitutive expression of the Pho regulon, regardless of environmental phosphate availability [4, 6, 152] (Fig. 1).

In addition to regulating genes involved in phosphate acquisition and metabolism, induction of the Pho regulon seems to have pleiotropic effects, as proteomic analysis of *E. coli* K-12 cultured under phosphate-limiting conditions reveals that up to 400 proteins are differentially expressed [133]. Microarray experiments performed on K-12 and pathogenic *E. coli* connected the Pho regulon directly or indirectly with multiple metabolic systems [8, 143, 153] (Table 1). Notably, the Pho regulon and bacterial virulence are connected, as its induction regulates pathogen-specific genes, and affects survival and virulence of many bacterial pathogens [6-8, 10, 147, 154-157].

Multiple stress responses and virulence attributes are affected by inactivation of the Pst system. Specifically, general, oxidative and acid stress responses are connected with the Pho regulon. The connection between the general and acid stress responses has consequences for the virulence of pathogenic *E. coli* (Lamarche et al., 2008b; Crepin et al., 2008). Among the virulence attributes altered by induction of the Pho regulon are a significant reduction in the amount of capsular antigen at the cell surface, resistance to the bactericidal effect of serum, to cationic antimicrobial peptides, and to acid and oxidative stresses, as well as the production of type 1 fimbriae [7-12, 158].

Pho regulon activation and ExPEC virulence

Extra-intestinal **p**athogenic ***E. c****oli* (ExPEC) are an important group of pathogenic *E. coli* that cause a diversity of infections in both humans and animals, including urinary tract infections (UTIs), meningitis, and septicaemia [159].

The role of the Pho regulon in virulence of ExPEC has been principally studied in the ExPEC strain 5131, which causes septicaemia in pigs, and the avian pathogenic *E. coli* (APEC) O78 strain χ7122, which causes colibacillosis in poultry [6-12, 160]. In APEC strain χ7122, the level of attenuation of virulence of mutant strains correlated directly with the level of activation of the Pho regulon [7]. However, selective capture of unique transcribed sequences (SCOTS) revealed that *phoB* was expressed during experimental infection of chicken with APEC strain χ7122 [161], suggesting that fine-tuning of the Pho regulon is required for virulence, while inappropriate or constitutive induction of the Pho regulon has deleterious effects.

Dissecting the specific contribution of both PhoBR and Pst systems

As mentioned above, the Pst system contributes to both regulation of the Pho regulon and high-affinity uptake of P_i. Until recently, this dual function for Pst made it difficult to explain the effects of Pst inactivation on the virulence of APEC, as it was unclear whether attenuation was due to constitutive activation of the PhoBR TCRS or the loss of Pst-mediated high-affinity phosphate uptake. It was observed that PhoB-mediated constitutive activation of the Pho regulon, rather than inactivation of the Pst system, was critical for the virulence of APEC [7]. A point mutation in

phoR, which constitutively activated the Pho regulon independently of P_i transport and inactivated the *pst* system, attenuated virulence and virulence attributes, such as sensitivity to hydrogen peroxide and serum, and production of type 1 fimbriae [7]. Interestingly, it was determined that the PhoB regulator is not required for virulence in APEC. This is in contrast with the implications of SCOTS analysis experiments, but accords with recent work by Pratt *et al*. [157] that showed that the attenuation of virulence of *V. cholerae* in a *pst* mutant was due to induction of the Pho regulon, not P_i transport per se.

Oxidative stress response

Oxidative stress is produced by bacterial metabolism (respiration), the immune system and exposure to host environmental factors such as metal ions. Oxidative stress induces damage to DNA, proteins and membranes, and can lead to cell death [162]. The expression of some genes whose products exhibit antioxidant activities were modulated in an APEC *pst* mutant [8]. The catalase *katE*, the superoxide dismutase *sodC*, the DNA protection protein *dps* and the small regulatory RNA *oxyS* were among the differentially expressed genes. However, the *pst* mutants were more sensitive to agents generating reactive oxygen intermediates (ROI) than the parent strain χ7122 [7, 8]. In *E*. *coli* K-12, it was observed that the alkylhydroperoxide reductase *ahpCF*, the catalase *katG* and the pyruvate oxidase *poxB* are required to resist oxidative stress generated by glucose metabolism [163, 164]. Since the ability to resist oxidative stress is crucial for full virulence in ExPEC [165], the sensitivity of *pst* mutants to oxidative stress could explain, at least in part, their attenuation.

Bacterial cell surface modification

Under conditions of phosphate starvation, bacteria have been shown to modify their phospholipids by substituting them with phosphorus-free lipids [6, 166]. A strong influence of the Pst system and the associated Pho regulon in modifications of lipid A structure and cell surface perturbations has been demonstrated in strain χ7122. Indeed, mutation in the Pst system results in structural modifications of lipid A, including a reduced amount of its hexa-acylated-1-pyrophosphate form [12]. The outer monolayer of the outer membrane of most gram-negative bacteria is composed of lipopolysaccharides, which includes the lipid A. These are involved in outer membrane integrity, forming a protective barrier against various environmental stresses

[167-169]. The change in lipid A structure in *pst* mutants is exemplified by increased sensitivity of the mutant to serum complement, vancomycin and cationic antimicrobial peptides (CAMPs) [7, 9, 10, 12].

In addition to being involved in the biosynthesis of lipid A, deletion of the Pst system leads to an imbalance in cyclopropane (CFA) and unsaturated fatty acids (UFA), and increases outer membrane permeability [11]. Microarray analysis has also revealed that lipid A modification and the differential expression of genes belonging to the biosynthesis of enterobacterial common antigen and LPS biosynthesis, occurred in a *pst* APEC mutant [8, 12]. Since membrane integrity is required for resistance to environmental stresses, membrane perturbation in Pst mutants may explain the decrease of such virulence traits in pathogenic *E. coli*.

Adhesin production and adherence

Type 1 and F9 fimbriae mediate adherence of *E. coli* to host cells and are involved in biofilm formation [14, 170, 171]. Type 1 fimbriae play a key role in virulence [50, 55-57, 59, 172]. In APEC *pst* mutants, it was observed that expression of these fimbriae was repressed [7, 8], as no fimbriae were found on their surface [8]. Since type 1 fimbriae were preferentially expressed in air sacs, the primary site of infection of APEC [173, 174], a decrease in fimbrial production in the *pst* mutant may contribute to reduced APEC colonisation and virulence. Similarly, in uropathogenic *E*. *coli* (UPEC), inactivation of the *pst* system also repressed expression of type 1 fimbriae and altered virulence (Crépin et al., submitted).

Intestinal pathogenic *E. coli*

Intestinal pathologies mostly result diarrhoea, with some instances leading to more severe diseases. Diseases associated with *E. coli* include enteric infections caused by different pathotypes of diarrhoeagenic *E. coli*, such as enterotoxigenic, enteropathogenic and enterohaemorrhagic *E. coli* (ETEC, EPEC and EHEC respectively). Diarrhoeagenic EPEC and EHEC produce a characteristic attaching and effacing (A/E) lesion on the brush border of infected intestinal enterocytes that is characterised by localised destruction (effacement) and intimate bacterial attachment [1]. These pathotypes pose a significant risk to human and animal health world-wide. As a cause of animal (and zoonotic) disease, attaching and effacing *E*. *coli*

(AEEC) infections have a great impact on human food safety (with animals such as cattle serving as reservoirs of some human pathogenic strains), animal welfare, economic production (costly outbreaks of post-weaning diarrhoea in pigs, risk of trade barriers), and environmental biosafety.

Pst system and adhesion of Intestinal Pathogenic *E. coli* strains

In the *in vitro* organ culture (IVOC) model, insertion of a transposon into the *pstS* gene impairs the ability of a porcine EPEC strain to attach to piglet ileal enterocytes [175]. Deletion of the *pst* operon in the EPEC strain LRT9 (O111: abH2) impairs its adherence to Hep-2 intestinal cells [155]. It has been hypothesised that the reduced adherence was due to the down-regulation of the *bfp* operon and *eae*, which code for the bundle forming pili and the adhesin intimin, respectively. Bfp is involved in the formation of microcolonies and intimin is involved in intimate adherence [155]. Their positive regulator genes, *perA* and *perC*, were also repressed [155]. Similarly, in the atypical EPEC strain E128012 (O114:H2), which lacks Bfp, adherence of a *pst* mutant to Hep-2 and T84 intestinal cells was reduced [154]. *Citrobacter rodentium* causes attaching and effacing intestinal lesions in mice, and C56BL/6 mice excrete lower numbers of the *pst* mutant [154]. It was suggested that the Pho regulon represses uncharacterized adhesins required for virulence [154]. Indeed, activation of the Pho regulon (PhoBR) by deletion of the *pst* system represses these adhesins, while adherence to HT-29 intestinal epithelial cells by EHEC O157:H7 ATCC strain 43894 downregulates *phoBR* and *pstS* [176]

These observations indicate that disruption of the *pst-phoU* locus not only activates the Pho regulon but, also induces various effects on adherence. In EPEC and EHEC, an intact Pst system seems to be required for full virulence by facilitating intestinal colonization.

Activation of the Pho regulon during adaptation by EHEC to environment

The role of the Pst system (Pho regulon) in the virulence of EHEC has not been well studied. Information about the induction of the Pho regulon by environmental stress mainly emanates from transcriptional studies. Exposure of *E. coli* O157:H7 strain EDL933 to sodium benzoate, a bacteriostatic and fungistatic agent, led to the up-regulation of *pst* and other genes of the Pho regulon [177]. It was suggested that the Pst system could function as an efflux pump for sodium

benzoate, as observed for *Mycobacterium smegmatis* [178]. Exposure of *E. coli* O157:H7 strains TW14359 and Sakai to oxidative stress up-regulated transcription of the *pst* operon as well as PhoBR [179]. Under acid shock conditions, the Pho regulon response regulator *phoB* was induced in EHEC O157:H7 strain FDA518 [180]. PhoB activates *asr*, a gene encoding an acid shock protein required for survival in moderately acidic conditions in *E*. *coli* K-12 [146], and to induce other acid shock proteins [8, 143, 153]. Using *in vivo*-induced antigen technology (IVIAT), PhoE and PhoA, an outer membrane porin involved in the transport of various anions and a periplasmic alkaline phosphatase, respectively, were identified among the *E. coli* O157:H7 proteins reacting with sera from patients previously diagnosed with haemolytic and uraemic syndrome [181]. This further indicates that members of the Pho regulon are expressed by pathogenic *E. coli* during infection.

EHEC virulence factors regulated by PhoBR

Candidate genes with putative PhoB binding sites were identified in EHEC O157:H7 Sakai as well as strain EDL933 [147]. In the Sakai strain, one of these loci was confirmed to be directly regulated by PhoB using a genomic library fused to *lacZ*. Yoshida et al. [182] have demonstrated that the gene cluster *esc0540-0544* is positively regulated by the Pho regulon. This gene cluster is homologous to the *siiCA-DA* operon of *Escherichia fergusonii*, which encodes a putative RTX toxin and its cognate type I secretion system (T1SS) [182]. Although the function of this putative RTX toxin in *E. coli* O157:H7 has not yet been established, it was suggested that P_i sensing could regulate such a secretion system in EHEC.

Concluding remarks

Pathogenic *E. coli* must survive in nutritionally disparate environments, including some ecosystems where P_i can be limited [157]. The Pho regulon is required for survival, not only to control phosphate homeostasis, but also to take part in a complex network important for both bacterial virulence and stress responses. Inappropriate expression or repression of this system may have significant consequences on bacterial virulence. Mutations in the Pst system or PhoB result in multiple effects, including alteration of *E. coli* virulence traits. Although these effects may be due to a deficiency in phosphate uptake under some conditions, PhoBR TCRS regulation is likely to be responsible for the multiple effects that are observed in Pst and PhoB *E.*

coli mutants. PhoB-mediated constitutive activity of the Pho regulon plays a major role in attenuation of virulence and associated traits in these mutants. Changes in adhesin expression, as well as altered capacity to adhere to cells and tissues, are affected by the activation of the Pho regulon. More in depth investigation needs to be done to characterize the functions associated with virulence genes of *E. coli* that are under the control of the Pho regulon.

Every year, the poultry industry sustains significant financial losses due to the high morbidity and mortality caused by APEC. Some ExPEC strains are capable of infecting both poultry and mammals, supporting the zoonotic potential of certain APEC strains [183, 184]. EPEC and EHEC infections have an impact on human food safety, animal welfare, economic production and environmental biosafety. With antimicrobial resistance of bacterial pathogens on the increase, it is important to develop novel preventive and therapeutic strategies. Designers of new attenuated vaccine strains may be able to take advantage of the fact that the degree of attenuation in *pst* and *phoBR* mutants can vary with the degree of constitutive activation of the Pho regulon [7]. Drugs inducing the Pst system or directly affecting PhoBR activity may also be useful as therapeutic agents to compromise bacterial virulence and facilitate elimination of the pathogen by host defences [185-190].

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Conflict of interest statement

The authors declare no conflict of interest

Erratum

In paragraph 2 in page 32, we indicated that phosphourus is the third most important element. In fact, phosphourus is the fifth most important element behind carbon, oxygen, nitrogen and hydrogen.

Table 1. Attributes affected by the induction of the Pho regulon

Strains	Virulence	Condition[a]	Altered systems	Phenotype	References
K-12					
MG1655	NA[b]		Oxidative stress	Sensitive	[163, 164]
	NA		Acid stress	Sensitive	[163]
W3110	NA		Acid stress	NA	[143, 153]
ANCK10	NA		Acid stress	NA	[146]
ExPEC					
ExPEC 5131	Attenuated		Cell surface modification	Serum and CAMPs[c] sensitive	[9, 11, 12]
APEC χ7122	Attenuated		Oxidative stress	Sensitive	[7, 8]
			Acid stress	Sensitive	[10]
			Type 1 and F9 fimbriae	Afimbriate	[7, 8]
			Cell surface modification	Serum and CAMPs[c] sensitive	[7, 10, 12]
CFT073	Attenuated		Type 1 fimbriae production	Reduced mouse bladder colonization	(Crépin et al., submitted)

Strains	Virulence	Condition[a]	Systems affected	Phenotype	References
EPEC					
LRT9	NA		Adhesin production	Impaired adhesion onto HEp-2 cells	[155]
86-1390	NA		?	Impaired attachment onto piglet ileal explants	[175]
E128012	NA		Adhesin production	Impaired attachment onto HEp-2 cells	[154]
C. rodentium ICC169	Attenuated			Impaired colonization of intestine	[154]
EHEC					
EDL933	NA	Exposure to sodium benzoate	Pst system; PhoBR		[177]
TW14359	NA	Exposure to oxidative stress	Pst system		[179]
Sakai	NA	Exposure to oxidative stress	Pst system; PhoBR; PhoA		[179]
			Putative RTX-toxin protein; Cognate type I secretion system		[182]
FDA518	NA	Acid stress	PhoB		[180]
Isolated from previously diagnosed HUS[d] patient	NA	Exposure to convalescent sera	PhoE; PhoA		[181]

[a]Condition under which the Pho regulon is induced; [b] Not applicable; [c]CAMPs, cationic antimicrobial peptides; haemolytic and uraemic syndrome

Figure 1

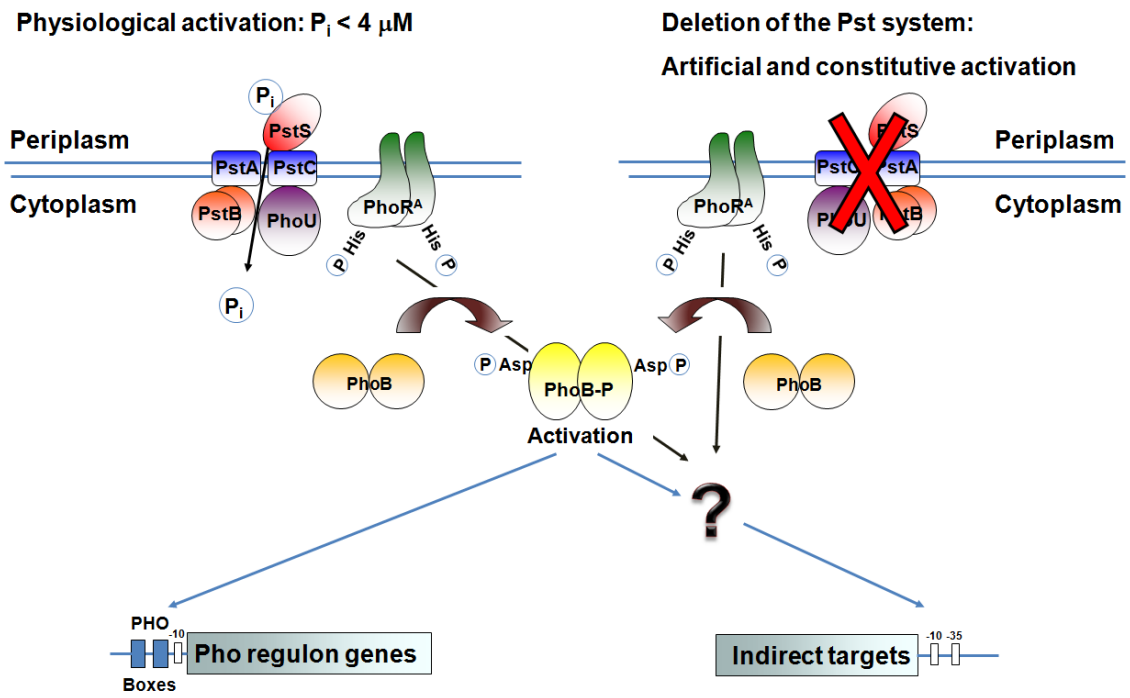

Figure 1. Induction of the Pho regulon by phosphate starvation and inactivation of the Pst system. Adapted from Lamarche et al. [6], with kind permission of John Wiley and Sons. Under phosphate starvation, PhoR autophosphorylates a histidine residue. The phosphoryl group is then transferred onto an aspartate residue of PhoB. This phosphotransfer activates PhoB, which can then bind to Pho boxes and activate transcription of Pho-regulated genes. PhoB or PhoR can also act indirectly on gene expression by regulating other regulators. Furthermore, mutations in any of the genes of the *pst* operon result in constitutive expression of the Pho regulon, regardless of environmental phosphate availability. Abbreviations: P_i, inorganic phosphate; PstS, periplasmic P_i-binding protein; PstA et PstC, integral membrane channel proteins; PstB, ATP-binding protein; PhoU, regulatory protein; PhoB, RR; PhoR, sensor protein (A, autophosphorylated; P, phosphorylated).

3.5. Le système Pst, le SRDC PhoBR, et la virulence d'autres souches bactériennes

Plusieurs méthodes de criblage de gènes induits *in vivo* ont démontré que les gènes associés au régulon Pho sont requis dans l'établissement de l'infection. Ces méthodes passent par la technologie des biopuces à ADN, DFI, l'IVET et son dérivé RIVET, le SCOTS et le STM (Tableau 3).

Tableau 3. Identification des gènes exprimés *in vivo* dépendant de PhoB à l'aide de modèles d'infections

Espèces	Modèle d'infection [a]	Sélection [b]	Références
Brucella melitensis	Septicémie murine (BALB/c)	STM	[191]
Burkholderia cenocepacia	Infection pulmonaire chronique chez le rat (Sprague-Dawley)	STM	[192]
Campylobacter jejuni	Infection intestinale aviaire (SPF Light Sussex)	STM	[193]
	Infection de cellules intestinales humaines (INT407)	Biopuce ADN	[194]
Corynebacterium pseudotuberculosis	Macrophages murins (J774A.1)	DFI	[195]
Escherichia coli	Infection du tractus urinaire murin (CBA)	STM	[55]
	Septicémie murine (ICR)	IVET	[196]
	Infection extraintestinale aviaire (Leghorn SPF)	SCOTS	[161]
Erwinia amylovora	Maladie nécrotique de plante (Poire immature, Pyrus communis L. cv. 'Bartlett')	IVET	[197]
Erwinia chrysanthemi	Maladie de plante (Feuille d'épinard)	IVET	[198]
Listeria monocytogenes	Septicémie murine (ICR Swiss)	IVET	[199]
	Macrophages murins (P388D1)	Biopuce ADN	
Klebsiella pneumoniae	Intranasal murin (C57Bl/6j)	STM	[200]

Espèces	Modèle d'infection [a]	Sélection [b]	Références
Mycobacterium tuberculosis	Poumon murin (BALB/c and BALB/c$^{SCID/SCID}$)	Biopuce ADN	[201]
Proteus mirabilis	Infection du tractus intestinal murin (CBA)	STM	[202]
Salmonella Typhimurium	Macrophages murins (RAW 264.7)	DFI	[203]
Salmonella Typhi	Monocytes humains (THP-1)	SCOTS	[204]
Shigella flexneri	Cellules intestinales humaines (INT407)	DFI	[205]
Streptococcus pneumoniae	Septicémie et pneumonie murine (BALB/c)	STM	[206]
	Bactériémie et transport nasopharyngiale murine (Swiss Webster)	STM	[207]
	Infection du tractus respiratoire murin (CD-1)	DFI	[208]
	Inflammation de l'oreille moyenne murine (Gerbille mongolienne)		
	Chambre intrapéritonéale murine (CD-1)		
Vibrio cholerae	Allaitement murin (CD-1)	STM	[209]
	Infection du tractus intestinal murin	RIVET	[210]
Yersinia enterocolitica	Septicémie et infection intestinale murin (BALB/c)	STM	[211]
Yersinia pestis	Infection du tractus respiratoire murin (C57BL/6)	Biopuce ADN	[212]

[a] Entre parenthèses, les souches animales ou lignées cellulaires utilisées dans les études. [b] STM, signature-tagged mutagenesis. DFI, differential fluorescence induction. IVET, *in vivo* expression technology. SCOTS, selective capture of transcribed sequences. RIVET, recombination-based *in vivo* expression technology. (Adapté de réf. [6]).

Chez *Vibrio cholerae*, il a été montré que PhoB est tardivement induit lors d'une infection [210] et que PhoB et le système Pst sont requis pour la virulence dans le modèle intestinal murin et de lapin [157, 213, 214]. PhoB régule négativement l'expression du pilus TCP et de la toxine

cholérique (CT) [157]. Ces derniers codent pour deux facteurs de virulence impliqués, respectivement, dans la diarrhée et la colonisation du petit intestin. L'induction de PhoB inhibe donc la production du pilus TCP et de la toxine CT, inhibant ainsi la colonisation du petit intestin et la diarrhée caractéristique. De plus, PhoB régule positivement l'opéron *acgAB*, codant pour deux protéines impliquées dans le métabolisme du c-di-GMP (voir section 4.2.) [215]. Comme le pilus TCP et la toxine CT sont requis dans les étapes précoces de l'infection et AcgAB dans les étapes tardives, l'induction de PhoB doit être finement régulée afin de permettre à *V*. *cholerae* de coloniser l'hôte. De plus, le système Pst est impliqué dans la formation des biofilms [213], qui est requis pour la virulence de *V.* cholerae [216]. Chez *Proteus mirabilis*, une diminution de la colonisation du tractus urinaire de souris est observé chez des mutant *pstS* et *pstC* [156, 202]. Tout comme chez *V*. *cholerae*, le système Pst est requis dans la formation des biofilms chez *P*. *mirabilis* [217]. Chez *Edwardsiella tarda*, bactérie impliquée dans des infections extraintestinales chez les poissons et les mammifères, la mutation des gènes *pstS*, *pstC* et *pstB* diminue la production de protéines impliquées dans la formation du translocon du système de sécrétion de type III, atténue sa capacité à se multiplier à l'intérieur des phagocytes, à résister à l'effet bactéricide du sérum et par conséquent, diminue sa virulence [218]. De plus, l'inactivation de *phoU* abolit la sécrétion des effecteurs du système de sécrétion de type VI [219]. Finalement, la mutation de *pstS* chez *S*. *flexneri* diminue la capacité de la souche mutante à former des plages de lyses au niveau des cellules intestinales [220].

En résumé, ces études montrent que le système Pst et le régulateur PhoB, en plus de leur rôle respectif dans le transport du P_i et la régulation de l'homéostasie du phosphate, sont impliqués dans la régulation de la pathogenèse bactérienne. Bien que leur rôle ait été plus étudié chez *E*. *coli* et *V*. *cholerae*, les mécanismes moléculaires reliant le régulon Pho et la virulence ne sont pas clairement établis.

4. Les petites molécules régulatrices ppGpp et c-di-GMP

4.1. Le ppGpp

Faisant face à un stress nutritionnel, les bactéries réaffecteront rapidement leurs ressources cellulaires et ce, en bloquant la synthèse d'ADN, des ARN stables, des protéines ribosomiques et membranaires. En contrepartie, elles produiront rapidement des facteurs cruciaux dans la

résistance aux stress, la glycolyse et la biosynthèse des acides aminés. Ce processus se nomme réponse stringente et est gouverné par l'alarmone guanosine tétraphosphate (ppGpp) [221]. Globalement, le ppGpp module la transcription génique en régulant directement l'activité de l'ARN polymérase et indirectement les facteurs sigma (σ) [222, 223]. Sa synthèse dépend des enzymes RelA et SpoT, codant respectivement pour une GDP pyrophosphokinase / GTP pyrophosphokinase et une guanosine 3'-diphosphate 5'-triphosphate 3'-diphosphatase. Ainsi, RelA est impliqué dans sa biosynthèse tandis que SpoT gouverne sa biosynthèse ainsi que sa dégradation (Fig. 7).

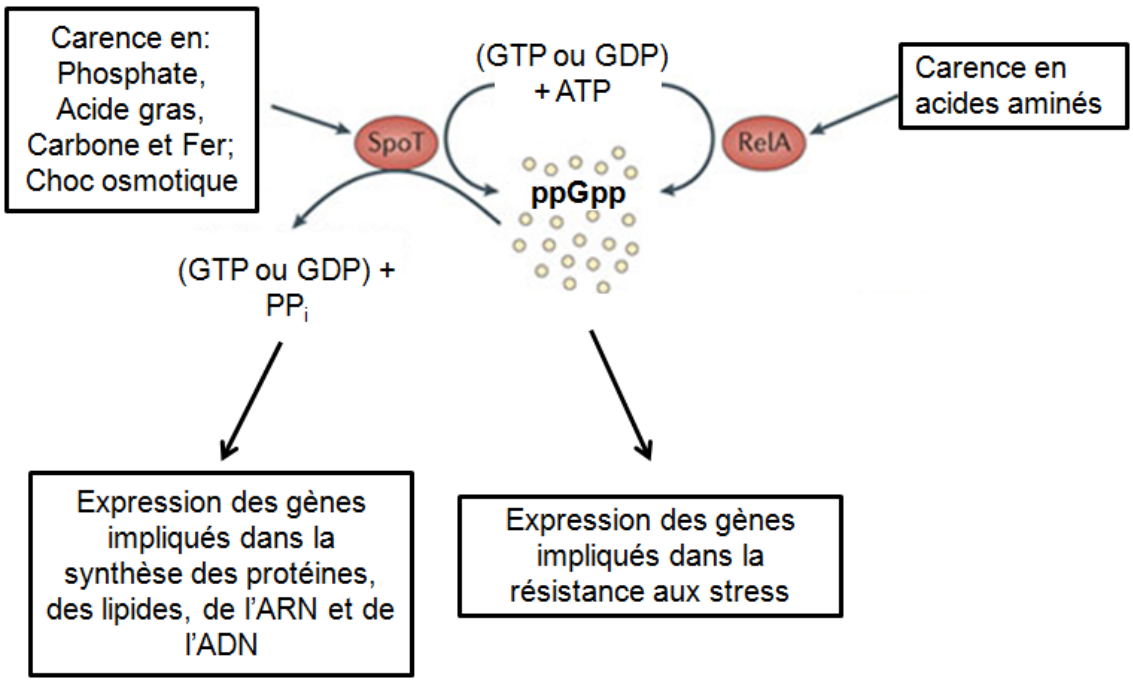

Figure 7. Métabolisme du ppGpp selon les conditions environnementales. En conditions de stress nutritionnels, les bactéries s'adaptent à ces conditions, notamment, en produisant du ppGpp. Celle-ci modulera l'expression des gènes répondant à ces stress. RelA induit la synthèse du ppGpp tandis que SpoT possède une double fonction, i.e. synthèse et dégradation. Pour plus d'informations, se référer au texte. Figure modifiée de la réf. [171]

En plus d'être impliqué dans la réponse au stress nutritionnel, le ppGpp est aussi impliqué dans la virulence et ce, autant chez les bactéries Gram-négative que Gram-positive [224]. Plus particulièrement, chez *E. coli*, le ppGpp régule l'adhérence aux tissus hôtes [78, 79, 225]. Ainsi, chez les UPEC, il a été montré que l'accumulation de ppGpp active la transcription de *fimB*, donc des fimbriae de type 1 [78, 79], contribuant ainsi à la virulence. Aussi, chez *E. coli* K-12,

l'inactivation du système Pst diminue la concentration intracellulaire en ppGpp [226, 227]. Étant donné que l'inactivation du système Pst chez les UPEC atténue leur virulence [55, 228], nous pouvons émettre l'hypothèse que cette atténuation peut être causée, en partie, par une diminution de la concentration intracellulaire en ppGpp, qui se traduirait par une diminution de l'expression des fimbriae de type 1.

4.2. Le c-di-GMP

Le c-di-GMP se compose de deux molécules de cGMP jointes par un lien phosphodiester 3' - 5'. Il fut identifié, pour la première fois, chez *Gluconacetobacter xylinus* et il fut démontré que le c-di-GMP est impliqué dans la régulation de la biosynthèse de la cellulose chez cette espèce [229]. De plus, le c-di-GMP est impliqué dans la régulation de plusieurs processus cellulaires tels la motilité, la formation des biofilms, la virulence ainsi que les intéractions cellules-cellules (Fig. 8) [230-232]. La régulation de ces processus cellulaires par le c-di-GMP s'effectue tant au niveau transcriptionel, traductionnel que post-traductionnel, dénotant ainsi la complexité des intéractions régulatoires de cette molécule.

La concentration intracellulaire de c-di-GMP est contrôlée par deux types d'enzymes, les diguanylates cyclases (DGC) et les phosphodiestérases (PDE). Les DGC convertissent deux molécules de GTP en c-di-GMP, tandis que les PDE clivent le composé cyclique en GMP. L'activité des DGC et PDE est, respectivement, conférée par les domaines protéiques GGDEF ainsi que EAL et HD-GYP [230-232] (Fig. 8).

En raison de l'explosion des découvertes concernant le c-di-GMP et ce, chez diverses espèces bactériennes, je ne m'attarderai qu'aux sujets étroitement reliés à ce projet de thèse, i.e. la régulation des adhésines ainsi que la virulence.

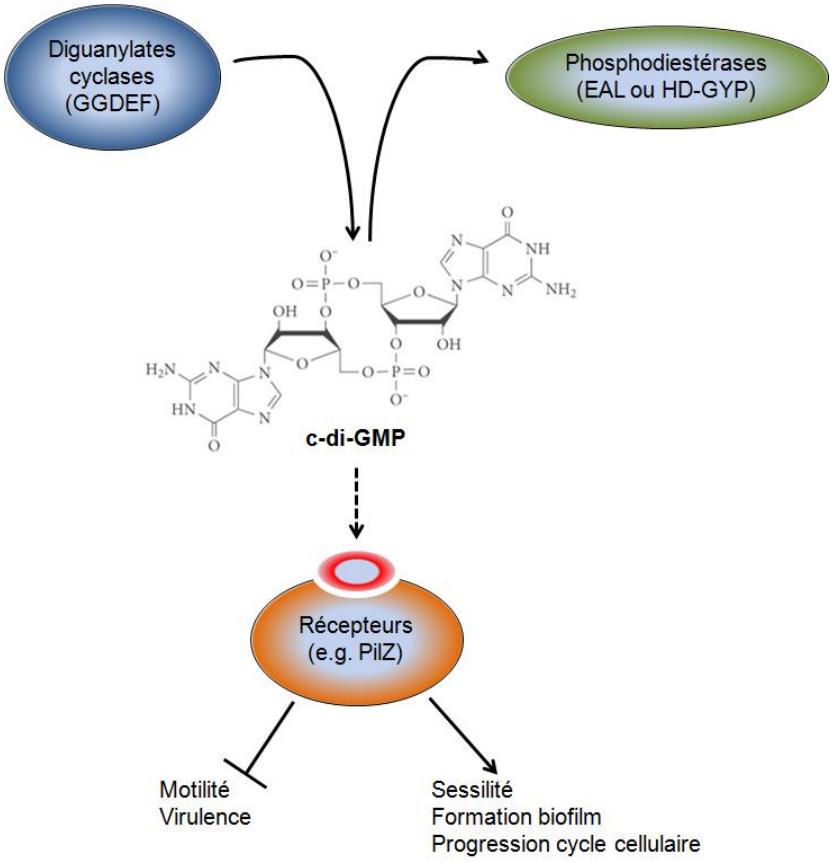

Figure 8. Composants des voies de signalisations du c-di-GMP. Au niveau cellulaire, les diguanylates cyclases, possédant un domaine GGDEF, activent la biosynthèse du c-di-GMP tandis que sa dégradation est favorisée par les phosphodiestérases, possédant un domaine EAL ou HD-GYP. Suite à l'intéraction du c-di-GMP à différents récepteurs, comme les protéines possédant un domaine PilZ, le c-di-GMP altère la motilité ainsi que la virulence tandis qu'il stimule la sessilité, la formation des biofilms ainsi que le cycle cellulaire. Fig. adaptée de la réf. [233]

Régulation des adhésines par le c-di-GMP

Il a été mis en évidence que le c-di-GMP est impliqué dans la régulation d'adhésines incluant les fimbriae. Ainsi, chez *P. aeruginosa*, une augmentation de la concentration intracellulaire en c-di-GMP active l'expression du groupe de gènes *cupA1-A5* [234], gènes codant pour un système de fimbriae putatif impliqué dans la formation de biofilms [235]. De plus, la surexpression du régulateur PvrR, régulateur contenant un domaine EAL, réduit la concentration de c-di-GMP et donc, réprime la production des fimbriae CupA ainsi que la formation de biofilm [234]. Chez, *P. fluorescens* Pf0-1, une carence en phosphate active la PDE RapA et par conséquent, diminue la concentration intracellulaire en c-di-GMP, inhibant ainsi la formation de biofilm [236, 237]. Cette réduction de la concentration en c-di-GMP est due à la surexpression de la PDE RapA. Cette surexpression inhibe la sécrétion de l'adhésine LapA, adhésine essentielle dans la formation

des biofilm chez *P*. *fluorescens* [238]. En bref, l'activation du régulon Pho, chez *P*. *fluorescens* entraîne une diminution de la concentration intracellulaire du c-di-GMP, induite par la surexpression de RapA, compromettant ainsi la sécrétion de LapA et par conséquent, l'abolition de la formation des biofilms par cette bactérie [236].

Le c-di-GMP et la virulence

Plusieurs groupes ont mis en évidence le rôle du c-di-GMP dans la virulence des souches pathogènes. Chez *Yersinia pestis*, l'inactivation du gène EAL *hmsP* augmente la production des polysaccharides de surface et par le fait même, atténue la virulence dans le modèle bubonique et pneumonique murin [239]. Chez *V*. *cholerae*, des concentrations élevées en c-di-GMP réduisent la colonisation du petit intestin de souris et réprime l'expression de l'activateur *toxT*, qui lui, régule positivement la toxine CT et le pilus TCP [240]. De plus, la PDE VieA régule positivement l'expression de *toxT* et par conséquent, est requis pour la virulence de *V*. *cholerae* [241]. Chez *P*. *aeruginosa*, il a été montré que le c-di-GMP régule l'expression des systèmes de sécrétion de type 3 (T3SS) et type 6 (T6SS). Ainsi, des concentrations élevés en c-di-GMP réprime le T3SS et active le T6SS tandis que de faibles concentrations entraînent la régulation inverse [242]. Comme le T3SS est impliqué dans l'infection aigüe (motilité) et que le T6SS est associé aux infections chroniques (formation de biofilm) [242-244], la concentration intracellulaire en c-di-GMP doit être finement régulé afin de ne pas nuire à l'infection. Par exemple, la production de cellulose, induite par l'augmentation intracellulaire en c-di-GMP, chez *Salmonella enterica* serovar Typhimurium, inhibe la capacité de la bactérie à envahir les cellules gastro-intestinales HT-29 [245]. Bien que l'étude du c-di-GMP soit en pleine expansion, chez *E*. *coli*, cependant, très peu d'études traitent du rôle du c-di-GMP dans la virulence. Chez la souche adhérente-invasive LF82, souche isolée de l'iléon d'un patient atteint de la maladie de Crohn, il a été démontré qu'une augmentation de la concentration intracellulaire en c-di-GMP réprime l'expression des fimbriae de type 1, altérant ainsi l'adhésion et l'invasion des cellules épithéliales intestinales [246]. Finalement, en plus de diminuer l'adhésion bactérienne aux cellules de la vessie, la production de cellulose, induite par le c-di-GMP, réduit la colonisation des reins d'une souche UPEC clinique [103]. D'autres études sont requises afin de déterminer les mécanismes moléculaires reliant le c-di-GMP et la virulence.

Problématique

Il est connu et bien documenté que l'inactivation du système Pst atténue la virulence de souches pathogènes. Par contre, les mécanismes moléculaires reliant le système Pst, le régulon Pho et la virulence ne sont pas encore clairement établis. Ainsi, chez *E*. *coli*, plusieurs observations et hypothèses ont été émises. Chez les souches ExPEC d'origine porcine et aviaire, l'atténuation de virulence serait due à une perturbation membranaire [7-12]. Chez les souches EPEC, cette atténuation serait reliée à une altération de l'adhésion au niveau du tractus intestinal [154, 155]. Jusqu'à récemment, le rôle du système Pst dans la virulence des souches UPEC n'était pas connu [55, 228]. Par contre, en 2008, des études de transcriptomique menées dans notre laboratoire ont permis de mettre en évidence que l'inactivation du système Pst réprime l'expression des fimbriae de type 1 chez la souche APEC χ7122 (Annexe 1) [8]. Par contre, ces fimbriae ne sont pas requis dans la virulence chez cette souche (Charles M. Dozois, résultats non publiés). Cependant, chez les UPEC, les fimbriae de type 1 sont essentiels à l'établissement de l'infection. Comme le système Pst est requis pour la virulence des souches UPEC et qu'il semble réprimer l'expression des fimbriae de type 1, nous avons émis l'hypothèse que les fimbriae de type 1 reliaient le système Pst et la virulence chez les UPEC.

Objectifs spécifiques :

1. Caractériser le rôle du système Pst dans la virulence de la souche UPEC CFT073 ainsi que dans l'expression des fimbriae de type 1.

2. Identifier les mécanismes moléculaires reliant le système Pst et les fimbriae de type 1 chez la souche UPEC CFT073.

3. Développer une méthode de complémentation simple-copie dans le chromosome et ce, à l'aide du transposon Tn7 afin d'éviter la perte des vecteurs plasmidiques de complémentation lors d'infection *in vivo*, causée par une absence de pression de sélection. Ceci est un objectif satellite et accessoire à ce projet de thèse.

ARTICLES

Article #2

Decreased Expression of Type 1 Fimbriae by a *pst* mutant of uropathogenic *Escherichia coli* Reduces Urinary Tract Infection.

Short title: Inactivation of Pst reduces UPEC *fim* expression

Sébastien Crépin, Sébastien Houle, Marie-Ève Charbonneau, Michaël Mourez, Josée Harel and Charles M. Dozois (2012). Infection and Immunity 80(8) : 2802-2815

A) Objectifs et approche utilisée

1- L'étude publiée dans le journal Infection and Immunity avait pour objectif de déterminer le rôle du système Pst dans la virulence de la souche UPEC CFT073 ainsi que dans la régulation des fimbriae de type 1. Les résultats présentés montrent que la délétion du système Pst réprime l'expression des fimbriae de type 1 et ce, autant *in vitro* qu'*in vivo*. De plus, phénotypiquement, nous avons montré que, contrairement aux autres souches ExPEC, la délétion du système Pst n'altère pas la membrane bactérienne de la souche UPEC CFT073. De ce fait, nous avons montré que l'atténuation de virulence du mutant Pst est causée majoritairement par la répression des fimbriae de type 1. Cette répression est directement reliée à l'expression différentielle des régulateurs de ces fimbriae.

2- L'étude a été réalisée en utilisant une approche multidisciplinaire regroupant des techniques de génétique bactérienne, de biologie moléculaire, le modèle murin d'UTI, de biologie cellulaire, de microscopie à fluorescence et électronique, de chromatographie en phase liquide à haute performance ainsi que des tests phénotypiques évaluant l'intégrité membranaire.

B) Contribution de l'étudiant

J'ai effectué presque la totalité des expérimentations. Sébastien Houle m'a assisté avec les infections dans le modèle murin d'UTI et Marie-Ève Charbonneau a effectué les quantifications

de ppGpp. Aussi, j'ai écrit la totalité de l'article, qui a été corrigé par mes directeurs et collaborateurs. J'ai, de plus, soumis l'article à Infection and Immunity.

C) Résumé de l'article :

Le système spécifique de transport du phosphate (Pst) est codé par l'opéron *pstSCAB-phoU*. L'inactivation de Pst active constitutivement le régulon Pho et atténue la virulence des souches pathogènes. Cependant, les mécanismes moléculaires reliant le système Pst et la virulence ne sont pas connus. Chez la souche *E. coli* uropathogène (UPEC) CFT073, la délétion de *pst* atténue la virulence dans le modèle murin d'infection urinaire. De plus, l'expression des fimbriae de type 1 est réprimée chez le mutant *pst*. Cette répression est concomittante avec l'expression différentielle des régulateurs de ces fimbriae, soit les recombinases *fimB*, *fimE*, *ipuA* et *ipbA*. Une restauration de virulence est observée lorsque *fim* est constitutivement exprimé chez le mutant *pst*, confirmant le rôle crucial de ces fimbriae dans la virulence. En raison de la répression de *fim*, le mutant *pst* est moins apte à envahir les cellules épithéliales de la vessie. Puisque les fimbriae de type 1 jouent un rôle important dans la virulence des UPEC, notamment en contribuant à la colonisation et l'invasion de la vessie, la diminution de la colonisation de la vessie par le mutant Pst est principalement causée par la répression de ces fimbriae. Ainsi, l'élucidation des mécanismes impliqués dans la régulation des fimbriae de type 1, par l'activation du régulon Pho chez les UPEC, ouvrira de nouvelles avenues dans les approches thérapeutiques et préventives des infections du tractus urinaire.

Decreased Expression of Type 1 Fimbriae by a *pst* mutant of uropathogenic *Escherichia coli* Reduces Urinary Tract Infection.

Short title: Inactivation of Pst reduces UPEC *fim* expression

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ABSTRACT

The *pstSCAB-phoU* operon encodes the phosphate specific transport system (Pst). Loss of Pst constitutively activates the Pho regulon, and decreases bacterial virulence. However, specific mechanisms underlying decreased bacterial virulence through inactivation of Pst are poorly understood. In uropathogenic *Escherichia coli* (UPEC) strain CFT073, inactivation of *pst* decreased urinary tract colonization in CBA/J mice. The *pst* mutant was deficient in production of type 1 fimbriae and showed decreased expression of the *fimA* structural gene which correlated with differential expression of *fimB*, *fimE*, *ipuA* and *ipbA* genes encoding recombinases, mediating inversion of the *fim* promoter. The role of *fim* down-regulation in attenuation of the *pst* mutant was confirmed using a *fim* phase locked-ON derivative which demonstrated a significant gain in virulence. In addition, the *pst* mutant was less able to invade human bladder epithelial cells. As type 1 fimbriae contribute to UPEC virulence by promoting colonization and invasion of bladder cells, the reduced bladder colonization by the *pst* mutant is predominantly attributed to down-regulation of these fimbriae. Elucidation of mechanisms mediating the control of type 1 fimbriae through activation of the Pho regulon in UPEC may open new avenues for therapeutics or prophylactics against urinary tract infections.

INTRODUCTION

Pathogenic *Escherichia coli* comprise a diversity of strains associated with both intestinal and extra-intestinal infections [1]. Urinary tract infections (UTIs) are one of the most common bacterial infections, and uropathogenic *E. coli* (UPEC) are the predominant causal agent, representing up to 85% of community acquired UTIs [247]. In addition to causing UTIs, extra-intestinal pathogenic *E. coli* (ExPEC) are an important pathogen associated with neonatal meningitis and septicemia in humans as well as systemic infections in poultry and livestock [184, 248]. Many virulence factors associated with UPEC strains are important for establishing infection, and include adhesins, toxins, iron acquisition systems, and capsular antigens [14].

An important aspect of bacterial virulence is the capacity to rapidly adapt to changes and stresses encountered during infection of the host, since changes in host environment may serve as cues mediating regulation of expression of key virulence factors during infection [249, 250]. One of the mechanisms by which bacteria respond to environmental signals is through two-component signal transduction systems (TCSs). TCSs are composed of an inner-membrane histidine kinase sensor protein and cytoplasmic response regulator [151]. TCSs are important for bacterial adaptation and virulence [251, 252], and a number of TCSs have been identified to be important for pathogenic *E. coli*, e.g. BarA-UvrY, PhoPQ and QseBC [253-256].

The Pho regulon is controlled by the PhoBR TCS, in which PhoR is the sensor histidine kinase and PhoB the response regulator. PhoBR responds to phosphate limitation, i.e. when the extracellular phosphate concentration falls below 4 μM [4, 152]. In phosphate-limiting conditions, PhoBR induces genes belonging to the Pho regulon, which are involved in phosphate transport and metabolism [4, 152]. The *pstSCAB-phoU* operon belongs to the Pho regulon and encodes the phosphate specific transport (Pst) system, an ATP-binding cassette (ABC) transporter specific for inorganic phosphate (P_i). Mutations in the *pst* operon mimic phosphate starvation conditions, and result in constitutive expression of the Pho regulon regardless of environmental phosphate availability [4]. In addition to mimicking phosphate starvation, inactivation of the Pst system attenuated virulence of both pathogenic ExPEC and enteropathogenic *E. coli* (EPEC) strains [5, 7, 10, 154, 155]. In UPEC, inactivation of *phoU*, a gene of the *pst* operon involved in repression of the Pho regulon in phosphate-replete conditions [152], reduced colonization of the

murine urinary tract [55, 228]. However, the precise role of the Pst system in UPEC virulence has not yet been investigated. Nevertheless, in avian pathogenic *E. coli* (APEC), attenuation of a *pst* mutant was attributed to increased sensitivity to serum, acid, oxidative stress and cationic antimicrobial peptides [7, 8, 10, 12]. Also, an altered membrane homeostasis and a reduction in the production of the hexa-acyl pyrophosphate form of lipid A is observed in this mutant strain [11, 12]. Taken together, these results suggest that inactivation of the *pst* system could attenuate pathogenic *E. coli* by altering membrane composition and repressing production of surface structures including adhesins required for colonization of host tissues. Indeed, inactivation of the *pst* system also inhibited expression of type 1 fimbriae by avian pathogenic *E. coli* [7, 8]. However, the specific mechanisms by which induction of the Pho regulon inhibit expression of type 1 fimbriae have not been previously investigated.

Type 1 fimbriae mediate attachment of *E. coli* to host cells and tissues by mannose-specific receptors. Thereby, type 1 fimbriae are required to establish infection of ExPEC strains [13, 14]. In UPEC strains, type 1 fimbriae are expressed in the bladder, mediate bacterial adherence to urothelial cells, and promote formation of intracellular bacterial communities [58, 60, 61, 257]. The latter allows the persistence of UPEC strains in the host [13]. Type 1 fimbriae are encoded by the *fimAICDFGH* operon (*fim*), where *fimA* encodes the major subunit and *fimH*, encodes the mannose-specific adhesin [45]. Expression of the *fim* operon is governed by a phase-variable promoter (*fimS*) which is located on a 314-bp invertible element flanked by two 9-bp inverted repeats [62]. Phase-variable expression of type 1 fimbriae is mediated by the inversion of *fimS* to and from the ON- or OFF-orientations. Orientation of *fimS* is controlled by the FimB and FimE recombinases [67]. In this manner, FimE promotes switching of *fimS* to the OFF-orientation (from phase-ON to phase-OFF), while FimB mediates switching in both directions (from phase-OFF to phase-ON and phase-ON to phase-OFF), where the ON-orientation is favored [67, 258]. In addition to FimB and FimE, the CFT073 UPEC strain encodes FimBE-like recombinases, IpuA and IpbA (10). IpuA promotes switching in both directions, like FimB, whereas IpbA only promotes the switching in the ON-position (10). Furthermore, IpuA and IpbA are sufficient for switching *fimS* and influencing type 1 fimbriae expression either *in vitro* and *in vivo* [65, 66].

Inactivation of the Pst system has been shown to cause pleiotropic effects and loss of membrane integrity in APEC strains [7, 8, 10-12]. However, the precise role of the Pst system in

UPEC virulence has not been assessed. In this study, we investigated to what extent the Pst system affects UPEC virulence. We demonstrate that decreased expression of the type 1 fimbriae is directly linked to attenuated virulence of the *pst* mutant in the murine UTI model. Indeed, in the *pst* mutant, type 1 fimbriae are down-regulated *in vitro* and *in vivo*. This down-regulation is concomitant with differential expression of the recombinase genes *fimB* and *fimE*, and the *fim*-like recombinases *ipuA* and *ipbA*. Furthermore, the *pst* mutant has a decreased capacity to invade bladder cells. The altered phenotypes of the *pst* mutant are due to the decreased expression of type 1 fimbriae, as constitutive expression of the *fim* operon restored these phenotypes. Therefore, our work shows that deletion of the Pst system in UPEC CFT073 reduces colonization of the bladder and invasion of uroepithelial cells specifically by reducing the expression of type 1 fimbriae.

MATERIALS AND METHODS

Bacterial strains, plasmids and media

The *E. coli* strains and plasmids used in this study are listed in Table 1. Bacteria were grown in Luria-Bertani (LB) broth at 37°C and in human urine. Urine was collected from healthy women volunteers aged 20 to 40 who had no history of UTI or antibiotic use in the prior 2 months. Each urine sample was immediately filter sterilized (0.2 µm pore size), pooled and frozen at -80°C and was used within 2 weeks. 5637 bladder cells were grown in RPMI 1640 medium (Wisent Bioproducts) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate. Bacteria were also grown in MOPS minimal media (Teknova) supplemented with 0.4% glucose, 0.2% $(NH_4)_2SO_4$, 1.32 mM K_2HPO_4, and 1 µg/mL thiamine (high phosphate). The MOPS low phosphate contained 1 µM K_2HPO_4 [259]. Antibiotics and reagents were added as required at the following concentrations: kanamycin, 40 µg/ml; ampicillin, 100 to 200 µg/ml; chloramphenicol, 30 µg/ml; gentamicin, 15 µg/ml; amikacin sulfate salt, 250 µg/ml; diaminopimelic acid (DAP), 50 µg/ml; and 5-bromo-4-chloro-3 indolylphosphate di-sodium (XP), 40µg/ml.

Construction of mutants and complemented strains

All mutants were generated by the procedure described by Datsenko and Wanner using plasmids pKD3 and pKD13 as the template for chloramphenicol and kanamycin resistance

cassettes respectively [260]. Primers used are listed in Supporting information Table S1. Antibiotic cassettes flanked by FRT sequences were removed by transforming the mutant strains with pCP20 expressing the FLP recombinase [261].

The Δ*pstSCA* strain was complemented by inserting the *pstSCA* genes at the *att*Tn7 site of the chromosome, resulting in strain Δ*pstSCA* compl. The procedure was performed as described by Crepin, Harel and Dozois (submitted for publication). Briefly, the *pstSCA* genes and their native promoter were amplified with primers CMD1069 and CMD1070. The amplified product was then digested with XhoI and XmaI and ligated into the MCS of the mini-Tn7 encoding vector pGP-Tn7-Gm, generating vector pGP-Tn7-*pst*. Strain MGN-617 (pGP-Tn7-*pst*) was conjugated overnight with the Δ*pst* strain, containing the vector pSTNSK encoding the transposases *tnsABCD* required for transposition of Tn7 at the *att*Tn7 site, at 30°C on LB agar plates supplemented with DAP. After incubation, the bacterial lawn was suspended in 1 ml of PBS, washed twice in PBS, serially diluted, and spread on LB agar supplemented with gentamicin and incubated at 37°C. Colonies were verified for sensitivity to kanamycin and ampicillin, indicating likelihood of integration at *att*Tn7 and loss of the transposase containing plasmid pSTNSK. Tn7 insertion into the *att*Tn7 site was verified by PCR using primers CMD1070 and CMD1072 (Table S1). The *pst* complemented derivative was designated QT2117.

For the *fim* L-ON derivative strains, primers CMD1185 and 1186 (found in Supplemental Table 1) were used to amplify the *cat* cassette from pKD3. Using the same approach as described by Gunther *et al*. [262], point mutations in primer CMD1186 were introduced in order to block the promoter switch in the ON-position.

Experimental UTI in CBA/J mice

Experimental infections were carried out using either competitive co-infection or single-strain infection models as described previously [263, 264]. Prior to inoculation, strains were grown 16 h at 37°C with shaking (250 rpm) in 55 ml of LB media. For co-infection, cultures were centrifuged and pellets of the WT and derivative strains were mixed 1:1. Six-week-old CBA/J female mice were transurethrally inoculated with 20 µl of the 1:1 mixture containing $5X10^8$ CFU of UPEC CFT073 Δ*lacZYA* strain (QT1081) and $5X10^8$ CFU of either the CFT073 Δ*pstSCA* (QT1911)

strain or its complemented derivative (QT2117). At 48 h p.i., mice were euthanized; bladders and kidneys were aseptically removed, homogenized, diluted and plated onto MacConkey agar to determine bacterial counts.

In the single-strain experimental UTI model, mice were infected as described above but with a pure culture (10^9 CFU) of each strain, and at 6-, 24- and 48 h p.i., bacterial counts were determined from the bladders and kidneys. Bladders were bisected, one half was used to determine bacterial counts and the other half was resuspended in TRIzol Reagent (Invitrogen) for RNA extraction and subsequent analysis of bacterial gene expression.

Yeast cell aggregation and mannose-resistant hemagglutination (MRHA) assay

A yeast cell aggregation assay was performed as described elsewhere [8]. Briefly, cultures were grown at 37°C in LB broth or human urine to mid-log phase or for 24 h to 48 h in LB broth without shaking, to enhance expression of type 1 fimbriae. An initial suspension of approximately 2 X 10^{11} cells ml^{-1}, in PBS, was serially diluted two-fold in microtiter wells, and equal volumes of a 3% commercial yeast suspension, in PBS, were added to each of the wells. After 30 min of incubation on ice, yeast aggregation was monitored visually, and the agglutination titer was recorded as the most diluted bacterial sample giving a positive aggregation reaction. The MRHA assay was determined using O^- human red blood cells with bacteria grown to mid-log phase in LB broth. To inhibit type 1 fimbriae-dependent hemagglutination, a final concentration of 1.5 % α-D-mannopyranose was added to samples.

RNA extraction and quantification of gene expression

RNAs were extracted from bacterial cultures grown in LB broth or human urine, using the RiboPure™-Bacteria Kit (Ambion, Austin, TX), according to the manufacturer's recommendations, with the exception that DNAse I treatment was performed twice. RNA were also extracted from infected bladders at 6-, 24- and 48 h p.i. with TRIzol Reagent (Invitrogen), followed by DNase 1 treatment (Ambion).

The iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad) was used for qRT-PCR according to the manufacturer's instructions. The *tus* gene was used as a housekeeping control [8]. Each qRT-PCR run was done in triplicate, and for each reaction the calculated threshold cycle (*C*t) was normalized to the *C*t of the *tus* gene amplified from the corresponding sample. The fold-change was calculated using the $2^{-\Delta\Delta Ct}$ method [265]. Genes with fold change above or below the defined threshold of 2 is considered as differentially expressed. Primers used for qRT-PCR analysis are listed in Supporting Table S1.

Detection and quantification of the ON/OFF state of the *fimS* region

Detection of the orientation of the *fimS* region was performed as described previously [72, 266]. Briefly, the *fimS* region was PCR amplified with primers CMD1258 and CMD1259 (Table S1), giving a fragment of 650 bp. The PCR products were digested with HinfI and analyzed on a 2% agarose gel. Profiles of the *fimS* switch are dependent on its orientation, with the ON-orientation producing fragments of 128 bp and 522 bp, and the OFF-orientation generating fragments of 411 bp and 239 bp. Quantification of the percentage of ON-cells was performed as described by Wu and Outten [267]. The WT strain was cultured statically 48 h at 37°C to enhance production of type 1 fimbriae and was used as control of ON-position. The WT strain was also cultured 24h on LB agar plate at room temperature and was used as control of OFF-position.

Quantification of the orientation of the *fimS* switch was also performed by quantitative PCR (qPCR) with iQ™ SYBR® Green Supermix (Bio-Rad) according to the manufacturer's recommendations. qPCR was performed on 10 ng of gDNA extracted from bacteria grown under different culture conditions as described above or from 500 ng of total DNA extracted from infected bladder as described by Struve and Krogfelt [71]. Primers CMD1246 and CMD1248 were used to amplify the ON-orientation while the CMD1247 and CMD1248 primers amplified the OFF-orientation. The threshold cycle (C_T) of the ON-and OFF-orientation was normalized to the C_T of the *vat* gene (CMD96 and CMD97), an uninvertible element. Fold change was calculated using the $2^{-\Delta\Delta Ct}$ method [265].

Preparation of fimbrial extracts and western blotting

Preparation of fimbrial extracts and western blotting were performed as described previously [8], with anti-FimA serum from *E. coli* strain B_{AM} and F1C fimbriae-specific (anti-$F165_2$) antiserum [268].

Nucleotide extraction and quantification of ppGpp

Nucleotides were extracted essentially as described by Traxler *et al*. [269], with the exception that bacteria were grown for 16 h or to mid-log phase of growth in 20 ml of LB medium. ppGpp was quantified by an ÄKTA purifier system using a Mono Q 5/50 GL column (GE Healthcare). The chromatography conditions were followed according to Traxler *et al*. [269], with the exception that ppGpp was identified as a peak that eluted at ~54.6% of 1.5 M sodium formate buffer. Samples were run in duplicate for at least three separate time-course experiments. ppGpp standard was purchased from TriLink Biosciences. Detection of ppGpp was in the range of 20 nM to 100 mM. The CF5747 strain, which contains a *relA* inducible vector and CF1693, where *relA* and *spoT* genes were deleted, were used as positive and negative control, respectively [270, 271].

Adhesion and invasion assays

Human bladder epithelial cell line 5637 (American Type Culture Collection HTB-9) was grown to confluency in 24-well plates in RPMI 1640. UPEC CFT073 and its derivative strains were grown in LB medium at 37°C to mid-log phase of growth (O.D. 0.6). The bacterial cells were centrifuged, washed twice with PBS, resuspended in RPMI 1640 medium (Wisent Biocenter, St-Bruno, Canada) supplemented with 10% fetal bovine serum at 10^6 CFU ml^{-1} and added to each well. Bacteria-host cell contact was enhanced by a 5-min centrifugation at 600 x *g*. For invasion assays, at 2 h post-adhesion, cells were washed three times and a 2 h treatment with amikacin sulfate (250 µg/ml) was performed to kill extracellular bacteria. At 2 h post-treatment, cells were washed three times and lysed with PBS-0.1% sodium deoxycholate (DOC), serially diluted and plated onto LB agar plates. Quantification of cell-associated bacteria and intracellular bacteria (via amikacin protection assay) was performed as previously described [61]. To block adherence mediated by type 1 fimbriae, 1.5% α-D-mannopyranose was added to culture medium.

Resistance to serum, oxidative stress, polymixin B and vancomycin

Resistance to 90% human serum was performed as described by Lamarche *et al*. [10]. The serum was pooled from three independent persons not working with *E*. *coli*. A strain was considered resistant if there was an increase in bacterial counts or a decrease of less than 2-log and sensitive if there was a decrease of more than 2-log. Resistance to oxidative stress was performed as described by Crepin *et al*. [8]. Differences in resistance were assessed from the diameter of the inhibition zone of strains cultured to mid-log phase of growth in LB media. Resistance to polymixin B and vancomycin was performed as described by Lamarche *et al*. [12], with slight modifications. Strains were grown to mid-log phase of growth in LB broth, then 10^6 CFU ml^{-1} were mixed, in microwell plates, with final concentrations of polymixin B (0.4 to 0.6 µg ml^{-1}) or vancomycin (50 to 100 µg ml^{-1}) and were incubated for 3 h at 37°C. Bacterial growth was evaluated by spectrophotometry. The MIC was considered the lowest drug concentration that reduced growth >50% compared with growth in the absence of antibiotic.

Electron microscopy

Electron microscopy was performed as described previously [8] with bacterial cultures grown in LB broth to mid-log phase.

Statistical analyses

All data were analyzed by using GraphPad Prism 4 software. A Wilcoxon signed-rank test (two-tailed $P \leq 0.05$) was used to determine statistical significance for co-infections. In single-strain infection experiments, a Mann-Whitney test was used. All other statistical analyses were determined by the Student's *t*-test or One-way ANOVA with Bonferroni's Multiple Comparison Test.

RESULTS

The *pst* mutant was out-competed in a murine UTI co-challenge model

To characterize the role of the Pst system in UPEC strain CFT073, co-infection in the mouse UTI model was performed between the Δ*pstSCA* mutant (*pst* mutant) and the WT CFT073 Δ*lac*

strain; or CFT073 Δ*lac* and complemented strain (Δ*pst* compl.). The CFT073 Δ*lac* strain is as virulent as the CFT073 wild-type parent and presented no statistical difference in urinary tract colonization [264]. At 48 h post-infection (p.i.), the *pst* mutant was outcompeted 732-fold in bladder and 2649-fold in kidneys (Fig. 1A). Complementation with the *pstSCA* genes into the *att*Tn7 site significantly improved the competitive fitness in both the bladder and kidneys (Fig. 1A). The complemented strain regained competitive colonization in the bladder and kidneys, although it was still significantly (P=0.02) less competitive (about 3-fold difference compared to the WT) in the kidneys.

To determine whether the out-competed phenotype observed *in vivo* for the *pst* mutant was due to different growth kinetics, an *in vitro* competition assay was performed in human urine, which represents an *ex vivo* condition that may reflect nutrient availability and environmental conditions encountered in the bladder. Although the *pst* mutant is 2.4-fold out-competed at 6 h post-inoculation, there was no significant difference at 24- and 48 h (Fig. S1 in the supplemental material). Hence, the out-competition of the *pst* mutant was not the consequence of a growth defect.

As inactivation of the *pst* system constitutively activates the PhoBR two-component regulatory system, we determined whether the constitutive activation of *phoB* was responsible for the attenuated virulence of the *pst* mutant. To do so, co-infection between the *phoB* mutant and the WT CFT073 Δ*lac* strain; or the double *pst phoB* mutant and the WT CFT073 Δ*lac* strain were performed. The *phoB* knock-out mutant colonized the urinary tract as well as the WT strain (Fig. 1B). Similarly, the double mutant strain was as virulent as the WT strain (Fig. 1B). This phenotype was expected as deletion of *phoB* in the *pst* mutant abrogated the induction of the Pho regulon induced by deletion of the *pst* system [7]. Thereby, these results show that inappropriate and constitutive induction of the Pho regulon has a deleterious effect on pathogenicity.

Production and transcription of type 1 fimbriae is reduced in the *pst* mutant

We previously demonstrated that deletion of the Pst system inhibited production of type 1 fimbriae by APEC strain χ7122 [7, 8]. As type 1 fimbriae contributed to UPEC pathogenicity [13,

14], we quantified production of type 1 fimbriae in UPEC CFT073 strain and its derivative mutants. At mid-log phase of growth in LB broth (O.D. 0.6), the *pst* mutant was unable to agglutinate yeast, while the WT and complemented strain respectively agglutinated yeast at the same titer (Fig. 2A). As expected, expression of type 1 fimbriae was restored in the double *pst phoB* mutant (Fig. 2A). To corroborate that production of type 1 fimbriae was impaired in the *pst* mutant, western blotting against the type 1 fimbrial major sub-unit FimA was performed. Western blot confirmed an important reduction of FimA production by the *pst* mutant, compared to the WT CFT073, the complemented and the double Δ*pst* Δ*phoB* strains (Fig. 2B). Production of type 1 fimbriae, by yeast agglutination, was also performed on bacteria cultured in human urine. At mid-log phase of growth (O.D. ~ 0.2), the *pst* mutant did not agglutinate yeast, whereas the WT and the complemented strains agglutinated yeast at titer 3 (Fig. 2C). When cultured statically for 24 h at 37°C, the *pst* mutant agglutinated yeast only at the highest density cell suspension, whereas the WT and complemented strains produced type 1 fimbriae at an agglutination titer of 4.5 (Fig. 2C).

Since deletion of the *pst* system inhibited production of type 1 fimbriae, we investigated if repression of type 1 fimbriae occurs at the transcriptional or post-transcriptional level. To test this, expression of the *fimA* gene was evaluated by real-time quantitative reverse transcription PCR (qRT-PCR). At mid-log phase of growth in LB broth, transcription of *fimA* was down-regulated 11.7-times in the *pst* mutant (Fig. 2D). When bacteria were grown in human urine to mid-log phase and 24 h statically, expression of the *fimA* gene was also down-regulated 2.6- and 2.9-times, respectively, in the *pst* mutant (Fig. 2D). Although repression of *fimA* in the *pst* mutant grown in human urine was slightly reduced as compared with LB broth, this reduction correlated to yeast agglutination of cells grown in human urine (Fig. 2C). Again, complementation of the *pst* mutation restored *fimA* expression to WT level (Fig. 2D).

As constitutive induction of the Pho regulon, by inactivation of the *pst* system, repressed expression of *fim*, we tested whether physiological activation of the Pho regulon had the same effect. To do so, bacteria were grown in low phosphate (LP) medium and expression of *fim* was quantified by qRT-PCR. As for the *pst* mutant, physiological induction of the Pho regulon repressed expression of *fimA*, as no difference was observed between the WT and the *pst* mutant when grown in LP medium (Fig. S2A in the supplemental material). Furthermore,

expression of *fimA* was decreased 14.0-fold in the WT strain cultured in LP compared to following culture in high phosphate (HP) medium (Fig. S2B in the supplemental material). This is similar to what observed between the *pst* mutant and the WT strain grown in LB medium (Fig. 2D). These results indicate that induction of the Pho regulon, physiologically or by inactivation of *pst*, alters expression of type 1 fimbriae at the transcriptional level.

Hence, under certain growth conditions, the Δ*pst* mutant was impaired in its capacity to produce type 1 fimbriae. This suggests that the attenuation observed in the *pst* mutant could be due, at least in part, to a reduced production of type 1 fimbriae during infection.

Inactivation of the Pst system favors the OFF-orientation of the *fim* promoter

As expression of the type 1 fimbriae is down-regulated in the *pst* mutant, we asked whether its down-regulation correlated with the OFF-position of the phase-variable promoter (*fimS*). The orientation of *fimS* was evaluated in strains grown under agitation to mid-log phase in LB broth. Using the classical procedure described by Stentebjerg-Olesen *et al*. [272], only 1.3% of the *pst* mutant population were in the ON-position, whereas 4.9% and 6.0% of WT and *pst* complemented cells were in the ON-position respectively (Fig. 3A).

To confirm differences in orientation of the *fimS* switch, we also performed quantitative PCR (qPCR). By comparing the ON-position of different strains cultured to mid-log phase in LB broth, the quantity of cells in the ON-orientation for the *pst* mutant was 4.2-fold lower than the WT strain (Fig. 3B), which is similar to results obtained by *Hin*fI digestion of PCR products (3.8-fold, Fig. 3A). Furthermore, complementation of the *pst* mutant restored the ON-orientation to the parent level (Fig. 3B). Similar results were obtained for strains cultured in human urine (data not shown). Thereby, in the *pst* mutant, the preferential orientation of the *fim* promoter in the OFF-position in both LB medium and human urine correlated with the decrease in *fim* expression observed.

Expression of the *fimS* recombinases are decreased in the Pst mutant

To correlate the *fimS* OFF-position in the *pst* mutant, expression of the *fimB* and *fimE* recombinases was analyzed by qRT-PCR. Thus, at mid-log phase of growth in LB broth, *fimB* transcription was down-regulated 2.6-fold in the *pst* mutant (Fig. 4A). Expression of *fimB* was restored in the complemented strain. However, no difference was observed for the *fimE* gene (Fig. 4A). In addition to *fimB* and *fimE*, the CFT073 strain encodes the *fim*-like recombinases IpuA and IpbA. Similarly to *fimB*, these two *fim*-like recombinases were down-regulated in the *pst* mutant (Fig. 4B). Furthermore, expression of *fimB*, *fimE*, *fimA* and orientation of *fimS* are known to be controlled by multiple regulators, e.g. *crp*, *hns*, *cyaA*, *rcsB*, *qseB*, *papB*, *nanR*, *ihf, lrp*, *nagC* and *focB*. To determine whether these genes may influence expression of type 1 fimbriae in the *pst* mutant, expression of these regulators was quantified by qRT-PCR. However, none of these regulators was differentially expressed in the *pst* mutant (data not shown).

As FimB, IpuA and IpbA promote inversion of *fimS* to the ON-orientation, and therefore positively modulate *fim* expression [65, 67], the OFF-position of *fimS* and the concomitant decreased expression of type 1 fimbriae in the *pst* mutant could be due to the cumulative reduced expression of the *fimB*, *ipuA* and *ipbA* encoding recombinases.

Inactivation of the Pst system reduces *fimA* expression *in vivo*

To determine whether type 1 fimbrial expression is also down-regulated *in vivo*, a time course of single-strain infection in the murine UTI model was performed as the *pst* mutant was severely attenuated during competitive infection (Fig. 1A). Prior to mice inoculation, expression of type 1 fimbriae was analyzed by yeast agglutination assay. As shown in Fig. 5A, following overnight growth in LB broth, the *pst* mutant produced type 1 fimbriae as well as the WT strain. At 6 h p.i., the *pst* mutant colonized the mouse urinary tract as well as the WT CFT073 strain (Fig. 5B). However, transcription of *fimA* in the *pst* mutant was down-regulated 2.3-fold in the bladder (Fig. 5C). By contrast, at 24- and 48 h p.i., the *pst* mutant was attenuated 28- and 158-times in bladder, respectively (Fig. 5A). Likewise, *fimA* transcription was down-regulated 5.5- and 44.0-fold in the bladder at 24- and 48 h, respectively (Fig. 5B). Overall, although production of type 1 fimbriae is similar between the *pst* mutant and the WT strain prior to mice inoculation, *in vivo*,

expression of *fimA* was decreased at all times post-infection in the *pst* mutant, and differences were more marked at later time points.

Orientation of the *fim* promoter (*fimS*) was analyzed to correlate with expression of *fimA* in the *pst* mutant in infected bladder. At all time points, *fimS* was more frequently in the OFF-position in the *pst* mutant compared to the WT strain. This was most marked at 48 h when the ON-position of *fimS* was decreased 56.1-fold (Fig. 5D), which correlates with the concomitant down-regulation of *fimA* observed at that time (Fig. 5C). Expression of the *fim* recombinases *fimB* and *fimE* was at 6-, 24- and 48 h p.i. in the bladder. Expression of *fimE* was increased in the *pst* mutant at each p.i. time and this up-regulation was more marked at later times (Fig. 5E). Thus, compared to the WT strain, expression of *fimE*, in the *pst* mutant, was increased 2.2- and 3.9-fold at 24- and 48 h p.i., respectively (Fig. 5E).

Taken together, these results confirm that inactivation of *pst* represses expression of the type 1 fimbriae and could explain its attenuation.

Attenuation of the *pst* mutant is mainly due to the down-regulation of type 1 fimbriae.

As we previously demonstrated that decreased virulence of *pst* mutants in ExPEC strains may be the consequence of surface perturbations [7, 8, 10-12], we compared membrane integrity of the UPEC CFT073 *pst* mutant. Although the *pst* mutant demonstrated intermediate resistance to 90% human serum up to 2 h post-incubation (Fig. 6A), the *pst* mutant was as resistant as the WT strain to hydrogen peroxide, polymixin B and vancomycin (Fig. 6B, C and D, respectively) as no statistical difference was observed.

To test whether attenuation of the *pst* mutant is mainly the consequence of down-regulation of type 1 fimbriae, we constitutively induced expression of type 1 fimbriae by introducing point mutations into the right inverted repeat of *fimS*, which locked the promoter switch in the ON-position (L-ON). We then compared the capacity of the WT and Δ*pst fim* L-ON derivative strains to colonize the mouse urinary tract. At 48 h p.i., no significant difference in colonization of the bladder was observed between the CFT073 *fim* L-ON and the Δ*pst fim* L-ON mutant strains,

whereas the Δ*pst fim* L-ON strain was attenuated 2.5-times in kidneys (Fig. 6E). By comparing the colonization of the mouse urinary tract between the *pst* (Fig. 5B) and the *pst fim* L-ON mutant (Fig. 6E), we observed that the *fim* L-ON strain colonized the bladder and kidneys 1384- and 3-times more than the *pst* mutant. Thereby, these results show the major contribution of the down-regulation of type 1 fimbriae in attenuation of the *pst* mutant during infection, which is more marked in bladder, where type 1 fimbriae are required.

Concentration of ppGpp is diminished in the *pst* mutant

The alarmone ppGpp is a second messenger produced by nutritional starvation and initiates global physiological adaptation [273]. Its synthesis relies on RelA and SpoT, which encode the GDP pyrophosphokinase / GTP pyrophosphokinase and guanosine 3'-diphosphate 5'-triphosphate 3'-diphosphatase, respectively. In addition to its role in adaptation to starvation, ppGpp negatively regulates expression of type 1 fimbriae. Indeed, accumulation of ppGpp induces transcription of *fimB* and thus, *fimA* [78, 79]. As it has been previously observed that mutations in the *pst* system decreased the concentration of ppGpp [226, 227], we hypothesized that the down-regulation of *fimB* and the reduced production of type 1 fimbriae, *in vitro*, may be mediated by a decrease in ppGpp concentration in the *pst* mutant.

At mid-log phase of growth in LB medium, the mean intracellular pool of ppGpp in the WT CFT073 strain was 45.65 nM and that of the *pst* mutant was 15.02 nM, which represents a decrease of 3-fold (Fig. 7). Although complementation did not completely restore the concentration of ppGpp to WT level, no statistical difference was observed between the complemented strain and the WT parent (Fig. 7). These results demonstrate that during mid-log growth, the *pst* mutant produces less ppGpp than the WT strain and could explain, at least in part, the decreased expression of *fimB* and reduced expression of type 1 fimbriae in the *pst* mutant.

Deletion of *pst* genes induces expression of F1C fimbriae

It has been observed that deletion of the *fim* operon resulted in enhanced expression of other types of fimbriae [113]. In this manner, as the Δ*pst* strain colonized the bladder as well as the WT strain up to 6 h p.i., production of other adhesins could compensate for the loss of type 1 fimbriae and then mediate adherence to epithelial cells.

To test this possibility, adhesion assays using 5637 human bladder epithelial cells (ATCC HTB-9) were performed. The Δ*pst* mutant adhered to human bladder cells as well as the WT strain (Fig. 8A and B). Interestingly, the CFT073 Δ*fim* strain also adhered as well as the WT strain (Fig. 8A), which supports the hypothesis that other fimbriae or adhesins are produced that compensate for the loss of the type 1 fimbriae in the *pst* mutant. Locking ON the *fim* promoter increased adherence of the WT and the *pst* mutant to 42.94- and 48.17%, respectively (Fig. 8A). As type 1 fimbriae recognize mannosylated residues on host epithelial cells, addition of soluble mannose to culture medium will inhibit type 1 fimbriae-dependent adhesion to 5637 cells. Addition of 1.5% α-D-mannopyranose decreased adherence of all strains to 5637 bladder cells, except for the Δ*pst* and Δ*fim* mutants, to approximately 6.5% (Fig. 8A and B). However, addition of α-D-mannopyranose did not reduce adherence of either the *pst* mutant, or the Δ*fim* strain (Fig. 8A and B). Not surprisingly, deletion of *phoB* in the *pst* mutant restored the mannose-sensitive adhesion to bladder cells (Fig. 8A).

As the *pst* and *fim* mutants adhered to bladder cells independently of type 1 fimbriae, presence of fimbrial structures at the cell surface of the *pst* and *fim* mutant strains was analyzed by electron microscopy. Indeed, fimbriae were observed on their cell surface (Fig. 8C). This confirms that adherence of the *pst* mutant to bladder cells is independent of type 1 fimbriae and is mediated by other adhesins present at the cell surface.

In UPEC strain CFT073, it was previously shown that when type 1 fimbriae were phase locked-OFF, Pap fimbrial gene expression increased [113]. To determine if Pap fimbriae were up-regulated in the *pst* mutant, mannose-resistant haemagglutination (MRHA) of human erythrocytes was performed onto strains cultured in LB medium. As shown in Fig. 8D, no differences in MRHA between the WT and *pst* mutant were observed, which suggests that Pap

fimbriae were similarly expressed between these strains. To confirm the MRHA results, expression of the genes encoding the Pap fimbriae major sub-units *papA* and *papA*_2 was determined following adherence to 5637 bladder cells, which may reflect adhesion to bladder cells observed *in vivo*. Similarly to MRHA experiments, no difference was observed between strains (Fig. 8E). These results demonstrate that the mannose-resistant adherence of the *pst* mutant to bladder epithelial cells is not attributed to increased production of Pap fimbriae. It was also previously shown that expression of F1C fimbriae increased considerably in a strain lacking the *fim* and *pap* gene clusters [113]. Thereby, we investigated by qRT-PCR and western blot whether expression of F1C fimbriae was increased in this background. As shown in Fig. 8E and F, F1C fimbriae were 2-fold up-regulated in the *pst* mutant. Therefore, up-regulation of F1C fimbriae in the *pst* mutant could contribute to *in vitro* adherence of the *pst* mutant to bladder cells.

Deletion of *pst* genes reduces invasion of 5637 human bladder cells *in vitro*

It has previously been shown that some UPEC strains can invade bladder epithelial cells [36] and that epithelial cell invasion is mediated by type 1 fimbriae [50, 61]. We therefore hypothesized that the *pst* mutant may demonstrate reduced cell invasion. Indeed, cell invasion by the *pst* mutant was significantly reduced to 59% of the invasive capacity of the WT strain (Fig. 9). Similarly, for the Δ*fim* strain, invasion was decreased to 17.3% of the WT despite the fact that it adhered as well as the WT strain to bladder cells. Deletion of *phoB*, in the *pst* mutant, restored the invasion capacity of the Δ*pst* strain. Finally, invasion of the CFT073 *fim* L-ON and the Δ*pst fim* L-ON derivative strains increased to 693 and 779% respectively, confirming the role of type 1 fimbriae in bladder epithelial cell invasion.

Taken together, these results demonstrate that although expression of the *fim* operon is down-regulated in the *pst* mutant, expression of other adhesins can mediate adherence to bladder cells. However, decreased expression of type 1 fimbriae results in a reduced invasive capacity of the *pst* mutant and could explain the decreased colonization of the bladder.

DISCUSSION

In this study, we determined that the attenuation of the UPEC CFT073 *pst* mutant strain was primarily attributed to the down-regulation of type 1 fimbriae. We show that the deletion of the Pst system repressed transcription and production of type 1 fimbriae both *in vitro* and *in vivo*. As type 1 fimbriae are essential for bladder colonization and invasion [13, 14], down-regulation of the *fim* genes could explain the attenuated virulence of the *pst* mutant. Indeed, in the bladder, *fimA* expression was progressively reduced during infection.

We observed that the *fim* promoter had an increased bias for the OFF-position in the *pst* mutant *in vitro* and *in vivo*. In LB medium, the recombinase encoding genes *fimB*, *ipuA* and *ipbA*, were down-regulated in the *pst* mutant, whereas *fimE* was not differentially expressed. Since the recombinases FimB, IpuA and IpbA promote expression of the *fim* operon by orienting the *fimS* promoter to the ON-position [65, 67, 258], their down-regulation correlates with the down-regulation of the *fim* operon and the increase orientation of *fimS* to the OFF-position in the *pst* mutant. We hypothesized that the differential expression of *fimB*, in the *pst* mutant, was the major factor leading to decreased expression of type 1 fimbriae in the *pst* mutant. Indeed, overexpression of *fimB* in the *pst* mutant, restored production of type 1 fimbriae to levels similar of the *fim* locked-ON strain (data not shown).

In infected bladders, the inverse phenomenon regarding *fimB* and *fimE* gene expression was observed, i.e. *fimB* was not differentially expressed, whereas transcription of *fimE* was increased in the *pst* mutant. The differential expression of *fimB* and *fimE in vitro* and *in vivo* could be due to differences in culture conditions, i.e. LB medium vs bladder. It was previously observed that human urine decreased expression of *fimB* and increased that of *fimE* [94]. Furthermore, Sohanpal *et al*. [75] have demonstrated that the presence of *N*-acetyl-β-glucosaminidase, a lysosomal enzyme found in high concentrations in the upper urinary tract, repressed expression of *fimB*. By comparing expression of *fimB* and *fimE* in bacteria grown in either human urine or LB medium, we observed that in the WT and Δ*pst* strains, expression of *fimB* is slightly increased in human urine (Fig. S3 in the supplemental material). Furthermore, in accordance with Schwan *et al*. [94], expression of *fimE* was increased 8.63-fold in the WT strain cultured in human urine (Fig. S3 in the supplemental material). Furthermore, disruption of the *pst* system enhanced this

up-regulation as *fimE* was increased 26.3-fold in the Δ*pst* strain. As FimE reduces expression of *fim* by promoting the OFF-position [67, 258], its induction, in infected bladder, may also direct the orientation of the *fim* promoter to the OFF-position, leading to down-regulation of type 1 fimbriae in the *pst* mutant.

Constitutive expression of the *fim* operon in the *pst* mutant restored production of fimbriae at the cell surface and colonization of the bladder to the WT level. This observation is not in agreement with the hypothesis that attenuation of the *pst* is the consequence of pleiotropic effects and membrane perturbation described elsewhere [7, 8, 10-12]. In contrast to *pst* mutants of other ExPEC strains, the *pst* mutant of UPEC CFT073 did not demonstrate marked phenotypes associated with membrane perturbation. Indeed, the CFT073 *pst* mutant showed intermediate resistance to 90% human serum up to 2 h post-incubation and resisted oxidative stress, polymixin B and vancomycin to levels that were not significantly different compared to the WT parent. Therefore, in contrast to the APEC strain, the CFT073 *pst* mutant demonstrates less pleiotropic effects. As such, the effects of the *pst* mutation on down-regulation of type 1 fimbriae in strain CFT073 is the major cause of the reduced colonization of the urinary tract. However, as constitutive activation of the Pho regulon through inactivation of Pst results in a cascade of regulatory changes, it is likely that a number of regulatory changes which may or may not lead to a reduction in type 1 fimbrial expression, contribute to reduced virulence.

Production of ppGpp could be one of the mechanisms by which the Pho regulon influences expression of type 1 fimbriae. Indeed, it has been observed that accumulation of ppGpp activates transcription of *fimB* and concomitantly, *fimA* [78, 79]. In our current report, at mid-log phase of growth, the *pst* mutant was shown to produce 3-fold less ppGpp than the wild-type parent strain CFT073 (Fig. 7). As a decreased concentration in ppGpp alters the transcription of *fimB* and the *fim* operon [78, 79], we suggest that the down-regulation of *fimB* and consequently, the *fim* operon, could be due to the decreased concentration of ppGpp in the *pst* mutant.

In either competitive or single-strain infections, the *pst* mutant was attenuated in the murine UTI model. However, the *pst* mutant was less attenuated in single-strain infections. As adhesion to bladder cells, via type 1 fimbriae, induces inflammation and recruitment of neutrophils to bladder

[14], it is possible that in the competitive infection, the innate immune response is accentuated, due to the presence of the WT strain. This increased induction could result in the loss of fitness of the *pst* mutant in the competitive infection and may explain its pronounced attenuation at 48 h p.i. in both bladder and kidneys.

In vitro, the *pst* mutant adhered to human bladder cells as well as the WT strain, which correlates with the colonization of the mouse bladder up to 6 h p.i. However, compared to the WT strain, this adhesion was mannose-resistant. Electron microcopy demonstrated fimbriae at the cell surface of the *pst* mutant, indicating that other adhesins are expressed at the bacterial cell surface and could mediate adherence to bladder epithelial cells. Interestingly, the *fim* mutant strain also adhered to bladder cells as well as the WT, in a mannose-resistant manner, and produced fimbriae at its cell surface. In addition, the *pst* mutant had the same colonization kinetics of the Δ*fim* strain in the murine UTI model (Fig. S4 in the supplemental material), where bladder and kidney colonization of the Δ*fim* strain is in agreement with the study of Gunther *et al*. [262]. UPEC strain CFT073 contains genes predicted to encode 12 distinct fimbriae and many afimbrial adhesins [16]. As one type of fimbriae is predominantly expressed at a time [111, 113], they are coordinately expressed and are subjected to a regulatory cross-talk [118]. Thereby, it is not surprising to note the presence of other fimbriae at the cell surface of the *pst* and *fim* mutants. In this study, we observed that F1C fimbriae were increased in the *pst* mutant. Since F1C fimbriae mediate adhesion to bladder and kidney epithelial cells [274], the observed adhesion of the *pst* mutant could be attributed to F1C fimbriae. However, further experiments are required to determine the precise contribution of F1C fimbriae in the *pst* mutant.

In addition to mediating adherence, type 1 fimbriae are also involved in invasion of bladder epithelial cells [13, 36]. Although the *pst* mutant adhered to bladder cells as well as the WT strain, its invasion capability was reduced. This invasion defect is directly linked to the down-regulation of the *fim* genes, since constitutive expression of type 1 fimbriae in the *pst* mutant considerably increased invasion. It has been postulated that invasion of bladder cells contributes to UPEC pathogenesis; since invasion can provide refuge from both innate and adaptive host immune defenses [250, 275]. In this manner, intracellular UPEC can serve as a source for recurrent UTIs [13, 36]. Thereby, the decreased invasion rate of the *pst* mutant could explain, at

least in part, its virulence attenuation. Reduced bladder colonization observed in the *pst* mutant at 48 h p.i. could also be attributed to its invasion defect.

In previous reports, inactivation of different TCS in *E. coli* reduced cell adherence and inhibited the expression of different types of fimbriae [80, 255, 256, 276]. In our case, by contrast, herein we demonstrate that induction of the PhoBR TCS, by either deletion of the *pst* system or phosphate starvation, results in repression of type 1 fimbriae. As the increase of antimicrobial resistance of bacterial pathogens is a major problem, it is important to develop novel preventive and therapeutic strategies. Since TCS are required for the adaptive response to environmental stimuli and they are absent from humans and other animals, they represent choice targets for therapeutic strategies. Indeed, a number of chemical inhibitors of TCSs have been identified by screening libraries of synthetic compounds [277, 278]. Furthermore, small chemical molecules targeting different virulence factors have been shown useful in preventing infection from different pathogens [185, 186, 188, 190]. In this manner, small molecules targeting the Pst system or PhoBR could be considered as therapeutic agents to constitutively activate the Pho regulon and potentially prevent such pathogens from causing infections.

ACKNOWLEDGMENTS

We thank Marie-Astrid Albert and Mélissa René (University of Montreal) for their technical advice regarding the adhesion assay and fluorescent microscopy and Matthew Blango (University of Utah) for his technical advice regarding the invasion assay. We also acknowledge Marc Drolet (University of Montreal) for sharing strains CF1693 and CF5747. SC was supported by scholarships from the Fonds Québécois de la Recherche sur la Nature et les Technologies and the Fondation Armand-Frappier. This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC, discovery grant RGPIN 250129-07) to CMD and (NSERC, discovery grant RGPIN SD-25120-09) to JH.

TABLE

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Characteristic(s)[a]	Source or reference
Strains		
BW25113	F$^-$ I$^-$ (*lacI*^{q} *rrnBT14 lacZWJ16 hsdR514 araBADAH33 rhaBADLD78*)	[260]
CF1693	MG1655 Δ*relA251::km* Δ*spoT207::cm*; Km^r, Cm^r	[271]
CF5747	MG1655 pALS13; Ptac -*relA*' (RelA 1-455), active RelA; Apr	[270]
CFT073	UPEC wild-type pyelonephritis strain	[16, 279]
χ7122	Avian pathogenic; O78:K80:H9; *gyrA* Nal^r	[280]
K3	χ7122 Δ*pstCAB*::*kan*	[10]
MG1655	F- lambda- *ilvG*- *rfb*-50 *rph*-1	[281]
MGN-617	*thi thr leu tonA lacY glnV supE* DΔ*asdA4 recA*::RP4 2-Tc::Mu [pir]; Km^r	[282]
ORN172	*thr-1 leuB thi-1 Δ(argF-lac)U169 xyl-7 ara-13 mtl-2 gal-6 rpsL tonA2 supE44 Δ(fimBEACDFGH)::kan pilG1*	[283]
QT1081	CFT073 Δl*acZYA* ::FRT	[264]
QT1324	CFT073 Δ*oxyR*::Km; Km^r	This study
QT1891	CFT073 Δ*pstSCA*::km; Km^r	This study
QT1911	CFT073 Δ*pstSCA*::FRT	This study
QT2063	CFT073 Δ*phoB*::FRT	This study
QT2085	MGN-617 + pGP-Tn7-*pst*	This study
QT2117	QT1911::Tn7T-Gm::*pstSCA*	This study
QT2138	CFT073 Δ*fimAICDFGH*::km; Km^r	This study
QT2207	QT1911 + pSTNSK	This study
QT2285	CFT073 *fimS* phase L-ON	This study
QT2305	QT1911 *fimS* phase L-ON	This study
QT2356	CFT073 + pKEN2	This study

QT2357	QT1911 + pKEN2	This study
S17-1(λpir)	λ*pir* lysogen of S17.1 (Tp^r Sm^r *thi pro hsdR$^-$M$^+$ recA* RP4::2-Tc::Mu-*km*::Tn7)	[284]
Salmonella enterica serovar Typhimurium SL1344	*rpsL hisG*	[285]
Plasmids		
pCP20	FLP helper plasmid Ts replicon; Ap^r Cm^r	[260]
pGP704	oriR6K mobRP4; Ap^r	[286]
pGP-Tn7-Gm	pGP704::Tn7T-Gm; Ap^r Gm^r	This study
pSTNSK	pST76-K::*tnsABCD*; Km^r	This study
pGP-Tn7-*pst*	pGP-Tn7-Gm::*pstSCA*	This study
pKD3	Template plasmid for the amplification of the *cat* gene bordered by FRT sites	[260]
pKD13	Template plasmid for the amplification of the km cassette bordered by FRT sites	[260]
pKD46	λ-Red recombinase plasmid Ts replicon; Ap^r	[260]
pKEN2	high copy phagemid constitutively expressing GFP, Ap^r	[287]
pST76-K	oriSC101 ts; Ap^r; Km^r	[288]
pTNS2	oriR6K; encodes the TnsABC+D specific transposition pathway; Ap^r	[289]
pUC18-mini-Tn7-Gm	pUC18-mini-Tn7-Gm (Gm^r on mini-Tn7; for gene insertion in Gm^s bacteria)	[289]

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance; Sm, streptomycin resistance; Tp^r, trimethoprim resistance.

FIGURES

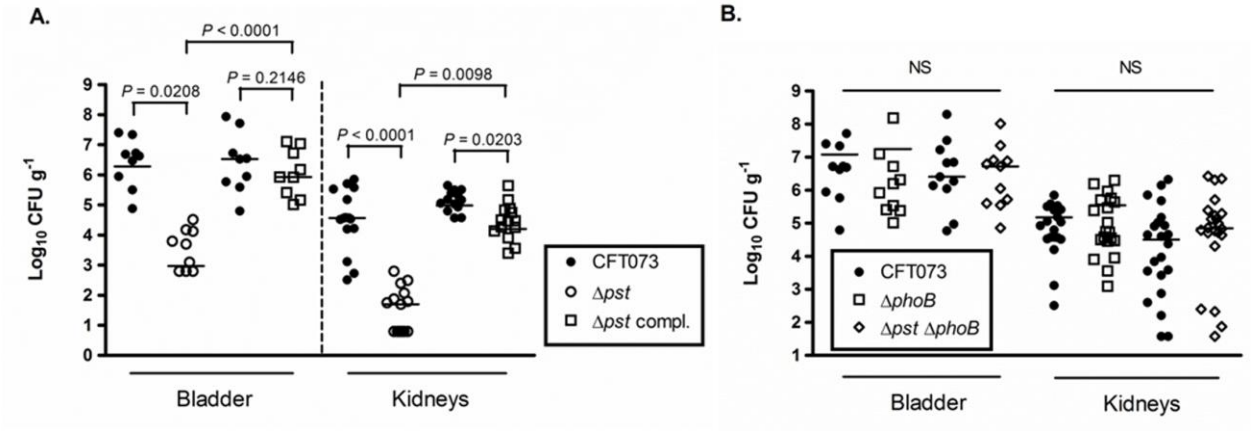

Figure 1. Inactivation of *pst* in uropathogenic *E. coli* CFT073 reduces competitive colonization of the mouse urinary tract. A. CBA/J mice were co-infected with a 1:1 ratio of CFT073 Δ*lac* and either the Δ*pst* mutant or the Δ*pst* complemented strain. B. CBA/J mice were co-infected with CFT073 Δ*lac* and Δ*phoB* or Δ*pst* Δ*phoB*. Results are presented as the Log_{10} CFU g^{-1}. Each data point represents a sample from an individual mouse and horizontal bars indicate the medians. Each kidney was sampled separately. A Wilcoxon signed-rank test (two-tailed) was used to determine statistical significance. The Mann-Whitney test was used to determine the statistical differences between the *pst* mutant and the complemented derivative strain.

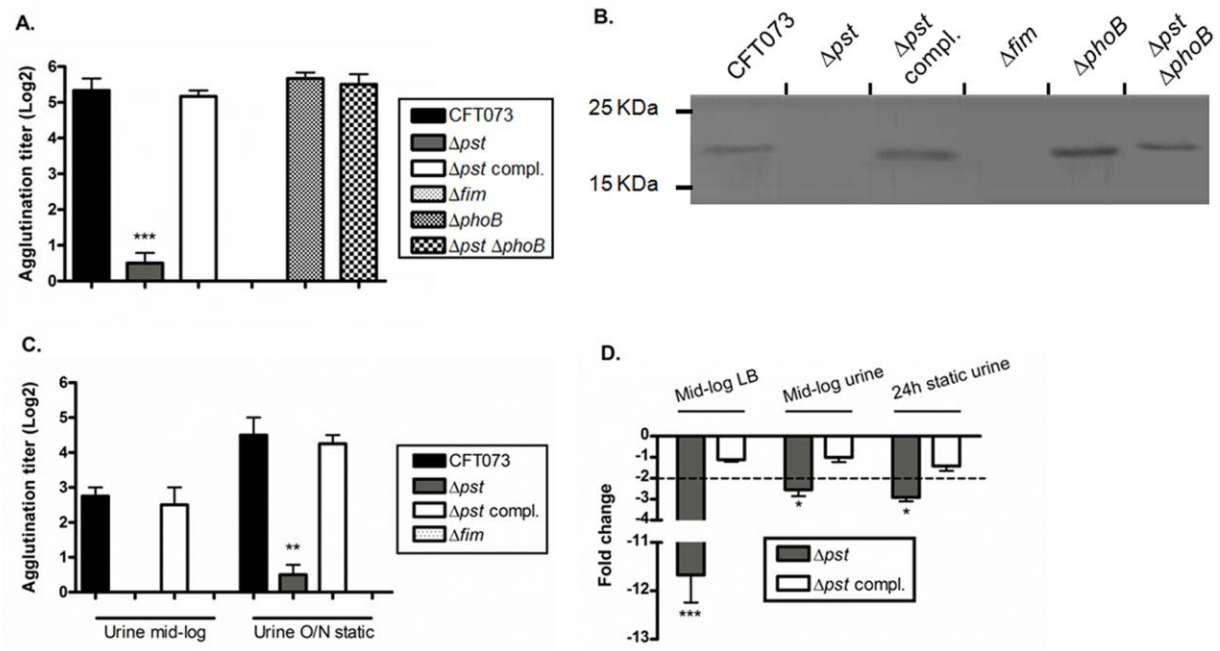

Figure 2. Production and transcription of type 1 fimbriae by uropathogenic *E. coli* CFT073 and derivative strains. (A) Production of type 1 fimbriae in strains cultured to mid-log phase of growth in LB broth. The Δ*fim* strain was used as negative control and showed no agglutination. (B) Western blot of fimbrial extracts of strains cultured to mid-log phase of growth in LB broth. (C) Production of type 1 fimbriae in strains cultured in human urine. (D) Expression of *fimA* in Δ*pst* and Δ*pst* compl. strains compared to WT CFT073 strain. The dashed line corresponds to the cut-off of a significant difference in expression. Results are the mean values and standard deviations of four biological experiments. Statistical significance was calculated by the Student's *t*-test, * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0001$.

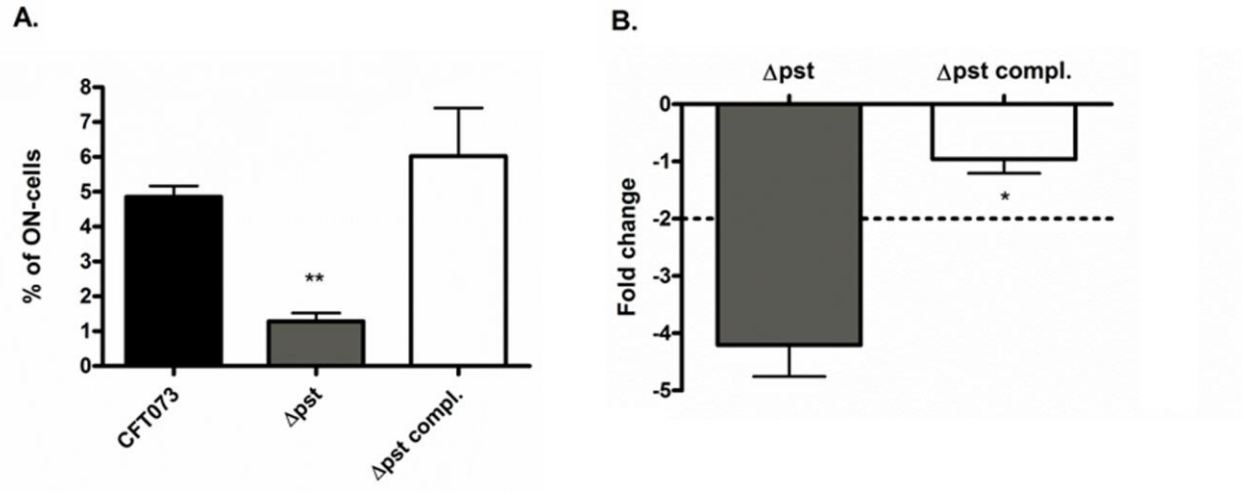

Figure 3. Effect of inactivation of *pst* on orientation of the *fim* promoter (*fimS*) *in vitro*. (A) Quantification of the percentage of cells with *fimS* in the ON-orientation. (B) ON- orientation of *fimS* was calculated by qPCR and was compared to the WT strain. The dashed line corresponds to the cut-off of a significant difference in expression. Results are the mean values and standard deviations of three biological experiments. Statistical significance was calculated by the Student's *t*-test, * $P < 0.05$; ** $P < 0.001$.

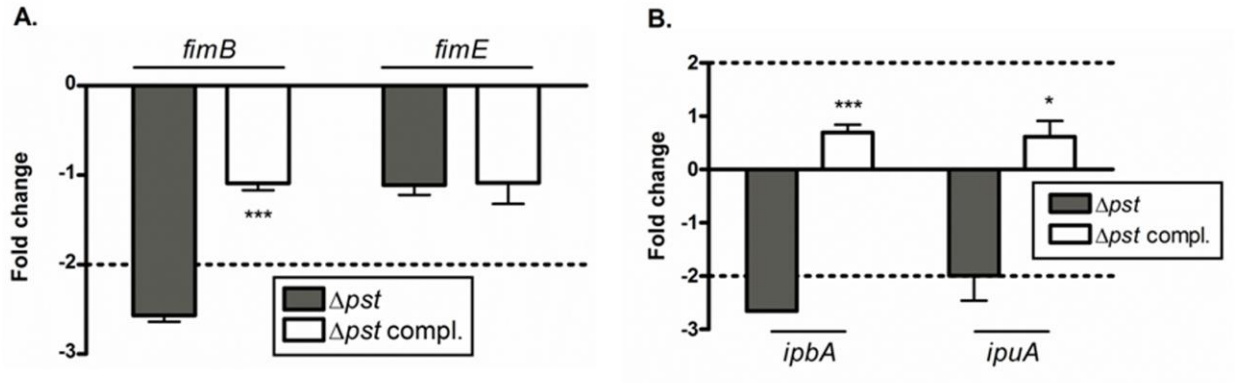

Figure 4. Effect of inactivation of *pst* on *fimS* regulators. Expression of the *fimB* and *fimE* recombinases (A) and *fim*-like recombinases *ipbA* and *ipuA* (B) compared to the WT strain. The dashed line corresponds to the cut-off of a significant difference in expression. Results are the mean values and standard deviations of three biological experiments. Statistical significance was calculated by the Student's *t*-test, * $P < 0.05$; *** $P < 0.0001$.

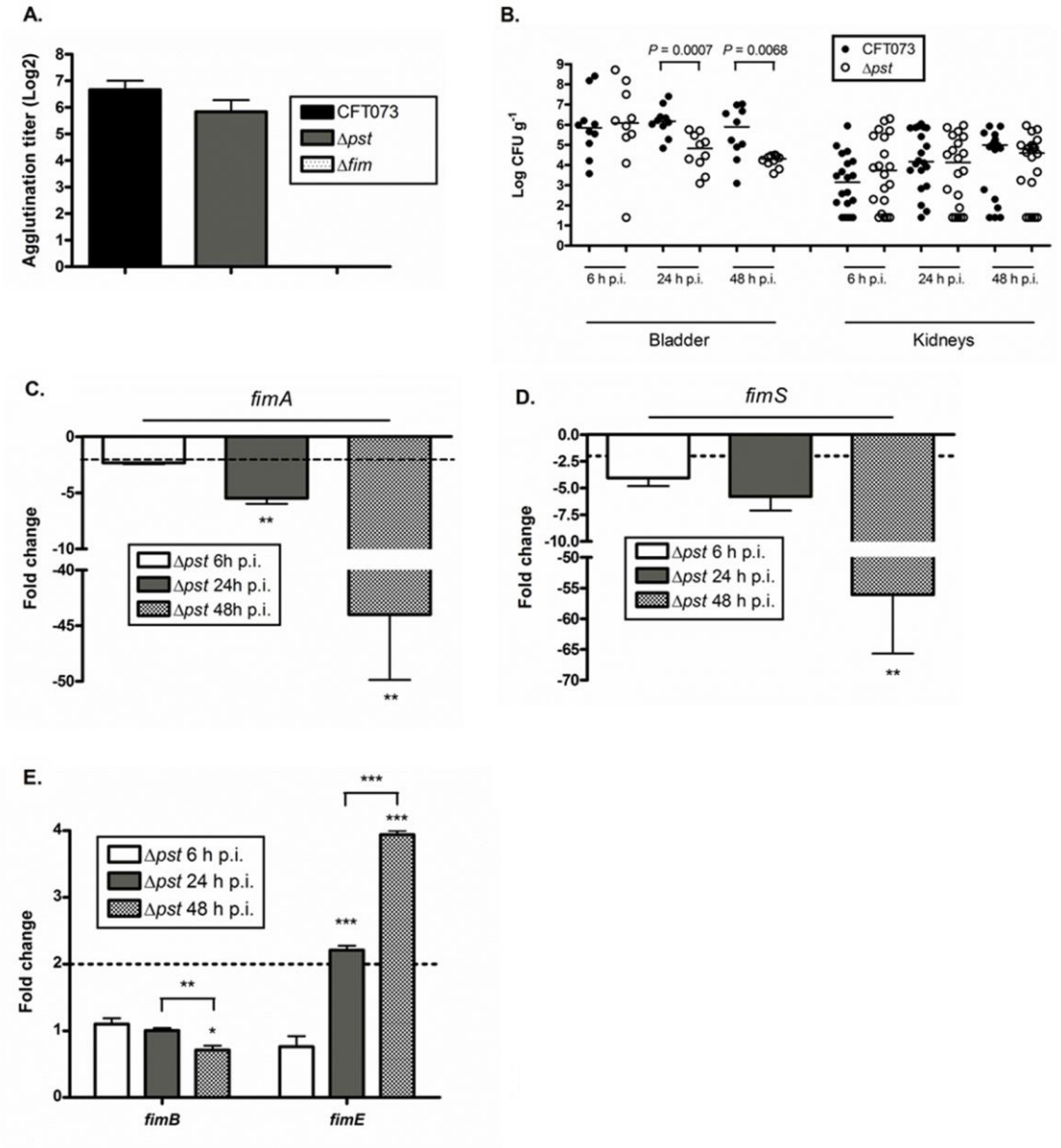

Figure 5. Inactivation of *pst* reduces colonization and results in differential expression of *fim* genes by uropathogenic *E. coli* CFT073 in the mouse bladder. (A) Production of type 1 fimbriae *in vitro* prior to inoculation. The Δ*fim* mutant was used as negative control since it did not mediate yeast agglutination. (B) Time course infection of CBA/J mice. Bacterial numbers are presented as the Log_{10} CFU g^{-1}. Each data point represents a sample from an individual mouse and horizontal bars indicate the median values. Each kidney was sampled separately. (C) Expression of *fimA* in infected bladders in the *pst* mutant compared to the WT strain. (D) Orientation of the *fim* promoter (*fimS*) in infected bladders in the *pst* mutant compared to the WT strain. (E) Transcription of the *fim* recombinases *fimB* and *fimE* in infected bladders in the *pst* mutant compared to the WT strain. The dashed line corresponds to the cut-off of a significant difference in expression. All results shown are the mean values and standard deviations of each bladder infected with the WT and the *pst* mutant strain. Statistical significance was calculated by the Student's *t*-test (A, C, D and E), * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0001$ and by the Mann-Whitney test (B).

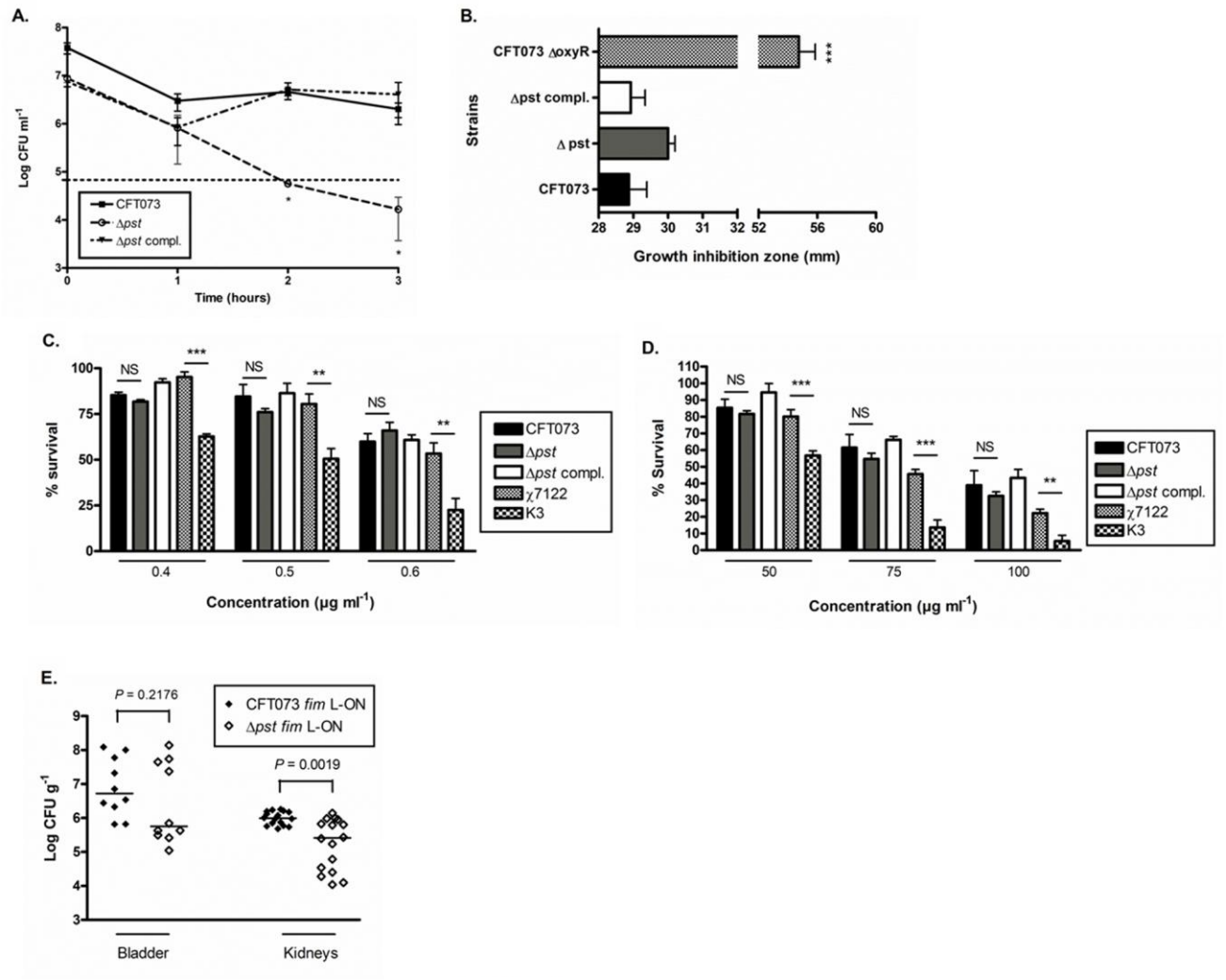

Figure 6. Altered expression of type 1 fimbriae is linked to attenuation of the *pst* mutant. (A) Resistance to 90% human serum. The MG1655 was used as negative control and was sensitive at time 1h. The dashed line corresponds to the intermediate resistance limit. (B) Resistance to hydrogen peroxide. The CFT073Δ*oxyR* was used as negative control. (C) Resistance to polymixin B. The χ7122 and K3 strains were used as positive and negative controls, respectively. (D) Resistance to vancomycin. The χ7122 and K3 strains were used as positive and negative controls, respectively. (E) Mono-infection of CBA/J mice with CFT073 and Δ*pst fim* L-ON derivative strains. Bacterial numbers are presented as the Log_{10} CFU g^{-1}. Each data point represents a sample from an individual mouse and horizontal bars indicate the median values. Results from A-D are the mean values and standard deviations of four biological experiments. Statistical significance was calculated by Student's *t*-test (A-D), * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0001$, and by the Mann-Whitney test (E).

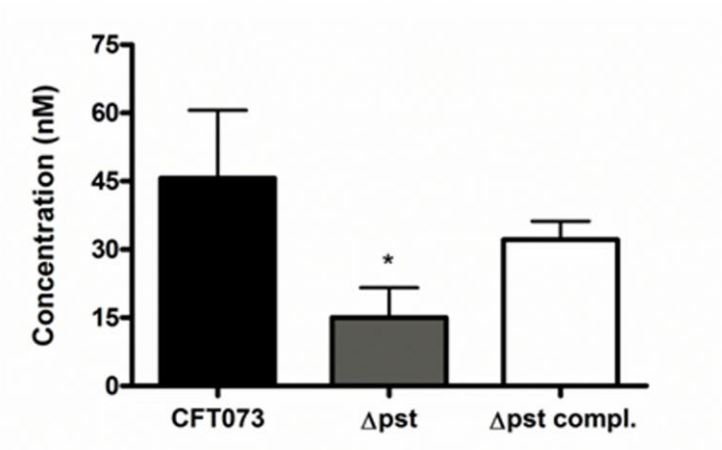

Figure 7. Production of ppGpp in the *pst* mutant. (A) Production of ppGpp in strains grown to mid-log phase of growth in LB medium. All results shown are the mean values and standard deviations of three biological experiments. Statistical difference was calculated by one-way ANOVA with Bonferroni's Multiple Comparison Test

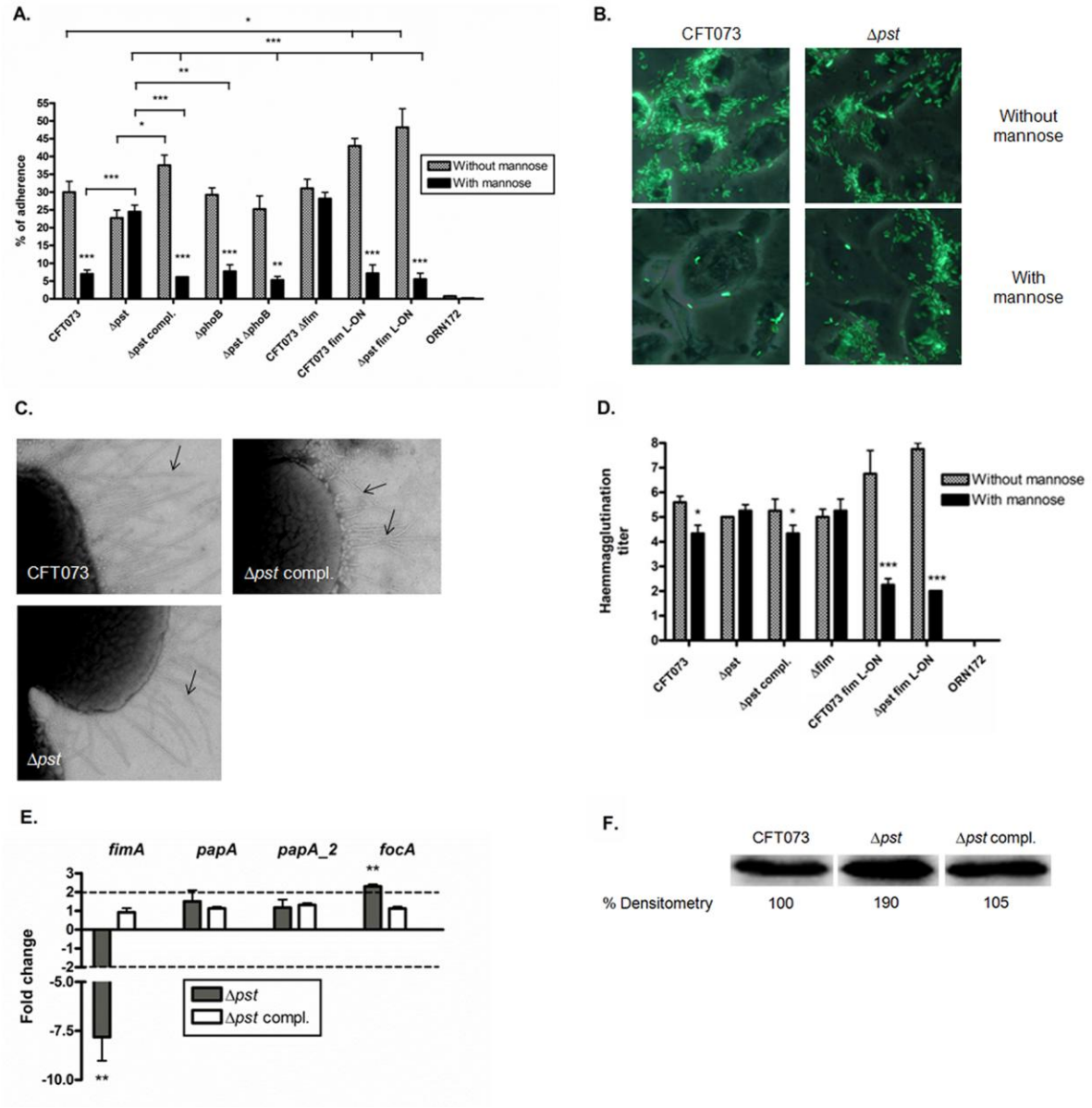

Figure 8. Effect of inactivation of *pst* and production of type 1 fimbriae on adherence of uropathogenic *E. coli* CFT073 to human bladder epithelial cells *in vitro*. (A). Adherence of strain CFT073 and its derivatives to human 5637 bladder epithelial cells in presence or absence of 1.5% α-D-mannopyranose. The *fim*-negative *E. coli* K-12 strain ORN172 strain was used as negative control. (B) Fluorescence microscopy of infected 5637 cells with CFT073-GFP and Δ*pstSCA*-GFP strain (magnification, X200). Images are a representative field. (C) Electron microscopy of CFT073, Δ*pstSCA* and Δ*fim* strains, respectively, at 12 000X. Images show a typical field of view of bacteria. Arrows show fimbriae on cell surfaces. (D) Production of Pap fimbriae by haemagglutination assay. (E) Transcription of *fimA*, *papA* major sub-units (*papA* and *papA_2*) and *focA* (F1C fimbriae-encoding) from bacteria adhered to 5637 bladder cells compared to the WT CFT073 strain. The dashed line corresponds to the cut-off of a significant difference in expression. (F) Western blot of fimbrial extracts using F1C-specific (anti-$F165_2$) antiserum. Densitometry of band from the WT CFT073 strain was considered as 100%. Bands and densitometry are from a representative gel. All results shown are the mean values and standard

deviations of four biological experiments. Statistical significance was calculated by the Student's *t*-test (A, D, E and F), * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0001$.

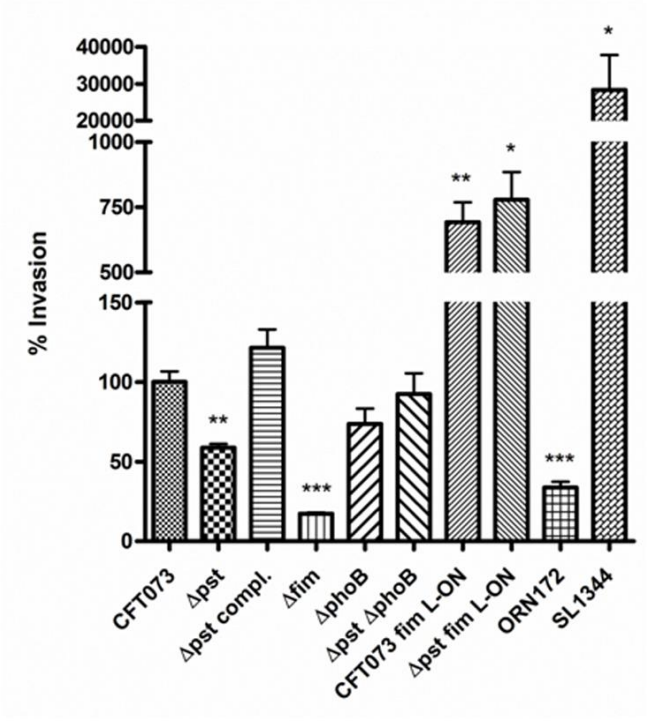

Figure 9. Invasion assay of 5637 bladder cells with UPEC CFT073 and derivative strains. The *Salmonella* Typhimurium SL1344 strain was used as a positive control for invasion. All results shown are the mean values and standard deviations of four biological experiments. Statistical significance was calculated by the Student's *t*-test, * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0001$.

Supplemental Table

Table S1. Primers used in this study

Primers	Direction	Characteristic(s)	Sequence 5'→ 3'
CMD1045	Forward	Amplification of kanamycin gene from pKD13 vector (used with CMD1046)	TCT CGT AGC CAA CAA ACA ATG CTT TAT GAA TCC TCC CAG GAG ACA TTA TGA TTC CGG GGA TCC GTC GAC C
CMD1046	Reverse		AAG GCG TTT ACG CCG CAT CCA GCA AGG TGT TAA CCG TGT TTA TTC TTC GCT GTA GGC TGG AGC TGC TTC G
CMD1067	Forward	Contains a MfeI restriction site; Amplification of the left and right Tn7 arms (used with CMD1068)	TGC GGT CAA TTG TAC CGC ACA GAT GCG TAA GGA GAA
CMD1068	Reverse	Contains a PspXI restriction site	AAC GCC GCT CGA GTT TAT AGT CCT GTC GGG TTT CGC CA
CMD1069	Forward	Contains a XhoI restriction site; *pstSCA* genes amplification (used with CMD1070)	TAT CTC GAG AAT CAA ACA TCC TGC CAA CTC CAT GT
CMD1070	Reverse	Contains a XmaI restriction site	TCG CCC GGG ATG CAC TTC GCG AAT CAG GCT TAC
CMD1072	Forward	*glmS-pstA* region amplification (used with CMD1070)	CAC CAA TCT TCT ACA CCG TTC CGC
CMD1258	Forward	Amplification of the *fimS* region (used with CMD1259)	TCG TTT TGC CGG ATT ATG GG
CMD1259	Reverse		AGT GAA CGG TCC CAC CAT TAA CC
CMD1246	Forward	Amplification of a *fimE*-IE region (used with CMD1248)	ACC GTA ACG CAG ACT CAT CCT CAT
CMD1247	Reverse	Amplification of a IE-*fimA* region (used with CMD1248)	TGA ACG GTC CCA CCA TTA ACC G
CMD1248	Forward or Reverse		TCA CAT CAC CTC CGC TAT ATG T
CMD96	Forward	Amplification of *vat* gene region (used with CMD97)	AAC GGT TGG TGG CAA CAA TCC
CMD97	Reverse		AGC CCT GTA GAA TGG CGA GTA
CMD1186	Reverse	Amplification of ON-orientation of *fimS* (used with CMD1185)	[§] AAAGTTTGTGCGCGATGCTTTCCTCT ATGAGTCAAAATAGATCTCCTTGATGG GAATTAGCCATGGTCC
CMD1185	Forward		TGGGAAAGAAATAATCTCATAAACGAA AAATTAAAAAGAGAAGAAGTTTGGTGT AGGCTGGAGCTGCTTC

CMD1135	Forward	Amplification of the *cat* gene from the pKD3 vector to phase locked-ON the *fim* (*fimS*) promoter (used with CMD1136)	CGA CTG CCC ATG TCG ATT TAG TTT TTT TAA AGG AAA GCA GCA TGT GTA GGC TGG AGC TGC TTC G
CMD1136	Reverse		ACC TGC ATT AGC AAT GCC CTG TGA TTT CTT TAT TGA TAA ACA AAA GTC ACA TTC CGG GGA TCC GTC GAC C
CMD1045		Amplification of the *km*^{r} cassette from pKD13 to delete the *pstSCA* genes (used with CMD1046)	TCT CGT AGC CAA CAA ACA ATG CTT TAT GAA TCC TCC CAG GAG ACA TTA TGA TTC CGG GGA TCC GTC GAC
CMD1046			AAG GCG TTT ACG CCG CAT CCA GCA AGG TGT TAA CCG TGT TTA TTC TTC GCT GTA GGC TGG AGC TGC TTC G
CMD1415	Forward	Contains a BspH1 restriction site; Amplification of *fimB* (used with CMD1416)	CAGACCTCATGAAGAATAAGGCTGATA ACAAA
CMD1416	Reverse	Contains a Pst1 restriction site	CTA TCT CTG CAG CTA TAA AAC AGC GTG ACG CTG TCG
CMD1417	Forward	Contains a BspH1 restriction site; Amplification of *fimE* (used with CMD1418)	GTACTTCATGAGTAAACGTCGTTATCT TACC
CMD1418	Reverse	Contains a Pst1 restriction site	CAA TAA CTG CAG TCA AAC TTC TTC TCT TTT TAA TTT
CMD1453	Forward	*relA* gene amplification (used with CMD1254)	TTGCCTGGCTGCGTAAACTGATTG
CMD1454	Reverse		TGTCGTTGATTTGCCTGCGGGATCAA
CMD1455	Forward	*spoT* gene amplification (used with CMD1256)	AAGCGGTGAACCCTATATCACGCA
CMD1456	Reverse		AGGTGGCGGGTGTATCTTCAATCA
qRT-PCR			
CMD1254	Forward	*fimE* gene amplification (used with CMD1255)	TCGGCATGGGATGCGTATTAGTGA
CMD1255	Reverse		TTTCCAGTTAGCACGTTCCTGGGT
CMD1256	Forward	*fimB* gene amplification (used with CMD1257)	ACGGAGTTGAAGGAGACTGGCTTT
CMD1257	Reverse		GACACCATTGTCAGCCAGAGCAAA
CMD1267	Forward	*fimA* gene amplification (used with CMD1268	ACC GTT CAG TTA GGA CAG GTT CGT
CMD1268	Reverse		CGA GAG CCA GAA CGT TGG TAT

CMD1419	Forward	*ipuB* gene amplification (used with CMD1420)	TGCTTAGGCATGGTTGTGGGTATG
CMD1420	Reverse		ACACATCACCCTCCCATACTCGTT
CMD1421	Forward	*ipuA* gene amplification (used with CMD1422)	CCCGTGGTTATTTGTTTCCCGAAC
CMD1422	Reverse		ACGGGTATCCACACCATTATCTGC
CMD1423	Forward	*ipbA* gene amplification (used with CMD1424)	ACGGAGTTGAAGGAGACTGGCTTT
CMD1424	Reverse		GACACCATTGTCAGCCAGAGCAAA
CMD1425	Forward	*leuX* gene amplification (used with CMD1426)	CCGAAGTGGCGAAATCGGTAGA
CMD1426	Reverse		TGCCGAAGGCCGGACTCGAA
CMD1427	Forward	*ihf* gene amplification (used with CMD1428)	TTAGCAAGCGGGATGCCAAAGAAC
CMD1428	Reverse		TTACGTCCCGGGCGTTGATTCTTA
CMD1429	Forward	*hns* gene amplification (used with CMD1430)	AACGAACGTCGCGAAGAAGAAAGC
CMD1430	Reverse		AGCTTTGGTGCCAGATTTAACGGC
CMD1431	Forward	*papB* gene amplification (used with CMD1432)	TCTGGTATTTCTCGCAGACCTCCT
CMD1432	Reverse		TGTTGCCCGGCTCTATGTCTGAAA
CMD1433	Forward	*focB* gene amplification (used with CMD1434)	TCCGGCTCCATGTCTGAAGAACAA
CMD1434	Reverse		GCGCCCAAGCGTTGTACTGAAATA
CMD1435	Forward	*rscB* gene amplification (used with CMD1436)	TACATCAAGCGCCATTTCCCAAGC
CMD1436	Reverse		AAATCGGTCGGTGCACCTTGTTTC
CMD1437	Forward	*crp* gene amplification (used with CMD1438)	TAAAGGCTCTGTGGCAGTGCTGAT
CMD1438	Reverse		TACGTTCCTGGCCCTCTTCAAACA
CMD1439	Forward	*cyaA* gene amplification (used with CMD1440)	TGAAGAGTACTTTGGTGCCAGCCT
CMD1440	Reverse		TCATGCAGTATGGATCGAGGCCAA
CMD1441	Forward	*qseB* gene amplification (used with CMD1442)	GCGAACCCTTAACGCTGAAACCAA
CMD1442	Reverse		CTTCAATCAGTTTGCGCGGCAGTA
CMD1443	Forward	*nanR* gene amplification (used with CMD1444)	AATCTTCATGGCGATCCACGTTGC
CMD1444	Reverse		GCGTCAACGATCGCAATATGCTGT

CMD1445	Forward	*nagC* gene amplification (used with CMD1446)	GCAACTTTGGCTGCCTGGAAACTA
CMD1446	Reverse		CCTTTGTTCGCGGCTTTGCAGATA
CMD1447	Forward	*papA* gene amplification (used with CMD1448)	ATTTGATGGTGCGACAGCAACAGG
CMD1448	Reverse		TCTGTTACAGGGTTGCCACTACCA
CMD1449	Forward	*papA*_2 gene amplification (used with CMD145)	CGGGTGAAATTTGATGGAGCCACT
CMD1450	Reverse		AGGCACCTTCAGCTACATTCTTGC
CMD1451	Forward	*focA* gene amplification (used with CMD1452)	TGTCTGCTGGTGCAGGAATTGTCT
CMD1452	Reverse		ACAACCTTGCCGGTATGGTCAGTA

[§] Underlined nucleotides in red of primers CMD1186 were exchanged from the original sequence to lock *fimS* in the ON-phase.

Supplemental Figures

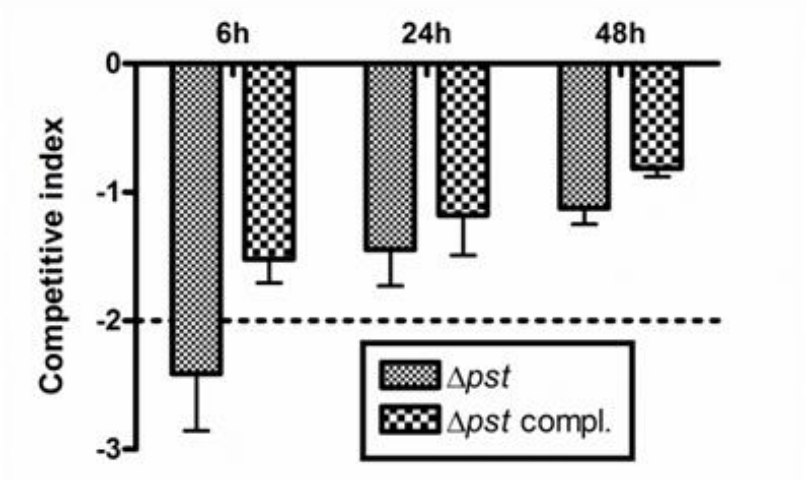

Figure S1. *In vitro* competitive assay of WT CFT073 with Δ*pst* and Δ*pst* compl. derivative strains. The dashed line corresponds to the cut-off of significant out-competition. A strain was considered as out-competed if the Competitive index showed more than a 2-fold difference. All results shown are the mean values and standard deviations of four biological experiments. Statistical significance was calculated by Student's *t*-test

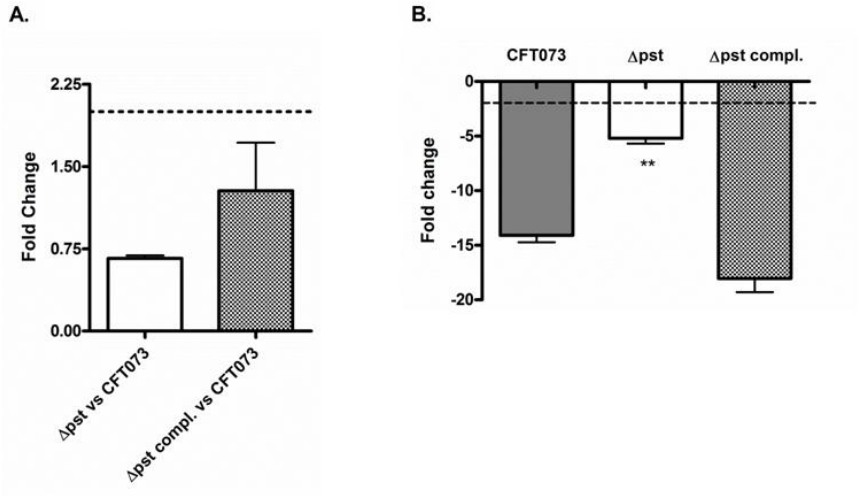

Figure S2. Expression of *fimA* during phosphate starvation. (A) Comparison of *fimA* transcription between WT strain CFT073 and its derivatives grown in MOPS LP medium. (B) Comparison of *fimA* expression in strains grown in MOPS LP vs MOPS HP media. The dashed line corresponds to the cut-off of a significant difference in expression All results shown are the mean values and standard deviations of four biological experiments. Statistical significance was calculated by Student's *t*-test, ** $P < 0.005$.

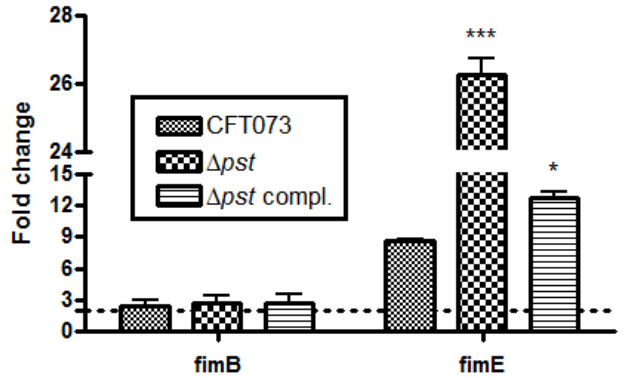

Figure S3. Comparative expression of *fimB* and *fimE* among strains cultured in human urine and LB broth. The dashed line corresponds to the cut-off of a significant difference in expression in human urine compared to LB broth. All results shown are the mean values and standard deviations of four biological experiments. Statistical significance was calculated by Student's *t*-test, * $P < 0.05$., *** $P < 0.001$

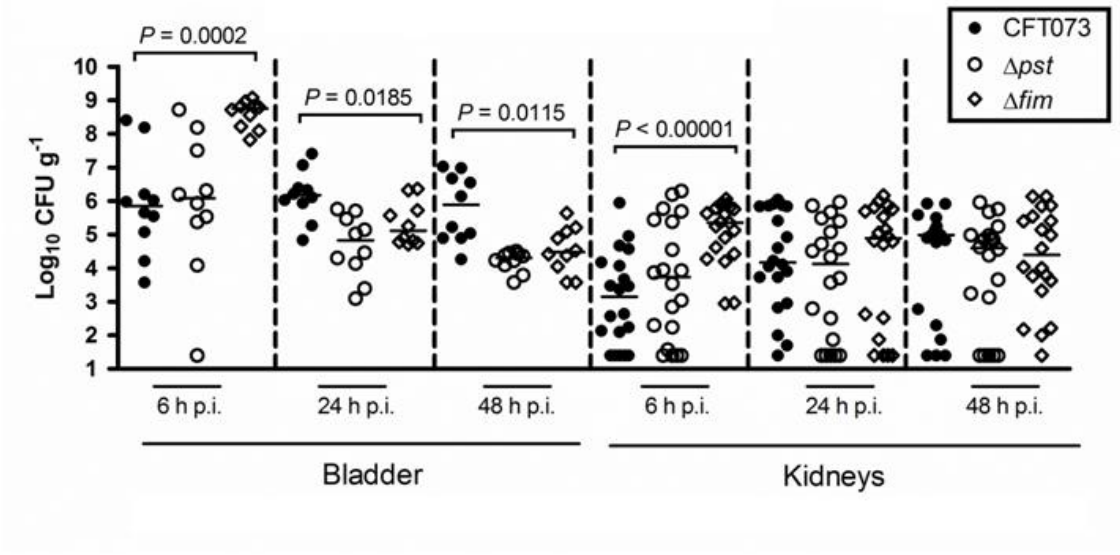

Figure S4. Comparative colonization of the murine urinary tract by CFT073, Δ*pst* and Δ*fim* strains. Results are presented as the Log_{10} CFU g^{-1}. Each data point represents a sample from an individual mouse and horizontal bars indicate the medians. Each kidney was sampled separately. The Mann-Whitney test was used to determine the statistical differences between the *fim* mutant and the WT strain.

Article #3

Altered regulation of the Diguanylate Cyclase YaiC reduces production of Type 1 Fimbriae in a Pst Mutant of Uropathogenic *E. coli* CFT073

Short title: The c-di-GMP pathway decreased expression of type 1 fimbriae

Sébastien Crépin, Gaëlle Porcheron, Sébastien Houle, Christine Martin, Josée Harel, Charles M. Dozois (2012) Article en préparation et sera soumis à Molecular Microbiology au cours de l'automne

A) Objectif et approche utilisée

1- Dans l'article #2, nous montrons que l'inactivation du système Pst atténue la virulence de la souche UPEC CFT073. Cette atténuation est la conséquence de l'induction constitutive du SRDC PhoBR qui elle, mène à la répression des fimbriae de type 1, facteur de virulence essentiel à l'établissement de l'infection chez les UPEC. Aussi, nous avons émis l'hypothèse que l'effet de PhoB, sur la répression des fimbriae de type 1, est indirecte. Notre hypothèse était donc que l'activation constitutive de PhoBR, causée par l'inactivation du système Pst, induit un/des répresseur(s), connu(s) où non, des fimbriae de type 1. Donc, l'objectif de cette étude en préparation était de déterminer les mécanismes moléculaires reliant le système Pst (régulon Pho) et l'expression des fimbriae de type 1. Pour ce faire, une banque de mutants à l'aide du transposon aléatoire *Tn*10 a été construite chez le mutant *pst*. Nous avons par la suite criblé les mutants pour leur capacité à produire les fimbriae de type 1. Dans le présent article, nous décrivons plus spécifiquement l'effet du système Pst sur le gène *yaiC*, impliqué dans la biosynthèse du c-di-GMP, ainsi que son rôle dans la régulation des fimbriae de type 1.

2- L'étude a été réalisée en utilisant une approche multidisciplinaire regroupant des techniques de génétique bactérienne, de biologie moléculaire, le modèle murin d'UTI, de biologie cellulaire, de chromatographie en phase liquide à haute performance et de spectrométrie de masse.

B) Contribution de l'étudiant

J'ai réalisé, avec l'aide de mes directeurs, toutes les étapes de conceptions de l'étude. J'ai réalisé toutes les expérimentations, à l'exception du dosage du c-di-GMP, qui a été réalisée par le groupe du Dre Martin. Sébastien Houle m'a assisté dans les infections dans le modèle murin d'UTI. J'ai écrit la totalité de l'article, qui a été corrigé par mes directeurs et mes collaborateurs.

C) Résumé de l'article :

Le système Pst code pour le système de transport spécifique du phosphate (Pst). En plus d'activer constitutivement le système de régulation à deux composants PhoBR, l'inactivation du système Pst atténue la virulence des souches pathogènes. Chez les souches UPEC, la répression des fimbriae de type 1 contribue majoritairement à cette atténuation. Cependant, les mécanismes moléculaires reliant le système Pst et les fimbriae de type 1 ne sont pas connus. Afin d'élucider ces mécanismes, une banque de mutants, à l'aide du transposon aléatoire *Tn*10, a été construite chez le mutant *pst*. Nous avons par la suite criblé les mutants pour leur capacité à produire les fimbriae de type 1. Parmi ces clones, le gène *yaiC*, codant pour une diguanylate cyclase, relie le système Pst et les fimbriae de type 1. Chez le mutant *pst*, l'induction d'*yaiC* réprime l'expression des fimbriae de type 1 et ce, en modulant l'expression des recombinases *ipuA* et *ipbA*. De plus, l'inactivation d*'yaiC*, chez le mutant *pst*, rétabli l'adhésion aux cellules urothéliales ainsi que la virulence du mutant *pst*. En introduisant des mutations spécifiques, nous montrons que *yaiC* est directement, et positivement, régulé par PhoB puisque *yaiC* se trouve sur le même transcrit que l'opéron *phoA-psiF*. Plus précisément, la transcription de *yaiC* est dépendante du promoteur du gène *phoA*. L'induction de *yaiC*, chez le mutant *pst*, est reliée à l'accumulation du c-di-GMP chez le mutant Pst. De plus, cette accumulation semble être reliée à la répression des fimbriae de type 1. Ainsi, nos résultats suggèrent qu'un des mécanismes par lequel l'inactivation du système Pst réprime l'expression des fimbriae de type 1 est via le système à deux composant PhoBR, qui active la transcription de *yaiC*, entraînant ainsi l'accumulation du c-di-GMP, ce qui cause l'inhibition de l'expression des fimbriae de type 1 et donc, atténue la virulence dans le modèle murin d'UTI.

Altered regulation of the Diguanylate Cyclase YaiC reduces production of Type 1 Fimbriae in a Pst Mutant of Uropathogenic *E. coli* CFT073

Short title: Inactivation of Pst deregulates the c-di-GMP pathway and decreases expression of Type 1 fimbriae

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SUMMARY

The *pst* gene cluster encodes the phosphate specific transport system (Pst). Inactivation of the Pst system constitutively activates the two-component regulatory system PhoBR and attenuates virulence of pathogenic bacteria. In uropathogenic *E*. *coli* strain CFT073, attenuation by inactivation of *pst* is predominantly attributed to the decreased expression of type 1 fimbriae. However, the molecular mechanisms connecting the Pst system and type 1 fimbriae are unknown. To address this, a transposon library was constructed in the *pst* mutant, and clones tested for a regain in type 1 fimbriae production. Among them, the diguanylate cyclase encoded by *yaiC* (*adrA*) was identified to link the Pst system and type 1 fimbrial expression. In the *pst* mutant, *yaiC* was induced and decreased expression of type 1 fimbriae by predominantly altering expression of the FimBE-like recombinases *ipuA* and *ipbA*, affecting at the same time, the inversion of the *fim* promoter. In the *pst* mutant, inactivation of *yaiC* restored *fim*-dependent adhesion to bladder cells and virulence. Interestingly, expression of *yaiC* was activated by PhoB since transcription of *yaiC* was linked to the PhoB-dependent *phoA-psiF* operon. As YaiC is involved in c-di-GMP biosynthesis, an increased accumulation of c-di-GMP was observed in the *pst* mutant. Hence, results suggest that one mechanism by which deletion of the Pst system reduces expression of type 1 fimbriae is through PhoBR-mediated activation of *yaiC*, which in turn increases accumulation of c-di-GMP, represses the *fim* operon and consequently, attenuates virulence in the mouse urinary tract infection model.

INTRODUCTION

An important aspect of bacterial physiology and virulence is the capacity to sense environmental signals. Sensing the environmental changes leads to adaptation; since genes responding to these changes will be specifically and coordinately regulated. Two-component signal transduction systems (TCSs) are one of the mechanisms by which bacteria respond to environmental signals. TCSs comprise an inner-membrane histidine kinase sensor protein and a cytoplasmic response regulator [151].

The TCS PhoBR comprises PhoR, which encodes the sensor histidine kinase, and PhoB, the response regulator, and responds to phosphate limitation, i.e. when the extracellular phosphate concentration falls below 4 μM [4, 152]. Thereby, PhoBR regulates genes belonging to the Pho regulon, such as those mediating phosphate transport and metabolism. Genes belonging to the Pho regulon possess PhoB-binding specific DNA sequences, known as Pho boxes, located within their promoter [4, 144, 147, 152]. During phosphate limitation, the TCS PhoBR is activated, and PhoB binds to Pho boxes to induce or repress gene expression [4, 152]. The periplasmic alkaline phosphatase (PhoA), which catalyses the hydrolysis and transphosphorylation of a wide variety of phosphate monoesters, and the phosphate specific transport (Pst) system, an ATP-binding cassette (ABC) transporter specific for inorganic phosphate (P_i), are among members of the Pho regulon [4, 152]. In addition to being involved in transport of P_i, the Pst system negatively regulates the activity of PhoBR, as disruption of Pst constitutively activates PhoBR regardless of environmental phosphate availability [4, 152]. Thus, inactivation of the *pst* system mimics phosphate limiting conditions. Moreover, the Pst system is also linked with pathogenicity, as its deletion attenuates virulence of pathogenic strains [5, 6]. Recently, we demonstrated that attenuation of an uropathogenic *Escherichia coli* (UPEC) *pst* mutant is mainly attributed to the decreased expression of type 1 fimbriae [290].

In UPEC strains, type 1 fimbriae are a key virulence factor and are required to establish infection [13, 14]. Type 1 fimbriae are expressed in the bladder and in addition to promoting adhesion to bladder cells and its colonization, they are also involved in invasion of bladder cells [58, 60, 61, 257]. Type 1 fimbriae are encoded by the *fimAICDFGH* operon (*fim*), where *fimA* encodes the major subunit and *fimH*, the mannose-specific adhesin [45]. Expression of the *fim* operon

depends on a promoter located on an invertible element (*fimS*) [62]. Expression of type 1 fimbriae is therefore subjected to phase variation. Expression of type 1 fimbriae is mediated by the switching of *fimS* between the ON- and OFF-orientations. Orientation of *fimS* is mainly controlled by the FimB and FimE recombinases [67]. FimB mediates switching in both directions, from phase-OFF to phase-ON and phase-ON to phase-OFF, where the ON-orientation is favored, while FimE promotes switching to the OFF-orientation, i.e. from phase-ON to phase-OFF [67, 258]. In addition to FimB and FimE, the CFT073 UPEC strain encodes FimBE-like recombinases, IpuA and IpbA (*fimX* in UPEC UTI89 strain) [65, 66]. IpuA promotes switching like FimB, whereas IpbA only promotes the switching to the ON-position [65, 66]. Furthermore, IpuA and IpbA are sufficient for switching *fimS* and influencing type 1 fimbriae expression *in vitro* and *in vivo* [65, 66].

Cyclic di-GMP (c-di-GMP) is a bacterial second messenger that controls various processes, e.g. flagellar motility, biofilm formation, the cell cycle and virulence [291]. c-di-GMP is synthesized by diguanylate cyclase proteins, which contain GGDEF domains, and is degraded by phosphodiesterase proteins, which contain EAL or HD-GYP domains. c-di-GMP acts via a variety of receptors containing PilZ or I-site domains [292] and acts by influencing the transcriptional, translational and post-translational regulation [230]. Its role in cellular processes is well studied in *Salmonella*, *Vibrio*, *Yersinia* and *Pseudomonas* species. In *Salmonella*, AdrA (homolog of YaiC in *Escherichia coli*) is one of the major diguanylate cyclases. By activating the biosynthesis of c-di-GMP, AdrA induces the formation of biofilm by increasing the production of cellulose through the induction of the *bcs* (bacterial cellulose synthesis) operon [293-296]. AdrA is positively regulated by the curli biosynthesis regulator CsgD [293, 297, 298]. On its side, CsgD is regulated by more than 10 transcription factors and small regulatory RNAs [101, 116, 299-303], each responding to specific environmental conditions. Cellulose production, along with curli, forms an extracellular matrix favorable to surface adhesion, cellular aggregation, persistence in the environment and biofilm formation [101].

As mentioned above, deletion of the *pst* system attenuated virulence of the UPEC CFT073 strain by decreasing expression of the type 1 fimbriae [290]. An *in silico* analysis showed that genes involved in regulation of, or in biosynthesis of type 1 fimbriae do not possess Pho boxes in their promoter regions [147]. Given that the specific mechanisms by which induction of the

Pho regulon (PhoBR) inhibits expression of type 1 fimbriae are unknown and that the Pho regulon seems to act indirectly, the genes linking the Pho regulon and type 1 fimbriae were investigated herein by the construction of a transposon library in the *pst* mutant. In this study, we show that YaiC is one of the mediators in the *pst* mutant contributing to decreased expression of type 1 fimbriae. Indeed, *yaiC* is induced in the *pst* mutant and is likely a repressor of type 1 fimbriae. In the *pst* mutant strain, *yaiC* was activated by PhoB since it formed an operon with *phoA-psiF* and its transcription depended on the PhoB-dependent promoter of the *phoA-psiF* operon. An accumulation of c-di-GMP is observed in the *pst* mutant and is concomitant with the increased expression of *yaiC*. Thereby, the c-di-GMP pathway alters the expression of type 1 fimbriae and may contribute to the attenuated virulence of the *pst* mutant by down-regulating expression of type 1 fimbriae.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids and media

E. coli strains and plasmids used in this study are listed in Table 1. Bacteria were grown in Luria-Bertani (LB) broth at 37°C. Bacteria were also grown in MOPS minimal medium (Teknova) supplemented with 0.4% glucose, 0.2% $(NH_4)_2SO_4$, 1.32 mM K_2HPO_4, and 1 µg/mL thiamine (high phosphate). MOPS low phosphate medium contained 1 µM K_2HPO_4 [259]. 5637 bladder cells were grown in RPMI 1640 medium (Wisent Bioproducts) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate. Antibiotics and reagents were added as required at the following concentrations: kanamycin, 40 µg/ml; ampicillin, 100 to 200 µg/ml;

chloramphenicol, 30 µg/ml; gentamicin, 15 µg/ml; diaminopimelic acid (DAP), 50 µg/ml; 5-bromo-4-chloro-3 indolylphosphate di-sodium (BCIP), 40µg/ml and isopropyl β-D-1-thiogalactopyranoside (IPTG); 500 µM.

Transposon mutagenesis

Transposon mutagenesis was performed as described by Simms *et al*. [304]. Briefly, the donor strain MGN-617 (pLOF/Km) and recipient strain CFT073 Δ*pst* strains were cultured O/N at 37°C in LB broth with appropriate antibiotics and supplements. Cultures were gently mixed at a 1:4

donor/recipient ratio, placed onto LB agar plates supplemented with DAP and IPTG and incubated 5 h at 37°C. Following incubation, the bacterial lawn was suspended in 1 ml of PBS, washed twice in PBS, serially diluted, and spread onto LB agar plates supplemented with kanamycin and incubated O/N at 37°C to select the recovery of kanamycin-resistant transposon mutants of CFT073 Δ*pst*. Transconjugants were then screened for susceptibility to ampicillin (100 µg/ml) to confirm loss of the pLOF/Km vector.

Evaluation of type 1 fimbriae production

Production of type 1 fimbriae by transposon mutants was quantified by a modified yeast agglutination assay. The transposon mutants were cultured in 96-well microtiter plates to mid-log phase of growth. Following centrifugation, the pellet was suspended in 40 µl of PBS and transferred to other microtiter wells containing equal volumes of a 3% commercial yeast suspension, in PBS. After 30 min of incubation on ice, yeast aggregation was monitored visually, and the agglutination titer was recorded as the most diluted bacterial sample giving a positive aggregation reaction. Results were confirmed by classical yeast aggregation assay [8, 290], where a suspension of approximately 2 X 10^{11} cells ml^{-1} were grown at 37°C in LB broth to mid-log phase was serially diluted two-fold in microtiter wells with an equal volumes of a 3% commercial yeast suspension, in PBS. Agglutination titers represent the last 2-fold dilution showing agglutination. As an example, an agglutination titer of 4 corresponds to a 16-fold dilution.

Site-specific integration of Tn*10*

Transposon site-specific integration was identified as described by Nichols *et al*. [305] and as shown in Fig. 1. Chromosomal DNA isolated from the transposon mutants was digested with RsaI. The fragmented DNA was self-ligated with T4 DNA ligase (Fermentas) and a first round of inverted PCR, with primers Pri1 and Pri2 (Table S1 in supplemental material), was performed onto the circularized DNA. The PCR product was diluted 1/10 and 1 µl was used as template for a second round of inverted PCR using Pri3 and Pri4. PCR products from this second round were gel electrophoresed and fragments were sequenced (Genome Quebec Innovation Centre, McGill University). Blastn was used to identify the site-specific integration site in the genome of the CFT073 Δ*pst* strain.

Construction of non-polar mutants and complemented strains

All mutants were generated by the procedure described by Datsenko and Wanner using plasmid pKD3 as kanamycin resistance cassette [260]. Primers used are listed in Table S1 in Supplemental material. Antibiotic cassettes flanked by FRT sequences were removed by transforming the mutant strains with pCP20 expressing the FLP recombinase [261].

The Δ*pstSCA* Δ*yaiC* (DMY) strain was complemented as described by Crépin *et al*. [306], by inserting the *yaiC* gene or *phoA-psiF yaiC* genes, with their respective promoters into the chromosomal *att*Tn7 site, resulting in strain DMY compl. (*att*Tn7::*yaiC*; QT2222) and DMY compl. *A-C* (*att*Tn7::*phoA-psiF-yaiC*; QT2244), respectively. Briefly, *yaiC* and its native promoter were amplified with primers CMD1156 and CMD1157 (Table S1) and cloned into the XmaI and XhoI sites of pGP-Tn7-Gm, creating vector pGP-Tn7-Gm-*yaiC*. The *phoA-psiF-yaiC* region, with the promoter of *phoA*, were amplified with primers CMD1260 and CMD1261 and cloned into the XmaI and XhoI sites of pGP-Tn7-Gm, creating vector pGP-Tn7-Gm-*AC*. The DMY strain (pSTNSK) was conjugated overnight with strain MGN-617 (pGP-Tn7-Gm-*yaiC* or pGP-Tn7-Gm-*AC*) at 30°C on LB agar plates supplemented with DAP. After incubation, the bacterial lawn was suspended in 1 ml of PBS, washed twice in PBS, serially diluted, and spread on LB agar supplemented with gentamicin and incubated at 37°C. Colonies were verified for sensitivity to kanamycin and ampicillin, the expected phenotype for integration at *att*Tn7 and loss of the transposase containing plasmid pSTNSK. Tn7 insertion into the *att*Tn7 site was PCR verified using primers CMD1070 and CMD1072 (Table S1).

Changing nucleotides in the promoter (Pho box) of *phoA* was performed as described by Posfai *et al*. [288, 307]. The *phoA* gene and its upstream promoter (Pho box) were amplified with primers CMD1245 and CMD1336 and were cloned in pGEM-T vector (Promega), creating vector pGEM::P*phoA*. Exchange of 13 nucleotides into the Pho box of *phoA* was performed according to the QuikChange® Site-Directed Mutagenesis Kit (Stratagene) with primers CMD1334 and CMD1335. The exchanged nucleotides were confirmed by sequencing the vector with M13-specific primers. The resulting plasmid (pGEM::P*phoA* PM) was digested with SacI and SphI and cloned into the respective sites of pSG76-C, creating vector pSG76C::P*phoA*PM. Plasmid pSG76C::P*phoA*PM was introduced in strain CFT073 Δ*pst*, which contained the p-PIRK vector, and grown 5h at 30°C. This step allowed the replication of the pSG76C::P*phoA*PM vector as the

π protein is provided by p-PIRK. Following growth at 30°C, the bacterial suspension was spread on LB-Cm plates and grown 5h at 42°C and O/N at 37°C. This step was done to inhibit replication of pSG76C::P*phoA*PM, while selecting for integration by Cm selection, as the helper plasmid is unable to replicate at these temperatures. In this manner, pSG76C::P*phoA*PM integrates by single-crossover recombination at the homologous site, i.e. the *phoA* gene and its promoter (Pho box) of the CFT073 Δ*pst* strain. The double-crossover was achieved by introducing the pST76-ASceP vector in the resulting strain. Expression of SceP from pST76-ASceP introduces a double strand-break into pSG76C::P*phoA*PM, stimulating intramolecular recombination between the mutant and the WT allele [288]. Loss of pST76-ASceP is induced by growing the bacteria at 37°C or 42°C. The resulting strain was named P*phoA* PM.

Experimental UTI in CBA/J mice

Experimental infections were carried out using co-infection models as described by Hagberg *et al*. and Sabri *et al*. [263, 264]. Prior to inoculation, strains were grown 16 h at 37°C with shaking (250 rpm) in 55 ml of LB medium. Cultures were then centrifuged and pellets of the WT and derivative strains were mixed 1:1. Six-week-old CBA/J female mice were transurethrally inoculated with 20 µl of the 1:1 mixture containing $5X10^8$ CFU of UPEC CFT073 Δ*lacZYA* strain (QT1081) and $5X10^8$ CFU of either CFT073 Δ*pstSCA* (QT1911) strain or CFT073 Δ*pstSCA* Δ*yaiC* (DMY, QT2065). The CFT073 Δ*lac* strain is as virulent as the CFT073 wild-type parent and presented no statistical difference with the WT strain [264]. Furthermore, the Δ*lac* strain provided a differential Lac-negative phenotype on MacConkey agar plates. At 48 h p.i., mice were euthanized; bladders and kidneys were aseptically removed, homogenized, diluted and plated onto MacConkey agar to determine bacterial counts.

RNA extraction and quantification of gene expression

RNAs from bacterial cultures grown in LB broth or MOPS minimal medium to mid-log phase were extracted using TRIzol reagent (Invitrogen), according to the manufacturer's recommendations, with the exception that DNAse I treatment was performed twice. The iScript cDNA synthesis kit and the SsoFast Evagreen Supermix kit (Bio-Rad) were used for qRT-PCR according to the manufacturer's instructions. The *tus* gene was used as a housekeeping control [8]. Each qRT-PCR run was done in quadruplicate, and for each reaction the calculated

threshold cycle (*C*t) was normalized to the *C*t of the *tus* gene amplified from the corresponding sample. The fold-change was calculated using the $2^{-\Delta\Delta Ct}$ method [265]. Genes with fold change above or below the defined threshold of 2 is considered as differentially expressed.

Quantification of the ON/OFF state of the *fimS* region

Quantification of the orientation of the *fimS* switch was performed by quantitative PCR (qPCR) with iQ™ SYBR® Green Supermix (Bio-Rad) according to Crepin *et al*. [290]. The qPCR was performed on 10 ng of gDNA extracted from bacteria grown to mid-log phase of growth in LB broth. Primers CMD1246 and CMD1248 were used to amplify the ON-orientation while the CMD1247 and CMD1248 primers amplified the OFF-orientation. The threshold cycle (C_T) of the ON-and OFF-orientation was normalized to the C_T of the *vat* gene (amplified with primers CMD96 and CMD97), an uninvertible element. Fold change was calculated using the $2^{-\Delta\Delta Ct}$ method [265]. Fold change above or below the defined threshold of 2 was considered as differentially oriented.

Adhesion assay

Human bladder epithelial cell line 5637 (American Type Culture Collection HTB-9) was grown to confluency in 24-well plates in RPMI 1640. UPEC CFT073 and its derivative strains were grown in LB medium at 37°C to mid-log phase of growth (O.D. 0.6). The bacterial cells were centrifuged, washed twice with PBS, resuspended in RPMI 1640 medium (Wisent Biocenter, St-Bruno, Canada), supplemented with 10% fetal bovine serum, at 10^6 CFU ml^{-1} and added to each well. Bacteria-host cell contact was enhanced by a 5-min centrifugation at 600 x *g*. At 2h post-adhesion, cells were washed three times and lysed with PBS-0.1% sodium deoxycholate (DOC), serially diluted and plated onto LB agar plates. Quantification of cell-associated bacteria was performed as previously described [61, 308]. To block adherence mediated by type 1 fimbriae, 1.5% α-D-mannopyranose was added to culture medium.

Determination of intracellular c-di-GMP levels

c-di-GMP was quantified as described by Waters *et al*. [309]. Bacteria were grown in LB medium to mid-log phase of growth. After cell lysis, supernatants were collected and analyzed by liquid

chromatography-tandem mass spectrometry on a Finnigan TSQ Quantum DiscoveryMax on a Quattro-Micro triple quadrupole mass spectrometer (Waters Corporation, Milford), coupled with an Alliance HPLC system (Waters Corporation, Milford). Masslynx software from Waters was used for instrument control, data acquisition and data processing. c-di-GMP was detected by using selected reaction monitoring (SRM) in negative ionization mode at m/z 689.0/150.1. SRM was used for simultaneous tracking of m/z 6893344 at 32 eV and *m/z* 6893150 at 45 eV, which gave a signal ratio of 1:0.4. The mass spectrometry parameters were as follows: cone energy 45 eV, collision energy 42 eV, capillary 3300 V; desolvation gas 500 l/h and cone gas 0 l/h. Temperature source was 120°C and temperature desolvation was 450°C. The interscan delay was 0.01 sec. Purified c-di-GMP (BIOLOG, Germany) was used to generate the standard curve. Data are given as the c-di-GMP concentration in ng per ml per unit of optical density at 600 nm and are the mean of three independent experiments. Each sample was quantified in duplicate and showed less than 10% variation between duplicates.

Expression of GGDEF and EAL genes from inducible promoter

Gene *ydaM* (GGDEF) was amplified with primers CMD1461 and CMD1462 and *yhjH* (EAL) with primers CMD1463 and CMD1464. The respective gene was cloned into NcoI and PstI sites of pTRC99a (Pharmacia Biotech). Gene expression was induced by adding 50 µM of IPTG in the culture broth.

RESULTS

Screening for genes involved in repression of type 1 fimbriae in the pst *mutant*

We previously demonstrated that constitutive activation of PhoBR, by disrupting the Pst system, decreased expression and production of the type 1 fimbriae and attenuated virulence [7, 8, 290]. However, the molecular mechanisms connecting the Pho regulon and regulation of type 1 fimbriae have not yet been determined. Furthermore, an *in silico* search revealed that the *fimB*, *fimE* and *fimA* genes do not possess Pho box(es) in their promoter region [147], which suggests that PhoBR may indirectly affect type 1 fimbriae expression (Crépin *et al*., 2012b). To identify genes that could link the Pho regulon (Pst system and PhoBR) to expression of type 1 fimbriae, a transposon library was constructed in the *pst* mutant. Transposon mutants were screened for a restoration in production of type 1 fimbriae. As inactivation of the *pst* system constitutively

activates PhoBR and then, the alkaline phosphatase PhoA, induction of the Pho regulon can be monitored by plating clones onto LB agar plates supplemented with 5-Bromo-4-chloro-3-indolyl phosphate (BCIP), a chromogenic substrate of PhoA, as the *pst* mutant appears blue on these plates, whereas the WT strain remains white. Furthermore, as deletion of *phoB* in the *pst* mutant abrogated the activation of the Pho regulon induced by inactivating the *pst* system [7] and the double *pst phoB* mutant produced type 1 fimbriae as well as the WT strain [290], we tested our approach by firstly screening the production of type 1 fimbriae using white colonies. Surprisingly, one of the mutations identified in this screening was not an insertion in the *phoBR* regulatory genes, but in *phoA*. However, as PhoA encodes a periplasmic enzyme devoid of regulatory function, how could PhoA regulate type 1 fimbriae expression ? Therefore, in this study, we focused on the Tn*10* mutant with an insertion that disrupted the alkaline phosphatase gene *phoA*.

The GGDEF gene yaiC *contributes to repression of type 1 fimbriae in the* pst *mutant*

PhoA is a periplasmic enzyme catalyzing the hydrolysis of a wide variety of phosphomonoesters. Given that the transposon was inserted in the opposite orientation of the *phoA-psiF* operon (Fig. 2A), we hypothesized that the transposon had a polar effect on gene(s) downstream *phoA*, i.e. onto *psiF* or *yaiC* (Fig. 2A) as the transposon will block the transcription of downstream gene. This hypothesis is also based on the fact that PhoA does not possess known regulatory domains or functions. To test this possibility, in both WT and Δ*pst* strains, non-polar mutations were introduced in *phoA* as well as on downstream genes *psiF* and *yaiC*. Production of type 1 fimbriae was then evaluated by yeast agglutination on strains cultured to mid-log phase in LB both. As expected, mutations within *phoA*, *psiF* or *yaiC* in the WT strain had no effect on production of type 1 fimbriae (Fig. 2B), as the Pho regulon is not induced in LB broth. By contrast, production of type 1 fimbriae was partly restored in the double *pst yaiC* mutant (DMY) (Fig. 2B). The *yaiC* gene, an ortholog of *adrA* in *Salmonella enterica*, encodes a GGDEF domain protein, is immediately adjacent to the *phoA-psiF* operon and does not belong to that operon (Fig. 2A). To corroborate the yeast agglutination assay, qRT-PCR was performed on *fimA*. Expression of *fimA* was increased 2-fold in the DMY strain (Δ*pst* Δ*yaiC*) when compared to the Δ*pst* strain (Fig. 2C). These results suggest that induction of the Pho regulon in the *pst* mutant mediates repression of type 1 fimbriae through deregulated expression of *yaiC*, but that *phoA* itself does not seem to play a role in this process. Although expression of type 1 fimbriae is not

fully restored to wild-type level, statistical differences were observed between the Δ*pst* mutant and the DMY strain.

Expression of the genes encoding type 1 fimbriae is dependent on an invertible element (*fimS*), which contains the *fim* promoter [62]. This invertible element alternates between the ON- and OFF-orientation, which leads to activation and inhibition of type 1 fimbriae expression, respectively. To correlate increased *fim* expression in the DMY strain with orientation of *fimS*, its orientation was determined by qPCR. By comparing the ON-position of strains cultured to mid-log phase in LB broth, the ON-orientation of the DMY strain was 2.0-fold lower than the WT strain, which also corresponds to a 2.0-fold increase compared to the *pst* mutant, whose ON-position orientation is 4.2-fold less than the WT parent strain (Fig. 2D).

In vitro, we previously demonstrated that repression of *fimA*, in the *pst* mutant, is due to a bias towards the OFF-orientation of *fimS*, which is mainly linked to the repression of *fimB* and possibly, to the down-regulation of the Fim-like recombinases *ipuA* and *ipbA* [290]. To correlate the restoration in type 1 fimbriae and *fimS* orientation with expression levels of the recombinases, expression of *fimB*, *fimE*, *ipuA* and *ipbA* were quantified by qRT-PCR. Thereby, deletion of *yaiC*, in the Δ*pst* background, had no effect on *fimB* and *fimE* expression since no statistical difference was observed when compared to the single *pst* mutant (Fig. 2E). However, expression of *ipuA* and *ipbA* was restored in the DMY strain as they were induced 3.9- and 2.9-fold (Fig. 2E). Since IpuA and IpbA promote orientation of *fimS* in the ON-position, restoration of type 1 fimbriae expression could be attributed to increased expression of these two Fim-like recombinases.

These results show that *yaiC* is related with the expression of type 1 fimbriae in the *pst* mutant. Indeed, its deletion restores expression of *fimA*, increased expression of the *ipuA* and *ipbA* recombinases, and promoted orientation of the invertible promoter towards the ON-position.

yaiC *is induced in phosphate-limiting conditions*

As deletion of *yaiC*, in the Δ*pst* strain, restores the expression / production of type 1 fimbriae, this suggests that *yaiC* is induced in the *pst* mutant. To test this possibility, expression of *yaiC* was evaluated by qRT-PCR. As shown in Fig. 3A, compared to the WT strain, *yaiC* was induced 4.4-fold in the *pst* mutant. Since constitutive induction of the Pho regulon, by deleting the Pst system, induced expression of *yaiC*, we tested if physiological activation of the Pho regulon had the same effect. To do so, bacteria were grown in low (LP) and high phosphate (HP) broth, and expression of *yaiC* was analyzed by qRT-PCR. When grown in LP broth, compared to HP broth, expression of *yaiC* was 4.1- and 3.3-fold induced in WT and Δ*pst* compl. strains (Fig. 3B). Since disruption of the *pst* system constitutively activates the Pho regulon regardless of environmental phosphate availability [4], it was not surprising that there was no difference in *yaiC* expression between the LP and HP media in the *pst* mutant.

We previously showed that cultivation in phosphate-limiting conditions decreased expression of type 1 fimbriae (Fig. 3C and [290]). This was reflected by similar expression patterns of *fimA* between the CFT073 and Δ*pst* strains grown in LP broth. As deletion of *yaiC* in the *pst* mutant restored transcription of *fimA* in LB broth, we investigated whether expression of *fimA* was restored in either the Δ*yaiC* and Δ*pst* Δ*yaiC* mutants grown in LP broth. Indeed, compared with the WT strain grown in LP broth, *fimA* was induced 3.4- and 4.0-fold in CFT073 Δ*yaiC* and Δ*pst* Δ*yaiC* (DMY) mutants, respectively (Fig. 3C).

These results indicate that induction of the Pho regulon, physiologically or by inactivation of *pst*, increases expression of *yaiC* and consequently, repressed expression of type 1 fimbriae. Thereby, these results suggest that *yaiC* is a repressor of type 1 fimbriae.

Deletion of* yaiC *restores type 1 fimbriae-dependent adhesion of the Pst mutant

In vitro, we previously demonstrated that the *pst* mutant adheres to bladder cells as well as the WT strain. However, unlike the WT parent, adhesion of the *pst* mutant was *fim*-independent and involved other adhesins [290]. Nevertheless, production of other adhesins by the *pst* mutant was not sufficient to allow efficient colonization of the bladder, as the *pst* mutant is attenuated in the

UTI mouse model [290]. Since deletion of *yaiC*, in the *pst* mutant, restored expression of type 1 fimbriae, we asked to what extent adhesion of the double *pst yaiC* (DMY) mutant to bladder cells was dependent on type 1 fimbriae. To do so, adhesion to 5637 human bladder epithelial cells (ATCC HTB-9) was tested in the presence or absence of 1.5% α-D-mannopyranose. As type 1 fimbriae recognize mannosylated residues on host epithelial cells [310], addition of soluble mannose to culture medium will inhibit type 1 fimbriae-dependent adhesion to 5637 cells [311].

In the absence of mannopyranose, the WT strain adhered to 26.8% (Fig. 4A). Similarly, the *pst* and the DMY strains adhered to bladder cells to 21.0- and 22.0%, respectively (Fig. 4A). As previously observed [290], addition of mannopyranose to culture medium decreased adherence of the WT strain significantly to 5.8%, whereas it had no significant effect on adhesion of the Δ*pst* strain, which adhered at 17.4% (Fig. 4A). As expected, deletion of *yaiC*, in the Δ*pst* background, restored the type 1 fimbriae-dependent adherence to bladder cells since the presence of mannopyranose decreased the adherence of the DMY strain significantly to 10.7%, which was similar to that observed in the WT strain (Fig. 4A). Thereby, these results confirm that *yaiC* is involved in the negative regulation of type 1 fimbriae.

Deletion of* yaiC *restored urinary tract colonization by the Pst mutant

Since YaiC negatively regulates expression of type 1 fimbriae and these fimbriae are important for infection, we tested whether the double *pst yaiC* (DMY) restores the virulence of the *pst* mutant. To do so, CBA/J mice were co-infected with an equal ratio 1:1 of CFT073 Δ*lac* and Δ*pst* mutant or CFT073 Δ*lac* and DMY strain. As expected and as we previously observed [290], at 48 h post-infection (p.i.), the *pst* mutant was outcompeted 618- and 1323-fold in bladder and kidneys, respectively (Fig. 4B). On the other hand, the DMY mutant was only outcompeted 5- and 13-fold in bladder and kidneys, respectively (Fig. 4B). Although the DMY mutant did not colonize the bladder and kidneys as well as the WT strain, it had the same virulence profile of the Δ*pst* compl. strain (Fig. 4B). Thereby, we can conclude that deletion of *yaiC* significantly restored the virulence of the *pst* mutant.

In the pst *mutant, expression of* yaiC *is independent of CsgD*

Transcription of *yaiC* depends on CsgD, its only known direct regulator [312]. As *csgD* is regulated by more than 10 transcription factors and several environmental conditions [101, 116, 302], we tested whether induction of the Pho regulon influenced its transcription and subsequently, *yaiC*. To do so, qRT-PCR was performed on strains cultured to mid-log phase of growth at 37°C in LB broth. Interestingly, *csgD* was not differentially expressed between the WT, the Δ*pst* and Δ*pst* compl. strains (data not shown). In order to eliminate any potential role of CsgD in *yaiC* regulation, because *csgD* is also regulated at post-transcriptional level by sRNA [299-301, 303], a non-polar mutation in *csgD* was introduced in the *pst* mutant and expression of *yaiC* was monitored by qRT-PCR. As shown in Fig. 3A, deletion of *csgD*, in the Δ*pst* mutant, did not affect *yaiC* transcription, as it was similarly expressed in the single Δ*pst* mutant. Furthermore, we tested whether *csgD* had any effects on type 1 fimbriae expression. As shown in Fig. 2C, D and E, deletion of *csgD*, in the *pst* mutant, had no effect on *fimA*, *fimB* and *fimE*, as well as orientation of *fimS* switch since their expression and orientation was not statistically different compared to the single *pst* mutant. These results are also reflected in the adhesion capacity of the double *pst csgD* (DMC) mutant to bladder epithelial cells, as this mutant adhered to bladder cells in a *fim* independent manner, similarly to the single *pst* mutant (Fig. 4A). Taken together, these results suggest that transcription of *yaiC* in the *pst* mutant seems to be independent of CsgD and in conditions in which the Pho regulon is induced, CsgD is not involved in regulation of type 1 fimbriae.

In the pst *mutant, yaiC is transcribed as part of the phoA-psiF operon and is dependent on the phoA promoter*

As expression of *yaiC* seems to be independent of CsgD in the *pst* mutant and as *yaiC* does not possess a Pho box in its promoter region [147], its induction in the *pst* mutant, and in phosphate starvation conditions, seems to be indirectly activated by the Pho regulon. Furthermore, since the transposon inserted into *phoA* (Fig. 2A) seems to have a polar effect on *yaiC*, we hypothesized that in the *pst* mutant, *yaiC* is co-transcribed as part of *phoA-psiF* operon (Fig. 2A). We reasoned this since *phoA* is positively regulated by PhoB and is strongly induced in a *pst* mutant [4, 8]. Furthermore, an *in silico* analysis showed that the intergenic region between the *phoA-psiF* operon and *yaiC* is only 100 nucleotides. To validate this hypothesis, RT-PCR was performed on the *phoA-psiF* and *yaiC* regions from strains cultured to mid-log phase of growth in

LB broth (Fig. 5A). First of all, as an operon control, the *phoA-psiF* region was reverse-transcribed with primers CMD1111 and CMD1112. An amplification product was observed in Δ*pst* and DMC (Δ*pst* Δ*csgD*) strains, whereas no band was observed for the WT strain (data not shown). These results were expected, since the Pho regulon is only activated in the *pst* and DMC (Δ*pst* Δ*csgD*) mutants. Thereafter, the *psiF-yaiC* region was reverse-transcribed with primers CMD1113 and CMD1114 and again, an amplification product was only observed for Δ*pst* and DMC (Δ*pst* Δ*csgD*) strains (data not shown), suggesting that *yaiC* is on the same transcript with *phoA-psiF*. To confirm this reverse-transcription, the *phoA-psiF-yaiC* region was reverse-transcribed with primers CMD1111 and CMD1114. As expected, no band was observed for the WT, Δ*pst* compl. and Δ*csgD* strains since the Pho regulon was not induced in these strains under this condition. However, a 1kb band, corresponding to the expected product size, was observed for the Δ*pst* and DMC (Δ*pst* Δ*csgD*) strains (Fig. 5B), confirming that *yaiC* is on the same transcript as the *phoA-psiF* operon.

As *yaiC* was co-transcribed as part of an operon with *phoA-psiF* in the *pst* mutant, we tested whether its transcription was dependent on the promoter of *phoA*, which is positively regulated by PhoB. In order to disrupt expression of *phoA-psiF* and therefore *yaiC*, we chromosomally changed 13 nucleotides (see experimental procedures and Fig. 5C) in the Pho box of *phoA* in the *pst* mutant. To confirm that the exchanged nucleotides inhibit *phoA* expression, qRT-PCR was performed on RNA sampled from the WT, the Δ*pst* and the P*phoA* PM strains cultured to mid-log phase of growth in LB broth. In the *pst* mutant, transcription of *phoA* was induced 99.0-fold (Fig. 5D). On the other hand, expression of *phoA* was abolished in the P*phoA* PM strain, as *phoA* was not differentially expressed when compared with the WT strain. Similarly, expression of *yaiC* was inhibited in the P*phoA* PM strain whereas in the single *pst* mutant, *yaiC* was increased 4.80-fold (Fig. 5D). These results strongly suggest that *yaiC* forms an operon with *phoA-psiF* and that its transcription is dependent on PhoB.

In addition, the P*phoA* PM strain also demonstrated a regain in expression of type 1 fimbriae. Compared to the WT parent strain, *fimA* expression in the P*phoA* PM strain is decreased 2.9-fold (Fig. 5D), which is similar to that observed in the DMY (Δ*pst* Δ*yaiC*) strain, where expression of type 1 fimbriae was decreased 4.8-fold (Fig. 2C), compared to the 9.9-fold decrease in the single *pst* mutant (Fig. 5D). Furthermore, adhesion of the P*phoA* PM mutant to bladder cells was

type 1 fimbriae-dependent (Fig. 4A). Indeed, adherence of the P*phoA* PM strain dropped from 25% to 12.0% in the presence of mannopyranose, which was similar to the type 1 fimbriae-dependent adherence of the double Δ*pst* Δ*yaiC* mutant, which dropped from 24% to 12.2% (Fig. 4A).

To further demonstrate the role of P*phoA* dependent expression of *yaiC* on expression of type 1 fimbriae in the *pst* mutant, we introduced either the *yaiC* gene alone, with its proximal *csgD*-dependent promoter, or the *phoA-psiF-yaiC* region, with the promoter of *phoA* (Pho box), at the *att*Tn7 site of strain DMY (Δ*pst* Δ*yaiC*). As expression of *yaiC* in the *pst* mutant depends on the *phoA* promoter, introduction of the *yaiC* alone from its native promoter should behave similarly to the DMY (Δ*pst* Δ*yaiC*) strain. However, introduction of *phoA-psiF-yaiC* to the DMY (Δ*pst* Δ*yaiC*) mutant should resemble the single *pst* mutant phenotype. To test this hypothesis, expression of *fimA* and adhesion properties of these two complemented strains were performed. As expected, introduction of *yaiC* in the DMY strain (DMY compl. *yaiC*) did not affect expression of *fimA* since no difference was observed with the DMY (Δ*pst* Δ*yaiC*) strain (Fig. 2C). However, when the *phoA-psiF-yaiC* region was introduced in the DMY strain (DMY compl. AC), expression of *fimA* was decreased (-13.5-fold) to levels similar to the single Δ*pst* mutant (Fig. 2C). Adhesion properties of these two complemented strains to bladder epithelial cells were also distinct, the DMY compl. *yaiC* demonstrated type 1 fimbriae-dependent adhesion, whereas the DMY compl. *AC* strain demonstrated type 1 fimbriae-independent adhesion to bladder epithelial cells (Fig. 4A). Thus, adhesion of the DMY compl. *AC* strain was similar to adhesion of the single *pst* mutant.

Taken together, these results demonstrate that, under conditions in which the Pho regulon is induced, *yaiC* is co-transcribed as part of an extended transcriptional unit which includes *phoA-psiF,* (the *phoA-psiF-yaiC* operon). Thus, in a *pst* mutant, *yaiC* expression is under the control of the *phoA* promoter and is consequently positively regulated by PhoB.

c-di-GMP influences the expression of type 1 fimbriae

It has been observed that the c-di-GMP pathway, specifically decreased expression of the YhjH phosphodiesterase EAL protein, in the adherent-invasive *E. coli* strain LF82, demonstrated

decreased adhesion and invasion and lowered expression of type 1 fimbriae [246]. Since the *yaiC* gene is involved in biosynthesis of the second messenger c-di-GMP [293, 295] and is induced in the *pst* mutant, we asked whether the *pst* mutant produces higher levels of c-di-GMP than the WT strain. This could explain the decreased expression of type 1 fimbriae in the *pst* mutant. To test this hypothesis, c-di-GMP was quantified. As shown in Fig. 6A, the *pst* mutant produced 91.2 ng ml^{-1} of c-di-GMP, whereas the WT strain produced 65.4 ng ml^{-1}. Although the difference was not statistically significant, the *pst* mutant produced 1.4-fold more c-di-GMP than the WT strain, corresponding to 25.8 ng ml^{-1} of c-di-GMP.

To confirm that c-di-GMP influences expression of type 1 fimbriae, the *ydaM* (GGDEF) and *yhjH* (EAL) genes were cloned into the inducible pTRC99a vector. Thereby, production of c-di-GMP, by YdaM, should decrease expression of type 1 fimbriae in the WT strain similarly to what is observed in the *pst* mutant, where induction of *yaiC* increased c-di-GMP concentration. On the other hand, induction of YhjH, in the *pst* mutant, will degrade c-di-GMP and should restore production of type 1 fimbriae similarly to the WT strain. These genes were then induced in the WT, Δ*pst*, Δ*yaiC* and the double *pst yaiC* mutants; and expression of type 1 fimbriae was quantified by yeast agglutination assay. Thereby, expressing *ydaM* in either the WT strain or DMY (Δ*pst* Δ*yaiC*) mutant inhibited expression of type 1 fimbriae to levels similar to the single *pst* mutant (Fig. 6B). As the *pst* mutant did not agglutinate yeast, it is not surprising to note that induction of *ydaM* had no effect. On the other hand, expressing the *yhjH* gene, in the *pst* mutant, restored the production of type 1 fimbriae to WT levels, while, not surprisingly, it had no effect in the WT, Δ*yaiC* and Δ*pst yaiC* strains (Fig. 6B).

These results show that although the increase in c-di-GMP concentration in the *pst* mutant was not statistically different compared to the WT strains, increased biosynthesis of c-di-GMP through activation of *yaiC* in the *pst* mutant could explain the diminished expression of type 1 fimbriae which results in a decreased capacity to colonize the urinary tract.

DISCUSSION

Inactivation of the Pst system not only constitutively activates the Pho regulon, it also attenuates virulence of pathogenic strains [5, 6]. Likewise, in extra-intestinal pathogenic *E*. *coli* from swine or poultry, attenuation seems to be mainly attributed to alteration in membrane integrity [8, 10-12]. In enteropathogenic *E. coli*, inactivation of the Pst system impairs adhesion to epithelial cells [154, 155]. In *Vibrio cholerae*, induction of PhoB represses virulence gene expression and induces genes involved in c-di-GMP metabolism [157, 215]. In UPEC, the precise role of the Pst system in virulence was unknown until recently [55, 228]. We have recently showed, in UPEC strain CFT073, that inactivation of Pst attenuates virulence by lowering colonization of the mouse urinary tract due to decreased expression of type 1 fimbriae [290]. In the current manuscript, we investigated the molecular mechanisms by which induction of the Pho regulon represses expression of type 1 fimbriae. Herein, we focused on the role of the *yaiC*-mediated pathway in reduced expression of type 1 fimbriae.

In the *pst* mutant, we observed decreased expression of type 1 fimbriae concurrently with up-regulation of the GGDEF gene *yaiC*. By knocking-out *yaiC*, in the *pst* mutant, expression of type 1 fimbriae was partly restored. The regain in expression of type 1 fimbriae in the double *pst yaiC* mutant is demonstrated by the increased type 1 fimbriae-dependent adhesion to bladder cells, which was similar to the WT strain. The loss of *yaiC* in the *pst* mutant also resulted in a regain in virulence in the UTI mouse model. Although the double *pst yaiC* mutant is somewhat outcompeted by the WT strain in bladder and kidneys, it behaves similarly to the Δ*pst* complemented strain. In the *pst* mutant, we previously demonstrated that repression of type 1 fimbriae is linked, *in vitro*, to the down-regulation of *fimB*, *ipuA* and *ipbA* and, *in vivo*, to the up-regulation of *fimE*. This differential expression promotes inversion of the *fim* promoter to the OFF-position [290]. As *fimB* and *fimE* were not differentially expressed in the double *pst yaiC* mutant, restoration of type 1 fimbrial expression could solely be attributed to the up-regulation of *ipuA* and *ipbA*, since these two genes are sufficient for switching the *fim* promoter and influencing type 1 fimbriae expression either *in vitro* or *in vivo* [65, 66]. As *yaiC* is induced in the *pst* mutant and its deletion restores expression of type 1 fimbriae, YaiC could be considered as a repressor of these fimbriae.

As *yaiC* does not possess a Pho box in its promoter region and CsgD is its sole known regulator, we hypothesized that the Pho regulon may affect *yaiC* by CsgD. However, we found that under conditions that activate the Pho regulon, expression of *yaiC* was dependent on PhoB instead of CsgD. Indeed, expression of *yaiC* was dependent on read-through of *yaiC* transcription from the Pho-regulated promoter of the *phoA-psiF* operon. Differential regulatory transcription from promoters which are active under distinct conditions has also been observed elsewhere. For instance, in *Borrelia burgdorferi*, *rpoS* possesses a short and a long transcript. The short transcript depends on RpoN, whereas the long transcript is dependent on a promoter found 1.5 kb upstream of the *rpoS* gene [313, 314]. Transcription of *rpoS* from this promoter is via read-through and includes, in order, the *flgI*, *flgJ* and *rpoS* genes. Furthermore, in *V*. *cholerae*, the *acgAB* operon is regulated via a read-through transcription from the upstream operon *alsDSO* [315]. However, under conditions that activate the Pho regulon, *acgAB* is regulated independently of *alsDSO* by PhoB [215].

As *yaiC* is involved in biosynthesis of c-di-GMP, phosphate starvation seems to be an activating signal of c-di-GMP metabolism. Indeed, cultivation under phosphate limiting conditions induced expression of *yaiC*. This induction was also observed in the *pst* mutant when cultured in LB broth. These results are in agreement with what was observed in *V*. *cholerae* and *Pseudomonas fluorescens*. Indeed, in *V*. *cholerae*, induction of the Pho regulon activates transcription of *acgAB* encoding a GGDEF and EAL protein, respectively [215]. Similarly, in *P*. *fluorescens*, the EAL gene *rapA* is induced under conditions that activate the Pho regulon [236]. In both cases, expression of the GGDEF and EAL genes is dependent on PhoB. In UPEC strain CFT073 *pst* mutant, induction of *yaiC* increased the intracellular concentration of c-di-GMP (Fig. 6A). As the conditions in which we observed the decreased expression of type 1 fimbriae are not optimal for the quantification of c-di-GMP, its concentration was low, even in the *pst* mutant. This could explain the minor difference in c-di-GMP levels observed between the *pst* mutant and the WT strain. To corroborate the quantification results, phenotypes related to c-di-GMP, like Congo red binding and Calcofluor fixation assays, were tested. The pink morphotype, on Congo red, and fluorescence, on Calcofluor, phenotypes are somewhat pronounced in the *pst* mutant, which denotes the production of cellulose (data not shown) [316], which depends on YaiC. These results are in agreement with the quantification of c-di-GMP, where the *pst* mutant produces more c-di-GMP than the WT strain. Functionality of *yaiC* in UPEC strain CFT073 was also tested by inducing its expression from the pBAD promoter in plasmid pBAD24. Following induction,

both the WT and the *pst* mutant turn red on Congo red (production of curli and cellulose [316]) and are bright fluorescent on Calcofluor (data not shown), confirming that *yaiC* is indeed functional. Further, the role of c-di-GMP in regulatory control of expression of type 1 fimbriae was confirmed by expressing the *ydaM* (GGDEF) or *yhjH* (EAL) in the WT and the *pst* mutant. In line with the role of c-di-GMP in down-regulation of type 1 fimbriae, their production was abolished by overexpressing *ydaM* (GGDEF) in the WT strain and conversely, type 1 fimbriae was restored in the *pst* mutant by overexpressing *yhjH* (EAL).

Many groups have reported that increased c-di-GMP levels attenuate virulence. For example, in *Yersinia pestis*, inactivation of the EAL gene *hmsP* increased the production of extracellular polysaccharide (EPS) and decreased virulence [239]. In *V*. *cholerae*, elevated c-di-GMP levels reduced colonization of the mouse small intestine and decreased expression of the major virulence gene transcriptional activator *toxT* [240]. Furthermore, the phosphodiesterase VieA is necessary for virulence in the mouse infant model and positively regulated expression of *toxT* and the cholera toxin *ctxAB* [241]. In *E*. *coli*, however, the role of c-di-GMP in virulence is not well studied. In the adherent-invasive *E. coli* LF82 strain, associated with Crohn's disease, it has been observed that the c-di-GMP pathway decreased adhesion and invasion by repressing expression of type 1 fimbriae [246]. Furthermore, in a clinical UPEC strain, it has been observed that production of cellulose decreased adhesion to bladder cells along with decreased kidney colonization [103]. In our current report, we demonstrated, for the first time, that the regulation of type 1 fimbriae by the Pho regulon implicates metabolism of c-di-GMP, and that the repression of type 1 fimbriae through c-di-GMP production underlies one of the mechanisms leading to virulence attenuation of the *pst* mutant in the UTI mouse model (Fig. 4B and [290].

According to the results shown in this study, under conditions in which the Pho regulon is activated, PhoB binds to the Pho box promoter of *phoA*, leading to the transcription of the *phoA-psiF* operon. Transcription from this promoter extends through to *yaiC*. Increased expression of *yaiC* leads to a higher intracellular concentration of c-di-GMP. Accumulation of c-di-GMP represses transcription of the *fimB*, *ipuA* and *ipbA* Fim-recombinases, leading to increased orientation of the *fim* promoter in the OFF-position, repressed expression of type 1 fimbriae (Fig. 7) and consequently, decreased UTI colonization and virulence. It remains to determine whether *yaiC* (c-di-GMP) directly influences the recombinases *fimB*, *ipuA* and *ipbA*, the *fim* promoter and

type 1 fimbriae and also, at what level (Fig. 7). As *yaiC* is on the same transcript as the *phoA-psiF* operon and is positively regulated by PhoB, *yaiC* could be considered as a new member of the Pho regulon.

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We are grateful to France Daigle (Université de Montréal) who kindly provided the pLOF-Km vector. We also thank the Metabolism Exploration: from genes to metabolites (PFEM) platform (INRA-Clermont-Ferrand) for quantification of the c-di-GMP. SC was supported by scholarships from the Fonds Québécois de la Recherche sur la Nature et les Technologies (FQRNT), the Fondation Armand-Frappier and the Centre de Recherche en Infectiologie Porcine (CRIP). This work was supported by grants to CMD from the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery grant (RGPIN 250129-07) and a Canada Research Chair; and a NSERC Discovery grant (RGPINSD-25120-09) to JH.

TABLE

Table 1. Bacterial strains and plasmids used in this study.

Arial	Characteristic(s)[a]	Source or reference
Strains		
CFT073	UPEC wild-type pyelonephritis strain	[16, 279]
MGN-617	*thi thr leu tonA lacY glnV supE* DΔ*asdA4 recA*::RP4 2-Tc::Mu [pir]; Km^r	[282]
ORN172	*thr-1 leuB thi-1 Δ(argF-lac)U169 xyl-7 ara-13 mtl-2 gal-6 rpsL tonA2 supE44 Δ(fimBEACDFGH)::kan pilG1*	[283]
QT1081	CFT073 Δ*lacZYA* ::FRT	[264]
QT1911	CFT073 Δ*pstSCA*::FRT	[290]
QT2117	QT1911::Tn7T-Gm::*pstSCA*; Gm^r	[290]
QT2138	CFT073 Δ*fimAICDFGH*::km; Km^r	[290]
QT2285	CFT073 *fimS* phase locked-ON; Cm^r	[290]
QT2334	CFT073 Δ*phoA*::FRT	This study
QT2335	CFT073 Δ*psiF*::FRT	This study
QT2336	QT1911 Δ*phoA*::FRT	This study
QT2337	QT1911 Δ*psiF*::FRT	This study
QT2065	QT1911 Δ*yaiC*::FRT	This study
QT2100	CFT073 Δ*yaiC*::FRT	This study
QT2222	QT1911::Tn7T-Gm::*yaiC*; Gm^r	This study
QT2244	QT1911::Tn7T-Gm::*phoA-psiF-yaiC*; Gm^r	This study
P*phoA* PM	QT1911 :: P*phoA* PM (mutations in promoter of *phoA*	This study
QT2099	QT1911 Δ*csgD*::FRT	This study
QT2141	CFT073 Δ*csgD*::FRT	This study
QT2209	MGN-617 + pIJ297; Ap^r Gm^r	This study
QT2208	MGN-617+ pIJ296: Ap^r Gm^r	This study
QT2087	MGN-617 +pLOF/Km; Ap^r Km^r	This study

QT2893	CFT073 + pTRC::*ydaM*; Ap^r	This study
QT2895	QT1911 + pTRC::*ydaM*; Ap^r	This study
QT2894	CFT073 + pTRC::*yhjH*; Ap^r	This study
QT2896	QT1911 + pTRC::*yhjH*; Ap^r	This study
QT2059	CFT073 + pIJ280; Ap^r	This study
QT2067	QT1911 + pIJ280; Ap^r	This study
Plasmids		
pCP20	FLP helper plasmid Ts replicon; Ap^r Cm^r	[260]
pSTNSK	pST76-K::*tnsABCD*; Km^r	[306]
pKD13	Template plasmid for the amplification of the km cassette bordered by FRT sites; Ap^r Km^r	[260]
pKD46	λ-Red recombinase plasmid Ts replicon; Ap^r	[260]
pGP-Tn7-Gm	pGP704::Tn7T-Gm; Ap^r Gm^r	[306]
pIJ280	pBAD24::*yaiC*; Ap^r	
pIJ297	pGP-Tn7-Gm::*yaiC*; Ap^r Gm^r	This study
pIJ296	pGP-Tn7-Gm::*phoA-psiF-yaiC* (AC); Ap^r Gm^r	This study
pGEM-T	TA cloning of PCR product; Ap^r	Promega
pSG76C	*Ori*R6K suicide vector possessing an I-SceI cleavage site; Cm^r	[288]
pGEM ::P*phoA*	pGEM-T :: P*phoA*-*phoA* (*phoA* with its promoter)	This study
pGEM::P*phoA* PM	pGEM-T :: P-*phoA* PM-*phoA*; introduction of mutations (PM) into the promoter of *phoA*;	This study
pSG76C:: P*phoA* PM	pSG76C :: P*phoA* PM-*phoA*	This study
pPIRK	Helper plasmid carrying the *pir* gene and the $pSC101^{ts}$ origin; Km^r	[288]
pST76-ASceP	$PSC101^{ts}$ origin suicide vector expression the I-SceI meganuclease; Ap^r	[307]
pLOF-Km	Tn10 based transposon vector delivery plasmid; Ap^r Km^r	[317]
pTRC::*ydaM*	pTRC99a::*ydaM*; Ap^r	This study
pTRC::*yhjH*	pTRC99a::*yhjH*; Ap^r	This study

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance.

FIGURES

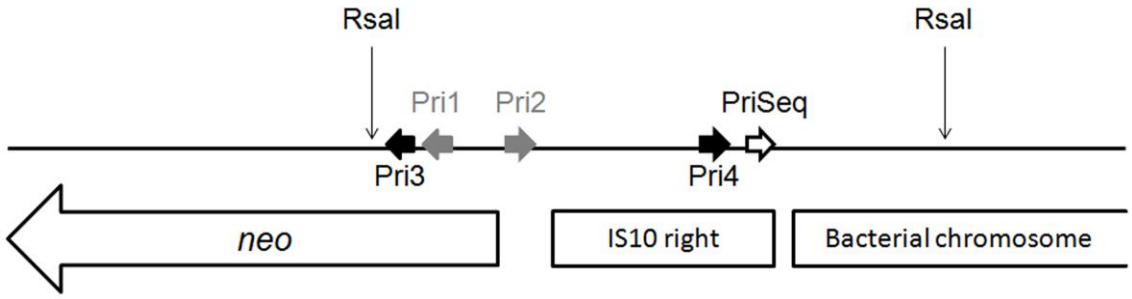

Figure 1. Identification of chromosomal site-specific integration of Tn*10*. Map of a portion of Tn*10* including *neo* (kanamycin resistance), IS*10*R (right) and the bacterial chromosome. The positions of the PCR primers (pri) used to identify the site-specific integration of Tn*10* are indicated. The first inverted-PCR round was performed with primers Pri1 and Pri2, while the second inverted-PCR round was performed with primers Pri3 and Pri4. The resulting amplification bands were sequenced with primer PriSeq.

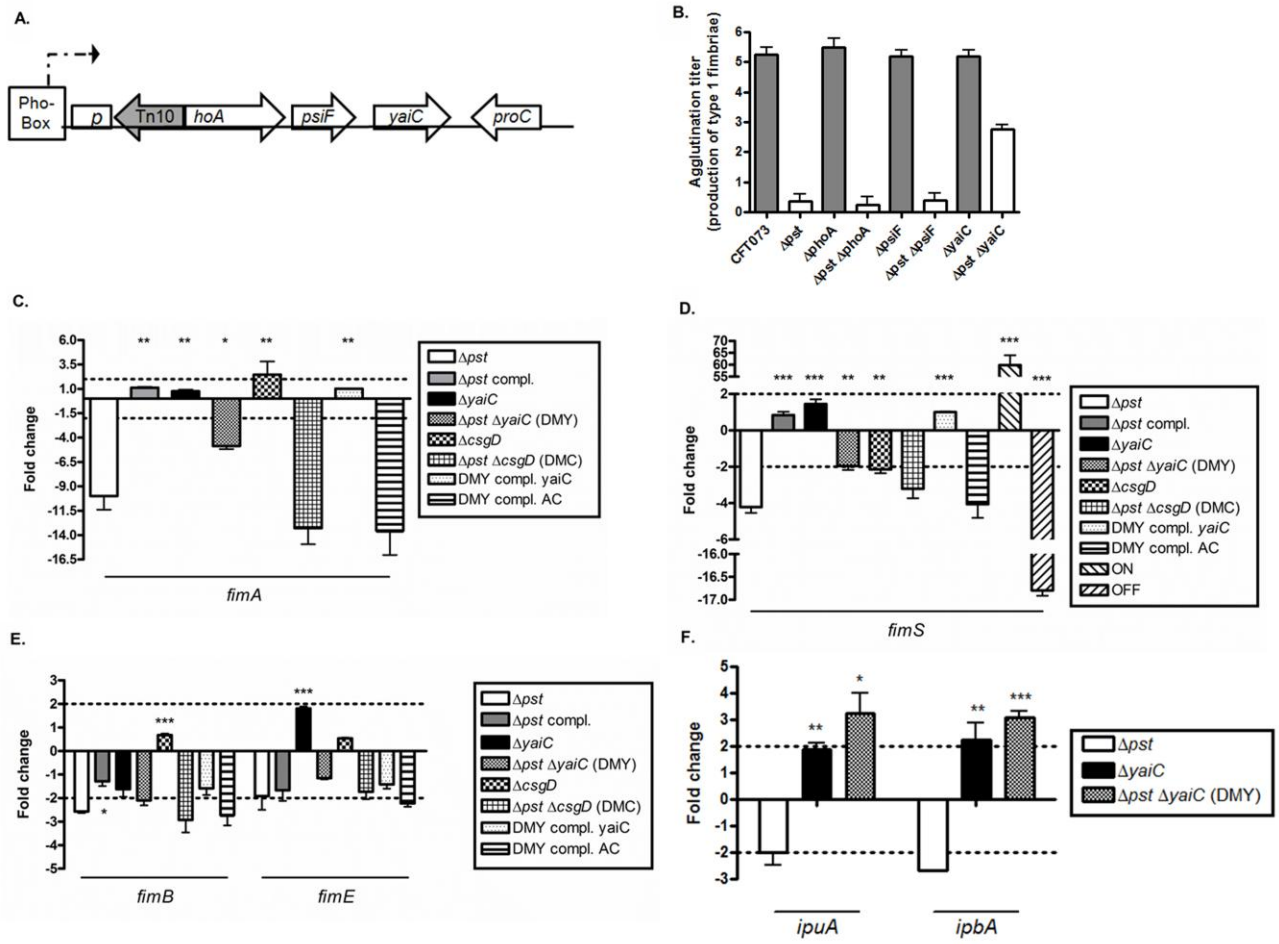

Figure 2. The *yaiC* gene alters expression of type 1 fimbriae. (A). Schematic representation of a transposon insertion which caused a regain in expression of type 1 fimbriae. (B) Quantification of type 1 fimbriae by yeast agglutination assay in non-polar mutants. The CFT073 Δ*fim* and Δ*pst* strains were used as negative controls since they did not agglutinate yeast. (C) Transcription of *fimA* in mutant strains compared to WT strain. (D) ON-orientation of *fimS* in mutant strain compared to WT strain. Expression of *fimB* and *fimE* (E); and *ipuA* and *ipbA* (F) in mutant strains compared to the WT strain. The dashed line corresponds to the cut-off of a significant difference in expression (C, E and F) and ON-orientation (D). All results shown are the mean values and standard deviations of four biological experiments. Statistical significance was calculated by the Student's *t*-test, * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0001$.

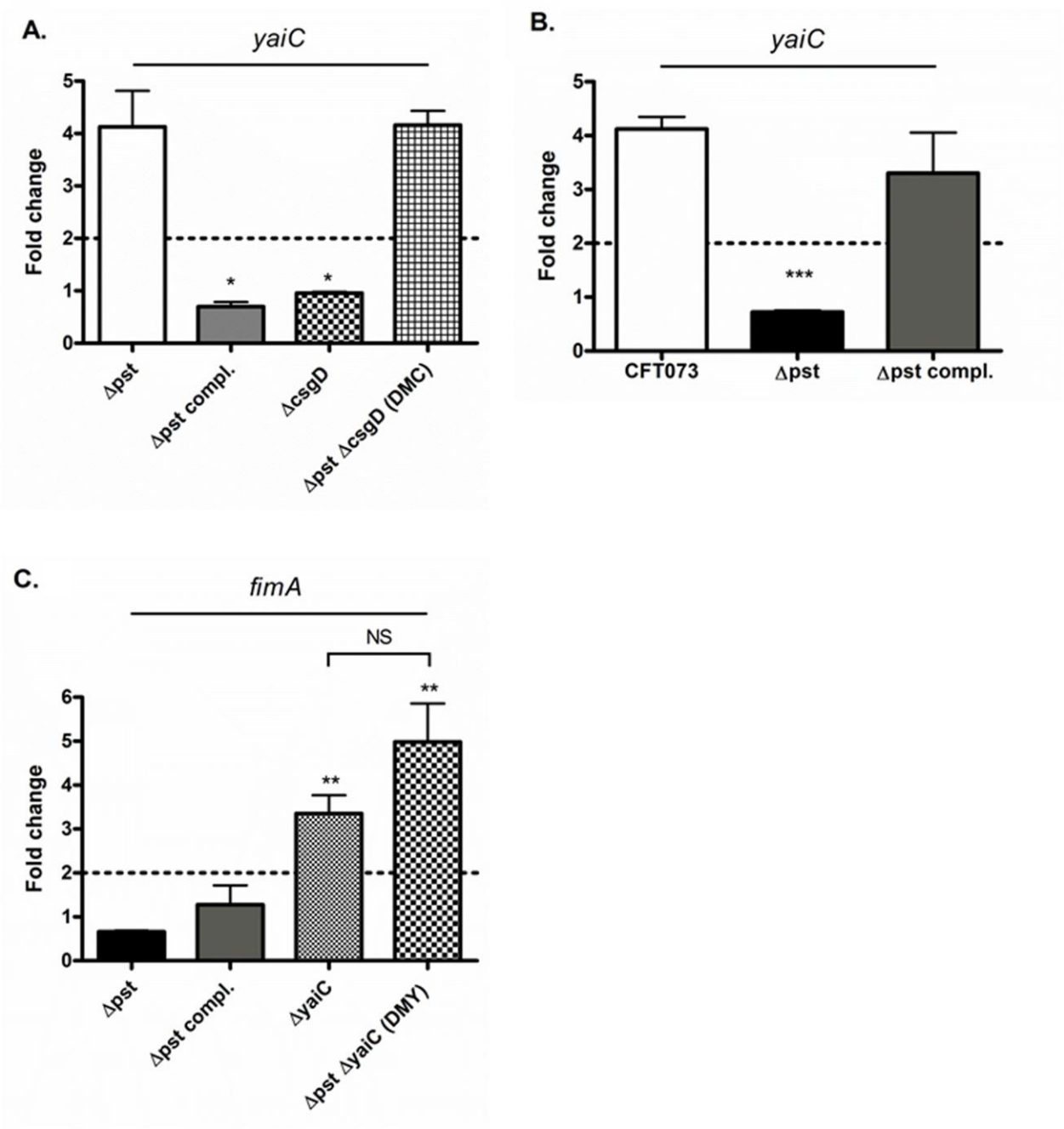

Figure 3. Expression of *yaiC* and *fimA* in phosphate-limiting conditions. (A) Expression of *yaiC* in strains grown in LB broth compared to WT strain (B) Expression of *yaiC* in strains grown in low-phosphate (LP) conditions. Expression in LP broth was compared to expression of the corresponding strains grown in high-phosphate (HP) (C) Expression of *fimA* in strains grown in LP broth. Expression was compared with those of the WT strain grown in LP broth. The dashed line corresponds to the cut-off of a significant difference in expression. All results shown are the mean values and standard deviations of four biological experiments. Statistical significance was calculated by the Student's *t*-test, * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0001$; NS, non significant.

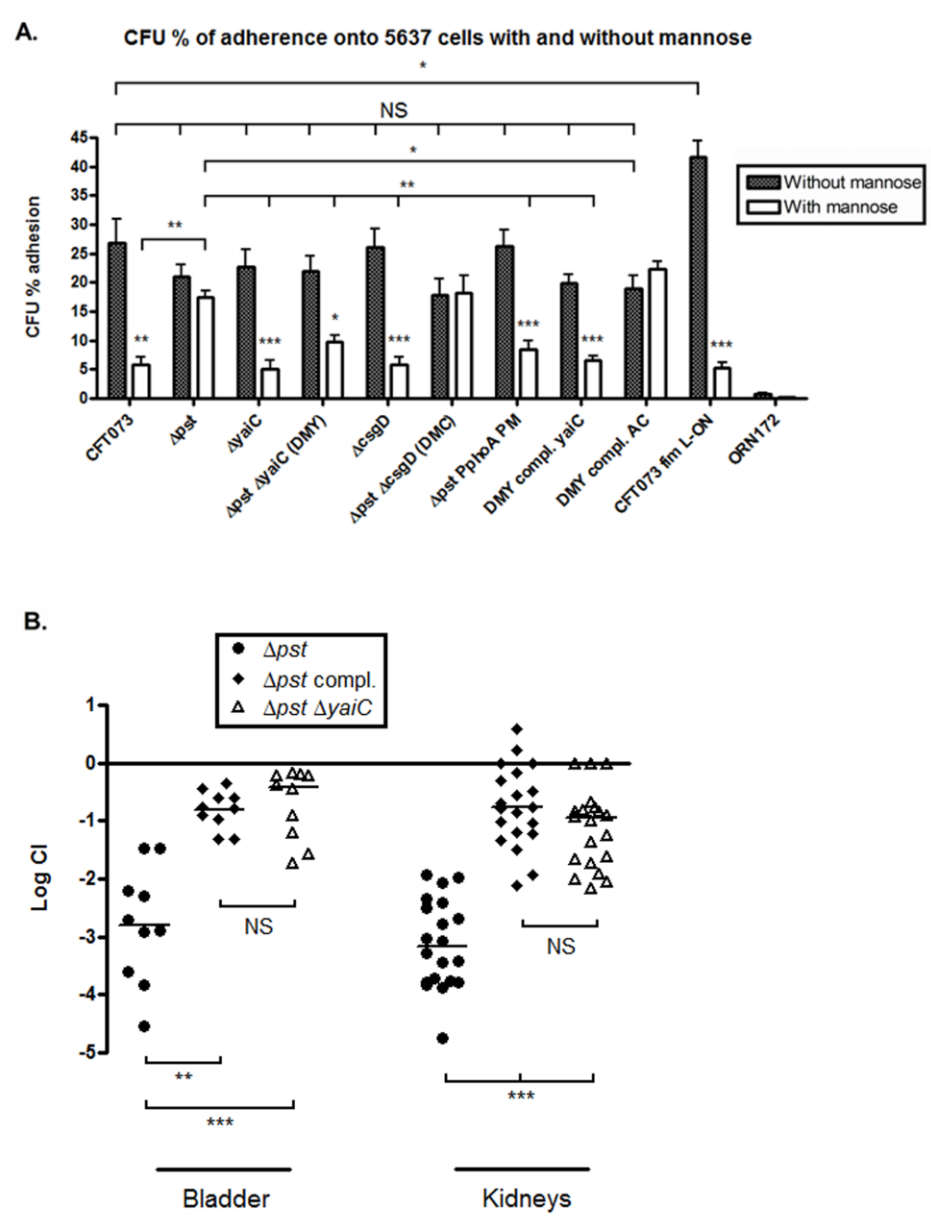

Figure 4. Adhesion and virulence of *yaiC* derivative strains. (A). Adherence of strain CFT073 and its derivatives to human 5637 bladder epithelial cells in the presence and absence of 1.5% mannopyranose. The CFT073 *fim*-locked ON strain and the *E. coli* K-12 *fim*-negative strain ORN172 were used as positive and negative controls, respectively. All results shown are the mean values and standard deviations of four biological experiments. Statistical significance was calculated by the Student's *t*-test, * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0001$; NS, non significant. (B) CBA/J mice were co-infected with a 1:1 ratio of CFT073 Δ*lac* and either the Δ*pst* mutant or the Δ*pst*Δ*yaiC* (DMY) strain. Results are presented as the Log_{10} CFU g^{-1}. Each data point represents a sample from an individual mouse and horizontal bars indicate the medians. Each kidney was sampled separately. A Wilcoxon signed-rank test (two-tailed) was used to determine statistical significance, ** $P < 0.005$; *** $P < 0.0001$; NS, Non significant

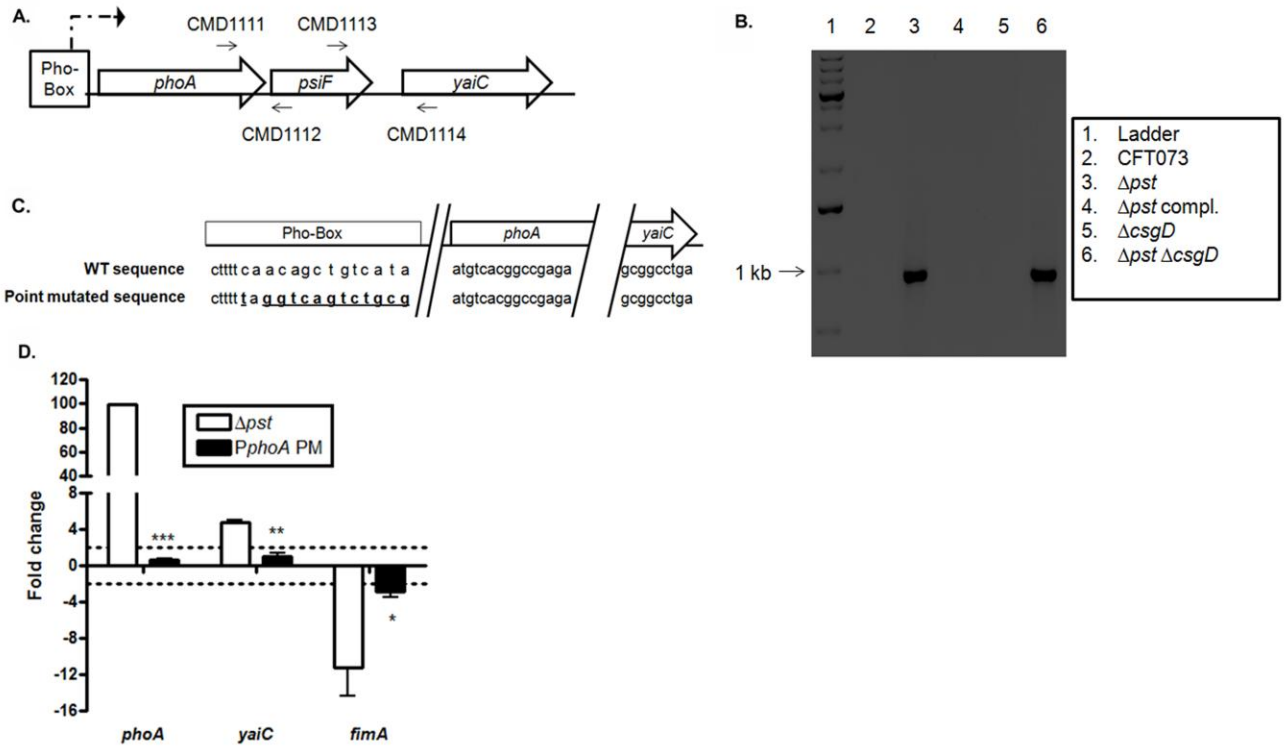

Figure 5. *yaiC* is transcribed as part of the *phoA-psiF* operon. (A) Schematic representation of how the RT-PCR experiments were performed. The *phoA-psiF* region was retro-transcribed and amplified with primers CMD1111 and CMD1112, the *psiF-yaiC* region with primers CMD1113 and CMD1114 and the *phoA-yaiC* region with primers CMD1111 and CMD1114. (B) Gel electrophoresis of RT-PCR products from amplification of the *phoA-psiF* region (primers CMD1111 and CMD1114). (C) Schematic representation of the WT and mutated promoter (Pho box) of *phoA*. Mutated sequence is represented by the bold and underlined nucleotides. (D) Expression of *phoA*, *yaiC* and *fimA* in the *pst* mutant and its derivative *PphoA* PM mutant. Expression was compared to the WT strain. The dashed line corresponds to the cut-off of a significant difference in expression. The calculated *C*t of *phoA*, *yaiC* and *fimA* was normalized to the *C*t of the *tus* gene amplified from the corresponding sample and compared with the WT strain. All results shown are the mean values and standard deviations of four biological experiments. Statistical significance was calculated by the Student's *t*-test (D), * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0001$.

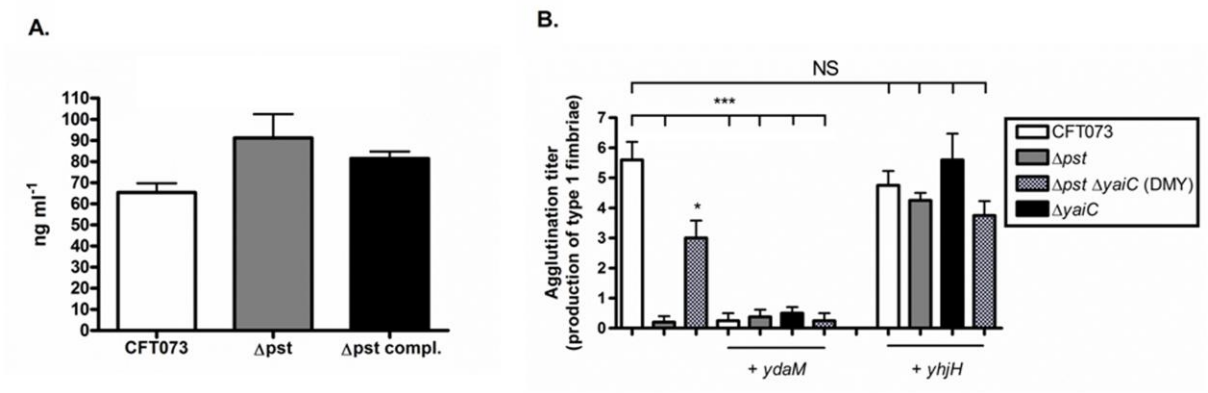

Figure 6. The c-di-GMP pathway influences expression of type 1 fimbriae. (A) Production of c-di-GMP in the WT, Δ*pst* and the Δ*pst* compl. strains. (B) Production of type 1 fimbriae by yeast agglutination assay in strains producing *ydaM,* from pTRC::*ydaM*, or *yhjH*, from pTRC::*yhjH*. All results shown are the mean values and standard deviations of three biological experiments. Statistical significance was calculated by the Student's *t*-test, * $P < 0.05$; *** $P < 0.0001$; NS, non significant.

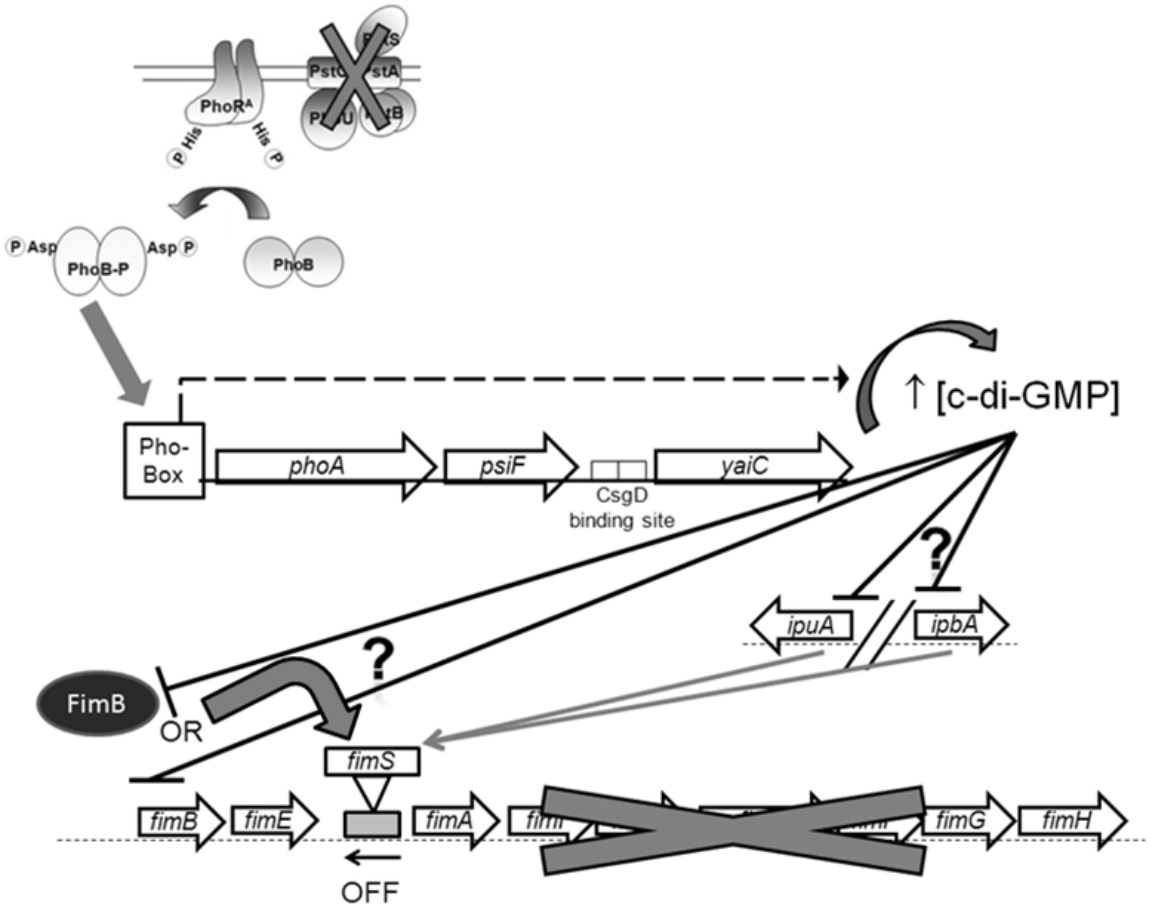

Figure 7. Model illustrating the interactions between the Pho regulon, *yaiC* and type 1 fimbriae. Arrows represent an activation/promotion, while ⊥ represents an inhibition. Induction of PhoBR leads to transcription of *yaiC* from the promoter of *phoA*, which leads to the biosynthesis of c-di-GMP, repression of *ipuA* and *ipbA* and then, reduced expression of the *fim* operon by promoting the OFF-orientation of *fimS*. It is not known (question mark) whether c-di-GMP directly or indirectly inhibits expression of *ipuA* and *ipbA*. Furthermore, c-di-GMP could inhibit the recombinase activity, instead of its transcription. As for *ipuA* and *ipbA*, it is not known (question mark) whether c-di-GMP directly or indirectly affects FimB or *fimB*.

SUPPORTING TABLE

Table S1. Primers used in this study.

Primers	Direction	Characteristic(s)	Sequence 5'→ 3'
CMD96	Forward	Amplification of *vat* gene (used with CMD97)	AACGGTTGGTGGCAACAATCC
CMD97	Reverse		AGCCCTGTAGAATGGCGAGTA
CMD1070	Reverse	Contains a XmaI restriction site; Amplification of the *glmS-pstA* region (used with CMD1072)	TCGCCCGGGATGCACTTCGCGAATCAG GCTTAC
CMD1072	Forward		CACCAATCTTCTACACCGTTCCGC
CMD1111	Forward	Amplification of the *phoA-psiF* region (used with CMD1112) or the *phoA-yaiC* region (used with CMD1114)	GCGCTAAATACCAAAGATGGCGCA
CMD1112	Reverse		ACAGGAGGTCATACGCTGTTGTTG
CMD1113	Forward	Amplification of the *psiF-yaiC* region (used with CMD1114)	TTGCCTGAAGAACAGCAAGTCTGC
CMD1114	Reverse		GAAACCAGCGTTGAAGCAATCGGT
CMD1156	Forward	Contains a XhoI restriction site; Amplification of *yaiC* and its promoter (used with CMD1157)	AAAGCCCTCGAGTACCTGATAGTGCTAA CGGGTGAG
CMD1157	Reverse	Contains a XmaI restriction site	TTCCGTCCCGGGGTGATCGAAGCGATG ACGAAGTGT
CMD1245	Reverse	Amplification of *phoA* and its upstream promoter (Pho box) (used with CMD1335)	AGCCATTTTCGTAGCTCACC
CMD1336	Forward		GATACGGAGCTGCTGCGCGATTAC
CMD1334	Reverse	Used with primer CMD1335 to introduce point mutations in the promoter of *phoA* (Pho box)	[§] CTTCGCAGACTGACCTAAAAAGATTAA CTTTTTACTGACGA
CMD1335	Forward		[§] TAGGTCAGTCTGCGAAGTTGTCACGG CCGAGACTTATAGTC
CMD1246	Forward	Amplification of ON-orientation of *fimS* (used with CMD1248)	ACCGTAACGCAGACTCATCCTCAT
CMD1247	Reverse	Amplification of OFF-orientation of *fimS* (used with CMD1248)	TGAACGGTCCCACCATTAACCG

CMD1248	Forward or Reverse	Primer binds to *fimS*. Alternates between forward and reverse according to the orientation of *fimS* (used with CMD1247 and CMD1248)	TCACATCACCTCCGCTATATGT
CMD1260	Reverse	Contains a XmaI restriction site; Amplification of the *phoA-psiF-yaiC* region with the promoter of *phoA* (Pho box) (used with CMD1261)	CAGCCCGGGTCGAAGCGATGACGAAGTGT
CMD1261	Forward		GGACTCGAGGCTGCTGCGCGATTACGTAA
CMD1461	Forward	Contains a BspHI restriction site; Amplification of *ydaM* (used with CMD1462)	TATCTGATTCATGACGTGGCTTTTTTGCGATCGG
CMD1462	Reverse	Contains a PstI restriction site	GACGCATCCTGCAGTTATGCCGCCAGCACGCGGTTGCG
CMD1463	Forward	Contains a BspHI restriction site; Amplification of *yhjH* (used with CMD1464)	CAGGACATTCATGATGATAAGGCAGGTTATCCAGC
CMD1464	Reverse	Contains a PstI restriction site	GAAAATGACTGCAGTTATAGCGCCAGAACCGCCGTATT

[§] Underlined nucleotides in red were altered from the original sequence to introduce point mutations into the promoter of *phoA* (Pho box).

Article #4

Chromosomal Complementation Using Tn7 Transposon Vectors in *Enterobacteriaceae*

Sébastien Crépin, Josée Harel and Charles M. Dozois (2012). Sous presse.

A) Objectifs et approche utilisée

1- La complémentation de gènes délétés s'effectue généralement à l'aide de plasmides recombinants. Bien que cette approche soit fonctionnelle *in vitro*, cette technique comporte plusieurs inconvénients. Dans un premier temps, la perte de pression de sélection, chez les modèles animaux et en industrie, mène à la perte du plasmide de complémentation. Aussi, le nombre de copie du plasmide, donc le nombre de copies du ou des gène(s) complémenté(s), excèdent toujours le nombre de copies chromosomiques, peut mener à des phénotypes aberrants. De plus, notre souche prototype, la souche UPEC CFT073, ne possède aucun plasmide et résulte à de laborieuses complémentations lors d'infection dans le modèle murin d'infection urinaire. Ainsi, l'étude sous presse à Applied and Environmental Microbiology avait pour objectifs de valider, développer et de simplifier une méthodologie permettant l'insertion chromosomique, en simple-copie, de fragments d'ADN chez notre souche CFT073. Aussi, nous avons montré que notre approche est fonctionnelle et efficace chez cinq espèces d'entérobactéries, i.e. *E. coli* pathogène, *Salmonella enterica*, *Klebsiella pneumoniae*, *Cronobacter sakazakii* et *Citrobacter rodentium*.

2- L'étude a été réalisée en utilisant une approche multidisciplinaire regroupant des techniques de génétique bactérienne, de biologie moléculaire et le modèle murin d'UTI.

B) Contribution de l'étudiant

J'ai réalisé, avec l'aide de mes directeurs, toutes les étapes de conceptions et réalisations des expérimentations. J'ai, de plus, soumis l'article à Applied and Environmental Microbiology. Ce manuscrit a été accepté le 9 juin 2012 et paraîtra dans le Vol. 78; No. 17

C) Résumé de l'article :

La complémentation de gènes mutés s'effectue généralement à l'aide de plasmides recombinants. L'utilisation du transposon Tn7 pour fin de complémentation a récemment été décrite. Ce système a pour avantage d'intégrer, en simple-copie, dans le chromosome des copies de fragments d'ADN et ce, sans avoir recours à une pression de sélection. Ce système a été utilisé chez diverses souches bactériennes, mais n'a jamais été démontré comme étant efficace chez les entérobactéries. Dans cette étude, nous avons développé, validé et simplifié ce système que nous avons évalué chez cinq différentes espèces d'entérobactérie. Pour ce faire, un site de clonage multiple (MCS) flanqué par les bras du transposon Tn7 a été cloné dans le vecteur suicide pGP704. D'une même manière, nous avons cloné les transposases *tnsABCD*, requises pour la transposition de Tn7, dans le vecteur thermosensible pST76K. De plus, des dérivés, contenant différentes cassettes de résistance aux antibiotiques, ont été construits. Afin d'évaluer l'efficacité d'intégration de Tn7 au site *att*Tn7, le gène *xylE*, codant pour une enzyme absente chez les entérobactéries, a été cloné dans le MCS de Tn7 et a été transposé dans quatre souches d'*E. coli* pathogènes, *Salmonella enterica* serovars Typhimurium and Typhi, *Klebsiella pneumoniae, Cronobacter sakazakii* et *Citrobacter rodentium*. Ces souches ont adéquatement intégré le gène *xylE* au site *att*Tn7. Globalement, nous avons, avec succès, adapté le système d'intégration employant le transposon Tn7 chez les entérobactéries. En plus d'être utile à des fins de complémentations de gènes, ce système peut être employé dans l'industrie biotechnologique, pour le développement de vaccin et dans l'expression génique.

Chromosomal Complementation Using Tn7 Transposon Vectors in *Enterobacteriaceae*

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Abstract

Genetic complementation in many bacteria is commonly achieved by re-introducing functional copies of the mutated or deleted genes on a recombinant plasmid. Chromosomal integration systems using the Tn7 transposon have the advantage of providing a stable single-copy integration that does not require selective pressure. Previous Tn7 systems have been developed although none have been shown to work effectively in a variety of Enterobacteria. Herein, we have developed several mini-Tn7 and transposase vectors to provide a more versatile system. Transposition of Tn7 at the chromosomal *att*Tn7 site was achieved by a classical conjugation approach, where the donor strain harbored the mini-Tn7 vector and the recipient strain possessed the transposase vector. This approach was efficient in 5 different pathogenic enterobacterial species. Thereby, this system provides a useful tool for single-copy complementation at an episomal site for research in bacterial genetics and microbial pathogenesis. Furthermore, these vectors could also be used for introduction of foreign genes of use for biotechnology applications, vaccine development, or for use in gene expression and gene fusion constructs.

Introduction

Determining the potential function of a gene is achieved through targeted or random mutagenesis approaches. A well established principle known as “Molecular Koch’s postulates”, was described by Stanley Falkow in the 80’s [318]. These postulates stipulate that a virulence trait should be associated to gene(s) function; specific inactivation of the associated gene(s) with the assumed virulence trait should attenuate virulence; and re-introduction of the wild-type gene(s) into the mutant strain should lead to the restoration of pathogenicity to the wild-type level [318].

Cloning genes on plasmids may often provide an effective means of genetic complementation. Plasmids used for either genetic complementation are valuable tools, particularly for *in vitro* studies [10, 155, 319-322]. However, plasmids may easily be lost in the absence of selection in conditions such as natural environments, animal models or for use in industries such as food production. Further, when complementation is to be tested, plasmid copy number and increased gene dosage exceed the chromosomal number. This could lead to aberrant phenotypes and limits the use of plasmids for successful demonstration of complementation.

The use of the Tn7 transposon is an elegant alternative. The Tn7 transposon integrates at the site-specific *att*Tn7, located downstream of the highly conserved *glmS* gene, which encodes an essential glucosamine-fructose-6-phosphate aminotransferase [323]. Tn7 integration at *att*Tn7 is mediated by the *tnsABCD* transposases [324], where TnsAB proteins recognize and excise the Tn7 fragment from the donor element, whereas TnsCD allow integration of Tn7 into the *glmS* transcriptional terminator (for reviews, see references [323, 325]). As Tn7 represents a “homing” transposon that recognizes a specific and conserved sequence in many bacteria, the Tn7 system has been developed as a tool to integrate DNA sequences into the chromosome of different gram-negative bacteria [289, 326-330], e.g. *Pseudomonas*, *Burkholderia* and *Yersinia* spp. Although this system provides many applications for a variety of bacteria, it comprises the use of ColE1-based suicide plasmids that can replicate readily in many Enterobacteria such as *Escherichia coli* and *Salmonella enterica* strains.

In this report, we validated, designed and simplified the efficacy of a Tn7-based cloning and delivery system that is optimized for use in enterobacterial species, including *E. coli* and *Salmonella*. This system was shown to be very effective for integration of recombinant genes in 5 different enterobacterial species, including 4 pathogenic *E. coli* strains, *Salmonella enterica* serovars Typhimurium and Typhi, *Klebsiella pneumoniae, Cronobacter sakazakii* and *Citrobacter rodentium*. We have also generated a number of vectors with different antibiotic-resistance markers to provide a more versatile system that could readily be used for genetic manipulations in strains that are naturally resistant to different antibiotics.

Materials and Methods

Bacterial strains, plasmids, primers and media

Strains and plasmids used in this study are listed in Table 1 while primers are listed in Table 2. Bacteria were grown in Luria-Bertani (LB) broth at 30°C or 37°C. Antibiotics and supplements were added as required at the following concentrations: kanamycin, 40 µg/ml; ampicillin, 100 µg/ml; chloramphenicol, 15 µg/ml; gentamincin, 15 µg/ml; trimethoprim, 10 µg/ml; diaminopimelic acid (DAP), 50 µg/ml; and 5-bromo-4-chloro-3-indolylphosphate (XP), 40 µg/ml

Construction of Tn7 and transposase vectors

The procedure was adapted for *E. coli* based on the system developed by Choi *et al*. [289]. Firstly, the mini-Tn7, containing a multiple cloning site (MCS) and a gentamicin resistance cassette (Gm^r), flanked by the two Tn7 ends, was amplified from pUC18-mini-Tn*7*-Gm with primers CMD1067 and CMD1068. The amplified product was digested with MfeI and PspxI (New England Biolabs) and then ligated into the suicide vector pGP704 previously digested with EcoRI and SalI, creating plasmid pGP-Tn7-Gm (Fig. 1A). Secondly, the Tn7 transposase encoding genes *tnsABCD* were excised from plasmid pTNS2 by SphI and XmaI digestion and were ligated into the same sites of the temperature-sensitive plasmid pST76-K, resulting in plasmid pSTNSK (Fig. 1B).

Derivatives of the mini-Tn7 pGP-Tn7-Gm vector were also constructed. The Gm^r cassette of pGP-Tn7-Gm was replaced by a *cat* (Chloramphenicol, Cm^r) or a *dhfrII* (trimethoprim, Tp^R)

cassette. The Cm^r resistance cassette was amplified from pFCM1 with primers CMD1466 and CMD1467. Replacement of the Gm^r cassette by a Cm^r *cat* cassette was achieved by the procedure described by Murphy and Campellone [331] using the pTP223 vector, creating the pGP-Tn7-Cm vector. Thereafter, the Cm^r cassette of pGP-Tn7-Cm was replaced by those of Tp^r. The Cm^r cassette was suppressed by digesting the pGP-Tn7-Cm vector with Bpu10I and BstBI. Following filling-in the unpaired ends with the Klenow fragment, the Tp^r cassette, obtained from the digestion of the pFTP1 vector with SmaI, was cloned into the blunt end sites of pGP-Tn7-Cm devoid of the Cm^r cassette, creating the pGP-Tn7-Tp vector. Similarly, derivatives of pSTNSK transposase vectors were constructed. The Cm^r and Tp^r cassettes were excised respectively from vectors pFCM1 and pFTP1 [289] with a XmaI digestion. The respective cassettes were cloned into the XmaI site of pSTNSK, creating respectively pSTNSK-Cm and pSTNSK-Tp.

The pGP-Tn7-Gm-*xylE* vector was constructed by cloning the *xylE* cassette, encoding the catechol 2,3-dioxygenase, previously isolated from pMEG685 by XhoI digestion, into the XhoI site of pGP-Tn7-Gm.

Delivery of Tn7 into *Enterobacteriaceae*

A classical mating (5 h or 18 h) using $2x10^7$ CFU ml^{-1} of donor strain *E. coli* SM10λ*pir*-derivative MGN-617, harboring the pGP-Tn7-Gm or derivative vectors, and $1x10^7$ CFU ml^{-1} of the recipient strains, carrying either pSTNSK or other Tn7 transposase-encoding plasmids, was performed at 30°C on LB agar plates supplemented with DAP. After incubation, the mating lawn was then serially diluted, spread onto LB Gm plates and incubated at 42°C for 4-5 h then for 18 h at 37°C. Colonies were then screened for resistance to Gm and sensitivity to Ap and Km. Since the Ap^r cassette is located outside of the Tn7 region on the vector, sensitivity to Ap denotes the proper integration of Tn7-Gm at *att*Tn7 instead of incorporation of the vector into the chromosome. Also, since the transposases are encoded on a temperature-sensitive plasmid, incubation at 42°C was undertaken to promote loss of the pSTNSK or derivative vectors from the recipient strain, which was denoted by sensitivity to Km. Furthermore, the use of LB Gm plates without DAP selected for growth of the recipient strain, as the MGN-617 donor strain is an *asd* mutant that requires DAP for growth.

Confirmation of integration of the Tn7 transposon at the established *att*Tn7 site located downstream of the *glmS* gene within different clones was verified by PCR in different enterobacterial strains using the primer-pairs listed in Table 2.

Alkaline phosphatase assay.

Alkaline phosphatase activity was performed as described by Crepin *et al*. [8]. Briefly, cells were grown in LB medium, were adjusted to an optical density 1.0 (OD_{600}), and 4 µg/ml of *p*-nitrophenyl phosphate was added to cells permeabilized by 50 µl of 1% sodium dodecyl sulfate (SDS) and 50 µl of chloroform. Color development was monitored at 420 nm and PhoA activity was expressed in Miller units (MU), calculated as follows: $1{,}000 \times [OD_{420} - (1.75 \times OD_{550})]/T$ (min) × V (ml) × OD_{600}, where T stands for the length of reaction time and V stands for the culture cell volume. Activity of PhoA in each strain was calculated at each hour throughout the growth curve.

Accession numbers

Vectors were sequenced and GenBank accession numbers are as follows: pGP-Tn7-Gm (JQ429758), pGP-Tn7-Cm (JQ429759), pGP-Tn7-Tp (JQ429760), pSTNSK (JQ436536), pSTNSK-Cm (JQ436537), pSTNSK-Tp (JQ436538).

Results and Discussion

Characteristics of the *tnsABCD* transposases and Tn7 transposon vectors

Herein, the chromosomal Tn7 integration systems described by Choi *et al*. [289, 326-328] were modified for practical use in Enterobacteriaceae*, including wild-type pathogenic* E*.* coli strains, Salmonella enterica, Klebsiella pneumoniae *and* Cronobacter sakazakii. As the pTNS2 [289] vector containing the *tnsABCD* genes encoding the Tn7 transposase system possesses an R6K origin of replication, which is of limited use in Enterobacteriaceae*,* we developed a more versatile vector that could be maintained in a variety of Enterobacteria. Thereby, the transposases *tnsABCD* genes were cloned into the thermosensitive vector pST76K (Fig. 1B). By using such a vector, bacteria can maintain the transposase system when grown at 30°C, but lose it following cultivation at either 37°C or 42°C.

The Tn7-containing vectors developed by Choi *et al.* [289] possess a pUC18 (ColE1) origin of replication. However, the ColE1 replicon is functional and gives plasmids of high-copy number in many enterobacteria such as *E. coli* and *Salmonella* [332]. To provide an efficient system that is amenable to use of Tn7 for single-copy integration of recombinant genes at the *att*Tn7 in a variety of enterobacteria, the modified mini-Tn7 transposon described in materials and methods was cloned into the λ*pir* dependent suicide vector pGP704, creating the pGP-Tn7-Gm plasmid. Furthermore, the Gm^r cassette is flanked by the Flippase Recognition Target (FRT) sites. These sites are recognized by the Flippase recombination enzyme (FLP), which can be introduced on vectors such as pCP20 [260]. By promoting reciprocal recombination across the inverted repeats (FRT), the resistance cassette can be excised from the chromosome by the FLP recombinase (data not shown). The pGP704 suicide plasmid was selected as it possesses the *mobRP4* region and the R6K origin of replication, and it is highly mobilizable and is an excellent suicide vector for the introduction of DNA into a variety of bacterial species in a non-replicating form [286].

As certain bacterial strains have innate or naturally acquired resistance to a variety of antibiotics, we also generated Tn7 system vector derivatives of pGP-Tn7-Gm in which the Gm^r cassette is replaced by a Cm^r and Tp^r cassette (Table 1). Further transposase encoding derivatives of pSTNSK were constructed by incorporating the Cm and Tp resistance cassette to this vector (Table 1).

The development of a two-plasmid system using a temperature sensitive-replicon to encode the transposase system and a mobilizable *pir*-dependent suicide vector for introduction of the Tn7 transposon system provides a simple means of introducing Tn7 flanking recombinant genes into the chromosome of a variety of enterobacterial strains through a simple bi-parental mating without the need of a helper plasmid, as previously described [289, 326-328, 333].

Transposition of Tn7 in *Enterobacteriaceae*

To determine the efficacy of the Tn7 system *in* Enterobacteriaceae, the xylE gene was cloned into pGP-Tn7-Gm, creating the pGP-Tn7-Gm-xylE vector. Introduction of the xylE gene provided a practical phenotypic reporter for screening, as colonies expressing *xylE* turn yellow after

exposure to a solution of catechol [334]. The uropathogenic *E*. *coli* (UPEC) strains CFT073 and 536, the enterohaemorrhagic O157:H7 *E*. *coli* strain EDL933, the avian pathogenic *E*. *coli* strain χ7122, *Salmonella* enterica serovar Typhimurium (strain SL1344) and serovar Typhi (strain Ty2a), *Klebsiella pneumoniae* strain KPPR1 and *Cronobacter sakazakii* BAA-894 were among strains tested for integration of the *xylE* gene at *att*Tn7 site. Mating and screening methodologies were performed as described in materials and methods. Following conjugation, 10^7 Gm-resistant CFU, from the bacterial lawn, were obtained (Table 3). Of those, 100 colonies were separately plated onto Gm, Ap and Km plates, as described in Materials and Methods.

Thereafter, clones were screened for their proper expression of *xylE*. By spraying a solution of catechol, strains harboring the Tn7-Gm-*xylE* fragment, at *att*Tn7 site, turned yellow, whereas the WT strains remained white (Fig. 2A). Proper integration of Tn7-Gm-*xylE* at *att*Tn7 was verified by PCR with primer pairs listed in Table 2. All the yellow clones correctly integrated the Tn7-Gm-*xylE* fragment at *att*Tn7, which is denoted by the presence of amplification products observed between 4- and 5 Kb (Fig. 2B). By cumulating the resistance, coloration and PCR screens, these strains incorporated the *xylE* gene at the *att*Tn7 site at an efficiency ranging from 80% to 96 % (Table 3).

Interestingly, although *E*. *coli* EDL933 and χ7122, *Salmonella* Typhimurium (SL1344) and Typhi (Ty2a); and *Cronobacter sakazakii* contained modified sites at *att*Tn7, due to differences in the *glmS* terminator loop or presence of additional genes such as fimbrial operons between *glmS* and *pstS* (Fig. 2C), the Tn7 transposon was still efficiently targeted to the *att*Tn7 site at the 3' end of the *glmS* gene (Fig. 2B). In these strains, we screened the Tn7 integration at *att*Tn7 site with primers homologous with the first gene found downstream of *glmS*. Proper amplification denoted that these genes adjacent to *glmS* in the WT strains were still present following integration at the *att*Tn7 sites.

Derivatives of the pSTNSK and pGP-Tn7-Gm vectors were also tested in UPEC strain CFT073. By using pSTNSK-Tp and pSTNSK-Cm transposase encoding vectors with pGP-Tn7-Gm, transposition of Tn7-Gm at the *att*Tn7 site occurred at a rate superior to 90 % (Table 3). The combination of pSTNSK with pGP-Tn7-Cm produced similar results. Although the pGP-Tn7-Tp

transposon vector was functional, its efficacy was considerably reduced, as only 10^2 Tp resistant colonies were obtained and among them, 15% integrated Tn7 at *att*Tn7, compared to 10^7 CFU and above 90% for pGP-Tn7-Cm or pGP-Tn7-Gm (Table 3). Although the pGP-Tn7-Tp vector is not as efficient as pGP-Tn7-Gm and pGP-Tn7-Cm, it can be a suitable alternative for use in strains that are resistant to both Gm and Cm.

Integration of Tn7 was also assayed in *Citrobacter rodentium*, a natural pathogen of mice used as model of infections for enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) [335]. Using the pGP-Tn7-Gm and pSTNSK vectors, 10^7 CFU were resistant to Gm and among them, 68% correctly integrated the Tn7-Gm fragment at *att*Tn7 (Table 3).

Thereby, these results validate that the Tn7 system we have developed can be applied to a variety of bacterial species. Compared with the previously described procedures [289, 326-329, 336], our method is simpler since transposition to *att*Tn7 does not require use of a helper plasmid. Indeed, it can be easily achieved by a classical conjugation procedure. Previously, a simple and convenient procedure using single vectors containing both the mini-Tn7 and transposases was described [330]. However, although this method was shown to be efficient in some enterobacterial strains such as *E. coli* K-12, it did not work well in our prototypal CFT073 and χ7122 pathogenic *E. coli* strains (data not shown). Furthermore, as these vectors only possess the Ap^r cassette, their use is limited since many clinical as well as environmental enterobacterial strains are increasingly resistant to beta-lactams and other antibiotics [337].

Due the presence of different combinations of antibiotic markers, vectors presented here can be useful in a large spectrum of species. They therefore provide an efficient means to introduce recombinant genes encoding reporter fusions, epitope-tagged or chimeric proteins or for complementation of specific mutations in strains by re-introduction of functional gene(s) at an episomal site.

Chromosomal complementation using Tn7 in UPEC strain CFT073

The UPEC CFT073 strain is an archetypal strain that has been used in a number of laboratories to investigate pathogenesis of *E. coli* urinary tract infections [279]. This strain does not contain any native plasmids [16], and trans-complementation by plasmids has been shown to be difficult in the absence of antibiotic selection, as plasmids may be rapidly lost without selective pressure.

The *pstSCAB-phoU* gene cluster encodes the phosphate specific transport system (Pst) and belongs to the Pho regulon. This regulon is controlled by the two-component regulatory system PhoBR, which activates genes involved in acquisition and metabolism of different kinds of phosphate groups in phosphate starvation conditions [4, 152]. The Pst system and the alkaline phosphatase PhoA are among the Pho regulon members. In addition to being involved in phosphate transport, the Pst system negatively regulates the Pho regulon, as its disruption constitutively activates PhoBR [4, 152]. Furthermore, the Pst system is also required for virulence as its inactivation attenuated virulence of pathogenic strains [5, 6]. Using the Tn7 transposon system we describe in this report, we have successfully complemented the virulence of a *pst* mutant in UPEC CFT073 strain by introducing these genes in single copy at the *att*Tn7 site [290].

By activating the PhoBR regulon through disruption of the Pst system, the alkaline phosphatase PhoA becomes constitutively expressed. Production of PhoA can be visualized by plating strains onto LB agar plates supplemented with 5-Bromo-4-chloro-3-indolyl phosphate (XP) [338]. Strains producing or not PhoA will appear blue and white, respectively. As observed in Figure 3A, the Pst mutant cultures appeared blue whereas the WT CFT073 strain remained white. Complementation of the *pst* mutant at *att*Tn7 restored the white phenotype of the *pst* mutant (Fig. 3A). Thereafter, quantification of PhoA in the WT, ∆*pst* and complemented strains was evaluated at various timepoints throughout the time by an alkaline phosphatase assay. As shown in Fig. 3B, the WT and complemented strain produced PhoA at a basal level, whereas this production was considerably higher in the *pst* mutant.

Stability of genes introduced in single copy using the Tn7 system.

Stability of integration of *xylE* at *att*Tn7 was evaluated following 14 passages, over a period of 7 days, in LB broth without selective pressure in CFT073 strain. As shown in Fig. 3C, at 7 days post-inoculation, 100% of colonies expressed *xylE* (yellow patches). Stability of the *pstSCA* genes inserted at the *att*Tn7, in the *pst* mutant, was also evaluated following passage in the murine model of UTI. As for *xylE*, 100% of colonies contain the *pstSCA* genes at *att*Tn7 (data not shown and [290]).

CONCLUSION

In this report, we have developed a series of practical vectors for the integration of Tn7 at *att*Tn7 site that was shown to be effective in a variety of enterobacterial species. This procedure is also versatile as several vectors with different selection markers have been constructed. Furthermore, integration of Tn7 at *att*Tn7 was shown to be efficient in a variety of *Enterobacteriaceae* including pathogenic *E*. *coli*, *Salmonella*, *Klebsiella*, *Cronobacter* and *Citrobacter* strains. In addition to serving as a chromosomal complementation method, integration of Tn7 at *att*Tn7 can be useful in biotechnology applications, vaccine development and in gene expression and gene fusion constructs.

Acknowledgements

The pUC18-mini-Tn*7*-*Gm*, pTNS2, pFTP1, pFCM1 and pFTC1 vectors were kindly provided from Eric Déziel (INRS-Institut Armand-Frappier) with the permission of Dr. Herbert P. Schweizer (Colorado State University). We also thank György Pósfai (Biological Research Center, Hungarian Academy of Sciences) for the gift of pST76-K and Kenan C. Murphy (University of Massachusetts Medical School) for providing the pTP223 vector. We are grateful to Sébastien Houle and Romain Coeurt (INRS-Institut Armand-Frappier) for their technical advice and assistance, respectively. SC was supported by the Fonds Québécois de la Recherche sur la Nature et les Technologies, the Fondation Armand-Frappier and the Centre de Recherche en Infectiologie Porcine (CRIP). This work was supported by grants to CMD from the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery grant (RGPIN 250129-07) and a Canada Research Chair; and a NSERC Discovery grant (RGPIN SD-25120-09) to JH.

TABLES

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)[a]	Source or reference
Strains		
Citrobacter rodentium		
ICC168 strain	Attaching and effacing mice pathogen	[339]
QT2787	*C. rodentium* ICC168 + pSTNSK; Km^r	This study
Cronobacter sakazakii		
BAA-894 strain	Isolated from a powdered formula used during an NICU outbreak	[340]
QT2765	*C. sakazakii* + pSTNSK-Tp, Km^r Tp^r	This study
E. coli		
DH5 α $\prod$	λ*pir* lysogen of DH5 α, Tc^r	[307]
MGN-617	*thi thr leu tonA lacY glnV supE* Δ*asdA4 recA*::RP4 2-Tc::Mu (pir); Km^r	[282]
S17-1(λ*pir*)	λ*pir* lysogen of S17.1 (Tp^r Sm^r *thi pro hsdR*$^-$*M*$^+$ *recA* RP4::2-Tc::Mu-*km*::Tn*7*)	[284]
QT2085	MGN-617 + pGP-Tn7-*pst*, Ap^r Gm^r	[290]
QT2740	MGN-617 + pGP-Tn7-Gm-*xylE*, Ap^r Gm^r	This study
Pathogenic *E. coli*		
536	UPEC wild-type pyelonephritis strain	[341]
QT2732	536 + pSTNSK, Km^r	This study
CFT073	UPEC wild-type pyelonephritis strain	[16, 279]
QT2496	CFT073 + pSTNSK, Km^r	This study
QT1911	CFT073 Δ*pstSCA::FRT*	[290]
QT2207	QT1911 + pSTNSK, Km^r	[290]
QT2651	QT1911 + pSTNSK-Cm, Km^r Cm^r	This study
χ7122	Avian pathogenic strain, O78:K80:H9 *gyrA*, Nal^r	[280]
QT2707	χ7122 + pSTNSK, Nal^r Km^r	This study
EDL933	Enterohemorrhagic *E. coli* (EHEC) O157:H7	[342]
QT2705	EDL933 + pSTNSK-Tp, Km^r Tp^r	This study

Klebsiella pneumoniae		
subsp. *pneumoniae* KPPR1 strain		ATCC 43816
QT2710	*K. pneumoniae* subsp. *pneumoniae* (KPPR1) + pSTNSK-Tp, Km^r Tp^r	This study
***Salmonella enterica* serovars**		
Typhi Ty2a	Vaccine strain of *S*. Typhi Ty2 strain	[343]
QT2774	*S*. Typhi Ty2a + pSTNSK, Km^r	This study
Typhimurium SL1344	Salmonella *enterica* subsp. enterica serovar Typhimurium	[285]
QT2706	SL1344 + pSTNSK-Tp, Km^r Tp^r	This study
Plasmids[b]		
pCP20	FLP helper plasmid Ts replicon; Ap^r Cm^r	[260]
pFCM1 (AY597271)	Chloramphenicol resistance FRT vector pFCM1, Ap^r Cm^r	[289]
pFTP1 (AY712951)	Trimethoprim resistance FRT vector pFTP1, Ap^r Tp^r	[289]
pGP704	ori*R6K*, *mobRP4*; Ap^r	[286]
pGP-Tn7-Cm (JQ429759)	pGP-Tn7-FRT::*Cm*, Ap^r Cm^r	This study
pGP-Tn7-FRT	pGP-Tn7-Gm::*FRT*, Ap^r	This study
pGP-Tn7-Gm (JQ429758)	pGP704::Tn7-*Gm*; Ap^r Gm^r	This study
pGP-Tn7-Gm-*xylE*	pGP-Tn7-Gm::*xylE*, Ap^r Gm^r	This study
pGP-Tn7-*pst*	pGP-Tn7-Gm::*pstSCA*, Ap^r Gm^r	(13)
pGP-Tn7-Tp (JQ429760)	pGP-Tn7-Cm::*Tp*, Ap^r Tp^r	This study
pMEG685	*xylE* cassette vector, Ap^r	[161]; Megan Health (St Louis, MO))
pST76-K (Y09897.1)	ori*SC101* ^{ts}; Km^r	[288]
pSTNSK (JQ436536)	pST76-K::*tnsABCD*; Km^r	This study
pSTNSK-Cm (JQ436537)	pSTNSK::*Cm*; Km^r Cm^r	This study

pSTNSK-Tp (JQ436538)	pSTNSK::*Tp*; Km^r Tp^r	This study
pTNS2 (AY884833)	T7 transposase expression vector, ori*R6K*, Ap^r	[289]
pTP223[c]	λ-red IPTG inducible vector, Tc^r	[331]
pUC18-mini-Tn*7*-*Gm* (AY619004)	pUC18-mini-Tn*7*-*Gm* (Gm^r on mini-Tn*7*T; for gene insertion in Gm^s bacteria), Ap^r Gm^r	[289]

[a] Ap^r, resistance to Ampicillin; Km^r, resistance to Kanamycine; Sm^r, resistance to Streptomycin; Tp^r, resistance to Trimethoprim; Gm^r, resistance to Gentamicin, Tc^r, resistance to Tetracycline; Cm^r, resistance to Chloramphenicol. [b] Genbank accession numbers are in parenthesis. [c] Addgene plasmid 13263.

Table 2. Primers used in this study

Strains or sources	**Gene primer sequence**	
Strains		
	Forward	**Reverse**
Citrobacter rodentium		
ICC168	*glmS* ACATCATTGAGATGCCGCACGTTG	*rod_40121* ACTGAGAAGCCGGAAGGTTGAGTT
Cronobacter sakazakii		
BAA-894	*glmS* TTGAAGAGGTTATCGCGCCGATCT	*ESA_04000* AAACGCGCTGAAGAGAAACAGCTG
Escherichia coli		
536	*glmS* CACCAATCTTCTACACCGTTCCGC	*pstS* AGATCAGTTTGGTGTACGCCAGGT
CFT073	*glmS* CACCAATCTTCTACACCGTTCCGC	*pstS* AGATCAGTTTGGTGTACGCCAGGT
χ7122	*glmS* GATCTTCTACACCGTTCCGC	*stgA* TTATTTCTTATATTCGACAGTAAAT
EHEC EDL933	*glmS* CACCAATCTTCTACACCGTTCCGC	IG[a] between *glmS* and z*5225* TCCACAACTATGAATTCGCGTAGA
Klebsiella pneumoniae		
subsp. *pneumoniae* KPPR1 strain	*glmS* ACATGCACATCATTGAGATGCCGC	*pstS* ATCTGCTTAACGCCACCAGAGGAA
Salmonella enterica **serovars**		
Typhi Ty2a	*glmS* ACATGCACATCATTGAGATGCCGC	*stgA* GTCAGGTCGATATGGAACTCGGTA
Typhimurium SL1344	*glmS* GGAGATTGTGGTGGCGCCGA	*sl3827* CCACGCCATCAGTGGTGGGG
Sources		
pUC18-mini-Tn*7*-Gm	CMD1067 TGCGGTCAATTGTACCGCACAGATGCGTAAGGAGAA	CMD1068 AACGCCGCTCGAGTTTATAGTCCTGTCGGGTTTCGCCA
pFCM1	CMD1466 TCCGGCCCTAGGCGAATTAGCTTCAA	CMD1467 CTACTGCCTAGGGCTCGAATTGGGGA

[a]IG; Intergenic region

Table 3. Integration of Tn7::*xylE* at the *att*Tn7 site[a]

Strains or plasmids	CFU[b]	% of integration[c]
Strains		
Citrobacter rodentium		
ICC168	10^7	68
Cronobacter sakazakii		
BAA-894	10^7	96
Escherichia coli		
536	10^6	97
CFT073	10^7	86
χ7122	10^6	96
EDL933	10^7	80
Klebsiella pneumoniae		
subsp. *pneumoniae* KPPR1	10^8	80
***Salmonella enterica* serovars**		
Typhi Ty2a	10^5	89
Typhimurium SL1344	10^6	96
Combination of vectors		
pGP-Tn7-Gm with:		
pSTNSK	10^7	86
pSTNSK -Tp	10^7	94
pSTNSK -Cm	10^6	91
pSTNSK with:		
pGP-Tn7-Gm	10^7	86
pGP-Tn7-Cm	10^7	98
pGP-Tn7-Tp	10^2	15

[a] The vectors used were pGP-Tn7-Gm-*xylE* and pSTNK for Tn7 and transposase vectors, respectively. [b] Following the O/N conjugation, the number of CFU resistant to Gm was calculated by spreading the conjugation layer onto Gm plates. [c] Integration of Tn7 at *att*Tn7 was evaluated by patching colonies onto Gm, Ap and Km plates. Numbers represent the % of colonies out of at least 100 that was exclusively resistant to Gm. Integration of Tn7::*xylE* to *att*Tn7 was PCR validated. All of the exclusively Gm resistant clones tested were positive.

FIGURES

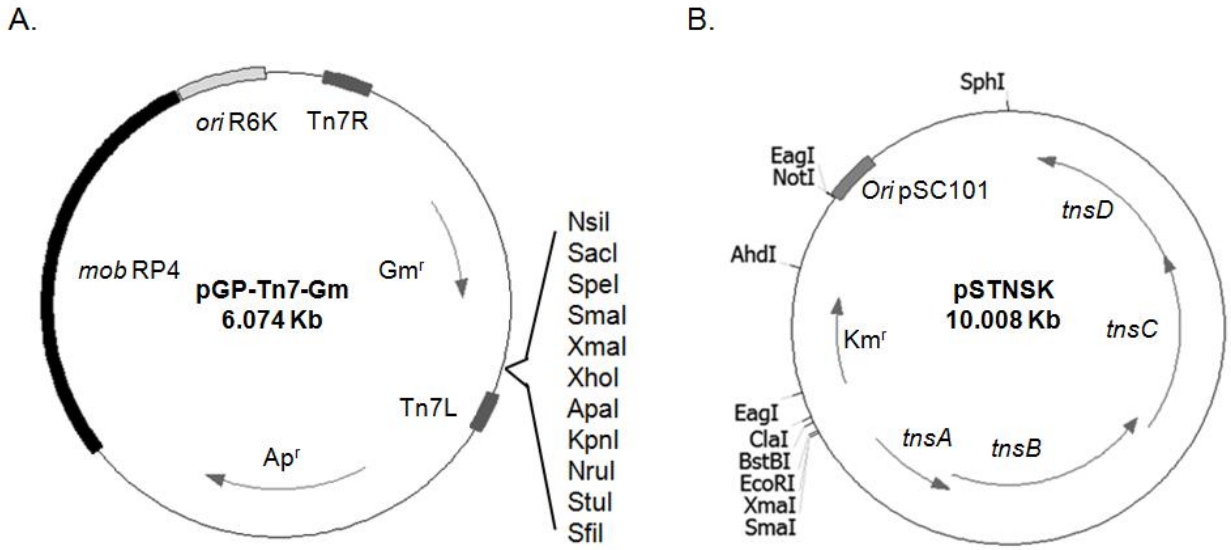

FIG. 1. Features of the mini-Tn7 and transposases vectors. (A) The mobilizable suicide vector pGP-Tn7-Gm contains the conjugative transfer Mob RP4 and the *ori* R6K. A multi-cloning site is integrated between the two Tn7 ends. Derivatives were constructed by replacing the Gm^r gene by the Cm^r and Tp^r gene, respectively. (B) The thermo-sensitive suicide vector pSTNSK contains the pSC101 origin and transposases *tnsABCD*. Derivatives were constructed by cloning respectively the Cm^r and the Tp^r gene into the XmaI site. Accession numbers are referenced in Materials and Methods. Abbreviations: Tn7L and Tn7R, left and right ends of Tn7, respectively.

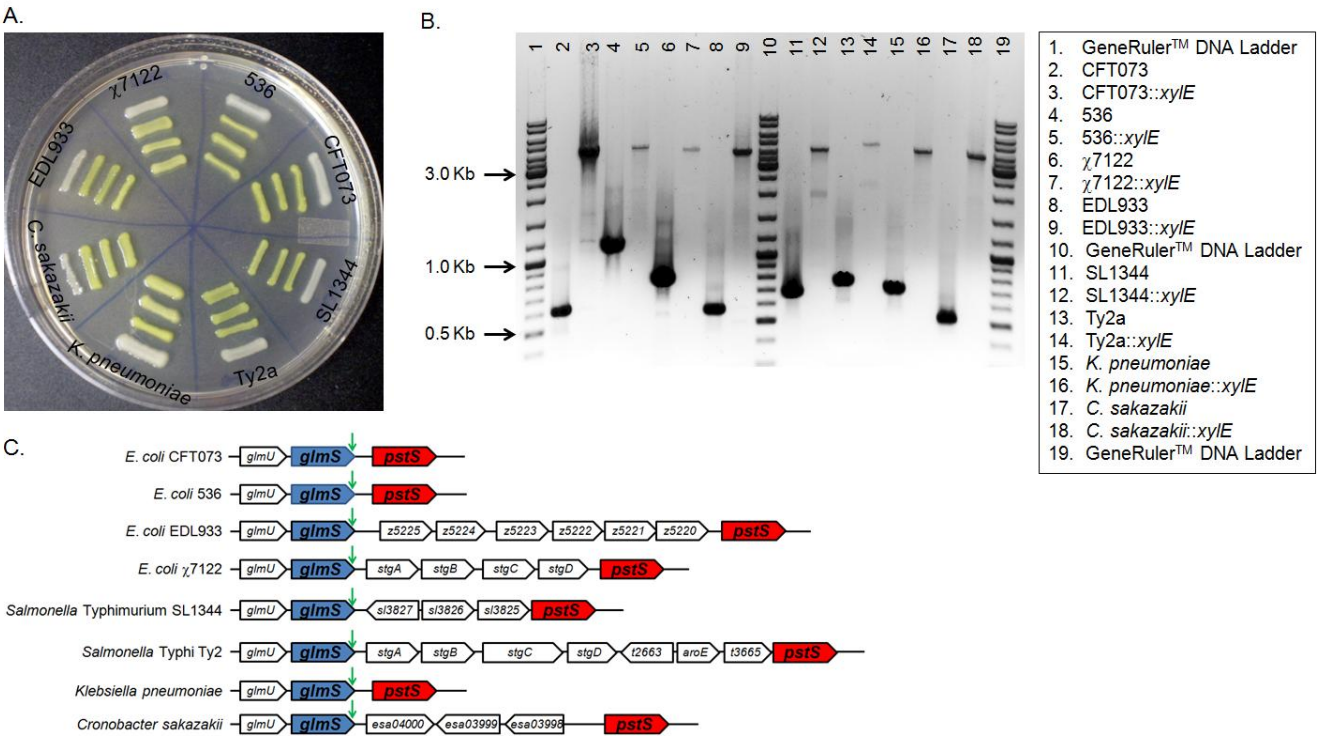

FIG. 2. Functionality of genes integration at *att*Tn7 with the transposon Tn7. (A) Expression of *xylE* gene following transposition at *att*Tn7. Following reaction with a solution of catechol, the WT strains remained white whereas clones in which *xylE* was integrated at *att*Tn7 appeared yellow. Specifications: *C. sakazakii* (*Cronobacter sakazakii* BAA-894), *E. coli* (536, CFT073, χ7122, EDL933), *K. pneumoniae* (*Klebsiella pneumoniae* subsp. *pneumoniae* KPPR1), Ty2a and SL1344 (*Salmonella enterica* serovars Typhi and Typhimurium, respectively) (B) Integration of *xylE* at *att*Tn7 was verified by PCR using the primer-pairs listed in Table 2. Specifications: same as in panel A. (C) Chromosomal visualisation of the *glmS-pstS* region in different strains tested. In *Salmonella* Typhi Ty2, *stgC* has been previously annotated as a pseudogene. However, it may encode the usher of the Stg fimbriae [344]. The green arrow represents the *att*Tn7 site. Length of ORFs and intergenic regions are not to scale.

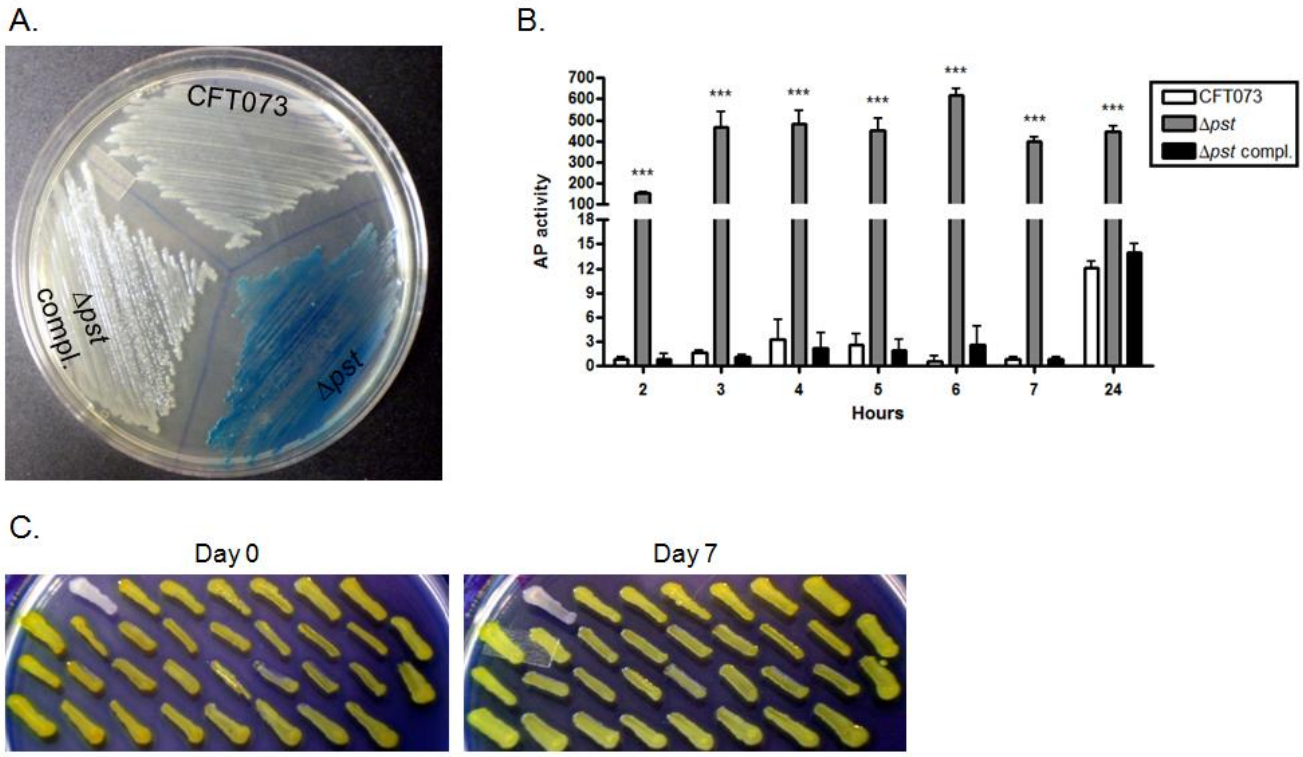

FIG. 3. Chromosomal complementation of *pst* mutant restores native production levels of PhoA. (A) On LB XP agar plates, the Δ*pst* was blue, whereas the WT and the complemented strains were white. (B) Production of PhoA was quantified by an alkaline phosphatase assay. As in panel A, the *pst* mutant massively produced PhoA, whereas in the WT and Δ*pst* compl. strains, production of PhoA was at the basal level. (C) Production of *xylE* from *att*Tn7 in strain CFT073 following passages without selection pressure over a 7 days period. The white patches represent the WT strain whereas the yellow ones, represent those producing *xylE* from *att*Tn7 site.

DISCUSSION

1. Discussion générale

En plus d'être impliqué dans le transport du P_i du milieu extracellulaire au cytoplasme, le système Pst est aussi impliqué dans la virulence bactérienne. Ainsi, l'inactivation du système Pst atténue la virulence d'une multitude de souches bactériennes, e.g. *E*. *coli*, *P*. *mirabilis*, *S*. *flexneri*, *V*. *cholerae*, etc. Ces dernières années, le rôle du système Pst dans la virulence a été plus particulièrement étudié chez *E*. *coli* et *V*. *cholerae*. Chez *E*. *coli*, plusieurs observations et hypothèses ont été décrites et émises afin d'expliquer l'atténuation de virulence. Ainsi, chez les souches EPEC, l'atténuation de virulence semble être causée par une diminution de l'adhérence aux cellules intestinales des mutants *pst* [154, 155]. Chez les souches ExPEC d'origines porcines et aviaires, cette atténuation semble être causée par une perturbation membranaire, rendant ainsi les souches mutantes sensibles à l'effet bactéricide du sérum, à l'acidité, au stress oxydatif et aux peptides cationiques antimicrobiens [7-12]. Cependant, les mécanismes moléculaires impliqués dans ces phénotypes ne sont toujours pas encore clairement définis.

Bien que les concentrations en P_i n'aient pas été directement quantifiées dans les organes testés, i.e. le modèle urinaire et aviaire, nous pouvons spéculer que celles-ci sont élevées. Cette affirmation se base sur les travaux menés par Bertrand *et al*. [197] ainsi que ceux présentés dans l'article #2 de cette thèse. Ainsi, les souches dans lesquelles le gène *phoB* a été délété sont aussi virulentes que les souches sauvages. Étant donné que la survie bactérienne à une carence en phosphate requiert l'activation du régulon Pho, donc la présence de PhoB, la virulence des mutants *phoB* révèle que les environnements rencontrés *in vivo* sont riches en phosphate. Ainsi, *in vivo*, le régulon Pho n'est pas induit. Cependant, ces résultats ont une certaines limitations puisque seule les infections du tractus urinaire (vessie et reins) chez les UPEC, et systémique (sang, poumon droit, foie, rate) chez les APEC, ont été testés. De plus, en induisant PhoBR, indépendamment du système Pst, Bertrand *et al* [197] ont montré que l'atténuation de virulence des mutants *pst* est donc associée à l'induction constitutive et inappropriée du régulon Pho plutôt qu'à son incapacité de transporter le phosphate.

Dans ce projet de thèse, nous avons montré que l'atténuation de virulence du mutant *pst* de la souche UPEC CFT073 est majoritairement causée par la répression des fimbriae de type 1 (Figures 2 et 5 de l'article #2). Cette répression diminue la capacité de la souche mutante à coloniser le tractus urinaire de souris et à envahir les cellules urothéliales. D'un point de vue

moléculaire, nous avons présenté que la répression des fimbriae de type 1 est directement reliée à l'expression différentielle de ses régulateurs, i.e. *fimB*, *fimE*, *ipuA* et *ipbA*. De plus, une diminution de la concentration intracellulaire en ppGpp, chez le mutant *pst*, semble être directement reliée à la répression de la recombinase *fimB* et parallèlement, à une diminution de l'expression des fimbriae de type 1. Aussi, nous avons observé que le mutant *pst* produit une plus grande quantité de c-di-GMP. Cette augmentation de la concentration intracellulaire en c-di-GMP est induite par l'activation de la diguanylate cyclase YaiC, menant ainsi à la répression des recombinases *ipuA* et *ipbA*, à la répression des fimbriae de type 1 et donc, à l'atténuation de virulence.

En combinant ces résultats, nous sommes en mesure de construire un modèle régulationnel reliant le système Pst, le SRDC PhoBR, les fimbriae de type 1 et la virulence comme présenté à la Fig. 7 de l'article #3. Ainsi, la mutation du système Pst, tout comme une carence en phosphate, active le SRDC PhoBR. L'activation de PhoB entraîne un changement de conformation, lui permettant de se fixer aux régions promotrices possédant une ou plusieurs boîtes Pho et ainsi induire la transcription des gènes faisant partie du régulon Pho. La fixation de PhoB au niveau du promoteur de *phoA* induit la transcription de l'opéron *phoA-psiF*. La transcription se poursuit au-delà de cet opéron puisque le gène *yaiC*, se trouvant en aval de *phoA-psiF*, est aussi induit et se trouve sur le même transcript que *phoA-psiF*. L'induction de *yaiC* entraîne l'accumulation intracellulaire en c-di-GMP, qui elle, mène à l'inhibition de la transcription ou de l'activité des recombinases FimB, IpuA et IpbA, entraînant ainsi l'orientation de *fimS* en phase fermée, menant à la répression des fimbriae de type 1 et par conséquent, à l'atténuation de virulence.

2. Différences entre les souches ExPEC aviaire (APEC) χ7122 et urinaire (UPEC) CFT073

Malgré que l'inactivation du système *pst* atténue la virulence de la souche aviaire (APEC) χ7122 et de la souche urinaire (UPEC) CFT073, les raisons de cette atténuation sont bien différentes et ce, même si les deux souches font partie de la même famille, i.e. les ExPEC. Ainsi, l'atténuation de virulence chez la souche χ7122 est principalement causée par une perturbation membranaire et à un effet pléïotrope [197, 198, 200-202], tandis que celle de la souche UPEC CFT073 est majoritairement attribuée à la répression des fimbriae de type 1 (Article #2).

Contrairement à la souche χ7122, la perméabilité membranaire ne semble pas être affectée chez le mutant *pst* de la souche CFT073. Ceci est reflété par une résistance similaire au stress oxydatif, à la polymyxine B et à la vancomycine (Fig. 6 de l'article #2) entre le mutant *pst* et la souche sauvage. Nous observons certes une augmentation de la sensibilité au sérum humain, mais cette sensibilité ne peut être entièrement attribuable à une perturbation membranaire. À cet effet, nous avons utilisé la souche MG1655 comme contrôle négatif. Cette dernière est extrêmement sensible au sérum humain puisqu'aucune colonie n'est comptée à 1 h post-incubation. Chez la souche MG1655, la sensibilité au sérum humain ne peut être associée à une perturbation membranaire, causée par une mutation quelquonque, puisque nous avons utilisé la souche sauvage. Cette sensibilité semble plus attribuable à une sensibilité au complexe du complément.

Le système du complément est l'effecteur majeur de la branche humorale du système immunitaire. C'est un système complexe ayant plusieurs fonctions, dont la lyse cellulaire (cellules de l'hôte, bactéries et virus), l'activation du système immunitaire (inflammation et production de cytokines) et l'opsonisation (favorise la phagocytose). Un des produits finaux du complément est la formation du complexe d'attaque membranaire (CAM). Ce complexe se fixe à la surface cellulaire et forme un pore, entraînant ainsi un débalancement du potentiel électrochimique et mène à la mort cellulaire [345]. La présence des LPS à la surface bactérienne permet, entre autres, à la bactérie de s'évader du système immunitaire de l'hôte. Il altère ainsi l'efficacité de la formation du CAM. Ainsi, les LPS influencent l'accessibilité du complément à la membrane bactérienne puisqu'ils distancent le CAM de celle-ci [346-349]. Comme la souche MG1655 possède un LPS de type rude, i.e. ne contient pas les chaînes oligosaccharidiques retrouvées à l'extrémité distale des LPS, sa membrane est donc facilement accessible au MAC, expliquant ainsi son extrême sensibilité au sérum humain observée à la Figure 6A de l'article #2. À cet égard, et selon les résultats de résistance au stress oxydatif, de polymyxine B et de vancomycine, j'en conclus que la sensibilité intermédiaire du mutant *pst* de la souche UPEC CFT073 au sérum humain peut être causée par une plus grande sensibilité, comparée à la souche sauvage, au complexe du complément, plutôt qu'à une perturbation membranaire proprement dite.

Plusieurs particularités, tant génétiques que physiologiques, peuvent expliquer que la mutation du même système (Pst) entraîne des effets distincts chez les souches APEC et UPEC. Ainsi, il a été montré que la souche APEC χ7122 n'est aucunement reliée phylogénétiquement à la souche UPEC CFT073 [350]. Cette souche fait partie du groupe phylogénétique B1 versus B2, pour la souche CFT073. Le groupe B1 rassemble généralement des souches virulentes chez les animaux autres que les primates, mais commensales chez l'homme, tandis que le groupe B2 inclus les souches ExPEC les plus virulentes chez l'homme [351, 352]. De plus, les sérotypes des deux souches sont différents, i.e. O78:K80:H9 pour la souche χ7122 [280] et O6:K2:H1 pour la souche CFT073 [353], dénotant ainsi une différence au niveau des lipopolysaccharides (O), de la capsule (K) et des flagelles (H). Aussi, l'hôte infecté diffère entre les deux souches, tout comme le type d'infection associé. Ainsi, la souche χ7122 infecte la volaille et est impliquée dans les cas d'inflammation des sacs aériens, de péricardite, de périhépatite et de péritonite menant à une septicémie mortelle [27, 183]. Pour sa part, la souche CFT073 infecte l'homme et est associée à l'infection du tractus urinaire (vessie et reins). Dans les cas sévères de pyélonéphrites, les bactéries se disséminent dans la circulation sanguine et peuvent causer une septicémie [354]. Ainsi, toutes ces particularités peuvent expliquer les différences de phénotypes entre les deux souches. Étant donné que le génome de la souche APEC χ7122 n'est pas encore annoté, nous ne pouvons que comparer les phénotypes des deux souches.

Aussi, en aucun cas je n'exclus la possibilité d'une réponse pléïotrope chez la souche CFT073. Par contre, cette réponse pléïotrope s'effectue au niveau régulationnel et semble aboutir, au final, à la répression des fimbriae de type 1. Nous avons identifié, à l'aide de la banque de transposon, que plusieurs systèmes semblent être influencés par l'inactivation du système *pst*. Ainsi, les flagelles (*flhC*), le transporteur ABC de la leucine (opéron *liv*), le locus de résistance multiple aux antibiotiques (*mar*) et plusieurs systèmes hypothétiques ont été identifiés (pour une description de ces systèmes, voir Annexe 2). Dans chacun des cas, l'élément commun est qu'ils répriment l'expression des fimbriae de type 1 chez le mutant *pst.*

3. Expression des fimbriae F1C

Comme mentionné dans la section Revue de littérature, la souche UPEC CFT073 possède au moins 12 systèmes génétiques distincts codant pour des fimbriae connus ou putatifs et plusieurs adhésines afimbriaires [16]. Puisqu'il a été démontré que les bactéries n'expriment qu'une adhésine à la fois [111], l'expression des adhésines est donc soumise à des mécanismes de co-régulation. Cela permet donc aux bactéries d'exprimer, au moment opportun, les fimbriae appropriés, permettant ainsi aux bactéries d'adhérer aux tissus hôtes et de s'adapter aux microenvironnements qu'elles rencontrent au cours de l'infection. De cette manière, il est suggéré que l'expression des fimbriae de type 1 active un ou des facteurs réprimant l'induction des fimbriae P et F1C [76, 77, 113]. Dans cette optique, le contraire est donc valable, i.e. la répression des fimbriae de type 1 réprime des répresseurs, activant ainsi l'expression d'autres fimbriae.

Dans le cas qui nous concerne, nous observons que l'inactivation du système *pst* réprime l'expression des fimbriae de type 1 autant *in vitro* qu'*in vivo*. D'autre part, nous observons que les fimbriae F1C sont induits chez le mutant *pst*, démontrant ainsi la co-régulation entre les adhésines. Cependant, les mécanismes moléculaires reliant le système Pst aux fimbriae F1C sont encore inconnus. Récemment, Simms *et al*. [355] ont démontré que la surexpression de *papX* (gène faisant partie de l'opéron *pap*), à partir d'un promoteur inductible, réprimait l'expression des fimbriae de type 1 et F1C. De plus, ils ont découvert que le gène *focX*, retrouvé en aval de l'opéron codant les fimbriae F1C, est un homologue quasi identique à *papX*. En effet, l'alignement de séquence des deux gènes montre une différence de seulement 11 nt. Cette différence se traduit par une modification de seulement 6 acides aminés [355]. Cela suggère que FocX est un répresseur des fimbriae de type 1 et des fimbriae F1C.

En considérant ces informations, je me suis demandé si les gènes *papX* et *focX* sont différentiellement exprimés chez le mutant *pst*, pouvant expliquer, en partie, la répression des fimbriae de type 1 et l'induction des fimbriae F1C. Pour ce faire, l'expression de ces deux gènes a été quantifiée par qRT-PCR chez la souche CFT073, le mutant *pst* et la souche complémentée. Comme montré à la Fig. 1 ci-dessous, et suite à la transformation logarithmique, nous remarquons que les gènes *papX* et *focX* sont respectivement réprimés 2,45 et 2,91 fois chez le mutant *pst*.

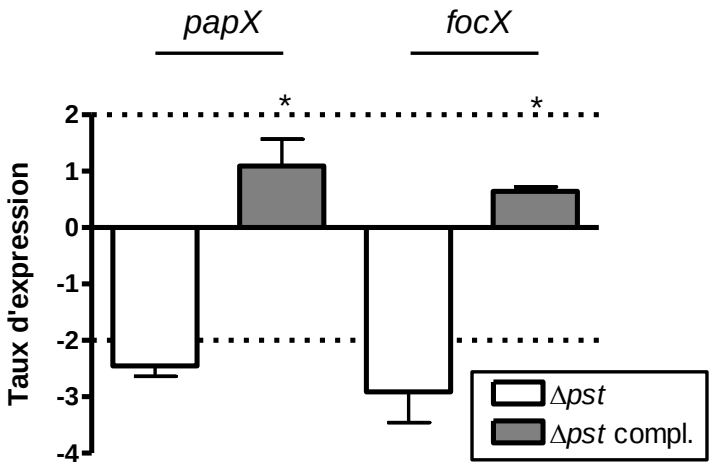

Figure 1. Expression différentielle des gènes *papX* et *focX*. L'expression de *papX* et *focX* chez les souches Δ*pst* et Δ*pst* compl. a été comparée à celle de la souche CFT073. Les lignes pointillées correspondent au seuil d'expression différentielle significative (± 2). Les résultats présentés sont la moyenne et l'écart-type de trois réplicats biologiques. Les différences statistiques ont été calculées à l'aide du test *t* de Student, * $P < 0,05$.

Ainsi, la répression de *papX* et *focX*, chez le mutant *pst*, peut expliquer l'induction des fimbriae F1C. Étant donné que les fimbriae F1C permettent, notamment, l'adhésion au niveau des cellules endothéliales de la vessie [51, 107], l'induction de ces derniers, causée par l'inhibition de ses répresseurs, peut être un effet compensatoire de la perte des fimbriae de type 1. De plus, une analyse *in silico* des promoteurs de ces deux gènes montre la présence d'une boîte Pho putative [147], suggérant ainsi que ces derniers sont directement régulés négativement par PhoB. Aussi, ce mécanisme régulationnel ne semble pas être unique à la souche CFT073 puisque la souche UPEC UTI89 possède également ces gènes, tout comme la présence d'une boîte Pho en amont de *papX* et *focX* [147]. Il serait donc intéressant de déterminer si ces gènes sont effectivement directement régulés par PhoB.

Étant donné qu'il a été démontré que PapX réprime l'expression des fimbriae de type 1 [355], nos résultats présentés à la Fig. 1 de la discussion semblent quelques peu contradictoires en ce qui concerne l'expression des fimbriae de type 1. Ainsi, nous montrons que chez le mutant *pst*, l'expression de *papX* est réprimée. Malgré cette répression, les fimbriae de type 1 sont tout de même réprimés chez cette souche (Article #2 et #3). Cependant, les mécanismes régulationnels des fimbriae de type 1, par PapX et FocX, ne sont pas encore définis. Étant donné que nous

observons, chez le mutant *pst*, une expression différentielle des régulateurs principaux des fimbriae de type 1, soit *fimB*, *fimE*, *ipuA* et *ipbA*, et que la répression de *papX* et *focX* n'affecte pas l'expression des fimbriae de type 1, je crois que ces derniers sont impliqués dans la régulation des fimbriae F1C plutôt que celle des fimbriae de type 1. Sinon, ils jouent un rôle accessoire dans cette régulation. Cependant, des analyses plus approfondies sont requises, comme l'induction de ces gènes et la création de mutants non-polaires, afin de déterminer les mécanismes moléculaires reliant le système Pst, le régulon Pho, PapX, FocX et les fimbriae F1C.

4. Les recombinases IpuA et IpbA

En plus d'être régulés par les recombinases FimB et FimE, les fimbriae de type 1 sont soumis à la régulation médiée par les recombinases IpuA et IpbA. Ces dernières se comportent similairement à FimB, i.e. qu'elles permettent le positionnement du promoteur des fimbriae de type 1 en phase ouverte [65, 66]. Cependant, le rôle précis, le niveau de régulation et la contribution d'IpuA et IpbA dans l'expression des fimbriae de type 1 ne sont pas connus. En effet, ces dernières ont été caractérisées chez une souche dépourvue des recombinases FimB et FimE [65]. Dans l'article #2, nous émettons l'hypothèse que IpuA et IpbA jouent un rôle plutôt accessoire dans la régulation des fimbriae de type 1. Cependant, selon les résultats présentés dans cette thèse, elles semblent jouer un rôle beaucoup plus important. En effet, dans l'article #2, nous démontrons qu'*in vitro*, les gènes *fimB*, *ipuA* et *ipbA* sont réprimés chez le mutant *pst*. Dans l'article #3, nous démontrons que l'inactivation du gène *yaiC*, chez le mutant *pst*, restaure l'expression des fimbriae de type 1. Celle-ci est, de plus, concomitante avec l'induction des recombinases *ipuA* et *ipbA*, suggérant fortement que la modulation de ces recombinases font partie d'un mécanisme par lequel le régulon Pho régule les fimbriae de type 1, comme suggéré à la Fig. 7 de l'article #3.

In vivo, à chacun des temps d'infection, nous affirmons que la répression des fimbriae de type 1, chez le mutant *pst*, est attribuable à l'induction de *fimE* puisque *fimB* n'est pas différentiellement exprimé. Par contre, il serait important de quantifier l'expression de *ipuA* et *ipbA*, *in vivo*, afin de déterminer leur contribution dans l'expression des fimbriae de type 1. Puisqu'ils régulent positivement l'expression des fimbriae de type 1 [52], nous devrions nous attendre à ce qu'ils soient réprimés chez le mutant *pst*. Aussi, l'induction de ces gènes chez le mutant *pst*, à partir

d'un promoteur inductible, nous permettrait de définir et de disséquer leur contribution dans la régulation des fimbriae de type 1. De plus, l'importance de ces deux recombinases, au-delà de FimB et FimE, pourrait être déterminée en introduisant des mutations non-polaires au niveau *d'ipuA* et *ipbA* chez le mutant *pst*. Ainsi, si ces dernières sont bel et bien importantes dans la régulation des fimbriae de type 1, ceux-ci devraient être encore plus réprimés chez les doubles mutants. Finalement, l'analyse *in silico* des promoteurs de *ipuA* et *ipbA* montre que seul *ipuA* semble posséder une boîte Pho [147]. Cependant, ce n'est pas parce que l'algorithme informatique ne reconnaît pas la présence d'une boîte Pho au niveau du promoteur qu'elle n'existe pas. À cet effet, il serait intéressant de déterminer si ces deux gènes sont directement régulés par PhoB. Pour ce faire, nous pourrions procéder à des expérimentations de retard sur gel. Cette procédure est une technique permettant l'étude des intéractions protéines-ADN ou protéines-ARN. Ainsi, en faisant intéragir PhoB, sous sa forme active, avec les régions promotrices de *ipuA* et *ipbA*, nous pourrions déterminer si PhoB active directement ou non ces deux gènes. La migration sur gel des régions promotrices seules ou en présence de PhoB nous permettra de déterminer si PhoB régule directement *ipuA* et/ou *ipbA* puisque l'intéraction entre PhoB et les régions promotrices entraînera un retard de migration, comparativement aux régions promotrices seules.

5. Rôle du ppGpp dans la régulation des fimbriae de type 1

L'effet de l'induction de PhoBR, causée par la délétion du système Pst, sur la transcription de l'opéron *fim* semble indirecte puisque aucune boîte Pho n'est retrouvée au niveau des promoteurs des gènes *fimB*, *fimE*, *fimA* [147]. Comme mentionné précédemment, ce n'est pas parce qu'aucune boîte Pho n'est pas identifiée par une approche *in silico*, que cette dernière n'existe pas. Par contre, selon les observations décrites à l'article #2 et #3, je suis porté à croire que l'effet du régulon Pho sur *fimB*, *fimE* et *fimA* est indirecte. Ainsi, il a été démontré que l'expression des fimbriae de type 1 est liée à la production de l'alarmone ppGpp [78, 79]. Étant donné que les fimbriae de type 1 sont essentiels à la colonisation de la vessie, que celle-ci est carencée en nutriments [356], nous pouvons spéculer que cet environnement est favorable à la production du ppGpp. En effet, l'accumulation de ppGpp active la transcription de *fimB* et donc, l'expression des fimbriae de type 1. De plus, il a été démontré que l'inactivation du système *pst*, chez une souche *E. coli* K-12, diminue la production de ppGpp [226, 227]. Selon ces observations, nous avons donc émis l'hypothèse qu'un possible lien entre le système Pst et

l'expression des fimbriae de type 1 pouvait passer par le ppGpp. Ainsi, comme montré à la Figure 7 de l'article #3, nous observons que le mutant *pst* produit moins de ppGpp que la souche sauvage. À cet effet, nous suggérons que la répression *in vitro* de *fimB*, et donc des fimbriae de type 1, pourrait être causée par une diminution de la concentration intracellulaire en ppGpp.

Cependant, ce ne sont que des résultats préliminaires puisque nous n'avons pas examiné plus en détails le rôle du ppGpp dans la régulation des fimbriae de type 1 chez le mutant *pst*. Ainsi, il serait intéressant d'induire l'expression de *relA* et/ou *spoT*, chez le mutant *pst*, afin d'activer la production de ppGpp et ainsi quantifier l'expression des fimbriae de type 1. Il serait aussi intéressant d'introduire des mutations non-polaires au niveau de *relA* et/ou *spoT* et de corréler l'expression des fimbriae de type 1. Aussi, étant donné que les fimbriae de type 1 sont aussi régulés par les recombinases *ipuA* et *ipbA* [65], il serait intéressant de déterminer l'effet du ppGpp sur l'expression / activité de ces deux recombinases et conséquemment, l'effet sur l'expression des fimbriae de type 1.

Le métabolisme du ppGpp dépend des enzymes RelA et SpoT, codant respectivement pour une GDP pyrophosphokinase / GTP pyrophosphokinase et une guanosine 3'-diphosphate 5'-triphosphate 3'-diphosphatase (Fig. 7 dans la revue de littérature) [221]. Il a été montré que le métablisme du ppGpp, lors d'une carence en phosphate, est contrôlé par SpoT [226, 227, 357]. Cependant, *spoT* n'est pas différentiellement exprimé chez le mutant *pst* (résultat non-publié). Ceci suggère donc que la concentration de ppGpp est contrôlée de façon post-transcriptionnelle. Dans le même ordre d'idée, il a été montré que lors d'un stress du métabolisme des acides gras, l'activité hydrolase de SpoT est inhibée, menant ainsi à l'accumulation du ppGpp [358]. Cette inhibition s'effectue par l'interaction directe de « l'acyl carrier protein (ACP) », un co-facteur central dans la biosynthèse des acides gras, et SpoT. Dans notre cas, nous observons que l'activation du régulon Pho inhibe la production de ppGpp. Donc, à l'instar du stress du métabolisme des acides gras, nous pouvons imaginer un mécanisme régulationnel semblable, i.e. un facteur X, induit par PhoB, inhiberait l'activité synthase de SpoT. L'identification de ce facteur pourrait se faire en procédant à des expérimentations de co-immunoprécipitation, en utilisant des anticorps dirigés contre SpoT, et de spectrométrie de masse. De plus, l'introduction de mutations ponctuelles au niveau du

domaine de l'activité synthase de SpoT, abolissant ainsi cette activité, nous permettrait de déterminer les mécanismes moléculaires reliant le ppGpp, SpoT et le régulon Pho.

6. Rôle du c-di-GMP dans la régulation des fimbriae de type 1

Comme pour le ppGpp, nous observons que le second messager c-di-GMP affecte l'expression des fimbriae de type 1. Ainsi, une augmentation de la concentration intracellulaire en c-di-GMP, chez le mutant *pst*, réprime l'expression des fimbriae de type 1. Malgré l'absence de différence significative entre le mutant *pst* et la souche sauvage, nous observons que le mutant *pst* produit 1,4 fois plus de c-di-GMP que la souche sauvage, ce qui correspond à 25.8 ng ml^{-1} de c-di-GMP. Plusieurs hypothèses peuvent expliquer l'absence de différence significative entre le mutant *pst* et la souche sauvage. Ainsi, il est très difficile de quantifier cette molécule puisqu'elle est finement régulée / produite de façon spatio-temporel, signifiant ainsi qu'elle agit localement et se retrouve dans des micro-compartiments [359]. De plus, les conditions dans lesquelles nous avons quantifié le c-di-GMP ne sont pas optimales, par rapport à ce qui a déjà été rapporté [309]. Ce sont pour ces raisons que nous avons choisi d'induire l'expression des gènes *ydaM* (GGDEF) et *yhjH* (EAL) à partir d'un promoteur inductible chez les différentes souches. De cette manière, nous avons pu contourner cette difficulté et ainsi confirmer que l'induction du c-di-GMP réprime l'expression des fimbriae de type 1.

Comme montré à la Fig. 2 (B, C et D) et 5D de l'article #3, l'inactivation de *yaiC*, chez le mutant *pst*, ne restaure pas entièrement l'expression des fimbriae de type 1. Cela signifie qu'il y a d'autres facteurs, en plus du c-di-GMP, qui sont impliqués dans la régulation des fimbriae de type 1 chez le mutant Pst. À cet effet, l'exploration de la banque de transposon décrite dans l'article #3 nous permettra de décortiquer en détail les mécanismes moléculaires impliqués dans la régulation des fimbriae de type 1. Aussi, il a récemment été démontré, chez *E. coli* K-12, que la formation des biofilms était dépendante de l'effet concerté entre le ppGpp et le c-di-GMP. Ainsi, la dégradation du ppGpp, par SpoT, mène à l'augmentation de la concentration intracellulaire en c-di-GMP médiée par la diguanylate cyclase YdeH [360]. Dans notre cas, nous remarquons similairement le même phénomène, i.e. une diminution de la production de ppGpp ainsi qu'une augmentation de la concentration intracellulaire en c-di-GMP, entraînant ainsi la répression des fimbriae de type 1. À cet effet, je me suis demandé si l'inactivation de la diguanylate cyclase *yaiC*, chez la souche CFT073 et le mutant *pst*, entraînait une augmentation

dans la concentration intracellulaire en ppGpp. Comme montré à la Fig. 2 ci-dessous, nous observons qu'il semble, en effet, exister un effet concerté entre le ppGpp et le c-di-GMP.

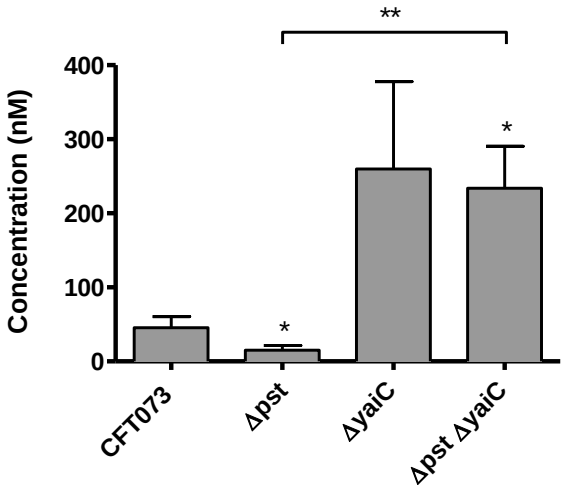

Figure 2. Production de ppGpp chez les mutants *yaiC*. Le ppGpp a été quantifié chez les souches se trouvant en milieu de phase exponentielle de croissance en bouillon LB. Les résultats présentés sont la moyenne et l'écart-type de trois réplicats biologiques. Les différences statistiques ont été calculées à l'aide du test *t* de Student, * $P < 0,05$; ** $P < 0,01$.

Selon les résultats présentés ci-haut, il semble que la production du c-di-GMP, chez le mutant *pst*, inhibe celle du ppGpp. La répression des fimbriae de type 1 pourrait donc être dûe à l'effet concerté de ces deux molécules. Il serait intéressant de déterminer la contribution de chacune de ces molécules dans l'expression des fimbriae de type 1. Pour ce faire, il serait intéressant de quantifier la production de ppGpp chez les souches produisant constitutivement du c-di-GMP. Cela nous permettrait de confirmer les résultats présentés à la Fig. 2 (ci-haut), i.e. que la production du c-di-GMP inhibe celle du ppGpp. De plus, il serait intéressant de déterminer l'expression des fimbriae de type 1 chez le mutant *pst* produisant constitutivement du ppGpp. Ces expérimentations nous permettraient de confirmer que les molécules jouent un rôle dans la régulation des fimbriae de type 1. Finalement, il serait intéressant d'inactiver l'activité hydrolase de SpoT chez le mutant *pst* et de quantifier l'expression des fimbriae de type 1. Cette souche devrait, en théorie, produire les fimbriae de type 1 en raison de la production constitutive du ppGpp. Ainsi, la construction de cette souche mutante nous permettrait de confirmer l'effet concerté du ppGpp et du c-di-GMP dans la régulation des fimbriae.

Il serait intéressant de déterminer à quel niveau le c-di-GMP agit sur la régulation des fimbriae de type 1. Comme suggéré dans le modèle proposé à la Fig. 7 de l'article #3, est-ce que le c-di-GMP agit directement sur la transcription de *ipuA* et *ipbA* ou agit-il sur l'activité de recombinase de FimB, IpuA et IpbA ? S'il s'agit d'une action indirecte, quel est le(s) facteur(s) impliqué dans cette régulation ? Nous pourrions identifier les mécanismes par lesquels le c-di-GMP régule l'expression des fimbriae de type 1 selon une approche similaire à celle présentée par Kevin G. Roelofs lors du congrès Cold Spring Harbor meeting on Microbial Pathogenesis and Host Response [361]. Ainsi, nous pourrions marquer radioactivement du c-di-GMP purifié, le mettre en contact avec les différentes souches et analyser l'interaction entre ce dernier et ses cibles potentielles. Ces dernières pourraient être identifiées en procédant à une extraction protéique, à une électrophorèse sur gel de polyacrylamide, à un autoradiogramme et à l'identification des protéines liant le c-di-GMP à l'aide de la spectrométrie de masse. Par la suite, le rôle de ces cibles dans le relais de l'information entre le c-di-GMP et la régulation des fimbriae de type 1 pourra s'effectuer en inactivant ces gènes.

Un travail colossal a été effectué afin de déterminer le rôle du c-di-GMP dans la virulence de *V. cholerae*. Ainsi, le groupe du Dr Andrew Camilli suggère que dans l'environnement, les gènes GGDEF, impliqués dans la biosynthèse du c-di-GMP, sont produits afin de favoriser la formation des biofilms et la survie dans les milieux aquatiques. Les bactéries sont par la suite ingérées, sous forme de biofilms, par l'hôte. Suite à l'ingestion, des signaux environnementaux, qui sont encore inconnus, inhibent l'expression des gènes GGDEF et activent celle des gènes EAL, menant ainsi à la dégradation du c-di-GMP. Ce changement d'expression de gènes induirait celle des gènes de virulence, dont le pilus TCP et la toxine cholérique (CT). Cela permettrait donc aux bactéries de survivre et de coloniser le petit intestin. Par la suite, un autre changement de régulation se produit, où les gènes tardifs sont induits, dont les gènes GGDEF, permettant ainsi la maintenance de l'infection au niveau intestinal. Cela permet aussi d'augmenter la survie bactérienne lors de la transition entre l'hôte et les milieux aquatiques. Suivant le relâchement dans l'environnement, les bactéries retrouvent leur mode biofilm et pourront infecter un nouvel hôte et recommencer un cycle infectieux [210].

Récemment, il a été montré chez *V*. *cholerae* que PhoB est induit tardivement lors de l'infection [210, 215]. Cette induction mène à la répression du pilus TCP et de la toxine CT [157], tandis qu'elle active celle des gènes EAL *acgA* et GGDEF *acgB* [215]. De cette manière, PhoB serait impliqué dans les étapes tardives de l'infection par *V*. *cholerae*, soit la maintenance de l'infection au niveau intestinal ainsi qu'à l'augmentation de la survie lors de la transition entre l'hôte et les milieux aquatiques. Ces données suggèrent donc que la concentration extracellulaire en P_i est limitante au niveau intestinal [215]. Ces résultats confirment l'hypothèse de Koch AL [362] stipulant que les bactéries transitant au travers du colon et dans les écosystèmes environnementaux sont confrontés à une carence chronique en phosphate.

De cette manière, nous pouvons extrapoler le modèle infectieux proposé par le groupe du Dr Camilli chez les UPEC. Ainsi, les UPEC résident principalement au niveau du tractus intestinal de l'homme et ce, de façon inoffensive [26]. Ainsi, comme suggéré par Patrice Moreau [163], il est possible que les conditions environnementales retrouvées au niveau du tractus intestinal activent PhoBR. Cette activation induirait l'accumulation du c-di-GMP, via YaiC, permettant ainsi aux bactéries de survivre et de persister au niveau de cette niche. Par la suite, le passage de la flore rectale à la vessie entraînerait un changement régulationnel menant à l'inactivation de PhoBR et donc, à la dégradation du c-di-GMP. Ce changement régulationel entraînerait l'induction des fimbriae de type 1 et par conséquent, la colonisation de la vessie. Ainsi, en inactivant le système *pst*, les mutants sont donc bloqués dans leur « mode intestinal » et sont donc inaptes à coloniser le tractus urinaire.

7. Conclusion

Dans ce projet de thèse, nous avions comme objectif principal de déterminer le rôle du système Pst dans la virulence de la souche UPEC CFT073. Plus précisément, nous voulions déterminer les mécanismes moléculaires reliant un système de transport du phosphate, le système Pst, et la virulence. En utilisant une approche multidisciplinaire, notamment la biologie moléculaire et les modèles animaux, nous avons mis en lumière quelques-uns de ces mécanismes. Ainsi, nous avons montré que l'atténuation de virulence des souches UPEC est principalement et directement due à la répression des fimbriae de type 1, plutôt qu'à une perturbation membranaire. L'inactivation du système *pst* altère l'expression des régulateurs des fimbriae de type 1, qui eux, sont régulés par l'effet synergique des seconds messagers ppGpp et c-di-GMP.

De plus, nous avions un objectif satellite, qui était de développer une méthode de complémentation simple-copie dans le chromosome. Nous avons testé, avec succès, notre approche chez 9 différentes souches d'*Enterobacteriaceae*. De cette manière, nous avons été en mesure de complémenter des mutations des gènes dans chacun des cas, nous retrouvons les phénotypes de la souche sauvage. Cela confirme donc la fonctionnalité de notre approche.

PERSPECTIVES

Précédemment, il a été mis en évidence que l'inactivation des systèmes de régulation à deux composants (SRDC) inhibe l'adhésion ainsi que l'expression de divers types de fimbriae chez *E. coli* [80, 255, 256, 276]. Dans notre cas, nous démontrons que l'induction de PhoBR, par l'inactivation du système Pst, réprime l'expression des fimbriae de type 1. En raison de l'augmentation croissante de la résistance aux antibiotiques, chez les souches pathogènes, il est important de développer de nouvelles stratégies préventives et thérapeutiques. Étant donné que les SRDC sont requis dans l'adaptation environnementale et qu'ils sont absents chez l'homme, ils représentent une cible de choix en terme de stratégies thérapeutiques. En effet, plusieurs molécules chimiques ont été développées et montrent un effet inhibiteur sur ces systèmes [277, 278]. Dans la même lignée, plusieurs molécules inhibent l'expression ou l'activité de divers facteurs de virulence, prévenant ainsi l'infection causée par ces pathogènes [185, 186, 188, 190]. De cette manière, le criblage de petites molécules inhibant le système Pst ou activant PhoBR peuvent être considérées comme des agents thérapeutiques puisque l'activation constitutive de PhoBR atténue la virulence.

Le Centre de Chimie Génomique de l'Université du Michigan à Ann Arbor possède une telle librairie chimique. Dans un premier temps, il serait intéressant de cribler, *in vitro*, l'effet de ses petites molécules sur l'activité du régulon Pho. Ensuite, parmi celles agissant sur l'activité du régulon Pho, il serait intéressant de déterminer si ces molécules agissent aussi *in vivo*, i.e. dans le modèle murin d'infection urinaire. Pour ce faire, nous pourrions infecter les souris à l'aide de la souche sauvage. Suite à l'infection, les molécules seraient inoculées (soit directement dans la vessie soit par ingestion) à la souris et nous pourrions déterminer la résorption ou non de l'infection.

En plus d'être utile pour la complémentation de gènes délétés, l'approche que nous avons développée sera aussi utile pour des applications biotechnologiques, des études d'expression génique et dans le développement de vaccins. Ainsi, chez une souche non-pathogène (commensale ou atténuée), nous pourrions induire, à partir du site *att*Tn7, l'expression d'un ou de plusieurs antigènes retrouvés uniquement chez des souches pathogènes. Par exemple, le groupe du Dr Harry Mobley ont déjà identifié 93 antigènes qui sont induits lors d'une infection urinaire [122]. De cette manière, nous pourrions intégrer un ou plusieurs de ces antigènes au site *att*Tn7 d'une souche non-pathogène (souche vaccinale) et expérimenter la procédure. Dans un premier temps, nous infecterions la souris avec la souche vaccinale. Nous testerions par la

suite la réponse immunitaire en quantifiant la production d'anticorps spécifiques aux antigènes exprimés par cette dernière. Finalement, nous pourrions déterminer l'efficacité de la vaccination en infectant les souris pré-immunisées avec la souche sauvage et ce, afin de quantifier son potentiel virulent. Ainsi, une bonne immunisation empêcherait l'instauration de l'infection par la souche sauvage. Globalement, avec cette approche, nous pourrions générer une protection contre des agents pathogènes d'importance en santé animale et/ou en santé publique.

BIBLIOGRAPHIE

1. Kaper JB, Nataro JP, Mobley HL: **Pathogenic *Escherichia coli***. *Nat Rev Microbiol* 2004, **2**(2):123-140.
2. Harvey Lodish AB, Paul Matsudaira, Chris A. Kaiser, Monty Krieger, Matthew P. Scott, Lawrence Zipursky, James Darnell: **Molecular Cell Biology, Fifth Edition**. 2004.
3. Wanner BL: **Gene regulation by phosphate in enteric bacteria**. *J Cell Biochem* 1993, **51**(1):47-54.
4. Wanner BL: **Phosphorus assimilation and control of the phosphate regulon, p. 1357–1381.** *In R C I Neidhardt, J L Ingraham, E E C Lin, K B Low, B Magasanik, W S Reznikoff, M Riley, M Schaechter, and H E Umbrager (ed), Escherichia coli and Salmonella: cellular and molecular biology, vol 1 American Society for Microbiology, Washington, DC* 1996.
5. Crepin S, Chekabab SM, Le Bihan G, Bertrand N, Dozois CM, Harel J: **The Pho regulon and the pathogenesis of *Escherichia coli***. *Vet Microbiol* 2011, **153**(1-2):82-88.
6. Lamarche MG, Wanner BL, Crepin S, Harel J: **The phosphate regulon and bacterial virulence: a regulatory network connecting phosphate homeostasis and pathogenesis**. *FEMS Microbiol Rev* 2008.
7. Bertrand N, Houle S, LeBihan G, Poirier E, Dozois CM, Harel J: **Increased Pho regulon activation correlates with decreased virulence of an avian pathogenic *Escherichia coli* O78 strain**. *Infect Immun* 2010, **78**(12):5324-5331.
8. Crepin S, Lamarche MG, Garneau P, Seguin J, Proulx J, Dozois CM, Harel J: **Genome-wide transcriptional response of an avian pathogenic *Escherichia coli* (APEC) pst mutant**. *BMC Genomics* 2008, **9**:568.
9. Daigle F, Fairbrother JM, Harel J: **Identification of a mutation in the *pst-phoU* operon that reduces pathogenicity of an *Escherichia coli* strain causing septicemia in pigs**. *Infect Immun* 1995, **63**(12):4924-4927.
10. Lamarche MG, Dozois CM, Daigle F, Caza M, Curtiss R, 3rd, Dubreuil JD, Harel J: **Inactivation of the pst system reduces the virulence of an avian pathogenic *Escherichia coli* O78 strain**. *Infect Immun* 2005, **73**(7):4138-4145.
11. Lamarche MG, Harel J: **Membrane homeostasis requires intact *pst* in extraintestinal pathogenic *Escherichia coli***. *Curr Microbiol* 2010, **60**(5):356-359.
12. Lamarche MG, Kim SH, Crepin S, Mourez M, Bertrand N, Bishop RE, Dubreuil JD, Harel J: **Modulation of hexa-acyl pyrophosphate lipid A population under *Escherichia coli* phosphate (Pho) regulon activation**. *J Bacteriol* 2008.
13. Hannan TJ, Totsika M, Mansfield KJ, Moore KH, Schembri MA, Hultgren SJ: **Host-pathogen checkpoints and population bottlenecks in persistent and intracellular uropathogenic *Escherichia coli* bladder infection**. *FEMS Microbiol Rev* 2012, **36**(3):616-648.
14. Nielubowicz GR, Mobley HL: **Host-pathogen interactions in urinary tract infection**. *Nat Rev Urol* 2010, **7**(8):430-441.
15. Stenutz R, Weintraub A, Widmalm G: **The structures of *Escherichia coli* O-polysaccharide antigens**. *FEMS Microbiol Rev* 2006, **30**(3):382-403.
16. Welch RA, Burland V, Plunkett G, 3rd, Redford P, Roesch P, Rasko D, Buckles EL, Liou SR, Boutin A, Hackett J *et al*: **Extensive mosaic structure revealed by the complete genome sequence of uropathogenic *Escherichia coli***. *Proc Natl Acad Sci U S A* 2002, **99**(26):17020-17024.
17. Dobrindt U: **(Patho-)Genomics of *Escherichia coli***. *Int J Med Microbiol* 2005, **295**(6-7):357-371.
18. Dozois CM, Curtiss R, 3rd: **Pathogenic diversity of *Escherichia coli* and the emergence of 'exotic' islands in the gene stream**. *Vet Res* 1999, **30**(2-3):157-179.

19. Hacker J, Kaper JB: **Pathogenicity islands and the evolution of microbes**. *Ann Rev Microbiol* 2000, **54**:641-679.
20. Organization WH: **Future directions for research on enterotoxigenic *Escherichia coli* vaccines for developing countries.** *Wkly Epidemiol Rec 81:97* 2006.
21. Taxt A, Aasland R, Sommerfelt H, Nataro J, Puntervoll P: **Heat-stable enterotoxin of enterotoxigenic *Escherichia coli* as a vaccine target**. *Infect Immun* 2010, **78**(5):1824-1831.
22. Hicks S, Candy DC, Phillips AD: **Adhesion of enteroaggregative *Escherichia coli* to pediatric intestinal mucosa in vitro**. *Infect Immun* 1996, **64**(11):4751-4760.
23. Nataro JP, Kaper JB: **Diarrheagenic *Escherichia coli***. *Clin Microbiol Rev* 1998, **11**(1):142-201.
24. Frank C, Faber MS, Askar M, Bernard H, Fruth A, Gilsdorf A, Hohle M, Karch H, Krause G, Prager R *et al*: **Large and ongoing outbreak of haemolytic uraemic syndrome, Germany, May 2011**. *Euro Surveill* 2011, **16**(21).
25. Scheutz F, Nielsen EM, Frimodt-Moller J, Boisen N, Morabito S, Tozzoli R, Nataro JP, Caprioli A: **Characteristics of the enteroaggregative Shiga toxin/verotoxin-producing *Escherichia coli* O104:H4 strain causing the outbreak of haemolytic uraemic syndrome in Germany, May to June 2011**. *Euro Surveill* 2011, **16**(24).
26. Alteri CJ, Mobley HL: ***Escherichia coli* physiology and metabolism dictates adaptation to diverse host microenvironments**. *Curr Opin Microbiol* 2012, **15**(1):3-9.
27. La Ragione RM, Woodward MJ: **Virulence factors of *Escherichia coli* serotypes associated with avian colisepticaemia**. *Res Vet Sci* 2002, **73**(1):27-35.
28. Ronald A: **Research Priorities.** *In: Stanton, S L, and Dwyer, P L (eds) Urinary tract infection in the female, Martin Dunitz, Ltd, London, UK* 2000.
29. Litwin MS, Saigal CS, Yano EM, Avila C, Geschwind SA, Hanley JM, Joyce GF, Madison R, Pace J, Polich SM *et al*: **Urologic diseases in America Project: analytical methods and principal findings**. *J Urol* 2005, **173**(3):933-937.
30. Johnson JR: **Treatment and prevention of urinary tract infections.** *In: T, M H L, and Warren, J W (eds), ASM Press, Washigton, D C* 1996.
31. Foxman B: **Recurring urinary tract infection: incidence and risk factors**. *Am J Public Health* 1990, **80**(3):331-333.
32. Ikaheimo R, Siitonen A, Heiskanen T, Karkkainen U, Kuosmanen P, Lipponen P, Makela PH: **Recurrence of urinary tract infection in a primary care setting: analysis of a 1-year follow-up of 179 women**. *Clin Infect Dis* 1996, **22**(1):91-99.
33. Foxman B, Zhang L, Tallman P, Palin K, Rode C, Bloch C, Gillespie B, Marrs CF: **Virulence characteristics of *Escherichia coli* causing first urinary tract infection predict risk of second infection**. *J Infect Dis* 1995, **172**(6):1536-1541.
34. Russo TA, Stapleton A, Wenderoth S, Hooton TM, Stamm WE: **Chromosomal restriction fragment length polymorphism analysis of *Escherichia coli* strains causing recurrent urinary tract infections in young women**. *J Infect Dis* 1995, **172**(2):440-445.
35. Lane MC, Alteri CJ, Smith SN, Mobley HL: **Expression of flagella is coincident with uropathogenic *Escherichia coli* ascension to the upper urinary tract**. *Proc Natl Acad Sci U S A* 2007, **104**(42):16669-16674.
36. Hunstad DA, Justice SS: **Intracellular lifestyles and immune evasion strategies of uropathogenic *Escherichia coli***. *Ann Rev Microbiol* 2010, **64**:203-221.
37. Dhakal BK, Kulesus RR, Mulvey MA: **Mechanisms and consequences of bladder cell invasion by uropathogenic *Escherichia coli***. *Eur J Clin Invest* 2008, **38 Suppl 2**:2-11.

38. Justice SS, Hung C, Theriot JA, Fletcher DA, Anderson GG, Footer MJ, Hultgren SJ: **Differentiation and developmental pathways of uropathogenic *Escherichia coli* in urinary tract pathogenesis**. *Proc Natl Acad Sci U S A* 2004, **101**(5):1333-1338.
39. Rosen DA, Hooton TM, Stamm WE, Humphrey PA, Hultgren SJ: **Detection of intracellular bacterial communities in human urinary tract infection**. *PLoS Med* 2007, **4**(12):e329.
40. Klemm P, Krogfelt KA: **Type 1-fimbriae of *Escherichia coli*. In Fimbriae: Adhesion, Genetics, Biogenesis, and Vaccines (Klemm, P., ed.), pp. 9–26, CRC Press, Boca Raton, FL.** 1994.
41. La Ragione RM, Cooley WA, Woodward MJ: **The role of fimbriae and flagella in the adherence of avian strains of *Escherichia coli* O78:K80 to tissue culture cells and tracheal and gut explants**. *J Med Microbiol* 2000, **49**(4):327-338.
42. Hahn E, Wild P, Hermanns U, Sebbel P, Glockshuber R, Haner M, Taschner N, Burkhard P, Aebi U, Muller SA: **Exploring the 3D molecular architecture of *Escherichia coli* type 1 pili**. *J Mol Biol* 2002, **323**(5):845-857.
43. Krogfelt KA, Bergmans H, Klemm P: **Direct evidence that the FimH protein is the mannose-specific adhesin of *Escherichia coli* type 1 fimbriae**. *Infect Immun* 1990, **58**(6):1995-1998.
44. Lowe MA, Holt SC, Eisenstein BI: **Immunoelectron microscopic analysis of elongation of type 1 fimbriae in *Escherichia coli***. *J Bacteriol* 1987, **169**(1):157-163.
45. Orndorff PE, Falkow S: **Organization and expression of genes responsible for type 1 piliation in *Escherichia coli***. *J Bacteriol* 1984, **159**(2):736-744.
46. Orndorff PE, Falkow S: **Nucleotide sequence of *pilA*, the gene encoding the structural component of type 1 pili in *Escherichia coli***. *J Bacteriol* 1985, **162**(1):454-457.
47. Russell PW, Orndorff PE: **Lesions in two *Escherichia coli* type 1 pilus genes alter pilus number and length without affecting receptor binding**. *J Bacteriol* 1992, **174**(18):5923-5935.
48. Hornick DB, Allen BL, Horn MA, Clegg S: **Fimbrial types among respiratory isolates belonging to the family Enterobacteriaceae**. *J Clin Microbiol* 1991, **29**(9):1795-1800.
49. McMichael JC, Ou JT: **Structure of common pili from *Escherichia coli***. *J Bacteriol* 1979, **138**(3):969-975.
50. Mulvey MA, Lopez-Boado YS, Wilson CL, Roth R, Parks WC, Heuser J, Hultgren SJ: **Induction and evasion of host defenses by type 1-piliated uropathogenic *Escherichia coli***. *Science* 1998, **282**(5393):1494-1497.
51. Mulvey MA: **Adhesion and entry of uropathogenic *Escherichia coli***. *Cell Microbiol* 2002, **4**(5):257-271.
52. Schembri MA, Klemm P: **Biofilm formation in a hydrodynamic environment by novel *fimh* variants and ramifications for virulence**. *Infect Immun* 2001, **69**(3):1322-1328.
53. Schembri MA, Christiansen G, Klemm P: **FimH-mediated autoaggregation of *Escherichia coli***. *Mol Microbiol* 2001, **41**(6):1419-1430.
54. Nishiyama M, Horst R, Eidam O, Herrmann T, Ignatov O, Vetsch M, Bettendorff P, Jelesarov I, Grutter MG, Wuthrich K *et al*: **Structural basis of chaperone-subunit complex recognition by the type 1 pilus assembly platform FimD**. *Embo J* 2005, **24**(12):2075-2086.
55. Bahrani-Mougeot FK, Buckles EL, Lockatell CV, Hebel JR, Johnson DE, Tang CM, Donnenberg MS: **Type 1 fimbriae and extracellular polysaccharides are preeminent uropathogenic *Escherichia coli* virulence determinants in the murine urinary tract**. *Mol Microbiol* 2002, **45**(4):1079-1093.

56. Connell I, Agace W, Klemm P, Schembri M, Marild S, Svanborg C: **Type 1 fimbrial expression enhances *Escherichia coli* virulence for the urinary tract**. *Proc Natl Acad Sci U S A* 1996, **93**(18):9827-9832.
57. Schaeffer AJ, Schwan WR, Hultgren SJ, Duncan JL: **Relationship of type 1 pilus expression in *Escherichia coli* to ascending urinary tract infections in mice**. *Infect Immun* 1987, **55**(2):373-380.
58. Smith SN, Hagan EC, Lane MC, Mobley HL: **Dissemination and Systemic Colonization of Uropathogenic *Escherichia coli* in a Murine Model of Bacteremia**. *mBio* 2010, **1**(5).
59. Snyder JA, Haugen BJ, Buckles EL, Lockatell CV, Johnson DE, Donnenberg MS, Welch RA, Mobley HL: **Transcriptome of uropathogenic *Escherichia coli* during urinary tract infection**. *Infect Immun* 2004, **72**(11):6373-6381.
60. Snyder JA, Lloyd AL, Lockatell CV, Johnson DE, Mobley HL: **Role of phase variation of type 1 fimbriae in a uropathogenic *Escherichia coli* cystitis isolate during urinary tract infection**. *Infect Immun* 2006, **74**(2):1387-1393.
61. Martinez JJ, Mulvey MA, Schilling JD, Pinkner JS, Hultgren SJ: **Type 1 pilus-mediated bacterial invasion of bladder epithelial cells**. *Embo J* 2000, **19**(12):2803-2812.
62. Abraham JM, Freitag CS, Clements JR, Eisenstein BI: **An invertible element of DNA controls phase variation of type 1 fimbriae of *Escherichia coli***. *Proc Natl Acad Sci U S A* 1985, **82**(17):5724-5727.
63. Blomfield IC: **The regulation of *pap* and type 1 fimbriation in *Escherichia coli***. *Adv Microb Physiol* 2001, **45**:1-49.
64. Blomfield IC, Kulasekara DH, Eisenstein BI: **Integration host factor stimulates both FimB- and FimE-mediated site-specific DNA inversion that controls phase variation of type 1 fimbriae expression in *Escherichia coli***. *Mol Microbiol* 1997, **23**(4):705-717.
65. Bryan A, Roesch P, Davis L, Moritz R, Pellett S, Welch RA: **Regulation of type 1 fimbriae by unlinked FimB- and FimE-like recombinases in uropathogenic *Escherichia coli* strain CFT073**. *Infect Immun* 2006, **74**(2):1072-1083.
66. Hannan TJ, Mysorekar IU, Chen SL, Walker JN, Jones JM, Pinkner JS, Hultgren SJ, Seed PC: **LeuX tRNA-dependent and -independent mechanisms of *Escherichia coli* pathogenesis in acute cystitis**. *Mol Microbiol* 2008, **67**(1):116-128.
67. Klemm P: **Two regulatory fim genes, *fimB* and *fimE*, control the phase variation of type 1 fimbriae in *Escherichia coli***. *Embo J* 1986, **5**(6):1389-1393.
68. McClain MS, Blomfield IC, Eisenstein BI: **Roles of *fimB* and *fimE* in site-specific DNA inversion associated with phase variation of type 1 fimbriae in *Escherichia coli***. *J Bacteriol* 1991, **173**(17):5308-5314.
69. Lane MC, Simms AN, Mobley HL: **complex interplay between type 1 fimbrial expression and flagellum-mediated motility of uropathogenic *Escherichia coli***. *J Bacteriol* 2007, **189**(15):5523-5533.
70. Lim JK, Gunther NWt, Zhao H, Johnson DE, Keay SK, Mobley HL: ***In vivo* phase variation of *Escherichia coli* type 1 fimbrial genes in women with urinary tract infection**. *Infect Immun* 1998, **66**(7):3303-3310.
71. Struve C, Krogfelt KA: ***In vivo* detection of *Escherichia coli* type 1 fimbrial expression and phase variation during experimental urinary tract infection**. *Microbiology* 1999, **145 (Pt 10)**:2683-2690.
72. Muller CM, Aberg A, Straseviciene J, Emody L, Uhlin BE, Balsalobre C: **Type 1 fimbriae, a colonization factor of uropathogenic *Escherichia coli*, are controlled by the metabolic sensor CRP-cAMP**. *PLoS Pathog* 2009, **5**(2):e1000303.

73. Lindberg S, Xia Y, Sonden B, Goransson M, Hacker J, Uhlin BE: **Regulatory Interactions among adhesin gene systems of uropathogenic *Escherichia coli***. *Infect Immun* 2008, **76**(2):771-780.
74. Blumer C, Kleefeld A, Lehnen D, Heintz M, Dobrindt U, Nagy G, Michaelis K, Emody L, Polen T, Rachel R *et al*: **Regulation of type 1 fimbriae synthesis and biofilm formation by the transcriptional regulator LrhA of *Escherichia coli***. *Microbiology* 2005, **151**(Pt 10):3287-3298.
75. Sohanpal BK, El-Labany S, Lahooti M, Plumbridge JA, Blomfield IC: **Integrated regulatory responses of *fimB* to N-acetylneuraminic (sialic) acid and GlcNAc in *Escherichia coli* K-12**. *Proc Natl Acad Sci U S A* 2004, **101**(46):16322-16327.
76. Holden NJ, Totsika M, Mahler E, Roe AJ, Catherwood K, Lindner K, Dobrindt U, Gally DL: **Demonstration of regulatory cross-talk between P fimbriae and type 1 fimbriae in uropathogenic *Escherichia coli***. *Microbiology* 2006, **152**(Pt 4):1143-1153.
77. Xia Y, Gally D, Forsman-Semb K, Uhlin BE: **Regulatory cross-talk between adhesin operons in *Escherichia coli*: inhibition of type 1 fimbriae expression by the PapB protein**. *EMBO J* 2000, **19**(7):1450-1457.
78. Aberg A, Shingler V, Balsalobre C: **(p)ppGpp regulates type 1 fimbriation of *Escherichia coli* by modulating the expression of the site-specific recombinase FimB**. *Mol Microbiol* 2006, **60**(6):1520-1533.
79. Aberg A, Shingler V, Balsalobre C: **Regulation of the *fimB* promoter: a case of differential regulation by ppGpp and DksA in vivo**. *Mol Microbiol* 2008, **67**(6):1223-1241.
80. Schwan WR, Shibata S, Aizawa S, Wolfe AJ: **The two-component response regulator RcsB regulates type 1 piliation in *Escherichia coli***. *J Bacteriol* 2007, **189**(19):7159-7163.
81. McVicker G, Sun L, Sohanpal BK, Gashi K, Williamson RA, Plumbridge J, Blomfield IC: **SlyA protein activates *fimB* gene expression and type 1 fimbriation in *Escherichia coli* K-12**. *J Biol Chem* 2012, **286**(37):32026-32035.
82. Donato GM, Kawula TH: **Phenotypic analysis of random *hns* mutations differentiate DNA-binding activity from properties of *fimA* promoter inversion modulation and bacterial motility**. *J Bacteriol* 1999, **181**(3):941-948.
83. Blomfield IC, Calie PJ, Eberhardt KJ, McClain MS, Eisenstein BI: **Lrp stimulates phase variation of type 1 fimbriation in *Escherichia coli* K-12**. *J Bacteriol* 1993, **175**(1):27-36.
84. Gally DL, Rucker TJ, Blomfield IC: **The leucine-responsive regulatory protein binds to the *fim* switch to control phase variation of type 1 fimbrial expression in *Escherichia coli* K-12**. *J Bacteriol* 1994, **176**(18):5665-5672.
85. Roesch PL, Blomfield IC: **Leucine alters the interaction of the leucine-responsive regulatory protein (Lrp) with the *fim* switch to stimulate site-specific recombination in *Escherichia coli***. *Mol Microbiol* 1998, **27**(4):751-761.
86. Dorman CJ, Bhriain NN: **Thermal regulation of *fimA*, the *Escherichia coli* gene coding for the type 1 fimbrial subunit protein**. *FEMS Microbiol Lett* 1992, **78**(2-3):125-130.
87. Gally DL, Bogan JA, Eisenstein BI, Blomfield IC: **Environmental regulation of the *fim* switch controlling type 1 fimbrial phase variation in *Escherichia coli* K-12: effects of temperature and media**. *J Bacteriol* 1993, **175**(19):6186-6193.
88. Dove SL, Smith SG, Dorman CJ: **Control of *Escherichia coli* type 1 fimbrial gene expression in stationary phase: a negative role for RpoS**. *Mol Gen Genet* 1997, **254**(1):13-20.

89. Old DC, Duguid JP: **Selective outgrowth of fimbriate bacteria in static liquid medium**. *J Bacteriol* 1970, **103**(2):447-456.
90. de Felice M, Lago CT, Squires CH, Calvo JM: **Acetohydroxy acid synthase isoenzymes of *Escherichia coli* K12 and *Salmonella typhimurium***. *Ann Microbiol* 1982, **133**(2):251-256.
91. Platko JV, Willins DA, Calvo JM: **The *ilvIH* operon of *Escherichia coli* is positively regulated**. *J Bacteriol* 1990, **172**(8):4563-4570.
92. Squires CH, De Felice M, Wessler SR, Calvo JM: **Physical characterization of the *ilvHI* operon of *Escherichia coli* K-12**. *J Bacteriol* 1981, **147**(3):797-804.
93. Lahooti M, Roesch PL, Blomfield IC: **Modulation of the sensitivity of FimB recombination to branched-chain amino acids and alanine in *Escherichia coli* K-12**. *J Bacteriol* 2005, **187**(18):6273-6280.
94. Schwan WR, Lee JL, Lenard FA, Matthews BT, Beck MT: **Osmolarity and pH growth conditions regulate *fim* gene transcription and type 1 pilus expression in uropathogenic *Escherichia coli***. *Infect Immun* 2002, **70**(3):1391-1402.
95. Lane MC, Mobley HL: **Role of P-fimbrial-mediated adherence in pyelonephritis and persistence of uropathogenic *Escherichia coli* (UPEC) in the mammalian kidney**. *Kid Int* 2007, **72**(1):19-25.
96. Bullitt E, Makowski L: **Structural polymorphism of bacterial adhesion pili**. *Nature* 1995, **373**(6510):164-167.
97. Kuehn MJ, Heuser J, Normark S, Hultgren SJ: **P pili in uropathogenic *E. coli* are composite fibres with distinct fibrillar adhesive tips**. *Nature* 1992, **356**(6366):252-255.
98. Leffler H, Svanborg-Eden C: **Glycolipid receptors for uropathogenic *Escherichia coli* on human erythrocytes and uroepithelial cells**. *Infect Immun* 1981, **34**(3):920-929.
99. Orndorff PE, Falkow S: **Identification and characterization of a gene product that regulates type 1 piliation in *Escherichia coli***. *J Bacteriol* 1984, **160**(1):61-66.
100. Olsen A, Jonsson A, Normark S: **Fibronectin binding mediated by a novel class of surface organelles on *Escherichia coli***. *Nature* 1989, **338**(6217):652-655.
101. Barnhart MM, Chapman MR: **Curli biogenesis and function**. *Ann Rev Microbiol* 2006, **60**:131-147.
102. Olsen A, Arnqvist A, Hammar M, Sukupolvi S, Normark S: **The RpoS sigma factor relieves H-NS-mediated transcriptional repression of *csgA*, the subunit gene of fibronectin-binding curli in *Escherichia coli***. *Mol Microbiol* 1993, **7**(4):523-536.
103. Kai-Larsen Y, Luthje P, Chromek M, Peters V, Wang X, Holm A, Kadas L, Hedlund KO, Johansson J, Chapman MR *et al*: **Uropathogenic *Escherichia coli* modulates immune responses and its curli fimbriae interact with the antimicrobial peptide LL-37**. *PLoS Pathog* 2010, **6**(7):e1001010.
104. Klemm P, Christiansen G, Kreft B, Marre R, Bergmans H: **Reciprocal exchange of minor components of type 1 and F1C fimbriae results in hybrid organelles with changed receptor specificities**. *J Bacteriol* 1994, **176**(8):2227-2234.
105. Klemm P, Jorgensen BJ, Kreft B, Christiansen G: **The export systems of type 1 and F1C fimbriae are interchangeable but work in parental pairs**. *J Bacteriol* 1995, **177**(3):621-627.
106. van Die I, Kramer C, Hacker J, Bergmans H, Jongen W, Hoekstra W: **Nucleotide sequence of the genes coding for minor fimbrial subunits of the F1C fimbriae of *Escherichia coli***. *Res Microbiol* 1991, **142**(6):653-658.
107. Khan AS, Kniep B, Oelschlaeger TA, Van Die I, Korhonen T, Hacker J: **Receptor structure for F1C fimbriae of uropathogenic *Escherichia coli***. *Infect Immun* 2000, **68**(6):3541-3547.

108. Backhed F, Alsen B, Roche N, Angstrom J, von Euler A, Breimer ME, Westerlund-Wikstrom B, Teneberg S, Richter-Dahlfors A: **Identification of target tissue glycosphingolipid receptors for uropathogenic, F1C-fimbriated *Escherichia coli* and its role in mucosal inflammation**. *J Biol Chem* 2002, **277**(20):18198-18205.
109. Spurbeck RR, Stapleton AE, Johnson JR, Walk ST, Hooton TM, Mobley HL: **Fimbrial profiles predict virulence of uropathogenic *Escherichia coli* strains: contribution of *ygi* and *yad* fimbriae**. *Infect Immun* 2011, **79**(12):4753-4763.
110. Korea CG, Badouraly R, Prevost MC, Ghigo JM, Beloin C: ***Escherichia coli* K-12 possesses multiple cryptic but functional chaperone-usher fimbriae with distinct surface specificities**. *Environ Microbiol* 2010, **12**(7):1957-1977.
111. Nowicki B, Rhen M, Vaisanen-Rhen V, Pere A, Korhonen TK: **Immunofluorescence study of fimbrial phase variation in *Escherichia coli* KS71**. *J Bacteriol* 1984, **160**(2):691-695.
112. Wullt B, Bergsten G, Samuelsson M, Gebretsadik N, Hull R, Svanborg C: **The role of P fimbriae for colonization and host response induction in the human urinary tract**. *J Infect Dis* 2001, **183 Suppl 1**:S43-46.
113. Snyder JA, Haugen BJ, Lockatell CV, Maroncle N, Hagan EC, Johnson DE, Welch RA, Mobley HL: **Coordinate expression of fimbriae in uropathogenic *Escherichia coli***. *Infect Immun* 2005, **73**(11):7588-7596.
114. Holden NJ, Uhlin BE, Gally DL: **PapB paralogues and their effect on the phase variation of type 1 fimbriae in *Escherichia coli***. *Mol Microbiol* 2001, **42**(2):319-330.
115. Schwan WR, Beck MT, Hultgren SJ, Pinkner J, Woolever NL, Larson T: **Down-regulation of the *kps* region 1 capsular assembly operon following attachment of *Escherichia coli* type 1 fimbriae to D-mannose receptors**. *Infect Immun* 2005, **73**(2):1226-1231.
116. Pesavento C, Becker G, Sommerfeldt N, Possling A, Tschowri N, Mehlis A, Hengge R: **Inverse regulatory coordination of motility and curli-mediated adhesion in *Escherichia coli***. *Gen Dev* 2008, **22**(17):2434-2446.
117. Holden NJ, Gally DL: **Switches, cross-talk and memory in *Escherichia coli* adherence**. *J Med Microbiol* 2004, **53**(Pt 7):585-593.
118. Wiles TJ, Kulesus RR, Mulvey MA: **Origins and virulence mechanisms of uropathogenic *Escherichia coli***. *Exp Mol Pathol* 2008, **85**(1):11-19.
119. Dhakal BK, Mulvey MA: **The UPEC pore-forming toxin alpha-hemolysin triggers proteolysis of host proteins to disrupt cell adhesion, inflammatory, and survival pathways**. *Cell Host Microb* 2012, **11**(1):58-69.
120. O'Hanley P, Lalonde G, Ji G: **Alpha-hemolysin contributes to the pathogenicity of piliated digalactoside-binding *Escherichia coli* in the kidney: efficacy of an alpha-hemolysin vaccine in preventing renal injury in the BALB/c mouse model of pyelonephritis**. *Infect Immun* 1991, **59**(3):1153-1161.
121. Smith YC, Rasmussen SB, Grande KK, Conran RM, O'Brien AD: **Hemolysin of uropathogenic *Escherichia coli* evokes extensive shedding of the uroepithelium and hemorrhage in bladder tissue within the first 24 hours after intraurethral inoculation of mice**. *Infect Immun* 2008, **76**(7):2978-2990.
122. Vigil PD, Alteri CJ, Mobley HL: **Identification of *in vivo*-induced antigens including an RTX family exoprotein required for uropathogenic *Escherichia coli* virulence**. *Infect Immun* 2011, **79**(6):2335-2344.
123. Vigil PD, Wiles TJ, Engstrom MD, Prasov L, Mulvey MA, Mobley HL: **The repeat-in-toxin family member TosA mediates adherence of uropathogenic *Escherichia coli* and survival during bacteremia**. *Infect Immun* 2012, **80**(2):493-505.

124. Lemonnier M, Landraud L, Lemichez E: **Rho GTPase-activating bacterial toxins: from bacterial virulence regulation to eukaryotic cell biology**. *FEMS Microbiol Rev* 2007, **31**(5):515-534.
125. Etienne-Manneville S, Hall A: **Rho GTPases in cell biology**. *Nature* 2002, **420**(6916):629-635.
126. Davis JM, Carvalho HM, Rasmussen SB, O'Brien AD: **Cytotoxic necrotizing factor type 1 delivered by outer membrane vesicles of uropathogenic *Escherichia coli* attenuates polymorphonuclear leukocyte antimicrobial activity and chemotaxis**. *Infect Immun* 2006, **74**(8):4401-4408.
127. Davis JM, Rasmussen SB, O'Brien AD: **Cytotoxic necrotizing factor type 1 production by uropathogenic *Escherichia coli* modulates polymorphonuclear leukocyte function**. *Infect Immun* 2005, **73**(9):5301-5310.
128. Doye A, Mettouchi A, Bossis G, Clement R, Buisson-Touati C, Flatau G, Gagnoux L, Piechaczyk M, Boquet P, Lemichez E: **CNF1 exploits the ubiquitin-proteasome machinery to restrict Rho GTPase activation for bacterial host cell invasion**. *Cell* 2002, **111**(4):553-564.
129. Guyer DM, Radulovic S, Jones FE, Mobley HL: **Sat, the secreted autotransporter toxin of uropathogenic *Escherichia coli*, is a vacuolating cytotoxin for bladder and kidney epithelial cells**. *Infect Immun* 2002, **70**(8):4539-4546.
130. Maroncle NM, Sivick KE, Brady R, Stokes FE, Mobley HL: **Protease activity, secretion, cell entry, cytotoxicity, and cellular targets of secreted autotransporter toxin of uropathogenic *Escherichia coli***. *Infect Immun* 2006, **74**(11):6124-6134.
131. Garenaux A, Caza M, Dozois CM: **The Ins and Outs of siderophore mediated iron uptake by extra-intestinal pathogenic *Escherichia coli***. *Vet Microbiol* 2012, **153**(1-2):89-98.
132. Garcia EC, Brumbaugh AR, Mobley HL: **Redundancy and specificity of *Escherichia coli* iron acquisition systems during urinary tract infection**. *Infect Immun* 2011, **79**(3):1225-1235.
133. VanBogelen RA, Olson ER, Wanner BL, Neidhardt FC: **Global analysis of proteins synthesized during phosphorus restriction in *Escherichia coli***. *J Bacteriol* 1996, **178**(15):4344-4366.
134. Harris RM, Webb DC, Howitt SM, Cox GB: **Characterization of PitA and PitB from *Escherichia coli***. *J Bacteriol* 2001, **183**(17):5008-5014.
135. Rao NN, Torriani A: **Molecular aspects of phosphate transport in *Escherichia coli***. *Mol Microbiol* 1990, **4**(7):1083-1090.
136. Willsky GR, Malamy MH: **Control of the synthesis of alkaline phosphatase and the phosphate-binding protein in *Escherichia coli***. *J Bacteriol* 1976, **127**(1):595-609.
137. Higgins CF: **ABC transporters: from microorganisms to man**. *Annu Rev Cell Biol* 1992, **8**:67-113.
138. Surin BP, Rosenberg H, Cox GB: **Phosphate-specific transport system of *Escherichia coli*: nucleotide sequence and gene-polypeptide relationships**. *J Bacteriol* 1985, **161**(1):189-198.
139. Oganesyan V, Oganesyan N, Adams PD, Jancarik J, Yokota HA, Kim R, Kim SH: **Crystal structure of the "PhoU-like" phosphate uptake regulator from Aquifex aeolicus**. *J Bacteriol* 2005, **187**(12):4238-4244.
140. Steed PM, Wanner BL: **Use of the rep technique for allele replacement to construct mutants with deletions of the *pstSCAB-phoU* operon: evidence of a new role for the PhoU protein in the phosphate regulon**. *J Bacteriol* 1993, **175**(21):6797-6809.

141. Liu J, Lou Y, Yokota H, Adams PD, Kim R, Kim SH: **Crystal structure of a PhoU protein homologue: a new class of metalloprotein containing multinuclear iron clusters**. *J Biol Chem* 2005, **280**(16):15960-15966.
142. Kubena BD, Luecke H, Rosenberg H, Quiocho FA: **Crystallization and x-ray diffraction studies of a phosphate-binding protein involved in active transport in *Escherichia coli***. *J Biol Chem* 1986, **261**(17):7995-7996.
143. Baek JH, Lee SY: **Novel gene members in the Pho regulon of *Escherichia coli***. *FEMS Microbiol Lett* 2006, **264**(1):104-109.
144. Blanco AG, Sola M, Gomis-Ruth FX, Coll M: **Tandem DNA recognition by PhoB, a two-component signal transduction transcriptional activator**. *Structure* 2002, **10**(5):701-713.
145. Kim SK, Kimura S, Shinagawa H, Nakata A, Lee KS, Wanner BL, Makino K: **Dual transcriptional regulation of the *Escherichia coli* phosphate-starvation-inducible *psiE* gene of the phosphate regulon by PhoB and the cyclic AMP (cAMP)-cAMP receptor protein complex**. *J Bacteriol* 2000, **182**(19):5596-5599.
146. Suziedeliene E, Suziedelis K, Garbenciute V, Normark S: **The acid-inducible *asr* gene in *Escherichia coli*: transcriptional control by the *phoBR* operon**. *J Bacteriol* 1999, **181**(7):2084-2093.
147. Yuan ZC, Zaheer R, Morton R, Finan TM: **Genome prediction of PhoB regulated promoters in *Sinorhizobium meliloti* and twelve *proteobacteria***. *Nucleic Acids Res* 2006, **34**(9):2686-2697.
148. Han JS, Park JY, Lee YS, Thony B, Hwang DS: **PhoB-dependent transcriptional activation of the *iciA* gene during starvation for phosphate in *Escherichia coli***. *Mol Gen Genet* 1999, **262**(3):448-452.
149. Gao R, Stock AM: **Biological insights from structures of two-component proteins**. *Annu Rev Microbiol* 2009, **63**:133-154.
150. Mizuno T: **Compilation of all genes encoding two-component phosphotransfer signal transducers in the genome of *Escherichia coli***. *DNA Res* 1997, **4**(2):161-168.
151. West AH, Stock AM: **Histidine kinases and response regulator proteins in two-component signaling systems**. *Trends Biochem Sci* 2001, **26**(6):369-376.
152. Hsieh YJ, Wanner BL: **Global regulation by the seven-component Pi signaling system**. *Curr Opin Microbiol* 2010, **13**(2):198-203.
153. Baek JH, Lee SY: **Transcriptome analysis of phosphate starvation response in *Escherichia coli***. *J Microbiol Biotechnol* 2007, **17**(2):244-252.
154. Cheng C, Tennant SM, Azzopardi KI, Bennett-Wood V, Hartland EL, Robins-Browne RM, Tauschek M: **Contribution of the *pst-phoU* operon to cell adherence by atypical enteropathogenic *Escherichia coli* and virulence of *Citrobacter rodentium***. *Infect Immun* 2009, **77**(5):1936-1944.
155. Ferreira GM, Spira B: **The *pst* operon of enteropathogenic *Escherichia coli* enhances bacterial adherence to epithelial cells**. *Microbiology* 2008, **154**(Pt 7):2025-2036.
156. Jacobsen SM, Lane MC, Harro JM, Shirtliff ME, Mobley HL: **The high-affinity phosphate transporter Pst is a virulence factor for *Proteus mirabilis* during complicated urinary tract infection**. *FEMS Immunol Med Microbiol* 2008, **52**(2):180-193.
157. Pratt JT, Ismail AM, Camilli A: **PhoB regulates both environmental and virulence gene expression in *Vibrio cholerae***. *Mol Microbiol* 2010, **77**(6):1595-1605.
158. Ngeleka M, Harel J, Jacques M, Fairbrother JM: **Characterization of a polysaccharide capsular antigen of septicemic *Escherichia coli* O115:K "V165" :F165 and evaluation of its role in pathogenicity**. *Infect Immun* 1992, **60**(12):5048-5056.

159. Dobrindt U, Hacker J: **Targeting virulence traits: potential strategies to combat extraintestinal pathogenic *E. coli* infections**. *Curr Opin Microbiol* 2008, **11**(5):409-413.
160. Lamarche MG, Harel J: **Growth Phase-dependent Membrane Perturbations Occur in an *Escherichia coli* Phosphate Specific Transport (Pst) Mutant Strain** *Soumis à Res Microbiol* 2008.
161. Dozois CM, Daigle F, Curtiss R, 3rd: **Identification of pathogen-specific and conserved genes expressed *in vivo* by an avian pathogenic *Escherichia coli* strain**. *Proc Natl Acad Sci U S A* 2003, **100**(1):247-252.
162. Seib KL, Wu HJ, Kidd SP, Apicella MA, Jennings MP, McEwan AG: **Defenses against oxidative stress in Neisseria gonorrhoeae: a system tailored for a challenging environment**. *Microbiol Mol Biol Rev* 2006, **70**(2):344-361.
163. Moreau PL: **The lysine decarboxylase CadA protects *Escherichia coli* starved of phosphate against fermentation acids**. *J Bacteriol* 2007, **189**(6):2249-2261.
164. Moreau PL, Gerard F, Lutz NW, Cozzone P: **Non-growing *Escherichia coli* cells starved for glucose or phosphate use different mechanisms to survive oxidative stress**. *Mol Microbiol* 2001, **39**(4):1048-1060.
165. Johnson JR, Clabots C, Rosen H: **Effect of inactivation of the global oxidative stress regulator *oxyR* on the colonization ability of *Escherichia coli* O1:K1:H7 in a mouse model of ascending urinary tract infection**. *Infect Immun* 2006, **74**(1):461-468.
166. Zavaleta-Pastor M, Sohlenkamp C, Gao JL, Guan Z, Zaheer R, Finan TM, Raetz CR, Lopez-Lara IM, Geiger O: ***Sinorhizobium meliloti* phospholipase C required for lipid remodeling during phosphorus limitation**. *Proc Natl Acad Sci U S A* 2010, **107**(1):302-307.
167. Frirdich E, Whitfield C: **Lipopolysaccharide inner core oligosaccharide structure and outer membrane stability in human pathogens belonging to the *Enterobacteriaceae***. *J Endotoxin Res* 2005, **11**(3):133-144.
168. Nikaido H: **Molecular basis of bacterial outer membrane permeability revisited**. *Microbiol Mol Biol Rev* 2003, **67**(4):593-656.
169. Bishop RE: **Fundamentals of endotoxin structure and function**. *Contrib Microbiol* 2005, **12**:1-27.
170. Dziva F, van Diemen PM, Stevens MP, Smith AJ, Wallis TS: **Identification of *Escherichia coli* O157 : H7 genes influencing colonization of the bovine gastrointestinal tract using signature-tagged mutagenesis**. *Microbiology* 2004, **150**(Pt 11):3631-3645.
171. van Diemen PM, Dziva F, Stevens MP, Wallis TS: **Identification of enterohemorrhagic *Escherichia coli* O26:H- genes required for intestinal colonization in calves**. *Infect Immun* 2005, **73**(3):1735-1743.
172. Teng CH, Cai M, Shin S, Xie Y, Kim KJ, Khan NA, Di Cello F, Kim KS: ***Escherichia coli* K1 RS218 interacts with human brain microvascular endothelial cells via type 1 fimbria bacteria in the fimbriated state**. *Infect Immun* 2005, **73**(5):2923-2931.
173. Dozois CM, Chanteloup N, Dho-Moulin M, Bree A, Desautels C, Fairbrother JM: **Bacterial colonization and in vivo expression of F1 (type 1) fimbrial antigens in chickens experimentally infected with pathogenic *Escherichia coli***. *Avian Dis* 1994, **38**(2):231-239.
174. Pourbakhsh SA, Boulianne M, Martineau-Doize B, Dozois CM, Desautels C, Fairbrother JM: **Dynamics of *Escherichia coli* infection in experimentally inoculated chickens**. *Avian Dis* 1997, **41**(1):221-233.
175. Batisson I, Guimond MP, Girard F, An H, Zhu C, Oswald E, Fairbrother JM, Jacques M, Harel J: **Characterization of the novel factor *paa* involved in the early steps of the**

adhesion mechanism of attaching and effacing *Escherichia coli*. *Infect Immun* 2003, **71**(8):4516-4525.
176. Kim Y, Oh S, Park S, Kim SH: **Interactive transcriptome analysis of enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 and intestinal epithelial HT-29 cells after bacterial attachment**. *Int J Food Microbiol* 2009, **131**(2-3):224-232.
177. Critzer FJ, D'Souza DH, Saxton AM, Golden DA: **Increased transcription of the phosphate-specific transport system of *Escherichia coli* O157:H7 after exposure to sodium benzoate**. *J Food Prot* 2010, **73**(5):819-824.
178. Bhatt K, Banerjee SK, Chakraborti PK: **Evidence that phosphate specific transporter is amplified in a fluoroquinolone resistant *Mycobacterium smegmatis***. *Eur J Biochem* 2000, **267**(13):4028-4032.
179. Wang S, Deng K, Zaremba S, Deng X, Lin C, Wang Q, Tortorello ML, Zhang W: **Transcriptomic response of *Escherichia coli* O157:H7 to oxidative stress**. *Appl Environ Microbiol* 2009, **75**(19):6110-6123.
180. Allen KJ, Lepp D, McKellar RC, Griffiths MW: **Examination of stress and virulence gene expression in *Escherichia coli* O157:H7 using targeted microarray analysis**. *Foodborne Pathog Dis* 2008, **5**(4):437-447.
181. John M, Kudva IT, Griffin RW, Dodson AW, McManus B, Krastins B, Sarracino D, Progulske-Fox A, Hillman JD, Handfield M *et al*: **Use of *in vivo*-induced antigen technology for identification of *Escherichia coli* O157:H7 proteins expressed during human infection**. *Infect Immun* 2005, **73**(5):2665-2679.
182. Yoshida Y, Sugiyama S, Oyamada T, Yokoyama K, Makino K: **Identification and characterization of novel phosphate regulon genes, *ecs0540-ecs0544*, in *Escherichia coli* O157:H7**. *Mol Genet Genomics* 2010, **284**(3):197-205.
183. Barnes HJ, J.-P. Vaillancourt, and W. B. Gross: **Colibacillosis, p. 631–652. In Y. M. Saif, H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald, and D. E. Swayne (ed.), Diseases of poultry, 11th ed. Iowa State Press, Ames, Iowa**. 2003.
184. Ron EZ: **Host specificity of septicemic *Escherichia coli*: human and avian pathogens**. *Curr Opin Microbiol* 2006, **9**(1):28-32.
185. Cegelski L, Marshall GR, Eldridge GR, Hultgren SJ: **The biology and future prospects of antivirulence therapies**. *Nat Rev Microbiol* 2008, **6**(1):17-27.
186. Cegelski L, Pinkner JS, Hammer ND, Cusumano CK, Hung CS, Chorell E, Aberg V, Walker JN, Seed PC, Almqvist F *et al*: **Small-molecule inhibitors target *Escherichia coli* amyloid biogenesis and biofilm formation**. *Nat Chem Biol* 2009, **5**(12):913-919.
187. Chauhan N, Calderone R: **Two-component signal transduction proteins as potential drug targets in medically important fungi**. *Infect Immun* 2008, **76**(11):4795-4803.
188. Garrity-Ryan LK, Kim OK, Balada-Llasat JM, Bartlett VJ, Verma AK, Fisher ML, Castillo C, Songsungthong W, Tanaka SK, Levy SB *et al*: **Small molecule inhibitors of LcrF, a Yersinia pseudotuberculosis transcription factor, attenuate virulence and limit infection in a murine pneumonia model**. *Infect Immun* 2010, **78**(11):4683-4690.
189. Mattmann ME, Blackwell HE: **Small molecules that modulate quorum sensing and control virulence in *Pseudomonas aeruginosa***. *J Org Chem* 2010, **75**(20):6737-6746.
190. Rasko DA, Moreira CG, Li de R, Reading NC, Ritchie JM, Waldor MK, Williams N, Taussig R, Wei S, Roth M *et al*: **Targeting QseC signaling and virulence for antibiotic development**. *Science* 2008, **321**(5892):1078-1080.
191. Lestrate P, Delrue RM, Danese I, Didembourg C, Taminiau B, Mertens P, De Bolle X, Tibor A, Tang CM, Letesson JJ: **Identification and characterization of *in vivo* attenuated mutants of *Brucella melitensis***. *Mol Microbiol* 2000, **38**(3):543-551.
192. Hunt TA, Kooi C, Sokol PA, Valvano MA: **Identification of *Burkholderia cenocepacia* genes required for bacterial survival *in vivo***. *Infect Immun* 2004, **72**(7):4010-4022.

193. Grant AJ, Coward C, Jones MA, Woodall CA, Barrow PA, Maskell DJ: **Signature-tagged transposon mutagenesis studies demonstrate the dynamic nature of cecal colonization of 2-week-old chickens by *Campylobacter jejuni***. *Appl Environ Microbiol* 2005, **71**(12):8031-8041.
194. Gaynor EC, Wells DH, MacKichan JK, Falkow S: **The *Campylobacter jejuni* stringent response controls specific stress survival and virulence-associated phenotypes**. *Mol Microbiol* 2005, **56**(1):8-27.
195. McKean S, Davies J, Moore R: **Identification of macrophage induced genes of *Corynebacterium pseudotuberculosis* by differential fluorescence induction**. *Microbes Infect* 2005, **7**(13):1352-1363.
196. Khan MA, Isaacson RE: **Identification of *Escherichia coli* genes that are specifically expressed in a murine model of septicemic infection**. *Infect Immun* 2002, **70**(7):3404-3412.
197. Zhao Y, Blumer SE, Sundin GW: **Identification of *Erwinia amylovora* genes induced during infection of immature pear tissue**. *J Bacteriol* 2005, **187**(23):8088-8103.
198. Yang S, Perna NT, Cooksey DA, Okinaka Y, Lindow SE, Ibekwe AM, Keen NT, Yang CH: **Genome-wide identification of plant-upregulated genes of *Erwinia chrysanthemi* 3937 using a GFP-based IVET leaf array**. *Mol Plant Microbe Interact* 2004, **17**(9):999-1008.
199. Dubail I, Berche P, Charbit A: **Listeriolysin O as a reporter to identify constitutive and *in vivo*-inducible promoters in the pathogen *Listeria monocytogenes***. *Infect Immun* 2000, **68**(6):3242-3250.
200. Lawlor MS, Hsu J, Rick PD, Miller VL: **Identification of *Klebsiella pneumoniae* virulence determinants using an intranasal infection model**. *Mol Microbiol* 2005, **58**(4):1054-1073.
201. Talaat AM, Lyons R, Howard ST, Johnston SA: **The temporal expression profile of *Mycobacterium tuberculosis* infection in mice**. *Proc Natl Acad Sci U S A* 2004, **101**(13):4602-4607.
202. Burall LS, Harro JM, Li X, Lockatell CV, Himpsl SD, Hebel JR, Johnson DE, Mobley HL: ***Proteus mirabilis* genes that contribute to pathogenesis of urinary tract infection: identification of 25 signature-tagged mutants attenuated at least 100-fold**. *Infect Immun* 2004, **72**(5):2922-2938.
203. Valdivia RH, Falkow S: **Fluorescence-based isolation of bacterial genes expressed within host cells**. *Science* 1997, **277**(5334):2007-2011.
204. Faucher SP, Porwollik S, Dozois CM, McClelland M, Daigle F: **Transcriptome of *Salmonella enterica* serovar Typhi within macrophages revealed through the selective capture of transcribed sequences**. *Proc Natl Acad Sci U S A* 2006, **103**(6):1906-1911.
205. Runyen-Janecky LJ, Payne SM: **Identification of chromosomal *Shigella flexneri* genes induced by the eukaryotic intracellular environment**. *Infect Immun* 2002, **70**(8):4379-4388.
206. Polissi A, Pontiggia A, Feger G, Altieri M, Mottl H, Ferrari L, Simon D: **Large-scale identification of virulence genes from *Streptococcus pneumoniae***. *Infect Immun* 1998, **66**(12):5620-5629.
207. Hava DL, Camilli A: **Large-scale identification of serotype 4 *Streptococcus pneumoniae* virulence factors**. *Mol Microbiol* 2002, **45**(5):1389-1406.
208. Marra A, Asundi J, Bartilson M, Lawson S, Fang F, Christine J, Wiesner C, Brigham D, Schneider WP, Hromockyj AE: **Differential fluorescence induction analysis of *Streptococcus pneumoniae* identifies genes involved in pathogenesis**. *Infect Immun* 2002, **70**(3):1422-1433.

209. Merrell DS, Hava DL, Camilli A: **Identification of novel factors involved in colonization and acid tolerance of *Vibrio cholerae***. *Mol Microbiol* 2002, **43**(6):1471-1491.
210. Schild S, Tamayo R, Nelson EJ, Qadri F, Calderwood SB, Camilli A: **Genes induced late in infection increase fitness of *Vibrio cholerae* after release into the environment**. *Cell Host Microb* 2007, **2**(4):264-277.
211. Darwin AJ, Miller VL: **Identification of *Yersinia enterocolitica* genes affecting survival in an animal host using signature-tagged transposon mutagenesis**. *Mol Microbiol* 1999, **32**(1):51-62.
212. Lathem WW, Crosby SD, Miller VL, Goldman WE: **Progression of primary pneumonic plague: a mouse model of infection, pathology, and bacterial transcriptional activity**. *Proc Natl Acad Sci U S A* 2005, **102**(49):17786-17791.
213. Mudrak B, Tamayo R: **The *Vibrio cholerae* Pst2 phosphate transport system is upregulated in biofilms and contributes to biofilm-induced hyperinfectivity**. *Infect Immun* 2012, **80**(5):1794-802.
214. von Kruger WM, Humphreys S, Ketley JM: **A role for the PhoBR regulatory system homologue in the *Vibrio cholerae* phosphate-limitation response and intestinal colonization**. *Microbiology* 1999, **145 (Pt 9)**:2463-2475.
215. Pratt JT, McDonough E, Camilli A: **PhoB regulates motility, biofilms, and cyclic di-GMP in *Vibrio cholerae***. *J Bacteriol* 2009, **191**(21):6632-6642.
216. Tamayo R, Patimalla B, Camilli A: **Growth in a biofilm induces a hyperinfectious phenotype in *Vibrio cholerae***. *Infect Immun* 2010, **78**(8):3560-3569.
217. O'May GA, Jacobsen SM, Longwell M, Stoodley P, Mobley HL, Shirtliff ME: **The high-affinity phosphate transporter Pst in *Proteus mirabilis* HI4320 and its importance in biofilm formation**. *Microbiology* 2009, **155**(Pt 5):1523-1535.
218. Srinivasa Rao PS, Lim TM, Leung KY: **Functional genomics approach to the identification of virulence genes involved in *Edwardsiella tarda* pathogenesis**. *Infect Immun* 2003, **71**(3):1343-1351.
219. Chakraborty S, Sivaraman J, Leung KY, Mok YK: **Two-component PhoB-PhoR regulatory system and ferric uptake regulator sense phosphate and iron to control virulence genes in type III and VI secretion systems of *Edwardsiella tarda***. *J Biol Chem* 2012, **286**(45):39417-39430.
220. Runyen-Janecky LJ, Boyle AM, Kizzee A, Liefer L, Payne SM: **Role of the Pst system in plaque formation by the intracellular pathogen *Shigella flexneri***. *Infect Immun* 2005, **73**(3):1404-1410.
221. Dalebroux ZD, Swanson MS: **ppGpp: magic beyond RNA polymerase**. *Nat Rev Microbiol* 2012, **10**(3):203-212.
222. Jishage M, Kvint K, Shingler V, Nystrom T: **Regulation of sigma factor competition by the alarmone ppGpp**. *Gen Dev* 2002, **16**(10):1260-1270.
223. Potrykus K, Cashel M: **(p)ppGpp: still magical?** *Ann Rev Microbiol* 2008, **62**:35-51.
224. Dalebroux ZD, Svensson SL, Gaynor EC, Swanson MS: **ppGpp conjures bacterial virulence**. *Microbiol Mol Biol Rev* 2010, **74**(2):171-199.
225. Nakanishi N, Abe H, Ogura Y, Hayashi T, Tashiro K, Kuhara S, Sugimoto N, Tobe T: **ppGpp with DksA controls gene expression in the locus of enterocyte effacement (LEE) pathogenicity island of enterohaemorrhagic *Escherichia coli* through activation of two virulence regulatory genes**. *Mol Microbiol* 2006, **61**(1):194-205.
226. Spira B, Silberstein N, Yagil E: **Guanosine 3',5'-bispyrophosphate (ppGpp) synthesis in cells of *Escherichia coli* starved for Pi**. *J Bacteriol* 1995, **177**(14):4053-4058.
227. Spira B, Yagil E: **The relation between ppGpp and the PHO regulon in *Escherichia coli***. *Mol Gen Genet* 1998, **257**(4):469-477.

228. Buckles EL, Wang X, Lockatell CV, Johnson DE, Donnenberg MS: **PhoU enhances the ability of extraintestinal pathogenic *Escherichia coli* strain CFT073 to colonize the murine urinary tract**. *Microbiology* 2006, **152**(Pt 1):153-160.
229. Ross P, Mayer R, Weinhouse H, Amikam D, Huggirat Y, Benziman M, de Vroom E, Fidder A, de Paus P, Sliedregt LA *et al*: **The cyclic diguanylic acid regulatory system of cellulose synthesis in *Acetobacter xylinum*. Chemical synthesis and biological activity of cyclic nucleotide dimer, trimer, and phosphothioate derivatives**. *J Biol Chem* 1990, **265**(31):18933-18943.
230. Mills E, Pultz IS, Kulasekara HD, Miller SI: **The bacterial second messenger c-di-GMP: mechanisms of signalling**. *Cell Microbiol* 2011, **13**(8):1122-1129.
231. Sondermann H, Shikuma NJ, Yildiz FH: **You've come a long way: c-di-GMP signaling**. *Curr Opin Microbiol* 2012.
232. Tamayo R, Pratt JT, Camilli A: **Roles of cyclic diguanylate in the regulation of bacterial pathogenesis**. *Ann Rev Microbiol* 2007, **61**:131-148.
233. Schirmer T, Jenal U: **Structural and mechanistic determinants of c-di-GMP signalling**. *Nat Rev Microbiol* 2009, **7**(10):724-735.
234. Meissner A, Wild V, Simm R, Rohde M, Erck C, Bredenbruch F, Morr M, Romling U, Haussler S: ***Pseudomonas aeruginosa cupA*-encoded fimbriae expression is regulated by a GGDEF and EAL domain-dependent modulation of the intracellular level of cyclic diguanylate**. *Environ Microbiol* 2007, **9**(10):2475-2485.
235. Vallet I, Olson JW, Lory S, Lazdunski A, Filloux A: **The chaperone/usher pathways of *Pseudomonas aeruginosa*: identification of fimbrial gene clusters (cup) and their involvement in biofilm formation**. *Proc Natl Acad Sci U S A* 2001, **98**(12):6911-6916.
236. Monds RD, Newell PD, Gross RH, O'Toole GA: **Phosphate-dependent modulation of c-di-GMP levels regulates *Pseudomonas fluorescens* Pf0-1 biofilm formation by controlling secretion of the adhesin LapA**. *Mol Microbiol* 2007, **63**(3):656-679.
237. Newell PD, Boyd CD, Sondermann H, O'Toole GA: **A c-di-GMP effector system controls cell adhesion by inside-out signaling and surface protein cleavage**. *PLoS Biol* 2011, **9**(2):e1000587.
238. Hinsa SM, Espinosa-Urgel M, Ramos JL, O'Toole GA: **Transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas fluorescens* WCS365 requires an ABC transporter and a large secreted protein**. *Mol Microbiol* 2003, **49**(4):905-918.
239. Bobrov AG, Kirillina O, Ryjenkov DA, Waters CM, Price PA, Fetherston JD, Mack D, Goldman WE, Gomelsky M, Perry RD: **Systematic analysis of cyclic di-GMP signalling enzymes and their role in biofilm formation and virulence in *Yersinia pestis***. *Mol Microbiol* 2011, **79**(2):533-551.
240. Tamayo R, Schild S, Pratt JT, Camilli A: **Role of cyclic Di-GMP during el tor biotype *Vibrio cholerae* infection: characterization of the *in vivo*-induced cyclic Di-GMP phosphodiesterase CdpA**. *Infect Immun* 2008, **76**(4):1617-1627.
241. Tischler AD, Camilli A: **Cyclic diguanylate regulates *Vibrio cholerae* virulence gene expression**. *Infect Immun* 2005, **73**(9):5873-5882.
242. Moscoso JA, Mikkelsen H, Heeb S, Williams P, Filloux A: **The Pseudomonas aeruginosa sensor RetS switches type III and type VI secretion via c-di-GMP signalling**. *Environ Microbiol* 2011, **13**(12):3128-3138.
243. Mougous JD, Cuff ME, Raunser S, Shen A, Zhou M, Gifford CA, Goodman AL, Joachimiak G, Ordonez CL, Lory S *et al*: **A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus**. *Science* 2006, **312**(5779):1526-1530.

244. Potvin E, Lehoux DE, Kukavica-Ibrulj I, Richard KL, Sanschagrin F, Lau GW, Levesque RC: ***In vivo*** **functional genomics of *Pseudomonas aeruginosa* for high-throughput screening of new virulence factors and antibacterial targets**. *Environ Microbiol* 2003, **5**(12):1294-1308.
245. Lamprokostopoulou A, Monteiro C, Rhen M, Romling U: **Cyclic di-GMP signalling controls virulence properties of *Salmonella enterica* serovar Typhimurium at the mucosal lining**. *Environ Microbiol* 2010, **12**(1):40-53.
246. Claret L, Miquel S, Vieille N, Ryjenkov DA, Gomelsky M, Darfeuille-Michaud A: **The flagellar sigma factor FliA regulates adhesion and invasion of Crohn disease-associated *Escherichia coli* via a cyclic dimeric GMP-dependent pathway**. *J Biol Chem* 2007, **282**(46):33275-33283.
247. TL G: **Urinary Tract Infections in Women. In: Litwin MS, Saigal CS, editors. Urologic Diseases in America. US Department of Health and Human Services, Public Health Service, National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases. Washington (D. C.): US Government Printing Office. pp. 587–620.** 2007.
248. Russo TA, Johnson JR: **Medical and economic impact of extraintestinal infections due to *Escherichia coli*: focus on an increasingly important endemic problem**. *Microbes Infect* 2003, **5**(5):449-456.
249. Miller JF, Mekalanos JJ, Falkow S: **Coordinate regulation and sensory transduction in the control of bacterial virulence**. *Science* 1989, **243**(4893):916-922.
250. Finlay BB, Cossart P: **Exploitation of mammalian host cell functions by bacterial pathogens**. *Science* 1997, **276**(5313):718-725.
251. Beier D, Gross R: **Regulation of bacterial virulence by two-component systems**. *Curr Opin Microbiol* 2006, **9**(2):143-152.
252. Calva E, Oropeza R: **Two-component signal transduction systems, environmental signals, and virulence**. *Microb Ecol* 2006, **51**(2):166-176.
253. Alteri CJ, Lindner JR, Reiss DJ, Smith SN, Mobley HL: **The broadly conserved regulator PhoP links pathogen virulence and membrane potential in *Escherichia coli***. *Mol Microbiol* 2011, **82**(1):145-163.
254. Hadjifrangiskou M, Kostakioti M, Chen SL, Henderson JP, Greene SE, Hultgren SJ: **A central metabolic circuit controlled by QseC in pathogenic *Escherichia coli***. *Mol Microbiol* 2011, **80**(6):1516-1529.
255. Kostakioti M, Hadjifrangiskou M, Pinkner JS, Hultgren SJ: **QseC-mediated dephosphorylation of QseB is required for expression of genes associated with virulence in uropathogenic *Escherichia coli***. *Mol Microbiol* 2009, **73**(6):1020-1031.
256. Palaniyandi S, Mitra A, Herren CD, Lockatell CV, Johnson DE, Zhu X, Mukhopadhyay S: **BarA-UvrY two-component system regulates virulence of uropathogenic *E. coli* CFT073**. *PloS one* 2012, **7**(2):e31348.
257. Wright KJ, Seed PC, Hultgren SJ: **Development of intracellular bacterial communities of uropathogenic *Escherichia coli* depends on type 1 pili**. *Cell Microbiol* 2007, **9**(9):2230-2241.
258. Gally DL, Leathart J, Blomfield IC: **Interaction of FimB and FimE with the *fim* switch that controls the phase variation of type 1 fimbriae in *Escherichia coli* K-12**. *Mol Microbiol* 1996, **21**(4):725-738.
259. Bougdour A, Wickner S, Gottesman S: **Modulating RssB activity: IraP, a novel regulator of sigma(S) stability in *Escherichia coli***. *Gen Dev* 2006, **20**(7):884-897.
260. Datsenko KA, Wanner BL: **One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products**. *Proc Natl Acad Sci U S A* 2000, **97**(12):6640-6645.

261. Cherepanov PP, Wackernagel W: **Gene disruption in *Escherichia coli*: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant**. *Gene* 1995, **158**(1):9-14.
262. Gunther NWt, Snyder JA, Lockatell V, Blomfield I, Johnson DE, Mobley HL: **Assessment of virulence of uropathogenic *Escherichia coli* type 1 fimbrial mutants in which the invertible element is phase-locked on or off**. *Infect Immun* 2002, **70**(7):3344-3354.
263. Hagberg L, Engberg I, Freter R, Lam J, Olling S, Svanborg Eden C: **Ascending, unobstructed urinary tract infection in mice caused by pyelonephritogenic *Escherichia coli* of human origin**. *Infect Immun* 1983, **40**(1):273-283.
264. Sabri M, Houle S, Dozois CM: **Roles of the extraintestinal pathogenic *Escherichia coli* ZnuACB and ZupT zinc transporters during urinary tract infection**. *Infect Immun* 2009, **77**(3):1155-1164.
265. Livak KJ, Schmittgen TD: **Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method**. *Methods* 2001, **25**(4):402-408.
266. Stentebjerg-Olesen B, Chakraborty T, Klemm P: **Type 1 fimbriation and phase switching in a natural *Escherichia coli fimB* null strain, Nissle 1917**. *J Bacteriol* 1999, **181**(24):7470-7478.
267. Wu Y, Outten FW: **IscR controls iron-dependent biofilm formation in *Escherichia coli* by regulating type I fimbria expression**. *J Bacteriol* 2009, **191**(4):1248-1257.
268. Harel J, Jacques M, Fairbrother JM, Bosse M, Forget C: **Cloning of determinants encoding F165(2) fimbriae from porcine septicaemic *Escherichia coli* confirms their identity as F1C fimbriae**. *Microbiology* 1995, **141 (Pt 1)**:221-228.
269. Traxler MF, Zacharia VM, Marquardt S, Summers SM, Nguyen HT, Stark SE, Conway T: **Discretely calibrated regulatory loops controlled by ppGpp partition gene induction across the 'feast to famine' gradient in *Escherichia coli***. *Mol Microbiol* 2011, **79**(4):830-845.
270. Svitil AL, Cashel M, Zyskind JW: **Guanosine tetraphosphate inhibits protein synthesis in vivo. A possible protective mechanism for starvation stress in *Escherichia coli***. *J Biol Chem* 1993, **268**(4):2307-2311.
271. Xiao H, Kalman M, Ikehara K, Zemel S, Glaser G, Cashel M: **Residual guanosine 3',5'-bispyrophosphate synthetic activity of *relA* null mutants can be eliminated by spoT null mutations**. *J Biol Chem* 1991, **266**(9):5980-5990.
272. Stentebjerg-Olesen B, Chakraborty T, Klemm P: **FimE-catalyzed off-to-on inversion of the type 1 fimbrial phase switch and insertion sequence recruitment in an *Escherichia coli* K-12 fimB strain**. *FEMS Microbiol Lett* 2000, **182**(2):319-325.
273. Dalebroux ZD, Svensson SL, Gaynor EC, Swanson MS: **ppGpp conjures bacterial virulence**. *Microbiol Mol Biol Rev* 2011, **74**(2):171-199.
274. Virkola R, Westerlund B, Holthofer H, Parkkinen J, Kekomaki M, Korhonen TK: **Binding characteristics of *Escherichia coli* adhesins in human urinary bladder**. *Infect Immun* 1988, **56**(10):2615-2622.
275. Mulvey MA, Schilling JD, Hultgren SJ: **Establishment of a persistent *Escherichia coli* reservoir during the acute phase of a bladder infection**. *Infect Immun* 2001, **69**(7):4572-4579.
276. Hung DL, Raivio TL, Jones CH, Silhavy TJ, Hultgren SJ: **Cpx signaling pathway monitors biogenesis and affects assembly and expression of P pili**. *EMBO J* 2001, **20**(7):1508-1518.
277. Stephenson K, Hoch JA: **Developing inhibitors to selectively target two-component and phosphorelay signal transduction systems of pathogenic microorganisms**. *Curr Med Chem* 2004, **11**(6):765-773.

278. Wiltshire C, Matsushita M, Tsukada S, Gillespie DA, May GH: **A new c-Jun N-terminal kinase (JNK)-interacting protein, Sab (SH3BP5), associates with mitochondria**. *Biochem J* 2002, **367**(Pt 3):577-585.
279. Mobley HL, Green DM, Trifillis AL, Johnson DE, Chippendale GR, Lockatell CV, Jones BD, Warren JW: **Pyelonephritogenic *Escherichia coli* and killing of cultured human renal proximal tubular epithelial cells: role of hemolysin in some strains**. *Infect Immun* 1990, **58**(5):1281-1289.
280. Provence DL, Curtiss R, 3rd: **Role of *crl* in avian pathogenic *Escherichia coli*: a knockout mutation of *crl* does not affect hemagglutination activity, fibronectin binding, or Curli production**. *Infect Immun* 1992, **60**(11):4460-4467.
281. Blattner FR, Plunkett G, 3rd, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF *et al*: **The complete genome sequence of *Escherichia coli* K-12**. *Science* 1997, **277**(5331):1453-1474.
282. Dozois CM, Dho-Moulin M, Bree A, Fairbrother JM, Desautels C, Curtiss R, 3rd: **Relationship between the Tsh autotransporter and pathogenicity of avian *Escherichia coli* and localization and analysis of the Tsh genetic region**. *Infect Immun* 2000, **68**(7):4145-4154.
283. Woodall LD, Russell PW, Harris SL, Orndorff PE: **Rapid, synchronous, and stable induction of type 1 piliation in *Escherichia coli* by using a chromosomal *lacUV5* promoter**. *J Bacteriol* 1993, **175**(9):2770-2778.
284. Simon R, Priefer U, A P: **A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram-negative bacteria**. *Bio/Technology* 1983, **1**:784–794.
285. Gulig PA, Curtiss R, 3rd: **Plasmid-associated virulence of *Salmonella typhimurium***. *Infect Immun* 1987, **55**(12):2891-2901.
286. Miller VL, Mekalanos JJ: **A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR***. *J Bacteriol* 1988, **170**(6):2575-2583.
287. Cormack BP, Valdivia RH, Falkow S: **FACS-optimized mutants of the green fluorescent protein (GFP)**. *Gene* 1996, **173**(1 Spec No):33-38.
288. Posfai G, Koob MD, Kirkpatrick HA, Blattner FR: **Versatile insertion plasmids for targeted genome manipulations in bacteria: isolation, deletion, and rescue of the pathogenicity island LEE of the *Escherichia coli* O157:H7 genome**. *J Bacteriol* 1997, **179**(13):4426-4428.
289. Choi KH, Gaynor JB, White KG, Lopez C, Bosio CM, Karkhoff-Schweizer RR, Schweizer HP: **A Tn7-based broad-range bacterial cloning and expression system**. *Nat Methods* 2005, **2**(6):443-448.
290. Crepin S, Houle S, Charbonneau ME, Mourez M, Harel J, Dozois CM: **Decreased Expression of Type 1 Fimbriae by a *pst* Mutant of Uropathogenic *Escherichia coli* Reduces Urinary Tract Infection**. *Infect Immun* 2012, **80**(8):2802-2815.
291. Hengge R: **Principles of c-di-GMP signalling in bacteria**. *Nat Rev Microbiol* 2009, **7**(4):263-273.
292. Ryjenkov DA, Simm R, Romling U, Gomelsky M: **The PilZ domain is a receptor for the second messenger c-di-GMP: the PilZ domain protein YcgR controls motility in enterobacteria**. *J Biol Chem* 2006, **281**(41):30310-30314.
293. Romling U, Rohde M, Olsen A, Normark S, Reinkoster J: **AgfD, the checkpoint of multicellular and aggregative behaviour in *Salmonella typhimurium* regulates at least two independent pathways**. *Mol Microbiol* 2000, **36**(1):10-23.

294. Romling U, Sierralta WD, Eriksson K, Normark S: **Multicellular and aggregative behaviour of *Salmonella typhimurium* strains is controlled by mutations in the agfD promoter**. *Mol Microbiol* 1998, **28**(2):249-264.
295. Simm R, Morr M, Kader A, Nimtz M, Romling U: **GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility**. *Mol Microbiol* 2004, **53**(4):1123-1134.
296. Zogaj X, Bokranz W, Nimtz M, Romling U: **Production of cellulose and curli fimbriae by members of the family *Enterobacteriaceae* isolated from the human gastrointestinal tract**. *Infect Immun* 2003, **71**(7):4151-4158.
297. Brombacher E, Baratto A, Dorel C, Landini P: **Gene expression regulation by the Curli activator CsgD protein: modulation of cellulose biosynthesis and control of negative determinants for microbial adhesion**. *J Bacteriol* 2006, **188**(6):2027-2037.
298. Brombacher E, Dorel C, Zehnder AJ, Landini P: **The curli biosynthesis regulator CsgD co-ordinates the expression of both positive and negative determinants for biofilm formation in *Escherichia coli***. *Microbiology* 2003, **149**(Pt 10):2847-2857.
299. Holmqvist E, Reimegard J, Sterk M, Grantcharova N, Romling U, Wagner EG: **Two antisense RNAs target the transcriptional regulator CsgD to inhibit curli synthesis**. *EMBO J* 2010, **29**(11):1840-1850.
300. Jorgensen MG, Nielsen JS, Boysen A, Franch T, Moller-Jensen J, Valentin-Hansen P: **Small regulatory RNAs control the multi-cellular adhesive lifestyle of *Escherichia coli***. *Mol Microbiol* 2012.
301. Mika F, Busse S, Possling A, Berkholz J, Tschowri N, Sommerfeldt N, Pruteanu M, Hengge R: **Targeting of *csgD* by the small regulatory RNA RprA links stationary phase, biofilm formation and cell envelope stress in *Escherichia coli***. *Mol Microbiol* 2012, **84**(1):51-65.
302. Ogasawara H, Yamada K, Kori A, Yamamoto K, Ishihama A: **Regulation of the *Escherichia coli csgD* promoter: interplay between five transcription factors**. *Microbiology* 2010, **156**(Pt 8):2470-2483.
303. Thomason MK, Fontaine F, De Lay N, Storz G: **A small RNA that regulates motility and biofilm formation in response to changes in nutrient availability in *Escherichia coli***. *Mol Microbiol* 2012.
304. Simms AN, Mobley HL: **Multiple genes repress motility in uropathogenic *Escherichia coli* constitutively expressing type 1 fimbriae**. *J Bacteriol* 2008, **190**(10):3747-3756.
305. Nichols BP, Shafiq O, Meiners V: **Sequence analysis of Tn10 insertion sites in a collection of *Escherichia coli* strains used for genetic mapping and strain construction**. *J Bacteriol* 1998, **180**(23):6408-6411.
306. Crepin S, Harel J, Dozois CM: **Chromosomal Complementation Using Tn7 Transposon Vectors in *Enterobacteriaceae***. *Appl Environ Microbiol* 2012.
307. Posfai G, Kolisnychenko V, Bereczki Z, Blattner FR: **Markerless gene replacement in *Escherichia coli* stimulated by a double-strand break in the chromosome**. *Nucleic Acids Res* 1999, **27**(22):4409-4415.
308. Leveille S, Caza M, Johnson JR, Clabots C, Sabri M, Dozois CM: **Iha from an *Escherichia coli* urinary tract infection outbreak clonal group A strain is expressed *in vivo* in the mouse urinary tract and functions as a catecholate siderophore receptor**. *Infect Immun* 2006, **74**(6):3427-3436.
309. Waters CM, Lu W, Rabinowitz JD, Bassler BL: **Quorum sensing controls biofilm formation in *Vibrio cholerae* through modulation of cyclic di-GMP levels and repression of vpsT**. *J Bacteriol* 2008, **190**(7):2527-2536.

310. Zhou G, Mo WJ, Sebbel P, Min G, Neubert TA, Glockshuber R, Wu XR, Sun TT, Kong XP: **Uroplakin Ia is the urothelial receptor for uropathogenic *Escherichia coli*: evidence from in vitro FimH binding**. *J Cell Sci* 2001, **114**(Pt 22):4095-4103.
311. Wellens A, Garofalo C, Nguyen H, Van Gerven N, Slattegard R, Hernalsteens JP, Wyns L, Oscarson S, De Greve H, Hultgren S *et al*: **Intervening with urinary tract infections using anti-adhesives based on the crystal structure of the FimH-oligomannose-3 complex**. *PloS One* 2008, **3**(4):e2040.
312. Romling U: **Characterization of the rdar morphotype, a multicellular behaviour in *Enterobacteriaceae***. *Cell Mol Life Sci* 2005, **62**(11):1234-1246.
313. Samuels DS: **Gene regulation in *Borrelia burgdorferi***. *Ann Rev Microbiol* 2011, **65**:479-499.
314. Samuels DS AC, Lybecker M, Drecktrach D, Hall L: **A tale a two transcripts: gene regulation in *Borrelia burgdorferi***. *Talk at FASEB meeting in Microbial Pathogenesis: Mechanisms of infectious disease* 2011.
315. Kovacikova G, Lin W, Skorupski K: **Dual regulation of genes involved in acetoin biosynthesis and motility/biofilm formation by the virulence activator AphA and the acetate-responsive LysR-type regulator AlsR in *Vibrio cholerae***. *Mol Microbiol* 2005, **57**(2):420-433.
316. Simm R, Fetherston JD, Kader A, Romling U, Perry RD: **Phenotypic convergence mediated by GGDEF-domain-containing proteins**. *J Bacteriol* 2005, **187**(19):6816-6823.
317. Herrero M, de Lorenzo V, Timmis KN: **Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria**. *J Bacteriol* 1990, **172**(11):6557-6567.
318. Falkow S: **Molecular Koch's postulates applied to microbial pathogenicity**. *Rev Infect Dis* 1988, **10 Suppl 2**:S274-276.
319. Caza M, Lepine F, Dozois CM: **Secretion, but not overall synthesis, of catecholate siderophores contributes to virulence of extraintestinal pathogenic *Escherichia coli***. *Mol Microbiol* 2011, **80**(1):266-282.
320. Kulesus RR, Diaz-Perez K, Slechta ES, Eto DS, Mulvey MA: **Impact of the RNA chaperone Hfq on the fitness and virulence potential of uropathogenic *Escherichia coli***. *Infect Immun* 2008, **76**(7):3019-3026.
321. Wang RF, Kushner SR: **Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli***. *Gene* 1991, **100**:195-199.
322. Wiles TJ, Bower JM, Redd MJ, Mulvey MA: **Use of zebrafish to probe the divergent virulence potentials and toxin requirements of extraintestinal pathogenic *Escherichia coli***. *PLoS Pathog* 2009, **5**(12):e1000697.
323. Peters JE, Craig NL: **Tn7: smarter than we thought**. *Nat Rev Mol Cell Biol* 2001, **2**(11):806-814.
324. Waddell CS, Craig NL: **Tn7 transposition: recognition of the attTn7 target sequence**. *Proc Natl Acad Sci U S A* 1989, **86**(11):3958-3962.
325. Craig NL: **Tn7, p. 423-456**. *In N Craig, R Craigie, M Gellert, and A Lambowitz (ed), Mobile DNA II, ASM Press, Washington, DC* 2002.
326. Choi KH, DeShazer D, Schweizer HP: **mini-Tn7 insertion in bacteria with multiple *glmS*-linked attTn7 sites: example *Burkholderia mallei* ATCC 23344**. *Nat Protoc* 2006, **1**(1):162-169.
327. Choi KH, Schweizer HP: **mini-Tn7 insertion in bacteria with secondary, non-*glmS*-linked attTn7 sites: example *Proteus mirabilis* HI4320**. *Nat Protoc* 2006, **1**(1):170-178.

328. Choi KH, Schweizer HP: **mini-Tn7 insertion in bacteria with single attTn7 sites: example *Pseudomonas aeruginosa***. *Nat Protoc* 2006, **1**(1):153-161.
329. LoVullo ED, Molins-Schneekloth CR, Schweizer HP, Pavelka MS, Jr.: **Single-copy chromosomal integration systems for *Francisella tularensis***. *Microbiology* 2009, **155**(Pt 4):1152-1163.
330. McKenzie GJ, Craig NL: **Fast, easy and efficient: site-specific insertion of transgenes into enterobacterial chromosomes using Tn7 without need for selection of the insertion event**. *BMC Microbiol* 2006, **6**:39.
331. Murphy KC, Campellone KG: **Lambda Red-mediated recombinogenic engineering of enterohemorrhagic and enteropathogenic *E. coli***. *BMC Mol Biol* 2003, **4**:11.
332. Lutz R, Bujard H: **Independent and tight regulation of transcriptional units in *Escherichia coli* via the LacR/O, the TetR/O and AraC/I1-I2 regulatory elements**. *Nucleic Acids Res* 1997, **25**(6):1203-1210.
333. Choi KH, Kim KJ: **Applications of transposon-based gene delivery system in bacteria**. *J Microbiol Biotech* 2009, **19**(3):217-228.
334. Inouye S, Nakazawa A, Nakazawa T: **Molecular cloning of TOL genes *xylB* and *xylE* in *Escherichia coli***. *J Bacteriol* 1981, **145**(3):1137-1143.
335. Borenshtein D, McBee ME, Schauer DB: **Utility of the *Citrobacter rodentium* infection model in laboratory mice**. *Curr Opin Gastroenterol* 2008, **24**(1):32-37.
336. Walters MS, Lane MC, Vigil PD, Smith SN, Walk ST, Mobley HL: **Kinetics of uropathogenic *Escherichia coli* metapopulation movement during urinary tract infection**. *mBio* 2012, **3**(1).
337. Bush K: **Alarming beta-lactamase-mediated resistance in multidrug-resistant *Enterobacteriaceae***. *Curr Opin Microbiol* 2010, **13**(5):558-564.
338. Brickman E, Beckwith J: **Analysis of the regulation of *Escherichia coli* alkaline phosphatase synthesis using deletions and phi80 transducing phages**. *J Mol Biol* 1975, **96**(2):307-316.
339. Barthold SW, Osbaldiston GW, Jonas AM: **Dietary, bacterial, and host genetic interactions in the pathogenesis of transmissible murine colonic hyperplasia**. *Lab Anim Sci* 1977, **27**(6):938-945.
340. Himelright I, Harris E, Lorch V, Anderson M: **From the Centers for Disease Control and Prevention. *Enterobacter sakazakii* infections associated with the use of powdered infant formula--Tennessee, 2001**. *Jama* 2002, **287**(17):2204-2205.
341. Hacker J, Knapp S, Goebel W: **Spontaneous deletions and flanking regions of the chromosomally inherited hemolysin determinant of an *Escherichia coli* O6 strain**. *J Bacteriol* 1983, **154**(3):1145-1152.
342. Riley LW, Remis RS, Helgerson SD, McGee HB, Wells JG, Davis BR, Hebert RJ, Olcott ES, Johnson LM, Hargrett NT *et al*: **Hemorrhagic colitis associated with a rare *Escherichia coli* serotype**. *N Engl J Med* 1983, **308**(12):681-685.
343. Hindle Z, Chatfield SN, Phillimore J, Bentley M, Johnson J, Cosgrove CA, Ghaem-Maghami M, Sexton A, Khan M, Brennan FR *et al*: **Characterization of *Salmonella enterica* derivatives harboring defined *aroC* and *Salmonella* pathogenicity island 2 type III secretion system (*ssaV*) mutations by immunization of healthy volunteers**. *Infect Immun* 2002, **70**(7):3457-3467.
344. Forest C, Faucher SP, Poirier K, Houle S, Dozois CM, Daigle F: **Contribution of the *stg* fimbrial operon of *Salmonella enterica* serovar Typhi during interaction with human cells**. *Infect Immun* 2007, **75**(11):5264-5271.
345. Taylor PW: **The mode of C5b-9 attack on susceptible Gram-negative bacteria. p. 129-138. In Bacteria, complement and the phagocytoc cell. Cabello, C. F., and Pruzzo, C. (eds). Springer-Verlag, New York.** 1988.

346. Burns SM, Hull SI: **Comparison of loss of serum resistance by defined lipopolysaccharide mutants and an acapsular mutant of uropathogenic *Escherichia coli* O75:K5**. *Infect Immun* 1998, **66**(9):4244-4253.
347. Joiner KA: **Complement evasion by bacteria and parasites**. *Ann Rev Microbiol* 1988, **42**:201-230.
348. Pages JM, Bolla JM, Bernadac A, Fourel D: **Immunological approach of assembly and topology of OmpF, an outer membrane protein of *Escherichia coli***. *Biochimie* 1990, **72**(2-3):169-176.
349. Sen K, Nikaido H: **Lipopolysaccharide structure required for in vitro trimerization of *Escherichia coli* OmpF porin**. *J Bacteriol* 1991, **173**(2):926-928.
350. Moulin-Schouleur M, Reperant M, Laurent S, Bree A, Mignon-Grasteau S, Germon P, Rasschaert D, Schouler C: **Extraintestinal pathogenic *Escherichia coli* strains of avian and human origin: link between phylogenetic relationships and common virulence patterns**. *J Clin Microbiol* 2007, **45**(10):3366-3376.
351. Clermont O, Bonacorsi S, Bingen E: **Rapid and simple determination of the *Escherichia coli* phylogenetic group**. *Appl Environ Microbiol* 2000, **66**(10):4555-4558.
352. Herzer PJ, Inouye S, Inouye M, Whittam TS: **Phylogenetic distribution of branched RNA-linked multicopy single-stranded DNA among natural isolates of *Escherichia coli***. *J Bacteriol* 1990, **172**(11):6175-6181.
353. Luo C, Hu GQ, Zhu H: **Genome reannotation of *Escherichia coli* CFT073 with new insights into virulence**. *BMC Genomics* 2009, **10**:552.
354. Al-Hasan MN, Eckel-Passow JE, Baddour LM: **Bacteremia complicating gram-negative urinary tract infections: a population-based study**. *J Infect* 2010, **60**(4):278-285.
355. Simms AN, Mobley HL: **PapX, a P fimbrial operon-encoded inhibitor of motility in uropathogenic *Escherichia coli***. *Infect Immun* 2008, **76**(11):4833-4841.
356. Hull RA, Hull SI: **Nutritional requirements for growth of uropathogenic *Escherichia coli* in human urine**. *Infect Immun* 1997, **65**(5):1960-1961.
357. Bougdour A, Gottesman S: **ppGpp regulation of RpoS degradation via anti-adaptor protein IraP**. *Proc Natl Acad Sci U S A* 2007.
358. Battesti A, Bouveret E: **Acyl carrier protein/SpoT interaction, the switch linking SpoT-dependent stress response to fatty acid metabolism**. *Mol Microbiol* 2006, **62**(4):1048-1063.
359. Christen M, Kulasekara HD, Christen B, Kulasekara BR, Hoffman LR, Miller SI: **Asymmetrical distribution of the second messenger c-di-GMP upon bacterial cell division**. *Science* 2010, **328**(5983):1295-1297.
360. Boehm A, Steiner S, Zaehringer F, Casanova A, Hamburger F, Ritz D, Keck W, Ackermann M, Schirmer T, Jenal U: **Second messenger signalling governs *Escherichia coli* biofilm induction upon ribosomal stress**. *Mol Microbiol* 2009, **72**(6):1500-1516.
361. Roelofs Kevin G VTL: **Differential radial capillary action of ligand assay: a novel method to determine protein-ligand interactions**. *Cold Spring Harbor meeting on Microbial Pathogenesis and Host Response* 2011, **(Cold Spring Harbor, 13-17 septembre 2011)**.
362. Koch. AL: **Why *Escherichia coli* should be renamed Escherichia ilei. *In: Phosphate metabolism and Cellular regulation in Microorganisms (Torriani-Gorini, A,, Rothman, F G Silver S, Wright, A and Yagil, E eds), pp 300-305 Amer Soc Microbiol, Washington, DC*** 1987.
363. VanBogelen RA, Abshire KZ, Pertsemlidis A, Clark RL, Neidhardt. FC: **Gene-protein database of *Escherichia coli* K-12, edition 6, p. 2067–2117. In F. C. Neidhardt, R.**

Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and Salmonella: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C. 1996.

364. Marc D, Arne P, Bree A, Dho-Moulin M: **Colonization ability and pathogenic properties of a fim- mutant of an avian strain of *Escherichia coli***. *Res Microbiol* 1998, **149**(7):473-485.
365. Bolstad BM, Irizarry RA, Astrand M, Speed TP: **A comparison of normalization methods for high density oligonucleotide array data based on variance and bias**. *Bioinformatics* 2003, **19**(2):185-193.
366. Blazejczyk M, M. Miron and R. Nadon.: **FlexArray: A statistical data analysis software for gene expression microarrays. Genome Quebec, Montreal, Canada**. *Genome Quebec, Montreal, Canada,* 2007, **http://genomequebec.mcgill.ca/FlexArray**
367. Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP: **Summaries of Affymetrix GeneChip probe level data**. *Nucleic Acids Res* 2003, **31**(4):e15.
368. Peterson JD, Umayam LA, Dickinson T, Hickey EK, White O: **The Comprehensive Microbial Resource**. *Nucleic Acids Res* 2001, **29**(1):123-125.
369. Sabri M, Caza M, Proulx J, Lymberopoulos MH, Bree A, Moulin-Schouleur M, Curtiss R, 3rd, Dozois CM: **Contribution of the SitABCD, MntH, and FeoB metal transporters to the virulence of avian pathogenic *Escherichia coli* O78 strain chi7122**. *Infect Immun* 2008, **76**(2):601-611.
370. Lymberopoulos MH, Houle S, Daigle F, Leveille S, Bree A, Moulin-Schouleur M, Johnson JR, Dozois CM: **Characterization of Stg fimbriae from an avian pathogenic *Escherichia coli* O78:K80 strain and assessment of their contribution to colonization of the chicken respiratory tract**. *J Bacteriol* 2006, **188**(18):6449-6459.
371. Laemmli UK: **Cleavage of structural proteins during the assembly of the head of bacteriophage T4**. *Nature* 1970, **227**(5259):680-685.
372. Dozois CM, Pourbakhsh SA, Fairbrother JM: **Expression of P and type 1 (F1) fimbriae in pathogenic *Escherichia coli* from poultry**. *Vet Microbiol* 1995, **45**(4):297-309.
373. Torriani A: **Influence of inorganic phosphate in the formation of phosphatases by *Escherichia coli***. *Biochim Biophys Acta* 1960, **38**:460-469.
374. Johnson TJ, Kariyawasam S, Wannemuehler Y, Mangiamele P, Johnson SJ, Doetkott C, Skyberg JA, Lynne AM, Johnson JR, Nolan LK: **The genome sequence of avian pathogenic *Escherichia coli* strain O1:K1:H7 shares strong similarities with human extraintestinal pathogenic E. coli genomes**. *J Bacteriol* 2007, **189**(8):3228-3236.
375. Hengge-Aronis R: **Signal transduction and regulatory mechanisms involved in control of the sigma(S) (RpoS) subunit of RNA polymerase**. *Microbiol Mol Biol Rev* 2002, **66**(3):373-395.
376. Loewen PC, Hu B, Strutinsky J, Sparling R: **Regulation in the rpoS regulon of *Escherichia coli***. *Can J Microbiol* 1998, **44**(8):707-717.
377. Storz G, Imlay JA: **Oxidative stress**. *Curr Opin Microbiol* 1999, **2**(2):188-194.
378. Altuvia S, Weinstein-Fischer D, Zhang A, Postow L, Storz G: **A small, stable RNA induced by oxidative stress: role as a pleiotropic regulator and antimutator**. *Cell* 1997, **90**(1):43-53.
379. Tjaden B, Goodwin SS, Opdyke JA, Guillier M, Fu DX, Gottesman S, Storz G: **Target prediction for small, noncoding RNAs in bacteria**. *Nucleic Acids Res* 2006, **34**(9):2791-2802.
380. Moreau PL: **Diversion of the metabolic flux from pyruvate dehydrogenase to pyruvate oxidase decreases oxidative stress during glucose metabolism in nongrowing *Escherichia coli* cells incubated under aerobic, phosphate starvation conditions**. *J Bacteriol* 2004, **186**(21):7364-7368.

381. Hommais F, Krin E, Coppee JY, Lacroix C, Yeramian E, Danchin A, Bertin P: **GadE (YhiE): a novel activator involved in the response to acid environment in *Escherichia coli***. *Microbiology* 2004, **150**(Pt 1):61-72.
382. Mates AK, Sayed AK, Foster JW: **Products of the *Escherichia coli* acid fitness island attenuate metabolite stress at extremely low pH and mediate a cell density-dependent acid resistance**. *J Bacteriol* 2007, **189**(7):2759-2768.
383. Foster JW: ***Escherichia coli* acid resistance: tales of an amateur acidophile**. *Nat Rev Microbiol* 2004, **2**(11):898-907.
384. Reitzer L: **Catabolism of Amino Acids and Related Compounds**. ***Escherichia coli and Salmonella: Cellular and Molecular Biology Online Version*** ***http://wwwecosalorg/ecosal/indexjsp*** 2005.
385. Deutscher J, Francke C, Postma PW: **How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria**. *Microbiol Mol Biol Rev* 2006, **70**(4):939-1031.
386. Vanderpool CK: **Physiological consequences of small RNA-mediated regulation of glucose-phosphate stress**. *Curr Opin Microbiol* 2007, **10**(2):146-151.
387. Vanderpool CK, Gottesman S: **Involvement of a novel transcriptional activator and small RNA in post-transcriptional regulation of the glucose phosphoenolpyruvate phosphotransferase system**. *Mol Microbiol* 2004, **54**(4):1076-1089.
388. Boos W: **Binding protein-dependent ABC transport system for glycerol 3-phosphate of *Escherichia coli***. *Methods Enzymol* 1998, **292**:40-51.
389. Bukau B, Horwich AL: **The Hsp70 and Hsp60 chaperone machines**. *Cell* 1998, **92**(3):351-366.
390. Mogk A, Deuerling E, Vorderwulbecke S, Vierling E, Bukau B: **Small heat shock proteins, ClpB and the DnaK system form a functional triade in reversing protein aggregation**. *Mol Microbiol* 2003, **50**(2):585-595.
391. Johnson DC, Dean DR, Smith AD, Johnson MK: **Structure, function, and formation of biological iron-sulfur clusters**. *Annu Rev Biochem* 2005, **74**:247-281.
392. Barron A, Jung JU, Villarejo M: **Purification and characterization of a glycine betaine binding protein from *Escherichia coli***. *J Biol Chem* 1987, **262**(24):11841-11846.
393. Boos W, Ehmann U, Bremer E, Middendorf A, Postma P: **Trehalase of *Escherichia coli*. Mapping and cloning of its structural gene and identification of the enzyme as a periplasmic protein induced under high osmolarity growth conditions**. *J Biol Chem* 1987, **262**(27):13212-13218.
394. Weber A, Kogl SA, Jung K: **Time-dependent proteome alterations under osmotic stress during aerobic and anaerobic growth in *Escherichia coli***. *J Bacteriol* 2006, **188**(20):7165-7175.
395. Lee H, Hsu FF, Turk J, Groisman EA: **The PmrA-regulated *pmrC* gene mediates phosphoethanolamine modification of lipid A and polymyxin resistance in *Salmonella enterica***. *J Bacteriol* 2004, **186**(13):4124-4133.
396. Nishino K, Hsu FF, Turk J, Cromie MJ, Wosten MM, Groisman EA: **Identification of the lipopolysaccharide modifications controlled by the *Salmonella* PmrA/PmrB system mediating resistance to Fe(III) and Al(III)**. *Mol Microbiol* 2006, **61**(3):645-654.
397. Raetz CR, Reynolds CM, Trent MS, Bishop RE: **Lipid A modification systems in gram-negative bacteria**. *Annu Rev Biochem* 2007, **76**:295-329.
398. Valvano MA: **Undecaprenyl phosphate recycling comes out of age**. *Mol Microbiol* 2008, **67**(2):232-235.
399. Ulett GC, Mabbett AN, Fung KC, Webb RI, Schembri MA: **The role of F9 fimbriae of uropathogenic *Escherichia coli* in biofilm formation**. *Microbiology* 2007, **153**(Pt 7):2321-2331.

400. Gross WB: **Colibacillosis, p. 138–44. In: Calnek BW, Barnes HJ, Beard CW, Reid WM, Yoder HW Jr, eds. Diseases of poultry. Ames, IO: Iowa State University Press,**. 1991.
401. Wilkinson SP, Grove A: **Ligand-responsive transcriptional regulation by members of the MarR family of winged helix proteins**. *Current issues in molecular biology* 2006, **8**(1):51-62.
402. Vila J, Soto S: **Salicylate increases the expression of *marA* and reduces *in vitro* biofilm formation in uropathogenic *Escherichia coli* by decreasing type 1 fimbriae expression**. *Virulence* 2012, **3**(3).
403. Newman EB, Lin R: **Leucine-responsive regulatory protein: a global regulator of gene expression in *E. coli***. *Ann Rev Microbiol* 1995, **49**:747-775.

ANNEXE

Annexe #1

Genome-wide transcriptional response of an avian pathogenic *Escherichia coli* (APEC) *pst* mutant

Sébastien Crépin, Martin G. Lamarche, Philippe Garneau, Julie Séguin, Julie Proulx, Charles M. Dozois et Josée Harel. (2008) BMC Genomics **9** :568

A) Contribution de l'étudiant

En tant que premier auteur, j'ai écrit la totalité de l'article. J'ai aussi effectué toutes les expérimentations de biopuces à ADN, de qRT-PCR ainsi que l'analyse des résultats. Ces expériences sont le cœur de l'article. Martin G. Lamarche a effectué les expérimentations préliminaires, Philippe Garneau a réalisé certaines analyses bioinformatiques, Julie Séguin a effectué les expérimentations concernant l'expression des fimbriae de type 1 tandis que Julie Proulx a effectué les expériences de stress oxydatif. L'article a été corrigé par Martin G. Lamarche, Charles M. Dozois et Josée Harel.

B) Résumé

Les souches pathogènes aviaires (APEC) sont associées aux infections extraintestinales aviaires. L'opéron *pstSCAB-phoU* code pour le système de transport spécifique du phosphate (Pst) et fait partie du régulon Pho. Un système Pst fonctionnel est requis pour la virulence, la résistance au sérum et à l'acidité. Afin de comprendre les mécanismes moléculaires reliant le régulon Pho et la virulence, nous avons entrepris une étude transcriptomique comparative entre la souche aviaire χ7122 et son mutant isogénique Pst (K3). Globalement, dans les conditions testées, 470 gènes sont différentiellement exprimés chez la souche K3 par un facteur d'au moins 1,5. En plus de l'induction des gènes impliqués dans le transport et le métabolisme de diverses sources de phosphate, l'induction de la réponse aux différents stress est observée chez la souche K3. Ainsi, les gènes de la réponse générale au stress ainsi que la réponse stringente sont différentiellement exprimés. L'expression différentielle de gènes impliqués dans la biosynthèse de l'enveloppe cellulaire suggère une modification membranaire. De plus, une

proportion similaire des gènes induits et réprimés est impliquée dans la réponse au stress oxydatif et la souche K3 montre une sensibilité accrue aux espèces réactives oxygénées. En accord avec les résultats de biopuces, la souche K3 présente peu de fimbriae à sa surface, agglutine 10-fois moins les levures et ne produit pas FimA. En conclusion, le régulon Pho n'est pas seulement un système régulationnel impliqué dans l'homéostasie du phosphate, mais fait partie d'un réseau complexe impliquant la réponse à différents stress ainsi que la virulence bactérienne.

Genome-wide transcriptional response of an avian pathogenic *Escherichia coli* (APEC) *pst* mutant

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ABSTRACT

Background: Avian pathogenic *E. coli* (APEC) are associated with extraintestinal diseases in poultry. The *pstSCAB-phoU* operon belongs to the Pho regulon and encodes the phosphate specific transport (Pst) system. A functional Pst system is required for full virulence in APEC and other bacteria and contributes to resistance of APEC to serum, to cationic antimicrobial peptides and acid shock. The global mechanisms contributing to the attenuation and decreased resistance of the APEC *pst* mutant to environmental stresses have not been investigated at the transcriptional level. To determine the global effect of a *pst* mutation on gene expression, we compared the transcriptomes of APEC strain χ7122 and its isogenic *pst* mutant (K3) grown in phosphate-rich medium.

Results: Overall, 470 genes were differentially expressed by at least 1.5-fold. Interestingly, the *pst* mutant not only induced systems involved in phosphate acquisition and metabolism, despite phosphate availability, but also modulated stress response mechanisms. Indeed, transcriptional changes in genes associated with the general stress responses, including the oxidative stress response were among the major differences observed. Accordingly, the K3 strain was less resistant to reactive oxygen species (ROS) than the wild-type strain. In addition, the *pst* mutant demonstrated reduced expression of genes involved in lipopolysaccharide modifications and coding for cell surface components such as type 1 and F9 fimbriae. Phenotypic tests also established that the *pst* mutant was impaired in its capacity to produce type 1 fimbriae, as demonstrated by western blotting and agglutination of yeast cells, when compared to wild-type APEC strain χ7122.

Conclusion: Overall, our data elucidated the effects of a *pst* mutation on the transcriptional response, and further support the role of the Pho regulon as part of a complex network contributing to phosphate homeostasis, adaptive stress responses, and *E. coli* virulence.

BACKGROUND

In *Escherichia coli* and many other bacterial species, the Pho regulon is activated when cells face phosphate limitation, whereas its expression is inhibited in excess of phosphate. The two-component system (TCS) PhoR/PhoB responds to environmental phosphate concentration variations and has been shown to control expression of at least 47 genes [4, 6]. PhoR is an inner membrane sensor protein that responds to periplasmic orthophosphate (P_i) concentrations and PhoB is the response regulator of the Pho regulon. PhoR is activated when the P_i concentration is below 4 μM. Under P_i-limited conditions, Phospho-PhoB binds to specific DNA sequences known as Pho-Boxes, located within Pho-dependent promoter regions to either induce or repress gene transcription. The *pstSCAB-phoU* operon encodes the Pst system and belongs to the Pho regulon. The Pst system encodes an ATP-binding cassette (ABC) transporter involved in the transport of P_i. Importantly, mutations in the Pst system result in constitutive expression of the Pho regulon, regardless of environmental phosphate availability, and affect virulence of many pathogenic bacteria [6].

Extraintestinal pathogenic *Escherichia coli* (ExPEC) strains are associated with various diseases, including urinary tract infections (UTIs), newborn meningitis (NBM), abdominal sepsis and septicemia [184]. In poultry, APEC strains are a frequent cause of extraintestinal infections, collectively called colibacillosis. In addition, *E. coli* O78 strains can also cause diseases in hosts other than poultry, such as swine, sheep, and humans [4].

We have previously shown that inactivation of the *pst* operon in porcine and avian ExPEC strains resulted in constitutive expression of the Pho regulon and rendered the strains avirulent [9, 10]. Moreover, it was reported that the *phoB* gene of the avian pathogenic *E*. *coli* (APEC) χ7122 strain is expressed during infection [161]. In accordance, in ExPEC strains, other PhoB regulated genes were shown to be expressed *in vivo* [55, 196]. A number of reports have described an association between the Pst system, the Pho regulon and bacterial virulence [6]. Although inactivation of *pst* genes has been shown to affect the virulence of a number of bacterial pathogens, the mechanisms underlying this attenuation have not been elucidated.

Microarray studies have been conducted to understand how *E. coli* K-12 responds to growth in phosphate-rich or phosphate-limited conditions in *phoB* mutant strains [143, 153]. Moreover,

proteome profiles of cells grown under phosphate-rich or phosphate-limited conditions revealed that the overall phosphate response of *E. coli* may comprise up to 400 genes [363]. However, these studies investigated non-pathogenic *E*. *coli* K-12 grown in phosphate-limiting conditions. To understand global responses resulting from a mutation in the Pst system, which constitutively activates the Pho regulon, and its relationship with APEC virulence, the transcriptional profile of the APEC χ7122 strain was compared with its isogenic Pst mutant (K3) using the Affymetrix GeneChip® *E. coli* Genome 2.0 Array. The Pho regulon is clearly not a simple regulatory circuit for controlling phosphate homeostasis; it is part of a complex network important for both bacterial virulence and the global stress response. Regulatory changes incurred due to inactivation of the Pst system resulted in modulation of genes involved in cell surface modifications, production of fimbrial adhesins, and protection against environmental stresses in the APEC mutant. These regulatory changes are likely to contribute to its reduced virulence and increased sensitivity to environmental stresses which may be encountered during host infection.

METHODS

Bacterial strains, media and growth conditions

The APEC strain χ7122, the χ7122 Δ*pstCAB* mutant (K3), and the complemented χ7122 Δ*pstCAB* mutant (CK3) were previously described [10, 280, 364]. The χ7122 Δ*fim* strain χ7279 was generated by allelic exchange using suicide vector pDM915 as described by Marc *et al*. [364]. *E*. *coli* strains were grown at 37°C in Luria-Bertani (LB) medium. Antibiotics or supplements were used at the following final concentration, when required: 40 µg/ml for nalidixic acid (Nal), 50 µg/ml for kanamycin (Kan), 12.5 µg/ml for chloramphenicol (Cm) and 40 µg/ml for 5-bromo-4-chloro-3-indolylphosphate di-sodium (XP).

Alkaline phosphatase assay

Alkaline phosphatase activity was measured as described previously with slight modifications [10]. Briefly, cells grown under different conditions were adjusted to an optical density of 1.0 at 600 nm (OD_{600}), and 4 µg/ml of *p*-nitrophenyl phosphate (Sigma) was added to cells permeabilized by 50 µl of 0.1% SDS and 50 µl of chloroform. Color development was monitored at 420 nm and alkaline phosphatase activity was expressed in Miller units (MU), calculated as follows: $1\,000 \times [OD_{420} - (1.75 \times OD_{550})] / T \text{ (min)} \times V \text{ (ml)} \times OD_{600}$].

RNA isolation

RNA extractions were performed using four biological replicates of cultures of strains χ7122 and K3. Briefly, overnight cultures grown at 37°C in LB broth were diluted 100-fold into 5 ml of LB broth and were allowed to grow to mid-log phase (OD_{600} 0.6). RNA samples were extracted with the RiboPure™-Bacteria Kit (Ambion, Austin, TX), according to the manufacturer's recommendations, with the exception that DNAse I treatment was performed twice. RNA concentration and purity were measured using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Quantitative RT-PCR (qRT-PCR) was performed on each RNA sample using a Smart Cycler (Cepheid, Sunnyvale, CA) to detect any DNA contamination. For this purpose, primers targeting the *rpoD* gene were used. RNAs were stored at -80°C for future use.

cDNA synthesis and biotinylation

Ten µg of RNA dissolved in 20 µl of RNAse-, DNAse- and pyrogen-free water (Sigma) were supplemented with 2 µl of the GeneChip® Eukaryotic Poly-A RNA Control Kit (Affymetrix, Santa Clara, CA) and converted into cDNA using SuperScript II and random hexanucleotide primers (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Following cDNA synthesis, 2 µl of 1 mg/ml of RNAse A (Sigma) were added to the reaction mix and samples were incubated at 37°C for 30 min to degrade remaining RNA. cDNAs were purified using Microcon YM-30 centrifugal filters (Milipore, Billerica, MA). Concentration and purity were measured using the Nanodrop ND-1000 spectrophotometer. A range between 3 to 7 µg of cDNAs was fragmented using DNAse I (Ambion, Austin, TX), at concentration of 0.5 U/µg of cDNA, and by incubating at 37°C for 10 min. Fragmentation was stopped by heating the reaction mixture at 98°C for 10 min. Fragmented cDNAs were 3' biotinylated using GeneChip DNA Labelling Reagent (Affymetrix, Santa Clara, CA) at 7.5 mM and 60 U of Terminal Deoxynucleotidyl Transferase (Promega, Madison, WI) at 37°C for 60 min. The reaction was stopped by adding 2 µl of 0.5 M EDTA (Sigma). A gel-shift assay on 14 % polyacrylamide gel using ImmunoPure NeutrAvidin (Pierce Chemical, Rockford, IL) was monitored to determine biotin incorporation.

DNA microarray hybridization and analysis

The cDNAs were hybridized onto the Affymetrix GeneChip® *E. coli* Genome 2.0 Array (Affymetrix, Santa Clara, CA) as recommended by the manufacturer (www.affymetrix.com). Hybridizations were performed at the Génome Québec Innovation Centre (McGill University, Montréal, Canada). Data were processed using the robust multiarray average algorithm (RMA) for normalization, background correction and expression value calculation [365]. Expression levels obtained from four independent biological replicates were compared using FlexArray 1.1 software [366]. Robustness of the data was further enhanced by the EB (Wright and Simon) algorithm and p-value calculation. Since the RMA algorithm diminished the false positive rate and compressed the fold change, a 1.5-fold change cut-off value was used for determination of the differentially expressed genes [367]. Functional classification was done according to the TIGR's Comprehensive Microbial Resource (CMR) [368]. Pathogen-associated ORFs were classified as such, since they represent sequences corresponding to one or more of the three pathogenic *E. coli* genomes (EDL933, Sakai and CFT073), but which are absent from the genome of *E. coli* K-12 strain MG1655.

Quantitative RT-PCR

The QIAGEN QuantiTect® SYBR® Green RT-PCR kit was used for qRT-PCR according to the manufacturer's instructions. Primer pairs were designed using the PrimerQuest software from Integrated DNA Technologies (http://www.idtdna.com/Scitools/Applications/Primerquest/) to yield PCR products varying between 80 to 200 bp. The *tus* gene was used as a housekeeping control. Each qRT-PCR run was done in triplicate and for each reaction, the calculated threshold cycle (*C*t) was normalized to the *C*t of the *tus* gene amplified from the corresponding sample. The fold-change was calculated using the $2^{-\Delta\Delta Ct}$ method [265]. Sequences of primers used for qRT-PCR analysis are available in additional file 1.

In silico search for Pho box(es)

We used the DNA Pattern search program available on the Colibri© website (http://genolist.pasteur.fr/Colibri/). The search was performed by using the pattern n[at]!c!c!g[at]!a!g!g!gn!g!g[tg][ta][ac]a[tc]nnn!g, where "n" represents any nucleotide, characters between square brackets "[ ]" means an ambiguity and "!" before a character indicates the

negation of this position. This pattern was designed based on different *E. coli* Pho box sequences presented by Blanco *et al*. [144]. We performed the DNA pattern search on the *E. coli* K-12 MG1655 whole genome, with no mismatch allowed, narrowing our search to the 200 bp upstream of predicted start codons. We also used the gene list containing predicted PhoB binding sites using the Pho box weight matrix elaborated by Yuan *et al*. [147]. Searches were performed against the *E. coli* MG1655, EDL933, Sakai, and CFT073 genomes which are represented on the Affymetrix GeneChip® *E. coli* Genome 2.0 array.

Sensitivity of *E. coli* strains to reactive oxygen intermediate (ROI)-generating agents.

Sensitivity to oxidative stress generating agents was determined by an agar overlay diffusion method on LB plates (1.5% agar) as described by Sabri *et al*. [369]. Briefly, overnight cultures grown in LB broth were adjusted to an OD_{600} of 0.5. Then, 100 µl of each culture were suspended in molten top agar (0.5% agar) and poured over the agar plates. Filter paper disks (6 mm diameter; Beckton Dickinson) were added to the surface of the solidified overlays and 10 µl of hydrogen peroxide (30%), plumbagin (53 mM), phenazine methosulfate (PMS) (15 mM) or phenazine ethosulfate (PES) (15 mM) were spotted onto the disks. The plates were then incubated overnight at 37ºC and following growth, the diameters of inhibition zones were measured.

Yeast Cell Aggregation Assay

The yeast aggregation assay was derived from a micro-hemagglutination assay in 96-well round-bottom plates [280]. Briefly, cultures were grown to mid-log phase (OD_{600} 0.6) in LB broth at 37°C with shaking (180 rpm) (conditions which were used for transcriptional analyses) or for 48 h without shaking at 37°C to enhance expression of type 1 fimbriae. Bacterial cells were centrifuged, and pellets were suspended in phosphate buffered saline (PBS, pH 7.4) to an initial suspension of approximately 2 X 10^{11} cells/ml. Samples were then serially diluted two-fold in microtiter wells, and equal volumes of a 3% commercial yeast suspension were added to each of the wells. After 30 min of incubation on ice, yeast aggregation was monitored visually, and the agglutination titer was recorded as the most diluted bacterial sample giving a positive aggregation reaction. The Δ*fim* type 1 fimbriae mutant strain χ7279 was used as a negative control.

Preparation of fimbrial extracts and Western blotting

Following growth of cultures with agitation at 37°C in LB to mid-exponential growth phase (OD_{600} 0.6), fimbrial extracts were prepared and Western blotting was performed as described previously [370]. Briefly, fimbrial extracts were separated by sodium dodecyl sulfate (SDS)-15% polyacrylamide gel electrophoresis in minigels as previously described by Laemmli [371]. Proteins were transferred to nitrocellulose membranes (Bio-Rad) using a Mini Trans-Blot electrophoretic cell (Bio-Rad) for 60 min at 100 V. The membrane was blocked with StartingBlock (Pierce) supplemented with 0.05% Tween 20 (Pierce). Incubations with primary (1:5000) and secondary (1:25 000) antibodies were carried out for 1 h at room temperature. SuperSignal West Pico chemiluminescent substrate (Pierce) was used for detection. Primary antibodies, raised against type 1 fimbriae from *E. coli* strain B_{AM} , were used, and react specifically with type 1 fimbriae from different APEC strains [372].

Electron microscopy

Cells for electron microscopy were grown as described above for microarray experiments. A glow-discharged Formvar-coated copper grid was placed onto a drop of bacterial culture for 1 min to allow the cells to adsorb. The excess of liquid was then removed using a filter paper, just before a drop of 1% phosphotungstic acid (negative stain) was placed onto the grid. Samples were left to air dry and viewed using a Phillips EM300 transmission electron microscope.

Microarray accession numbers

Microarray data are available at the National Center for Biotechnology Information Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE9178.

RESULTS AND DISCUSSION

Microarray design to identify differentially expressed genes in the APEC *pst* mutant

To assess the effects of a *pst* mutation as well as Pho regulon activation on APEC strain χ7122, a transcriptional profiling approach was used. Strain χ7122 and its isogenic *pst* mutant (K3) were grown in a phosphate-rich (LB) medium. In this medium, strains χ7122 and K3 grew well, and they showed similar growth curves (data not shown). The alkaline phosphatase activity of PhoA is commonly used to evaluate the activation state of the Pho regulon [373]. The Pho regulon induction reaches its maximal rate at mid-log phase of growth in strain K3 as determined by PhoA activity (200 MU), whereas it was repressed in the wild-type parent strain χ7122 (3 MU). Thus, in the K3 strain, the Pho regulon is highly activated even during growth in phosphate-rich LB medium.

The Affymetrix GeneChip *E. coli* genome 2.0 Array contains oligonucleotides corresponding to four *E. coli* genomes (*E. coli* K-12 MG1655, *E. coli* enterohemorrhagic EDL933 and Sakai and extraintestinal *E. coli* (ExPEC) CFT073). Avian pathogenic *Escherichia coli* (APEC), a frequent cause of extraintestinal infections in poultry, are categorized as ExPEC. Although the genome sequence of the χ7122 APEC strain is not yet available, it is now known that the APEC O1:K1 strain genome shares 90 % similarity with the CFT073 ORFs [374]. This suggests that probes on the GeneChip could represent at least in part the genome of χ7122. In addition, to determine the representative gene targets on the array that were present in strain χ7122, hybridization of χ7122 genomic DNA revealed that, among the 20,366 ORFs represented on the DNA array, 5751 were specific to the χ7122 genome. The non-K-12 ORFs were considered as pathogen-associated ORFs.

Significant transcriptional changes in the K3 *pst* mutant

The transcriptomic study identified 470 genes that were differentially expressed by at least 1.5-fold in the *pst* mutant strain K3 when compared to the APEC strain χ7122, with a p-value of ≤ 0.05 and an estimated false discovery rate (FDR) of 2.71%. Globally, 254 genes were up- and 216 were down-regulated. Specific subsets of differentially expressed genes of known

physiological relevance or putative function are discussed below and presented in Tables 1 and 2. A complete list of the differentially expressed genes is available online (see additional files 2 and 3). These tables contain genes that are discussed below and represent significant change at the transcriptional level occurring in Pho regulon activation. The known genes of the Pho regulon were induced varying from 145- to 2-fold. Besides those, the *gadW* gene showed the highest induction (6.8-fold), whereas the greatest repression was observed for the *yhcN* gene (-7.8-fold). Functional classification of the differentially expressed genes (Fig. 1) indicated predominant transcriptional changes among genes associated with cellular homeostasis and metabolism. Genes that were up-regulated included those encoding proteins of unknown function, transport and binding proteins, energy and central intermediary metabolism, and transcription (Table 1). Genes that were down-regulated also included the unknown function genes, as well as those involved with protein fate; protein synthesis; DNA metabolism; purine, pyrimidine, nucleoside and nucleotide pathways; and cell envelope proteins (Table 2). In addition, 5 small RNAs (sRNA) were differentially expressed. As expected, expression patterns of the genome-wide transcriptional response observed in the *pst* mutant in phosphate-rich medium shared similarities with transcriptomic analyses of non-pathogenic *E*. *coli* K-12 during phosphate starvation [143, 153]. However, in contrast to *E. coli* K-12 which demonstrated marked phosphate limitation by early stationary phase (OD_{600} of 0.9), the APEC *pst* mutant exhibited an early maximal induction of the Pho regulon following growth to mid-log phase (OD_{600} 0.6) in LB medium [153]. In addition, many genes associated with stress responses and metabolic functions were differentially expressed by the APEC *pst* mutant when compared to its wild-type parent.

In contrast to the many differentially expressed *E. coli* genes involved in general metabolic or transport functions, only a few confirmed virulence- or stress-response associated genes demonstrated significant transcriptional changes in the *pst* mutant. Of these, genes involved in acid and oxidative stress response were modulated. Furthermore, genes encoding type 1 and F9 fimbriae were down-regulated in strain K3. However, the majority of the pathogen-associated ORFs identified are of unknown function. Among the 470 differentially expressed genes, 18 were not found in *E. coli* K-12 strain MG1655 and were considered as pathogen-associated ORFs. Of these, 14 were specific to CFT073 and 4 to the EHEC (EDL933 and Sakai) genomes. Overall we identified 18 pathogen-associated ORFs that are influenced by constitutive

expression of the Pho regulon. However, the functions encoded by these ORFs are currently unknown.

Modulation of the pathogen-associated ORFs (Table 1 and 2) could contribute to the reduced virulence of strain K3 observed by Lamarche *et al*. [10]. Interestingly, the virulence-associated gene systems *sitABCD*, *iroBCDEN*, *iucABCD-iutA*, coding for metal transporters present in χ7122, that hybridized to the genomic array, were not differentially expressed in the attenuated *pst* mutant.

Blanco *et al*. showed that the C-terminal domain of PhoB interacts with a 22 bp region of dsDNA that consists of two direct repeats of 11 bp [144]. Each 11 bp repeat has a conserved 7 bp region (consensus, CTGTCAT) followed by a less conserved 4 bp segment. By *in silico* analysis and by using the list of genes identified in *E. coli* genomes by Yuan *et al*., a number of genes with predicted Pho boxes not associated with P_i metabolism were identified among the differentially expressed genes [147]. Ninety-one genes or transcriptional units possessed putative Pho box(es), including the known Pho regulon members (Tables 1 and 2). Differentially regulated genes such as those involved in amino acid acquisition (*glnHPQ*, *artJ* and *oppABC*), energy metabolism (*srlAEBD-gutM-srlR-gutQ*, *treA*, *talA*, *ulaABCDEF*, *ulaG* and *glpD*), acid resistance (*gadA* and *gadBC*) and F9 fimbriae biosynthesis (*ydeTSR* and *ydeQ*) possess putative Pho box(es). Distribution of putative Pho regulon members across different functional classes supports the hypothesis that the Pho regulon overlaps and interacts with several other control circuits [6]. However, further studies will be required to establish whether these genes are directly regulated by PhoB.

Validation of microarray results by qRT-PCR

Validation of microarray results was achieved using qRT-PCR. Fifteen genes representing a wide range of gene expression ratios (5 up-, 8 down-regulated and 2 non-differentially expressed genes) in K3 strain were selected for comparative qRT-PCR analysis (Table 3). Comparison of gene expression by microarray hybridizations and qRT-PCR demonstrated a very high level of concordance between the datasets, which is represented by a correlation coefficient of 0.94 and a Pearson correlation of 0.97 (Fig. 2).

Global stress response

Globally, the transcriptional profile of strain K3 indicated that in addition to up-regulating genes associated with scavenging pathways for phosphate acquisition and conservation, the *pst* mutant also demonstrated changes in expression of genes dealing directly with global stress. Many lines of evidence suggest that the Pho regulon and the stress response are interrelated [6]. The gene expression profile of strain K3 represents an exacerbated response in which the strain reacts to an inaccurate detection of phosphate-limiting conditions when, in fact, phosphate levels are actually abundant. This adaptive response entails the induction of different mechanisms to optimize the acquisition and the bioavailability of phosphate and to maintain the essential biochemical reactions active. In addition, during growth, the K3 mutant activates a generalized stress response adaptation.

Genes associated with the RpoS regulon

The Pho regulon and the RpoS regulon are interrelated regulatory networks of the bacterial adaptive response [6]. RpoS is a sigma factor implicated in the cellular response to many stresses. It is also implicated in the stationary phase and the induction of genes in nutrient-limiting environments [375]. The *rpoS* gene is expressed under a variety of growth conditions, but regulation and RpoS production is largely dependent on post-transcriptional stability [375, 376]. During exponential growth, in the K3 mutant, the *rpoS* gene was not differentially expressed. However, the RpoS-regulatory gene *iraP* was induced 4.2-fold. IraP encodes an anti-adaptor protein that enhances RpoS stability and accumulation by inhibiting its targeting to the ClpXP degradosome [259]. Moreover, the small regulatory RNA (sRNA) OxyS, that inhibits the translation of *rpoS*, was down-regulated (-1.82-fold). Bougdour and Gottesman have recently shown that the transcription of the *iraP* gene is promoted by ppGpp accumulation during phosphate starvation [357]. These results suggest that in strain K3, during exponential phase, the sigma factor RpoS stability is increased, leading to regulatory expression of numerous RpoS-dependent genes. Accordingly, 49 genes known to be regulated by the RpoS sigma factor are differentially expressed in strain K3 (Tables 1 and 2). Hence, in the exponential growth phase, strain K3 may alter its gene regulation to respond to phosphate limitation, which includes establishment of a general stress response. Quantification of the RpoS protein would further demonstrate the establishment of the RpoS response, at mid-log phase of growth, in the *pst* K3 mutant.

Oxidative stress response

During aerobic growth, the cytotoxic by-products of the molecular oxygen metabolism, collectively known as reactive oxygen species (ROS), must be eliminated to reduce oxidative stress. It is generally thought that the primary endogenous source of ROS is the respiratory chain, namely the NADH dehydrogenase II, which can leak electrons to oxygen, thereby producing superoxide anion radicals ($O_2^{-}\cdot$). Superoxide is normally detoxified through the activities of superoxide dismutases and catalases that dismute $O_2^{-}\cdot$ into molecular oxygen, hydrogen peroxide (H_2O_2) and eventually to H_2O. However, if they are not rapidly detoxified, superoxide radicals can damage iron–sulfur clusters in enzymes, thereby releasing Fe^{2+}, which can react with H_2O_2 and produce reactive hydroxyl radicals ($HO\cdot$). $HO\cdot$ can eventually initiate the oxidation of proteins, lipids and DNA [377]. Several genes that belong to the RpoS regulon are involved in defense against oxidative stress (*katE*, *dps*, *sodC* and *xthA*) and/or OxyR (which controls *oxyS*, *katG*, *ahpCF*, *gorA*, *grxABC*, *dps* and *fur*) and SoxR (which notably controls *sodA*, *nfo* and *fur*) (for a review, see reference [377]). In strain K3, some genes involved in the oxidative stress response were found to be differentially expressed. Among those, the *dps* (DNA protection protein and ferritin-like protein), *sodC* (superoxide dismutase Cu-Zn), *grxB* (Glutaredoxin 2), *katE* (catalase) and *sufABCDSE* (Fe-S cluster assembly proteins) genes were up-regulated from 1.95- to 1.75-fold. The down-regulated genes (from -2.19- to -1.54-fold) were represented by *grxA* (glutaredoxin 1), *trxB* (thioredoxin reductase), *trxC* (thioredoxin 2), *pqiA* (paraquat-inducible protein) and *ybjC* (predicted inner membrane protein). In addition, the sRNA OxyS regulator was down-regulated (-1.82) in strain K3. OxyS has been proposed to play a role in protecting cells against the damaging effects of elevated hydrogen peroxide concentrations by controlling translation of >40 genes associated with the oxidative stress response [378, 379]. Other, regulators involved in oxidative stress, such as OxyR and SoxRS, were not differentially expressed in strain K3.

Since expression of some genes whose products exhibit antioxidant activities is modulated, the viability to oxidative stress of K3 cells was tested using different reactive oxygen intermediate (ROI)-generating agents. We used H_2O_2 and various superoxide generators, such as plumbagin, phenazine methosulfate (PMS) and phenazine ethosulfate (PES) to evaluate the sensitivity of the K3 *pst* mutant and its wild-type parent strain χ7122. Strain K3 was more sensitive to oxidative stress than the parent strain χ7122, and wild-type resistance levels to all ROI

compounds, except for H_2O_2, were restored by complementation (strain CK3) (Table 4). These results show that the *pst* mutation affects bacterial resistance to oxidative stress, and modulation of certain genes implicated in the oxidative stress response is not sufficient to confer resistance to this stress. Based on the transcriptional data, strain K3 may already be subjected to increased oxidative stress during growth, and is likely less able to cope with additional stresses incurred from exogenous ROI-generating compounds. Furthermore, Moreau *et al.* have shown that glucose metabolism in non-growing cells starved for Pi generates oxidative stress [164, 380]. Since oxidative stress is increased during infection of the host, a decreased capacity to resist ROS could explain, at least in part, virulence attenuation observed for *pst* mutants [9, 10, 55, 228].

Acid stress response

The acid fitness island (AFI) contains genes encoding proteins that are known to provide protection against acid stress in *E. coli*. This broad acid response system helps the cell avoid self-imposed acid stresses that occur as a result of fermentation and enables the cell to survive to low pH conditions [381, 382]. The microarray data demonstrate that the expression of the AFI genes increased significantly in strain K3. This includes the glutamate decarboxylase gene *gadA* (2.57-fold), the multiple transcriptional regulator genes that control expression of the glutamate dependent acid resistance (GDAR) system, *gadE* (3.60-fold), *gadX* (2.15-fold) and *gadW* (6.84-fold), the two chaperones *hdeAB* (2.10-fold for the *hdeA* gene), the multidrug resistance efflux transporter *mdtE* (1.64-fold) and the transcriptional regulator *yhiF* (1.66-fold). In accordance, the *gadBC* operon (4.91-fold for the *gadB* gene), which encodes the glutamate-decarboxylase and an antiporter, respectively, were also up-regulated. Regulation of the AFI and GDAR systems is extremely complex and multifactorial. The expression of these systems is controlled by a large number of regulators, including, among others, the EvgAS two-component system, the specific transcriptional regulators (GadX, GadW, YdeO, and GadE) and the RpoS sigma factor [383]. Up-regulation of genes involved in acid resistance suggests that the *pst* mutant may be subjected to increased acid stress during exponential growth. The effect of the Pho regulon on induction of the acid resistance response is likely to be indirect, since no putative Pho box(es) were found upstream of these regulators.

Previous reports have described the relationship between the Pho regulon and genes involved in acid stress resistance [143, 146, 153, 163]. Transcriptional activation of the *gadA*/*BC*/E genes was observed in *E*. *coli* W3110 grown in low phosphate media [143, 153]. In spite of the induction of acid stress genes, strain K3 was previously shown to be more sensitive to acid stress [10]. It is possible that the *pst* mutant is already subjected to a decreased internal pH from an accumulation of organic acids produced by the glycolytic pathway, which could induce acid response genes during exponential growth [163]. As such, strain K3 which may already incur endogenous acid stress, may therefore be less able to cope when subjected to decreased external pH and acid stress, as observed by Lamarche *et al.* [10]. Based on these data, it would be interesting to compare the pH of the culture medium and the intracellular pH of the wild-type and *pst* mutant during growth, to assess whether induction of these acid response genes may be specifically due to increased acidity during growth.

Transport and binding proteins

In addition to Pho regulon members involved in the transport of phosphate and phosphorylated compounds, genes encoding amino acid and oligopeptide transport systems were up-regulated in strain K3. Indeed, the glutamine and the arginine transporters, encoded respectively by the *glnHPQ* operon and by the *artIQM*/*artJ* genes were up-regulated in strain K3 (1.57- and 1.74-fold, respectively). Down-regulation of the *argR* (-1.57-fold) gene, which encodes the transcriptional repressor of the arginine transporter, occurred at the same time as with the up-regulation of the arginine transporter *artIQM*/*artJ*. The oligopeptide transporter, encoded by the *oppABCDF* genes, was also induced (2.66-fold). This transporter is specific for small peptides of up to five amino acids. Besides their roles in proteins synthesis, amino acids can serve as nitrogen, carbon and ATP sources and they can protect cells from different stresses, such as acid stress [384]. Furthermore, genes encoding the glutamine, arginine and oligopeptide transporters possess putative Pho box(es), which suggest that the uptake of oligopeptides and amino acids via these transporters may be directly regulated by the Pho regulon.

Energy metabolism

Some metabolic genes, involved in the catabolism of different carbon sources, were up-regulated. The *srlAEBD-gutM-srlR-gutQ* operon, involved in glucitol transport and metabolism

and belonging to the phosphoenolpyruvate (PEP): carbohydrate phosphotransferase system (PTS), was induced in strain K3 (4.61-fold) [385]. In addition, up-regulation of genes enzymatically linked to the production of fructose-6-phosphate (*talA* and *nagB*) (1.57- and 1.8-fold, respectively) and glucose (*melA* and *treA*) (3.71- and 1.67-fold, respectively), and metabolism of dihydroxyacetone (*dhaKL*) (1.85-fold) and L-ascorbate (*ulaBDE* and *ulaG*) (2.46-fold) were also induced. These catabolic pathways link to the glycolytic pathway, and intermediates could take part in the tricarboxylic acid cycle. Interestingly, the small regulatory RNA (sRNA) SgrS, which inhibits synthesis of the PtsG glucose-specific PTS system and alleviates the effects of glucose-phosphate stress, was also up-regulated (1.98-fold), suggesting that the strain K3 has preferentially shifted to transport and metabolism of carbon sources other than glucose [386, 387].

On the other hand, repression of genes involved in catabolism of glycerol-3-phosphate (G3P) as a carbon source, such as the G3P transporter encoded by the *glpTQ* genes (-2.54-fold) and the glycerol-3-dehydrogenase *glpD* (-3.56-fold) was observed. The Glp system mediates utilization of G3P or its precursors, glycerol and glycerophosphodiesters. Since uptake of G3P by the GlpTQ transporter leads to P_i counter flow and an overall loss of P_i from the cell, it is not surprising that this system is repressed in strain K3 [388]. By contrast, genes encoding the other G3P-specific transporter Ugp were highly up-regulated (40.00-fold). Accordingly, the *ugp* genes are members of the Pho regulon. Under phosphate starvation conditions, G3P is transported by the Ugp transporter, which leads to the use of G3P as a phosphate source [4]. Identification of putative Pho box(es) upstream the *srl* and *ula* operons, the *talA*, *treA*, and *ulaG* genes denotes a possible direct connection between these catabolic pathways and the Pho regulon. Furthermore, the presence of putative Pho boxes upstream of the *glpD* gene suggests that the Pho regulon inhibits the use of G3P as carbon source. Thus, as the *pst* mutant falsely senses phosphate limitation, it may alter carbon uptake and utilization pathways in an effort to conserve phosphate sources. In order to specifically determine differences in carbon source utilization and transport by the *pst* mutant compared to its wild-type parent, strains could be compared for their capacity to metabolize and grow on different carbon sources.

Protein fate and synthesis

Many genes from the protein fate and synthesis functional classes were down-regulated in strain K3. These genes included those encoding components of the 30S and 50S ribosomal subunits and many chaperone genes involved in protein fate, such as those of the DnaK system and iron-sulfur cluster assembly. The *dnaKJ* (-3.23-fold), *grpE* (-1.68-fold) and *ibpAB* (-2.63-fold) genes comprise the DnaK system. This system is involved in many cytoplasmic events, such as folding of nascent polypeptide chains, rescue of misfolded proteins and assembly and disassembly of protein complexes [389, 390]. Iron-sulfur cluster complexes play several important roles in cellular processes, such as iron storage, electron transfer and regulation of enzyme activity and gene expression [391]. However, genes involved in iron-sulfur cluster assembly were down-regulated in strain K3. They were represented by *hscAB-fdx* (-1.81-fold) and *iscU* (-1.62-fold) genes. Repression of the DnaK system and iron-sulfur cluster assembly genes suggests a decrease in cellular processes occurring in strain K3. Down-regulation of chaperones and ribosomal components suggest a reduction in cellular processes and a potential increase of misfolded peptides, which could be deleterious for cells and may contribute, at least in part, to virulence attenuation of the APEC *pst* mutant strain. Such changes are likely to be indirectly influenced by the Pho regulon, since there were no putative Pho box(es) identified upstream of these genes.

Cell membrane components

In many bacteria, the Pho regulon was shown to be involved in regulation of some cell surface component modifications such as teichuronic acid, phosphate-free lipids, phospholipids and exopolysaccharides [6]. Among the differentially expressed genes in strain K3, 109 encode membrane components. Of these, 54 genes were up- and 55 were down-regulated (Tables 1 and 2, and additional files 2 and 3). Many of the up-regulated genes belong to the transport and binding protein functional class and include members of the Pho regulon that were increased by more than 15-fold. In contrast, the *proVWX* operon (-2.3 fold), involved in glycine betaine osmoprotector transport, demonstrated a decreased expression. This system is implicated in protection from osmotic shock [392]. Beside their role in energy metabolism, the *talA* (1.57-fold) and *treA* (1.67-fold) genes were also induced by osmotic stress under aerobic conditions [393, 394]. This suggests that the *pst* K3 strain was faced with osmotic stress, and down-regulation of the *proVWX* operon could reduce protection from this stress.

Among the down-regulated genes, 7 are involved in lipopolysaccharide (LPS) biosynthesis including lipid A modification. Indeed, the *rffCDGH* (-1.66-fold) and the *rfaJ* (-1.68-fold) genes, respectively involved in the enterobacterial common antigen (ECA) biosynthesis pathway and LPS-core biosynthesis, were down-regulated. However, we previously did not observe any changes in O-antigen and/or ECA profiles by SDS-PAGE and Western blot analyses [10, 12]. The results of this study suggest that subtle changes could indeed occur within the LPS structure in strain K3.

The *eptA* and *yeiU* genes, involved in lipid A modification, were also down-regulated (-1.68 and -1.57-fold, respectively). The *eptA* gene is the *Salmonella pmrC* ortholog and is involved in phosphoethanolamine (pEtN) covalent modification of lipid A [395]. The BasRS two-component system that regulates *eptA* was also down-regulated (-1.68-fold) in this study [395]. The *basRS* genes are orthologs of the *pmrAB* genes of *Salmonella enterica,* and PmrAB has been shown to be required for resistance to polymyxin B and other antimicrobial compounds [396]. In addition, we have determined that, in strain K3, the down-regulation of *yeiU* (*lpxT*), encoding an undecaprenyl pyrophosphate phosphatase involved in the biosynthetic origin of the lipid A 1-pyrophosphate, correlated with a decrease in the hexa-acylated 1-pyrophosphate form of lipid A compared to the APEC strain χ7122 [12]. Valvano MA and Raetz *et al*. have proposed that modification of lipid A might contribute to the fine-tuning of the lipopolysaccharide molecule, which is often the target of modifications that might provide adaptive advantages to changing environmental conditions [397, 398]. LPS modification could contribute to the phenotypes observed in the *pst* mutant, such as decreased resistance to serum, acid and cationic antimicrobial peptides [9, 10, 12]. Furthermore, down-regulation of LPS modification genes may be a strategy used by the bacteria to optimize the P_i availability. However, such cell surface modifications could play a role in the reduced virulence observed in *pst* mutants [12].

Reduced expression of fimbrial genes and production of type 1 fimbriae

Fimbriae are adhesive organelles of paramount importance for successful bacterial recognition and colonization of specific host tissues. Genes involved in type 1 and F9 fimbriae biosynthesis were down-regulated in the K3 strain (Table 2). Indeed, genes involved in the biosynthesis of

type 1 (major type 1 subunit *fimA*, the chaperone *fimC* and the *fimI* gene, whose function is not elucidated, and the regulator of type 1 fimbriae *fimB*) (-2.1 fold) as well as F9 fimbriae (*ydeTSR*, *ydeQ*, *c1936* and *c1935)* (-3.72- to -1.54-fold) were repressed in strain K3. Type 1 and F9 fimbriae are important virulence factors involved in colonization and biofilm formation [1, 53, 170, 171, 399]. In APEC strains, type 1 fimbriae are preferentially expressed in air sacs, which are the primary infection site [183]. F9 fimbriae are homologous to type 1 fimbriae but are immunologically and functionally distinct [399]. However, the production of F9 fimbriae and the possible role of these fimbriae in APEC infections have not yet been investigated.

In order to investigate whether the decreased expression of fimbrial genes correlated with decreased fimbrial production, strain χ7122, the *pst* mutant K3 and the complemented mutant CK3 were grown under conditions used for microarray analyses and were examined by electron microscopy. Indeed, very few or no fimbriae were observed at the surface of cells of the K3 mutant, compared to wild-type strain χ7122 and the complemented strain CK3 (Fig. 3 A,B,C). The type 1 fimbrial adhesin recognizes D-mannose receptors on cells, and mediates aggregation of yeast cells which are rich in mannose surface molecules [51].

When cultures of strains χ7122, the *pst* mutant K3, and the complemented CK3 were grown under conditions corresponding to those used in the microarray analyses, i.e. OD_{600} 0.6, the K3 strain did not demonstrate any yeast aggregation even at the most concentrated bacterial cell suspension (approx. 1 $X10^{11}$ cfu/ml), whereas χ7122 and the complemented mutant CK3 agglutinated yeast at titers at least 10-fold less (approx. 1 $X10^{10}$ cfu/ml) (Fig 3D). When strains were cultured for 48 h without agitation, to enhance production of type 1 fimbriae, the K3 mutant agglutinated yeast cells, but 51-fold less than wild-type strain χ7122 and the complemented mutant (Fig. 3D). Hence, the *pst* mutant was shown to be considerably less able to produce type 1 fimbriae compared to its wild-type parent even under different growth conditions. To determine whether decreased yeast agglutination was specifically due to a reduction in production of the type 1 fimbrial adhesin, cell surface extracts were tested for production of the type 1 major fimbrial subunit, FimA, by Western blotting, and this confirmed an important reduction of FimA production in K3 strain, compared to χ7122 and CK3 strains (Fig. 3E).

Several studies have provided evidence that the expression of type 1 fimbriae is altered in response to environmental stress conditions such as high osmolarity, pH and temperature, and that expression is induced upon entry into stationary phase [87, 94]. Putative Pho box(es) were identified upstream of the *ydeT* and *ydeQ* genes, which are involved in F9 fimbriae biosynthesis, whereas no putative Pho box(es) were observed upstream of the genes involved in type 1 fimbriae biosynthesis. Taken together, results indicate that in the *pst* mutant, the production of type 1 fimbriae is repressed, and this loss of adhesin expression could be indirectly controlled by PhoBR. It was previously shown that in APEC strains, type 1 fimbriae are preferentially expressed in the air sacs and lungs, where the air sacs are the primary site of initial infection [400]. Thus, in the K3 strain, in addition to other previously established phenotypes, down-regulation of adhesins such as type 1 fimbriae could affect the initial step of colonization and possibly contribute to decreased virulence.

CONCLUSION

In conclusion, by assessing the global transcriptional response of a *pst* mutant, we determined that the effects of this mutation resulted in up-regulation of members of the Pho regulon, which are involved in phosphate uptake and metabolism. Transcriptional analyses also demonstrated the induction of a general stress response in the *pst* mutant, including genes involved in adaptation to acid stress, oxidative stress, and the general stress response (notably RpoS-regulated genes). Other changes included down-regulation of genes associated with cell surface composition. Phenotypic tests confirmed a reduced capacity of the *pst* mutant to produce type 1 fimbriae and a decreased capacity to resist oxidative stress. Altogether, our data provide further support that the Pho regulon is an important part of a complex network that encompasses not only phosphate homeostasis, but also adaptive responses to stress and altered regulation of a diversity of genes including virulence factors such as those encoding fimbrial adhesins.

LIST OF ABBREVIATIONS

Pst system: Phosphate specific transport system, EDTA: ethylene diamine tetraacetic acid, cDNA: complementary DNA, ORF: Open Reading Frame, bp: base pair, Fe^{2+}: Ferrous iron, Fe-S: Iron-sulfur cluster, SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis.

AUTHORS' CONTRIBUTIONS

SC designed and performed the transcript profiling experiments, carried out downstream data analysis and drafted the manuscript. MGL constructed the K3 and CK3 strain, participated in the study design and revised the manuscript. PG executed the bioinformatics analysis and revised the manuscript. JS carried out the characterization of fimbriae experiments. JP performed the oxidative stress experiments. CMD supervised the characterization of fimbriae and oxidative stress experiments, performed data analysis and revised the manuscript. JH participated in the conception and supervised the design of the study, performed data analysis and revised the manuscript. All authors read and approved the final manuscript.

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FIGURES LEGENDS

Figure 1. Functional classification of the differentially expressed genes. White and black bars represent the up- and down-regulated genes, respectively. The x-axis represents the percentage of the functional class according to the number of genes that were up- and down-regulated.

Figure 2. Microarray results validation by qRT-PCR. Five up-regulated genes, eight down-regulated genes and two non-differentially expressed genes are presented. Mean $\log_2$ ratios of the qRT-PCR experiments are plotted against the mean $\log_2$ ratios of the microarray experiments. Numbers on the graph refer to genes listed in Table 3.

Figure 3. Production of type 1 fimbriae by the strain K3. A, B and C) Electron microscopy performed onto χ7122, K3 and CK3 strains, respectively, at 12 000X. Images show a typical field of view of bacteria and demonstrate that the K3 strain lacks fimbriae, compared to the χ7122 and CK3 strains. Arrow shows fimbriae on cell surface. D) Minimal bacterial titer allowing yeast agglutination. The χ7122 Δ*fim* (χ7279) strain was used as negative control and did not show agglutination (data not shown). The § symbol shows that at mid-log phase of growth, no agglutination was observed at the highest cell titer for the K3 strain, which was also observed for Δ*fim* strain χ7279 (data not shown). Asterisks indicate significant differences observed between bacterial concentration of the wild-type χ7122 and the *pst* mutant strain K3 (*P value* = 0.0001) as calculated by Student's t test. No difference was observed between the χ7122 and the CK3 strains. E) Western blot of protein extracts from strains χ7122 (lane 1), K3 (lane 2), CK3 (lane 3), and χ7279 (lane 4) using anti-FimA serum.

Tables

Table 1. Up-regulated genes in K3 strain

Functional class and gene name[a, b, c, d, e, f, g]	Operon [c, d, e, f, h]	Known or predicted function	Fold change
Transport and binding protein			
artI	***art**PI**QM***[d]	Arginine-binding periplasmic protein 1	1.74
artJ[c]		Arginine-binding periplasmic protein 2	1.78
feoB	***feo**A*[c]***B***	Ferrous iron transport protein	2.19
glnH[c]	***glnHPQ***[d]	Glutamine ABC transporter periplasmic-binding	1.57
mdtE[e]	***mdtE**F*	Multidrug resistance efflux transporter	1.64
oppA[c]	***oppABC**DF*	Periplasmic oligopeptide-binding protein	2.66
phnC [b, c]	***phnCDEFGHIJKLMNOP***	Phosphonates transport ATP-binding protein	145.81
phoE [b, c]		Outer membrane phosphoporin protein	85.15
potE	*speF-**potE***	Putrescine-ornithine antiporter	2.56
pstS[b, c]	***pstS**CA**B-phoU***	High-affinity phosphate-specific transport system	60.97
srlA[c]	***srlAEBD-gutM-srlR***[c]***-gutQ***	PTS system, glucitol/sorbitol-specific IIC2 component	4.61
ugpB [b, c, d]	***ugpBAEC*** [d]***Q***	sn-glycerol 3-phosphate transport system periplasmic binding protein	40.00
yeaN	***yeaN**O*	Putative amino acid / amine transport protein	2.91
yhiD[d, e]		Predicted Mg(2+) transport ATPase inner membrane	2.35

Energy metabolism

dhaK	***dhaKL****M*	Dihydroxyacetone kinase	1.85
grxB		Glutaredoxin 2	1.85
hcp	***hcp-hcr***	Hydroxylamine reductase	1.63
melA	***melAB***	Alpha-galactosidase	3.24
nagB	***nagB****ACD*	Glucosamine-6-phosphate deaminase	1.83
napH	***nap****FDAG****HB****C-* *ccmABCDEFGH*	Quinol dehydrogenase membrane component	2.56
nrfA	***nrfABCD****EFG*	Cytochrome c552 / nitrite reductase, formate-	3.74
talA[c, d]		Transaldolase	1.57
treA[c, d]		Periplasmic trehalase	1.67
ulaB	***ula****A****B***^{c}*C****DE****F*	L-ascorbate-specific enzyme IIB component of PTS	2.46
ulaG		Predicted L-ascorbate 6-phosphate lactonase	2.04
Protein fate			
hdeA[d, e]	***hdeAB***[d, e]	Acid-resistance protein	2.10
iraP [c,d]		Anti-adaptor protein for σ^{S} stabilization	4.42
Cellular processes			
amn [b, *c*]		AMP nucleosidase	6.08
katE[d]		Catalase HPII / hydroperoxidase HPII(III)	1.95
sodC[f]		Superoxide dismutase (Cu-Zn)	1.75
ycgV		Predicted adhesin	4.01
Transcription			
chaB	***chaB****C*	Cation transport regulator	1.75
gadE[e]		DNA-binding transcriptional activator	3.60

gadX[d, e]		DNA-binding transcriptional dual regulator	2.15
gadW[d, e]		DNA-binding transcriptional activator	6.84
iciA [b, c]		Chromosome replication initiation inhibitor protein	2.00
phoB [b, c]	***phoBR***	DNA-binding response regulator in two-component	36.23
yhiF[e]		Predicted transcriptional regulator	1.66
Central intermediary metabolism			
gadA[c, d, e]		Glutamate decarboxylase alpha	2.57
gadB[c, d, e]	***gadBC***	Glutamate decarboxylase beta	4.91
phoA [b, c]	***phoA-psiF***	Alkaline phosphatase	112.67
sufA[e]	***sufABCDSE***	Iron-sulfur cluster assembly scaffold protein	1.91
Cell enveloppe			
yibD[b]		Putative glycosyl transferase	45.95
Fatty acid and phospholipid metabolism			
cdh		CDP-diacylglycerol phosphotidylhydrolase	4.28
hdhA[d]		7-alpha-hydroxysteroid dehydrogenase	1.52
Unknown function			
c0754 [g]		Hypothetical protein	2.12
c0778 [g]	***c0778-speF-potE***	Hypothetical protein	2.43
c0902		Hypothetical protein	1.65
c1013		Hypothetical protein	1.83
c1153 [c g]		Hypothetical protein	2.35
c1435 [g]		Hypothetical protein	2.26
c2837 [g]		Hypothetical protein	1.53
c4182		Hypothetical protein	1.76

phoH [b,c]		Conserved protein with nucleoside triphosphate hydrolase domain	5.95
psiE [b, c]		Phosphate-starvation-inducible protein	1.84
ytfK [b]		Hypothetical protein	6.65
z0950 [g]		Unknown	1.58
Biosynthesis of cofactors, prosthetic groups and carriers			
iucD [g]	*iucABC**D**-iutA*	Lysine / ornithine N-monooxygenase	1.76
Regulatory function			
dps [d, f]		DNA protection during starvation conditions	1.87
isrA		Small antisense RNA	1.64
sgrS		Small antisense RNA	1.98
rybA		Small antisense RNA	1.59
rygC		Small antisense RNA	1.50
yddV	***yddV-dos***	Predicted diguanylate cyclase	1.80

[a] The first up-regulated gene of the operon is shown, [b] Members of the Pho regulon [c] Indicates the presence of Pho box in the gene promoter, [d] Genes belonging to RpoS regulon, [e] Genes involved in acid stress response, [f] Genes involved in oxidative stress response, [g] Pathogen-associated ORF, [h] Genes in bold are up-regulated in K3 strain.

Table 2. Down-regulated genes in K3 strain

Functional class and gene name[a, b, c, d, e, f]	Operon[b, c,d, e, g]	Known or predicted function	Fold change
Transport and binding protein			
dctA		C4-dicarboxylate transport protein	-2.03
fadL		Long-chain fatty acid transporter	-1.69
proV	**proVWX**	Glycine betaine transporter subunit	-2.30
Energy metabolism			
fucI[b]	*f**uc**PIK**UR*	L-fucose isomerase	-1.59
glpD[c]		Aerobic glycerol-3-phosphate dehydrogenase	-3.56
glpE[b]	***glpEGR***	Thiosulfate sulfurtransferase	-2.04
glpT	***glpTQ***	sn-glycerol-3-phosphate transporter	-2.54
glpX	***glp**FK**X***	Fructose 1,6-bisphosphatase II	-1.51
grxA[e]		Glutaredoxin 1	-2.19
trxB[b, e]		Thioredoxin reductase, FAD/NAD(P)-binding	-1.88
trxC[b, e]		Thioredoxin 2	-1.87
Protein fate			
c1935[f]		Chaperone protein (F9 fimbriae)	-3.72
dnaK	***dnaKJ***	Molecular chaperone	-3.23
grpE		Heat shock protein	-1.68
hscB	***hscBA**-**fdx**-iscX*	Co-chaperone with HscA	-1.81
hslO		Hsp33-like chaperonin	-1.59

hslV	***hslVU***	ATP-dependent protease peptidase subunit	-2.68
htpG [b]		Heat shock protein 90	-1.91
ibpA	***ibpAB***	Heat shock chaperone	-2.63
slpA [d]		FKBP-type peptidyl-prolyl cis-trans isomerase (rotamase)	-1.60
ydhJ	***ydhIJK***	Undecaprenyl pyrophosphate phosphatase	-2.05
Protein synthesis			
dinD		DNA-damage-inducible protein	-1.90
lexA	**lexA**-***dinF***	Regulator for SOS regulon	-2.14
nrdA	***nrdAB***	Ribonucleotide-diphosphate reductase alpha subunit	-1.58
recN		DNA repair protein / recombination and repair protein	-2.80
rplR	rplNXE-rpsNH-**rpl**F**R**-**rpsE**-**rpmD**-**rplO**-secY-rpmJ	50S ribosomal protein L18	-1.59
rplW	rpsJ-**rpl**CD**WB**-**rpsS**-**rplV**-rpsC-**rplP**-**rpmC**-**rpsQ**	50S ribosomal protein L23	-1.91
umuD	***umuDC***	DNA polymerase V, subunit D	-1.72
Purines, pyrimidines, nucleosides and nucleotides			

carA[b]	***carAB***	Carbamoyl-phosphate synthase small subunit	-2.02
guaB	***guaBA***	Inositol-5-monophosphate dehydrogenase	-2.33
purH[b]	***purHD***	Bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase / IMP cyclohydrolase	-3.01
purI		Phosphoribosylformyl-glycineamide synthetase	-3.33
purM	*purMN*	Phosphoribosylaminoimidazole synthetase	-2.62
pyrL	***pyrLB****I*	Orotate phosphoribosyltransferase	-1.96
Cell envelope			
bcsZ	***bcs****ABZC*	Endo-1,4-D-glucanase	-1.58
eptA		Predicted metal dependent hydrolase	-1.68
rfaJ	***rfa****QGPSBI****J****YZ-waaU*	Lipopolysaccharide 1,2-glucosyltransferase	-1.68
rffC	*rfe-****rff****A****CD****E****GH****MT-wzxE-wzyE-wzzE*	Lipopolysaccharide biosynthesis protein / TDP-fucosamine acetyltransferase	-1.66
yeiU	***yei****R****U***	Undecaprenyl pyrophosphate phosphatase	-1.57
Fatty acid and phospholipid metabolism			
fabB[b]		3-oxoacyl-(acyl carrier protein) synthase	-1.51

yieE[b]	***yieE****F*	Predicted phosphopantetheinyl transférase	1.53
Transcription			
basR	***basRS***[b]	DNA-binding response regulator in two-component regulatory system with BasS	-1.68
crl		Curlin genes transcriptional activatory protein	-2.52
fimB		Type 1 fimbriae regulatory protein / Inversion of on/off regulator of fimA	-1.66
pdhR[b]	***pdhR-aceEF-lpdA***	Transcriptional regulator for pyruvate dehydrogenase complex	-3.42
rpoD	*rpsU-dnaG****-rpoD***	RNA polymerase sigma factor	-1.65
Cellular processes			
c1936[f]		Type 1 fimbrial protein (homologue)	-3.23
fimA[c]	***fimAIC****DFGH*	Major type 1 subunit fimbrin (pilin)	-2.10
ydeQ[b]		Predicted fimbrial-like adhesin protein (F9 fimbriae)	-1.57
ydeR	***ydeRST***[b]	Hypothetical fimbrial-like protein (F9 fimbriae)	-2.21
Regulatory function			

oxyS [e]		Global regulatory RNA	-1.82
Central intermediary metabolism			
iscR	***iscR****SU**A*	NifU-like protein / Scaffold protein	-1.62
Unknown function			
c3759 [b, f]	*parC-**c3759***	Hypothetical protein	-1.72
pqiA	***pqiA****B*	Paraquat-inducible membrane protein	-1.70
yhcN		Unknown	-7.82
yjbC	***ybjC****-nfsA-rimK-ybjN*	Hypothetical protein	-1.54
ybcU		Bor protein homolog from lambdoid prophage	-1.62
z5852		Hypothetical protein	-1.52
Biosynthesis of cofactors, prosthetic groups and carriers			
thiC	***thiCEFSGH***	Thiamine biosynthesis protein	-3.30
Mobile and extrachromosomal element functions			
hfq	*yjeFE-amiB-**mutL-miaA-***	RNA-binding protein	-1.56
z3347 [f]		Unknown protein encoded within prophage CP-	-1.65
z1876 [f]		Putative endolysin of prophage CP-933X	-1.85

[a] The first down-regulated gene of the operon is shown, [b] Indicates the presence of Pho box in the gene promoter, [c] Genes belonging to RpoS regulon, [d] Genes involved in acid stress response, [e] Genes involved in oxidative stress response, [f] Pathogen-associated ORF, [g] Genes in bold are down-regulated in K3 strain.

Table 3. Genes used for microarray validation with qRT-PCR

No.	Gene	Gene title	Microarray Fold change (Log2)	qRT-PCR Fold change (Log2)
1	*phoA*	Alkaline phosphatase	6.816	7.600
2	*gadW*	DNA-binding transcriptional	2.773	2.303
3	*cdh*	CDP-diacylglycerol phosphotidylhydrolase	2.099	1.607
4	*ycgV*	Predicted adhesin	2.022	2.483
5	*yddV*	Diguanylate cyclase	0.851	0.890
6	*ydeQ*	Predicted fimbrial-like adhesin	-0.651	-0.573
7	*rpoD*	RNA polymerase sigma factor	-0.640	-1.087
8	*crl*	Curlin genes transcriptional activatory protein	-1.331	-1.037
9	*thiF*	Thiamine biosynthesis protein	-2.152	-1.787
10	*yhcN*	Hypothetical protein	-2.967	-5.680
11	*lexA*	Regulator for SOS regulon	-1.198	-2.377
12	*oxyS*	Oxidative stress regulator	-0.864	-1.777
13	*hfq*	RNA-binding protein	-0.638	-1.340
14	*yidB*	Hypothetical protein	-0.10	-0.013
15	*yeiR*	Predicted enzyme	-0.04	0.212

Table 4. Growth inhibition zone (mm) of APEC χ7122, K3 (*pst* mutant) and CK3 (complemented) strains to oxidative stress generating compounds[a]

Strain	**LB agar with:**			
	Plumbagin[b]	**H_2O_2**	**PES**	**PMS**
χ7122	8.5±0.1	19.2± 0.2	15± 0.4	15.7±0.4
K3	**10.7 ± 0.6[c]**	**23.0± 0.9**	**19.2±0.5**	**20.7±0.2**
CK3	8.7 ± 0.3	**21.9 ± 1.0**	15.1±0.3	16.7±0.2

[a] Data presented are the means ± the standard deviations of six independent experiments.

[b] Compounds used were 10 µl of plumbagin (53 mM), H_2O_2 (30% vol/vol), phenazine ethosulfate (15 mM) (PES), or phenazine methosulfate (PMS) (15 mM).

[c] Values indicated in bold text are significantly different ($P<0.05$) compared to the mean of the wild-type strain.

Figure 1

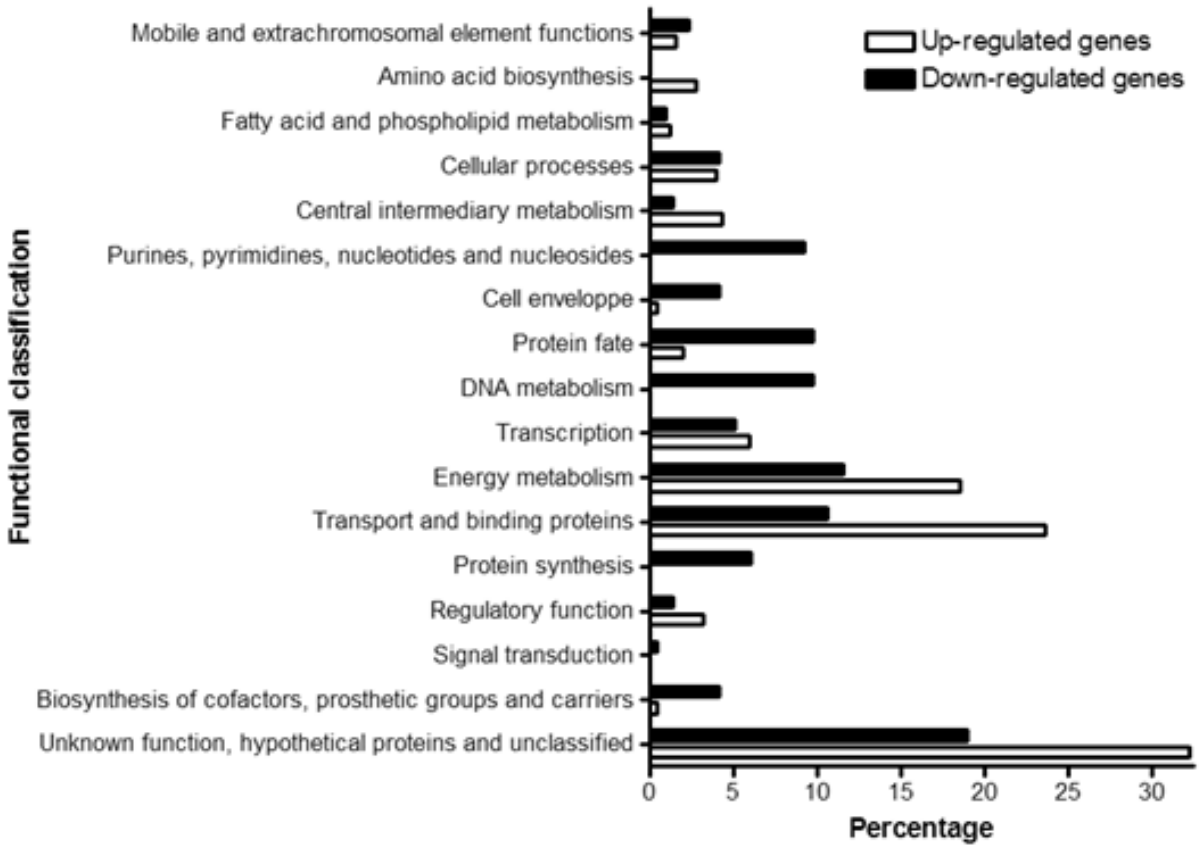

Mobile and extrachromosomal element functions
Amino acid biosynthesis
Fatty acid and phospholipid metabolism
Cellular processes
Central intermediary metabolism
Purines, pyrimidines, nucleotides and nucleosides
Cell enveloppe
Protein fate
DNA metabolism
Transcription
Energy metabolism
Transport and binding proteins
Protein synthesis
Regulatory function
Signal transduction
Biosynthesis of cofactors, prosthetic groups and carriers
Unknown function, hypothetical proteins and unclassified
Functional classification
Up-regulated genes
Down-regulated genes
0
5
10
15
20
25
30
Percentage

Figure 2.

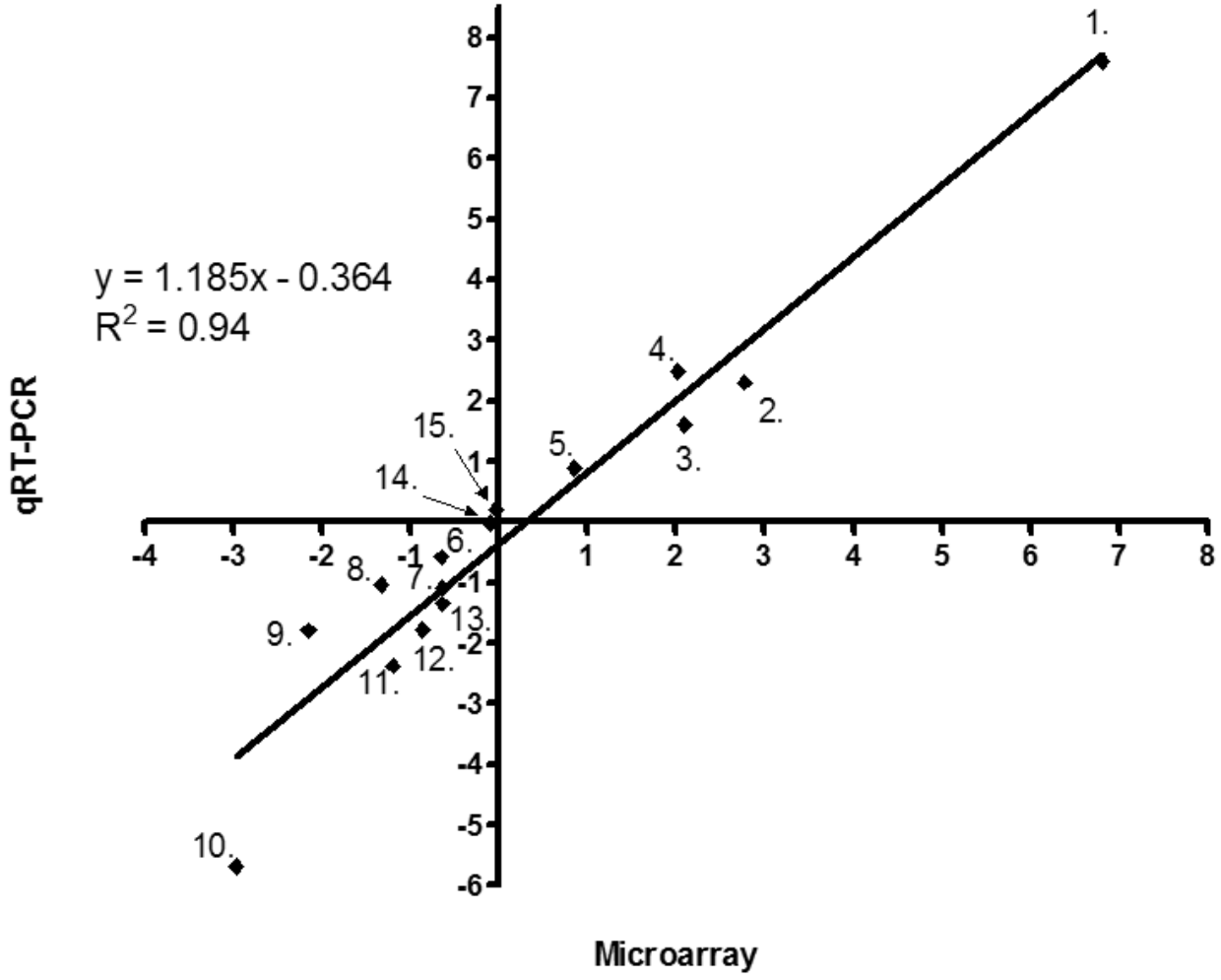
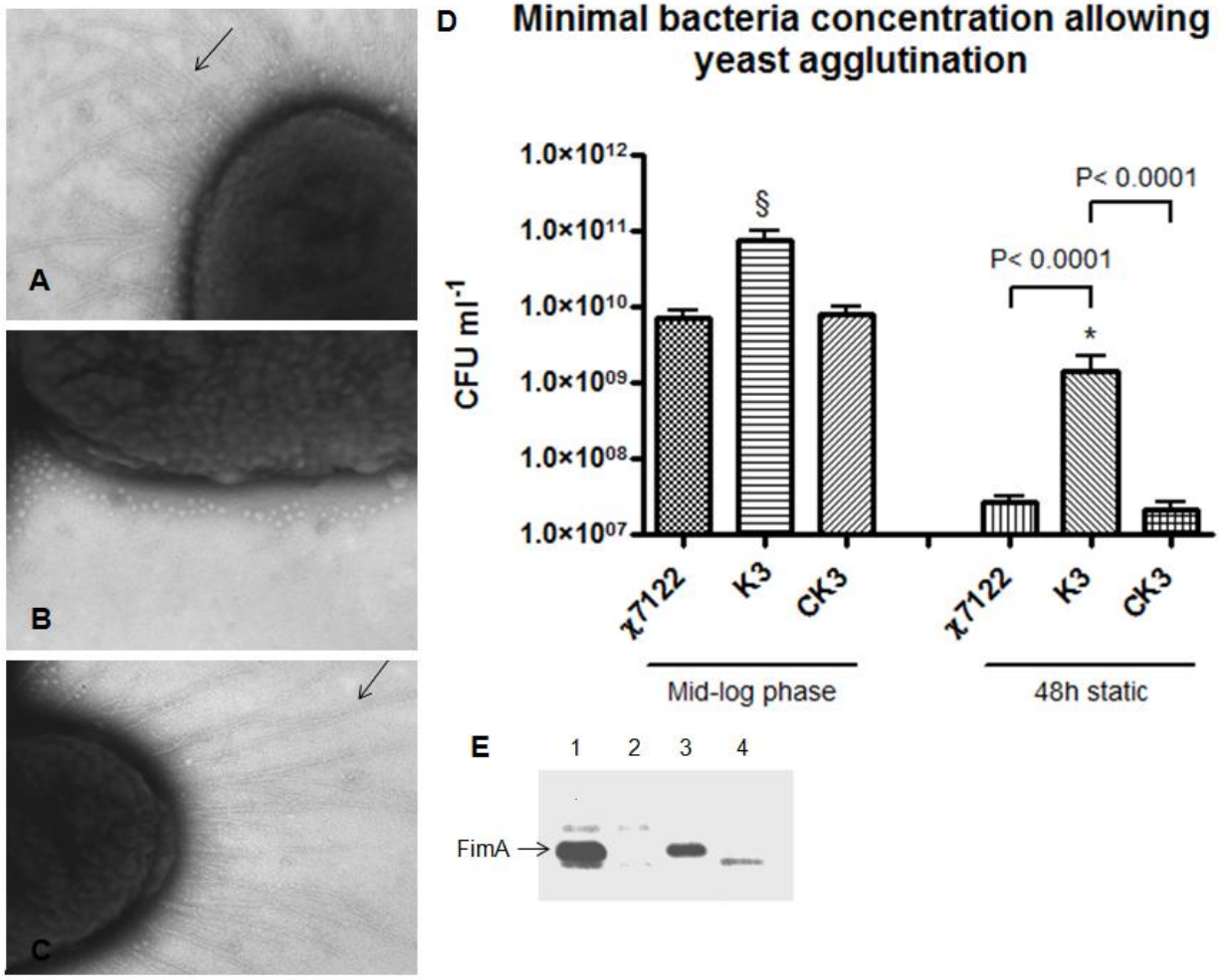

y = 1.185x - 0.364
R2 = 0.94
qRT-PCR
Microarray
1.
2.
3.
4.
5.
6.
7.
8.
9.
10.
11.
12.
13.
14.
15.

Figure 3.


A
B
C
D
Minimal bacteria concentration allowing yeast agglutination
CFU ml-1
1.0×10^12
1.0×10^11
1.0×10^10
1.0×10^09
1.0×10^08
1.0×10^07
§
*
P< 0.0001
P< 0.0001
χ7122
K3
CK3
χ7122
K3
CK3
Mid-log phase
48h static
E
1 2 3 4
FimA

Additional files

Additional file 1

File format: DOC

Title: List of primers used in the qRT-PCR experiments.

Description: Complete lists of primers used in the qRT-PCR experiments, including primer sequences are shown.

Additional file 2

File format: XLS

Title: Up-regulated genes in the K3 strain.

Description: The complete list of genes up-regulated by at least 1.5-fold with a p-value of ≤ 0.05 and an estimated false discovery rate (FDR) of 2.71%, in the *pst* K3 strain. Probe number, gene name, fold change in Log2, and p-value were provided.

Additional file 3

Format: XLS

Title: Down-regulated genes in the K3 strain.

Description: The complete list of genes down-regulated by at least 1.5-fold, with a p-value of $\leq$ 0.05 and an estimated false discovery rate (FDR) of 2.71%, in the *pst* K3 strain. Probe number, gene name, fold change in Log2, and p-value were provided.

Annexe #2

Identification des sites d'insertion du transposon aléatoire *Tn*10

L'objectif de l'article #3 était de déterminer les mécanismes moléculaires reliant le système Pst (PhoBR) et l'expression des fimbriae de type 1. Pour ce faire, nous avons construit une banque de mutants à l'aide du transposon aléatoire *Tn*10 chez le mutant *pst*. Nous avons choisi cette souche puisque les fimbriae de type 1 sont réprimés chez cette dernière. Ainsi, notre raisonnement se basait sur le fait que l'inactivation du système Pst induit l'expression d'un ou de plusieurs facteurs, qui lui (eux), réprime(nt) les fimbriae de type 1. De cette manière, l'inactivation de ce(s) facteur(s) inhiberait son effet répressif sur l'expression des fimbriae de type 1. La première étape de l'identification des sites d'intégration était de cribler les mutants obtenus pour leur capacité à produire les fimbriae de type 1.

Près de 5000 mutants ont été générés. Cependant, nous n'avons, pour le moment, criblé qu'une centaine d'entre eux. Au total, 21 mutants, comptant pour 14 gènes, montrent une restauration complète ou partielle de l'expression des fimbriae de type 1. Ainsi, un des régulateurs des flagelles (*flhC*), le transporteur ABC de la leucine (opéron *liv*), le locus de résistance multiple aux antibiotiques (*mar*) et plusieurs systèmes hypothétiques semblent relier le système Pst et l'expression des fimbriae de type 1 (Tableau 1). Dans les prochains paragraphes, je ne traiterai que des facteurs dont la fonction est connue, i.e. FlhC, Liv et Mar.

En raison de la régulation croisée entre les fimbriae de type et les flagelles [69], il n'est pas surprenant, et plutôt logique, de constater que l'inactivation de *flhC* (un des régulateurs des flagelles) restaure l'expression des fimbriae de type 1. Cette régulation croisée permet à la bactérie d'accomplir qu'une seule action à la fois, i.e. adhérer aux cellules hôtes (coloniser) ou nager vers d'autres sites d'infection. Au cours de ma première année de doctorat, nous avons testé la capacité de la souche mutante à nager. La quantification de cette nage fut difficile puisque la souche CFT073 croît rapidement et nage beaucoup. Nous étions donc limités dans le matériel utilisé et les conditions expérimentales. Malgré ces limitations, nous avons remarqué

qu'en effet, le mutant *pst* a tendance à nager plus que la souche sauvage, dénotant ainsi une activité accrue des flagelles. Cette observation est en accord avec les résultats d'infections *in vivo* présenté à la Figure 5B de l'article #2, où nous observons que malgré le fait que le mutant Pst soit atténué dans sa capacité à coloniser la vessie, dû à la répression des fimbriae de type 1, il colonise les reins au même niveau que la souche sauvage. Ces résultats sont en accord avec ce qui a été précédemment observé, i.e. que les flagelles promouvent le passage de la vessie aux reins [35].

Tableau 1. Site insertion du transposon *Tn*10 chez les mutants regagnant la production des fimbriae de type 1

Gènes ou région	Fonction	# d'insertions
c2440	Gène dont la fonction est inconnue. Homologie avec l'adhésine putative YeeJ	1
c2587	Gène hypothétique	1
c3183	Gène hypothétique	1
c3331	Protéine fonction inconnue	5
c4502	Antiporter putatif	1
c5297	Gène dont la fonction est inconnue	1
flhC	Activateur transcriptionnel des flagelles	1
livH	Perméase faisant partie du transporteur de la leucine, isoleucine et valine	1
marR	Régulateur du locus de résistance multiple aux antibiotiques	1
phoA	Phosphatase Alcaline	4
Région intergénique	Région intergénique entre les gènes *c5387* et *c5388*	1
ybhI	Gène hypothétique	1
yicI	Dégradation des oligosaccharides contenant des liens α-1,6-xylosidique	1
yjhS	Gène dont la fonction est inconnue	1

Nous avons aussi identifié un site d'intégration au niveau du gène *marR*. Ce gène code pour un régulateur négatif des systèmes de résistances multiples aux antibiotiques, de la réponse aux stress, des mécanismes métaboliques et des facteurs de virulence [401]. Étant donné que l'inactivation de *marR*, chez le mutant *pst*, restaure l'expression des fimbriae de type 1, cela suggère que *marR* est induit chez le mutant *pst*. Étant donné que MarR code pour un régulateur, il est possible que chez le mutant Pst, l'activation de MarR entraîne sa fixation au niveau des promoteurs de *fimA*, *fimB*, *ipuA* ou *ipbA*, entraînant ainsi leur répression et par conséquent, l'inhibtion de l'expression des fimbriae de type 1. Il serait donc intéressant de déterminer l'expression de *marR*, chez le mutant *pst*, afin de le relier avec l'expression des fimbriae de type 1. De plus, *marR* fait partie de l'opéron *marRAB*, où *marA* code pour un régulateur impliqué dans la régulation d'au moins 80 gènes [401]. De plus, il a récemment été montré que l'induction de *marA* inhibait l'expression des fimbriae de type 1 [402]. De cette manière, il est possible que l'intégration du transposon au niveau de *marR* ait un effet polaire sur l'expression de *marA* et que le phénotype d'expression des fimbriae de type 1 soit dûe à *marA* plutôt qu'à *marR*. Dans de futures expérimentations, il sera important d'introduire des mutations non-polaires au niveau de *marR* et *marA* afin de déterminer quel gène est impliqué dans la régulation des fimbriae de type 1.

Finalement, un site d'intégration au niveau du gène *livH* a aussi été identifié. Ce gène fait partie de l'opéron *livKHMGF* qui, avec le gène *livJ*, est impliqué dans le transport de la leucine, de l'isoleucine et de la valine [403]. Cet opéron est négativement régulé par le régulateur Lrp (leucine responsive protein). Ainsi, lorsque la concentration intracellulaire en leucine est élevée, Lrp fixe cet acidé aminé et s'apparie, notamment, aux promoteurs des gènes impliqués dans le transport et le métabolisme de la leucine. L'expression de ces derniers est donc inhibée en présence de leucine. Par contre, en condition de carence en leucine, les sites de fixation de la leucine de Lrp se retrouveront inocuppés et Lrp se détachera des promoteurs, induisant ainsi l'expression des gènes impliqués dans le métabolisme de la leucine [403]. L'introduction de mutations non-polaires au niveau de *livH* et de l'opéron *liv* chez le mutant *pst* donne les mêmes résultats, i.e. restauration de l'expression des fimbriae de type 1. Cela montre que cet opéron est impliqué, soit de façon directe ou indirecte, dans l'expression des fimbriae de type 1. En quantifiant l'expression de l'opéron *liv*, chez le mutant *pst*, nous observons que ce dernier est induit 2,5 fois. Ce résultat suggère donc que la répression induite par Lrp est inhibé chez le mutant *pst*.

En plus d'être impliqué dans la régulation négative du métabolisme de la leucine, Lrp régule aussi positivement l'expression des fimbriae de type 1. Ainsi, la fixation de Lrp, au niveau du promoteur (*fimS*) des fimbriae de type 1, est requise pour que c'est derniers soient transcripts [63]. Ainsi, selon les données obtenues concernant l'opéron *liv*, nous pouvons supposer que, chez le mutant *pst*, Lrp n'est plus fixé au niveau de *fimS*, entraînant ainsi la répression des fimbriae de type 1. Dans ce cas, il se peut qu'il y ait des signaux intracellulaires reliant le régulon Pho et le régulon Lrp.