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Virulence News & Views

Deformed wing virus: The main suspect in unexplained honeybee deaths worldwide

Comment on: Martin SJ, et al. Science 2012; 336:1304-6; PMID:22679096; http://dx.doi.org/10.1126/science.1220941

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The unexplained collapse of honeybee (*Apis mellifera*) colonies across the world continues to fascinate both the scientific and mainstream media alike. This is mainly due to the worldwide importance of honeybees in ecological and commercial sectors. We recently reported how the ectoparasitic mite, *Varroa destructor*, altered the viral landscape in the Hawaiian archipelago by decreasing the viral genetic diversity while increasing the prevalence of a particular virus species, deformed wing virus (DWV). Here we explain why DWV is now the most likely candidate responsible for the majority of the colony losses that have occurred across the world during the past 50 years.

Historically, extensive losses of honeybee colonies are not unusual and have occurred repeatedly over many centuries and locations (Oldroyd, PLoS Biol 2007). They all have in common the disappearance of adult worker bees, which can occur over periods of weeks or months. It is generally assumed that all honeybee collapses are considered to have the same underlying cause. Currently there are two main categories of colony collapse; those associated with the presence of the parasitic *Varroa destructor* (Varroa) mite (**Fig. 1**) and those where the mite is not directly involved, such as the recently observed "colony collapse disorder" (CCD) (e.g., Oldroyd, PLoS Biol 2007) and over-wintering colony losses (OCL) (Highfield et al., Appl Environ Microbiol 2009). Due to the economic importance of honeybees, considerable interest in honeybee toxicology and pathology have implicated various

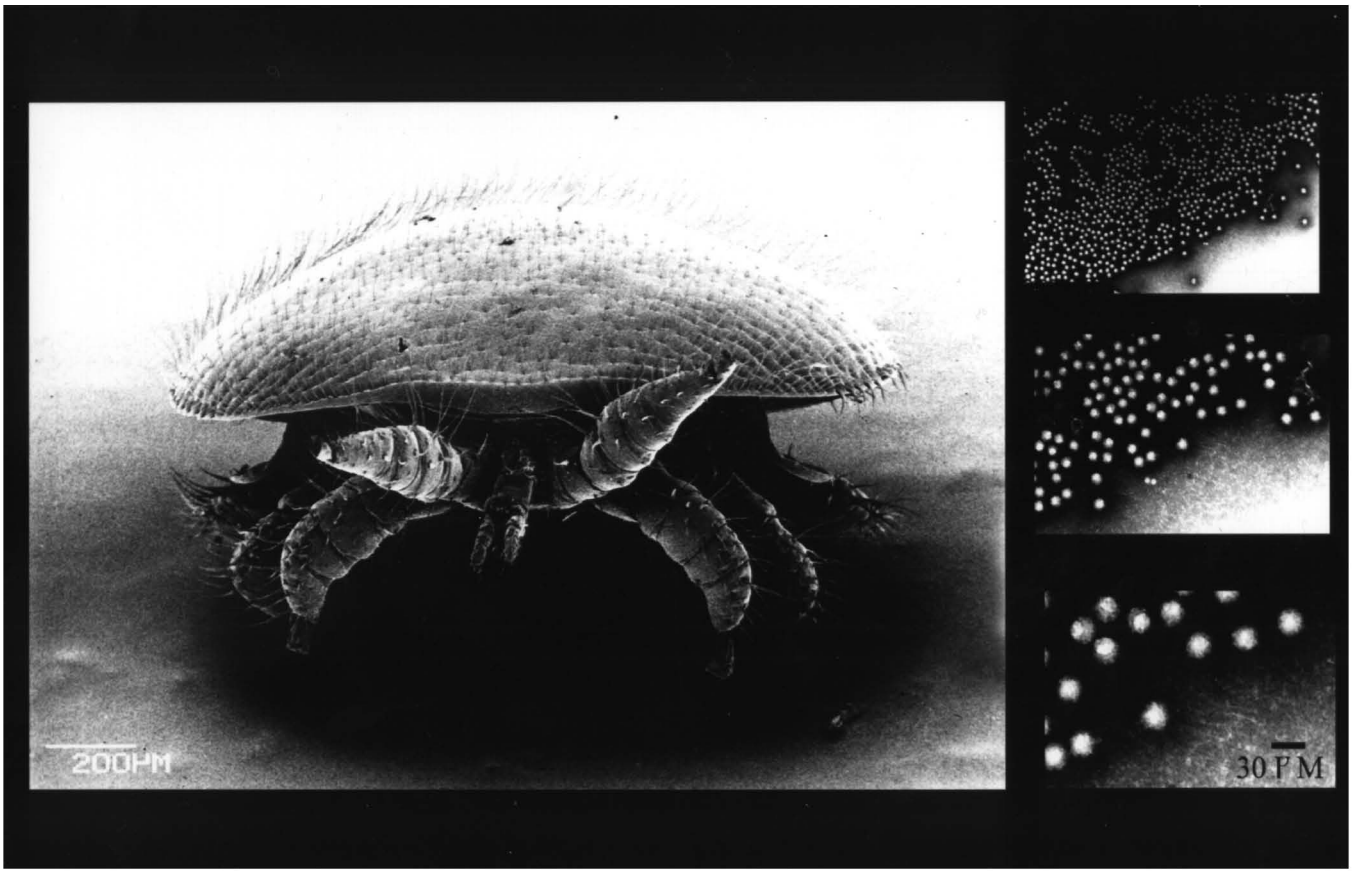

Figure 1. Electron micrographs of the ectoparasitic mite, *Varroa destructor*, and picorna-like virion particles of deformed wing virus (magnified series of three micrographs).

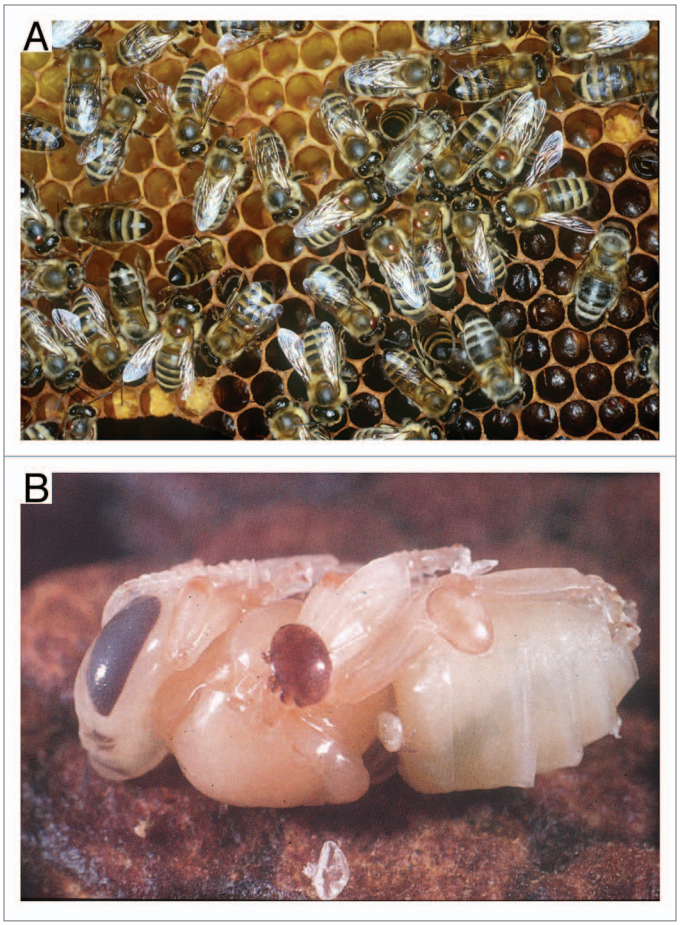

Figure 2. Photographs of *Varroa destructor* (brown reddish mites) feeding on (**A**) adult worker bees and (**B**) a developing pupa that was removed from its brood chamber.

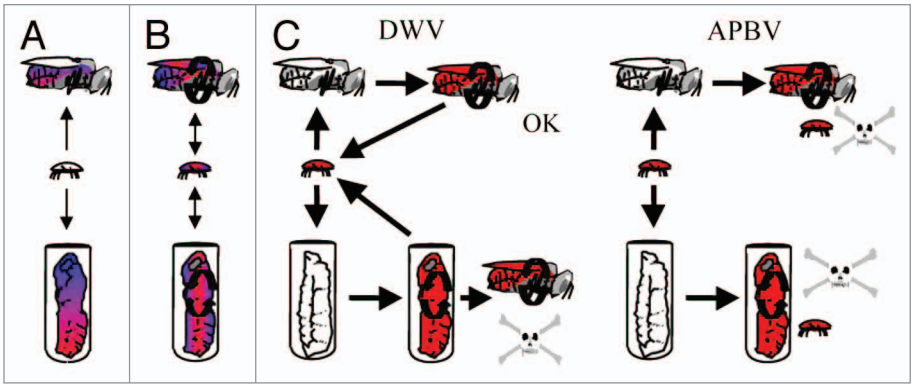

Figure 3. Role of Varroa in the transmission of deformed wing (DWV) and acute paralysis bee (APBV) viruses in the honeybee colony showing three basic phases. (**A**) Naturally diverse cloud of viral variants (red-blue) persisting at low levels in the honeybees. (**B**) Varroa feeds on an overtly infected bee introducing a new viral transmission route i.e., via injection. This over a period of years reduces the strain diversity selecting a genetically restricted cloud of highly virulent (red) strains. (**C**) The virulent DWV (red) strain selected by the Varroa-bee transmission cycle results in a reduced lifespan of bees infected as pupa, but not bees infected as adults, since they have a normal lifespan despite high viral loads. These adult bees become carriers, which aids the further spread of DWV to other colonies. However, in the acutely virulent ABPV, the virus quickly kills both the adult and pupa bee once the pathogen has been transmitted by the mite. This breaks the transmission cycle as the vector (mite) is also killed either by the adult bee dying away from the colony or been entombed within the brood cell.

pests, pathogens, pesticides and even mobile phones in honeybee colony collapses (discussed in Martin et al., Science 2012).

The collapse of millions of honeybee colonies associated with the global spread of Varroa has resulted in this pest being attributed to many of the overt colony collapses during the past 50 years. Numerous studies have revealed a complex association between Varroa, its honeybee host and a range of viral pathogens (e.g., Hung et al., Amer Bee J 1995; Martin J Appl Ecol 2001). However, Varroa has been ruled out as the cause of unexplained losses such as those attributed to CCD (Cox-Foster et al., Science 2007) and OCL (Highfield et al., Appl Environ Microbiol 2009). This is mainly due to the mite numbers remaining low in the collapsing colonies because of the various Varroa control measures applied by the beekeeper.

In honeybees, 18 viruses have been identified and physically characterized to date. Most resemble picorna-like viruses; 30 nm isometric particles containing single stranded positive sense RNA (**Fig. 1**). Five "species" have been routinely linked with honeybee colony collapses, in particular, acute bee paralysis virus (ABPV) and deformed wing virus (DWV) in Europe, and Kashmir bee virus (KBV), Israel acute paralysis virus (IAPV) and DWV in the US. It is now generally accepted that ABPV, IAPV and KBV all belong to the same cloud of ABPV variants (Baker and Schroeder, Virology J 2008; de Miranda et al., J Invert Pathol 2010) assigned to the family Dicistroviridae, order Picornavirales. ABPVs follow a classic acute-type infection strategy since relatively low loads (hundreds of viral particles per honeybee) can rapidly translate into overt symptoms of paralysis and ultimately death for the honeybee. Virulence is highly dependent on the mode of transmission and type of the variant present. Generally speaking, less than 100 APBV particles injected into a honeybee causes death within 4–8 days but 10,000,000,000 viral particles are required to kill a bee when introduced via an oral route i.e., feeding (Bailey and Gibbs, J Ins Pathol 1964). Modeling work (Sumpter and Martin, J Animal Ecol 2004) revealed that in the honeybee-Varroa-virus system, pathogens such as APBV with a high virulence can only kill a colony when very large mite populations (10,000–20,000) are present, since acutely virulent pathogens transmitted by the mites kill the adult bees on which they live (**Fig. 2A**) and the honeybee brood

(**Fig. 2B**) on which they reproduce. Therefore, the death of the developing brood prevents any subsequent mite reproduction, as the mites die, entombed within the brood cells. This along with adults dying outside the colony helps break the viral transmission cycle (**Fig. 3**). It is therefore our contention that despite the acute virulent nature of the ABPV variants, they have never been consistently linked with colony collapse even in the presence of the Varroa mite.

DWV on the other hand, is a more persistent pathogen (Highfield et al., Appl Environ Microbiol 2009) that has become synonymous with the death of mite-infested colonies across the world. This pathogen is currently assigned to the family Iflaviridae within the order Picornavirales. It is generally considered to be much less a virulent pathogen compared with the ABPV variants since adult bees can survive for weeks with very high virus loads (**Fig. 3**). Although in some bees, DWV can cause overt symptoms of wing deformity resulting in emerging bees that are unable to fly. The appearance of overt symptoms is far more common in Varroa infested colonies since the wing deformity is associated with the viral load being injected in the developing pupae by the mite (Gisder et al., J Gen Virol 2009). In the numerous viral surveys conducted across the world, DWV is always the most prevalent viral pathogen in honeybee colonies especially when exposed to Varroa mites. Despite prevalence levels that normally exceed 90% (e.g., Baker and Schroeder, J Invert Pathol 2008), incidence of overt DWV-associated colony collapse is now low because of the numerous husbanded practices that control mite numbers. Nonetheless, when DWV is injected into developing pupa by the feeding activities of Varroa this causes a reduction in adult lifespan of 50–75% (Martin, J Appl Ecol 2001). Therefore, when Varroa mite infestation exceeds 2,000–3,000 during the autumn period, sufficient over-wintering bees become infected with DWV causing the colony to collapse during the long winter period. This is mainly due to the fact that no new brood is produced over this period to replace the dying infected over-wintering bees (Martin, J Appl Ecol 2001). However, in Africanized bees that only inhabit tropical and sub-tropical regions of the Americas they don't suffer a similar fate despite being infested with both Varroa and DWV. This is because new bees are produced continuously throughout the year and this rapid turnover in bees results in mite populations needing to exceed 12,000 before a colony collapses (Sumpter and Martin, J Animal Ecol 2004). In addition, behavioral adaptations of Africanized bees help maintain mite populations to between 1,000 and 6,000 per colony (Martin and Medina, Trends Parasitol 2004). So in temperate regions the control of the Varroa numbers within a colony is widely regarded as being the only preventive tool against DWV-associated colony collapse.

A key discovery from the Hawaiian study was an improved understanding of the role of Varroa in changing the viral landscape. Varroa increased both DWV prevalence and titer among the honeybee population. While this was already a well-established fact (Carreck et al., J Apicultural Res 2010), our new discovery revealed that the Varroa-honeybee transmission cycle vastly reduced a wide range of naturally occurring DWV variants to only a small subset. Moreover, these remaining DWV variants can now be found almost universally throughout the world, which raises important questions around DWV virulence. For example, the Varroa-DWV association causes wing deformities in only a small proportion of honeybees. This is despite otherwise seemingly healthy asymptomatic honeybees containing extremely high DWV loads (levels that would typically be associated with wing deformity). However, in these asymptomatic bees, DWV can affect learning behavior (Iqbal and Mueller, Proc Biol Sci 2007), aggressiveness (Fujiyuki et al., J Virol 2004) and lifespan (Martin, J Appl Ecol 2001). In the absence of Varroa, DWV have been implicated in colony death in at least five cases to date. In addition, the DWV group of variants is now known to infect a wide range of insect hosts, including beetles, ants, other bees, wasps and hoverflies (Evison et al., PLoS ONE 2012). Their true impact within asymptomatic honeybees and other insects is largely unknown. The observations from the Hawaiian study indicate that the DWV quasi-species consists of a very large cloud of variants that are capable of infecting a wide range of hosts, and potentially tissue types that may help explain the wide range of observed pathologies. This further suggests that this pathogen is evolving at different rates resulting in varying levels of virulence that is not dependant on the Varroa-honeybee transmission cycle. Consequently, the introduction of Varroa into honeybee colonies may have been selected for particular virulent forms of DWV capable of causing colony collapse in effectively Varroa free colonies. This is however yet to be proven. If this link could be made then the common denominator for unexplained colony collapses worldwide such as OCL and potentially CCD could be due to the emergence of particularity virulent form(s) of DWV.

As history has shown us, in honeybees a highly virulent virus would only be transitory in nature. This is largely due to the limited exchange of bees between colonies, although current beekeeping practices would prolong such outbreaks as colonies are often maintained in artificially high densities. It is now vital that further studies into the role of DWV in the collapse of colonies, either dependent or independent of the mite-bee transmission cycle, investigate the link between clouds of DWV variants and their virulence, to fully understand the continual evolution of an emerging pathogen whose effect may extend well beyond honeybees.

Molecular blueprint of uropathogenic *Escherichia coli* virulence provides clues toward the development of anti-virulence therapeutics

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Effective treatment of many infections has progressively become more difficult due to the worldwide increase in antimicrobial resistance and the paucity of new antimicrobial development. Urinary tract infections (UTIs) are among the most common infections afflicting primarily women, and often resulting in recurrences or chronic infections that require frequent retreatment or long-term prophylaxis with antimicrobials. Either approach exposes patients to frequent antimicrobial use and its consequences. This is leading to a serious medical impasse requiring innovative therapeutic strategies. Uropathogenic *Escherichia coli* (UPEC) are the predominant UTI causative agent. This article highlights work that we have performed, distinguishing the contribution of two UPEC virulence determinants, the QseBC two-component system and type 1 pili, in pathogenesis. We discuss our findings on the impact of QseC disruption alone and in the presence of mannosides, orally bioavailable small-molecular weight inhibitors of the FimH adhesin that are highly efficacious in the treatment of UTI in a murine model. Our work provides insights toward the development of alternative therapeutics for UTIs.

Urinary tract infections (UTI) are among the most common bacterial infections, accounting for over 15 million cases annually and over 3.5 billion dollars in health care costs in the US alone, due to their prevalence and tendency toward recurrence and/or chronicity (Foxman, Nat Rev Urol 2010). More than 85% of UTI are caused by uropathogenic *Escherichia coli* (UPEC) (Griebling 2007, Chapter 18 in Urol Diseases in America). During infection, UPEC utilize the FimH adhesin at the tip of type 1 pili to bind mannosylated receptors on bladder epithelial cells, mediating colonization and invasion of the host bladder (Thankavel et al., J Clin Invest 1997; Martinez et al., EMBO J 2000; Hung et al., Mol Microbiol 2002; Bouckaert et al., Mol Microbiol 2005). Internalized UPEC avoid TLR-4 mediated exocytosis by escaping into the host cell cytoplasm, where they replicate into biofilm-like intracellular bacterial communities (IBC) (Anderson et al., Science 2003; Justice et al., Proc Natl Acad Sci U S A 2004; Bishop, et al., Nat Med 2007). Upon IBC maturation, bacteria detach from the IBC biomass, flux out of the infected cell and spread to naïve epithelial cells capable of re-initiating the IBC developmental cycle (Justice et al., Proc Natl Acad Sci U S A 2004). Thus, IBC formation allows bacteria to evade extracellular host defenses, while rapidly expanding in numbers (Anderson et al., Science 2003). Exfoliated bladder epithelial cells containing IBCs have been observed in urine obtained from women with recurrent UTIs but not in healthy controls or in cases of UTI caused by gram-positive pathogens (Rosen et al., PLoS Med 2007). Mutations in *fimH* are postulated to drive the evolution of UPEC strains and those mutations that confer a fitness advantage within the urinary tract are selected in this body habitat (Chen et al., Proc Natl Acad Sci U S A 2009). Recent studies have also demonstrated that higher IBC numbers correlate with higher frequency of chronic UTI, indicating that IBC formation is a critical determinant for persistence (Schwartz et al., Infect Immun 2011).

Antibiotics are currently the primary course of treatment for UTI; however, given the impact on microbiota and the rise in antibiotic resistant uropathogens, finding novel treatment strategies is becoming a pressing issue. Type 1 pili mediate colonization and invasion of the bladder and thus, previous studies in our lab focused on the development of innovative compounds, mannosides ZFH-02056 and ZFH-04269, which attenuate UPEC virulence in a murine model, by specifically blocking FimH binding to bladder epithelial cells, thereby preventing bacterial adherence, invasion and IBC formation (Han et al., J Med Chem 2010; Cusumano et al., Sci Transl Med 2011). In particular, we have shown that ZFH-02056 and ZFH-04269 dramatically reduce the bacterial bladder population when dosed orally in mice, showing great potential in treating chronic cystitis and preventing acute UTI (Cusumano et al., Sci Transl Med 2011). Moreover, ZFH-02056 potentiated TMP-SMZ efficacy against PBC-1, a strain clinically resistant to TMP-SMZ (Cusumano et al., Sci Transl Med 2011). Thus, by preventing bacterial invasion of bladder epithelial cells, mannosides compartmentalize UPEC to the bladder lumen, where antibiotic concentrations are well above the minimum inhibitory concentration (MIC), resulting in bacterial death (Cusumano et al., Sci Transl Med 2011). Translated to clinical practice, mannosides could be a cost-effective treatment that lowers the antibiotic resistance rate, which is as high as 30% in some cases (van der Starre et al., Curr Infect Dis Rep 2011). Consequently, among the ongoing efforts in our laboratories is to optimize and/or potentiate mannoside efficacy.

We have previously shown that the QseBC two-component system (TCS) is implicated in UPEC virulence gene regulation (Kostakioti et al., Mol Microbiol 2009; Hadjifrangiskou et al., Mol Microbiol 2011). More specifically, deletion of the QseC sensor kinase leads to aberrant activation of the QseB response regulator, which in turn interferes with the canonical progression of central metabolic processes, thereby impacting virulence gene expression and causing UPEC attenuation (Kostakioti et al., Mol Microbiol 2009; Hadjifrangiskou et al., Mol Microbiol 2011) (**Fig. 1A**). We have shown that QseC is a bi-functional sensor kinase/phosphatase and that its phosphatase activity is imperative for maintaining the equilibrium between QseB phosphorylated and dephosphorylated states (Kostakioti et al., Mol Microbiol 2009). In the absence of QseC, QseB can still become phosphorylated via an alternative phosphor-donor, but cannot become dephosphorylated and deactivated. Given that active QseB can upregulate its own expression (Clarke and Sperandio, Mol Microbiol 2005),

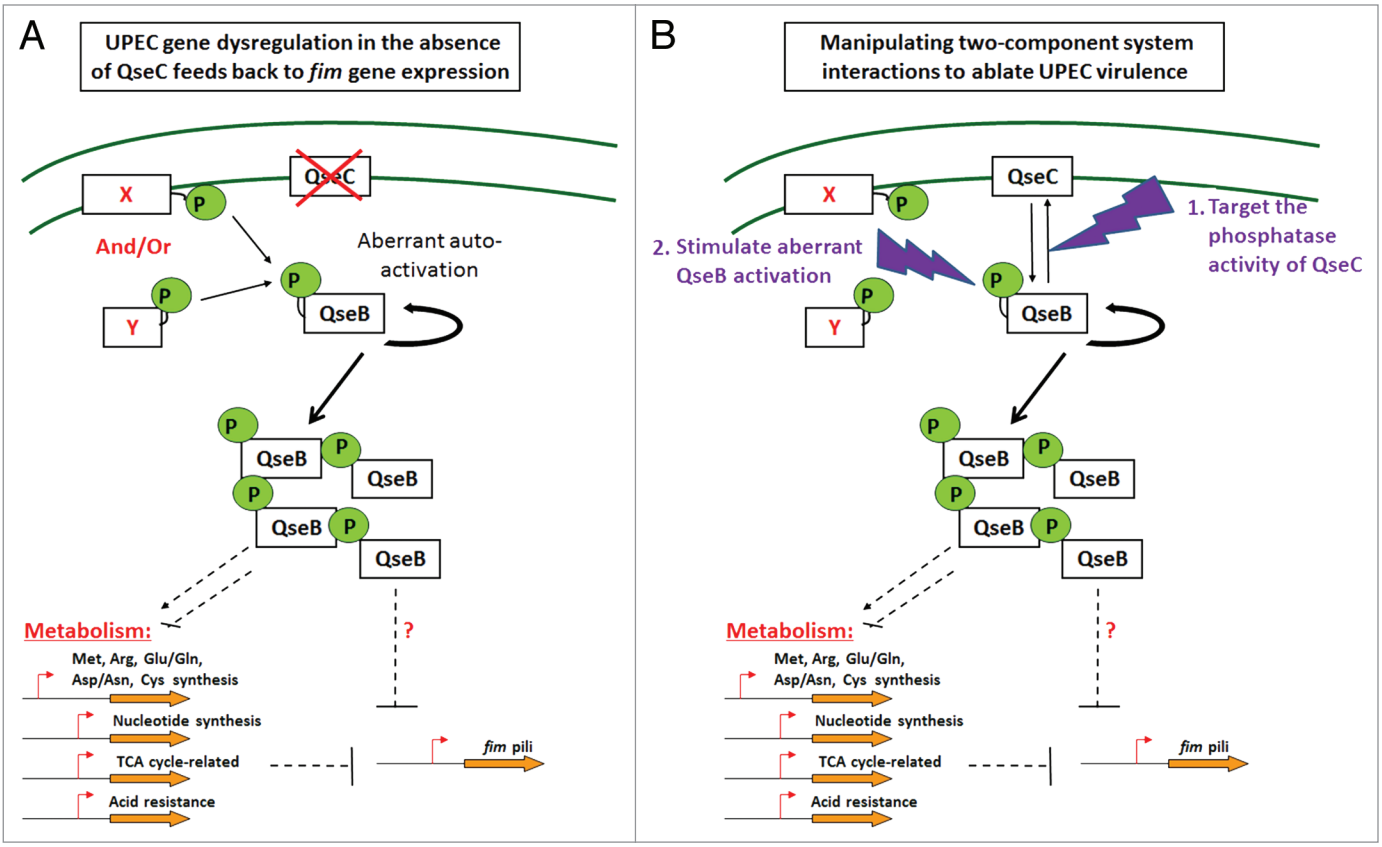

Figure 1. Two-component system interactions as drug targets. Model depicting perturbations in gene expression upon deletion of the *qseC* sensor in UPEC and how these perturbations could be exploited toward ablating UPEC virulence. (**A**) In the absence of QseC, the QseB response regulator becomes readily phosphorylated by another phosphodonor molecule that cannot adequately dephosphorylate and deactivate QseB. As a result, phosphorylated QseB accumulates at higher than normal levels, leading to dysregulation of multiple genes (representative examples are highlighted) including type 1 pili, and causing virulence attenuation. The observed downregulation of type 1 pili stems, at least in part, from downregulation of TCA cycle genes. We have currently no evidence that QseB directly modulates expression of type 1 pili. (**B**) The pathway linking QseBC to *fim* gene expression can be exploited for the development of alternative therapeutics: (1) deletion of the QseC sensor can be recapitulated by identifying inhibitors that interfere with the QseC phosphatase activity, thereby enabling the QseB over-activation; (2) irreversible QseB activation may be chemically stimulated in the presence of QseC.

deletion of QseC triggers a positive feedback loop that operates uncontrolled, leading to accumulation of phosphorylated QseB and dysregulation of target genes (Kostakioti et al., Mol Microbiol 2009) (**Fig. 1A**). Thus, ablation of QseC function tips the balance toward unidirectional QseB phosphorylation and activation, causing virulence attenuation. These attributes make QseC an attractive drug target (**Fig. 1**).

Interestingly, type 1 pili are among the factors downregulated upon *qseC* deletion as a result of the aberrant QseB activity (Kostakioti et al., Mol Microbiol 2009; Hadjifrangiskou et al., Mol Microbiol 2011). More specifically, in the absence of *qseC*, the invertible *fim* promoter element switches to the OFF orientation, resulting in reduced type 1 pili expression. The prominent role of type 1 pili in UPEC pathogenesis prompted us to investigate the extent to which the QseB-mediated effects in the *qseC* deletion mutant are attributed to the observed type 1 pili defects. In the work published in *Infection and Immunity* (Kostakioti et al., Infect Immun 2012), we discern the type 1 pilus-dependent and independent effects that contribute to the virulence attenuation of a UPEC *qseC* deletion mutant, using a murine model of experimental UTI. We performed these studies using isogenic parent and Δ*qseC* strains in which the *fim* promoter was genetically tethered in the ON configuration. We went on to show that these strains were not over-producing type 1 pili, indicating that besides promoter inversion, additional levels of transcriptional control exist that ensure proper expression of type 1 pili. Given that deletion of *qseC* only affects promoter inversion, the Δ*qseC* "locked-on" mutant expressed type 1 pili in a manner that was similar to the isogenic wild-type strain, while it retained the remaining reported Δ*qseC* phenotypic defects. Using these strains we demonstrated that although restored type 1 pilus expression in the Δ*qseC* mutant rescued the ability of this strain to colonize the host and initiate acute infection up to 16 h post-infection, it was unable to facilitate persistence in the urinary tract, exhibiting a diminished capacity to establish chronic infection. Moreover, the Δ*qseC* "locked-ON" mutant was rapidly outcompeted during acute infection when co-inoculated with a wild-type strain, indicating that type 1 pili-independent effects in the absence of QseC contribute to UPEC virulence past the acute checkpoint of infection.

In order to investigate the potential of co-targeting these UPEC virulence determinants, we assessed the effects of a prophylactic oral dose of one of our most potent mannosides,

ZFH-02056, on the ability of the *qseC* mutant to establish chronic infection. Our findings indicated that although either mannoside treatment or loss of QseC alone significantly reduced chronic colonization of the bladder by UPEC, loss of QseC in combination with mannoside treatment resulted in an additive effect thereby potentiating bacterial clearance from the bladder. Collectively, our work indicates that mannosides represent an interesting therapeutic compound for the treatment and prevention of UTIs and that compounds targeting QseC may represent an alternative that could act synergistically with mannosides for combating UTIs.

Many studies have focused on inhibiting virulence by interfering with bacterial signaling cascades, given that two-component systems constitute a central means of intercepting and translating environmental changes (Cegelski et al., Nat Rev Microbiol 2008; Rasko et al., Science 2008; Njoroge and Sperandio, EMBO Mol Med 2009). Inhibition of signal transduction systems poses an attractive means of anti-virulence therapy, by deprogramming optimal gene expression and ablating virulence. Previous studies investigating the potential of inhibiting the QseC kinase activity in enterohemorrhagic *E. coli* identified LED209 as a promising compound (Rasko et al., Science 2008). We have shown that in UPEC, LED209 does not impact virulence-associated gene expression and pathogenesis. These data are not surprising given that the phosphatase function of QseC is critical for maintaining QseB activity under control in both UPEC and EHEC, and our analyses revealed that LED209 has no inhibitory effect on the phosphatase activity of QseC. Thus targeting QseC phosphatase activity would be an optimized strategy to decouple normal gene expression in QseC-bearing pathogens (**Fig. 1B**). Other possible intervention points in the QseBC pathway that could be exploited for the development of novel therapeutics may also be considered, such as stimulation of irreversible QseB activation.

Our findings identify a new area of research targeting the QseC phosphatase function, either on the enzymatic level or in its interaction with QseB. As is the case with FimH inhibitors, identifying specific QseC inhibitors would constitute a new and exciting avenue for the development of therapeutics that could be used to potentiate or replace current strategies. Such studies are currently underway in our laboratories.

Turning up Francisella pathogenesis: The LPS thermostat

Comment on: Li Y, et al. Proc Natl Acad Sci U S A 2012; 109:8716-21; PMID:22586119; http://dx.doi.org/10.1073/pnas.1202908109

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The ability of microbes to adapt in response to an environmental change, such as the infection of a warm-blooded host is an important mechanism in pathogenesis. One major mechanism by which microbes respond to an environmental change is through the modification or remodeling of their membranes. Lipid A, or endotoxin, the bioactive component of lipopolysaccharide (LPS) is the major component of the outer leaflet of the outer membrane of Gram-negative bacteria. Lipid A, the membrane anchor of LPS is a β-(1'-6)-linked glucosamine disaccharide backbone with amide linked fatty acids at the 2 and 2' positions, ester linked fatty acids at the 3 and 3' positions and terminal phosphate moieties at the 1 and 4' positions. Early steps in lipid A synthesis are conserved across Gram-negative species (Raetz pathway) though species-specific structural modifications are also observed. Modification of lipid A in response to environmental stressors such as pH, cation concentration, oxygen saturation and osmolarity have been shown in a number of bacterial species to contribute to condition-specific membrane phenotypes. Temperature has also been shown to play an important role in lipid A structure supported by the presence of specific genes for growth at high temperatures in *Escherichia coli* (*htrB*/*lpxM*) and temperature dependent hypoacylation in *Yersinia pestis*. The ability of bacterial species to alter or modify their lipid A in response to specific environmental cues is important for pathogenesis.

In our recent manuscript, we demonstrated the importance of temperature as an environmental cue in outer membrane remodeling of the intracellular pathogen, *Francisella novicida*. Francisella has been identified in many environmental niches and a wide array of hosts and vectors including humans, lagomorphs, arthropods and amoebae. Throughout its complex and varied life cycle, Francisella adapts to a range of temperatures that alter the composition of its lipid A. Specifically, after growth at lower temperatures normally encountered in arthropods or water environments (~25°C), Francisella synthesizes a lipid A with predominately shorter fatty acids (16 carbons in length). At higher temperatures, like those encountered in warm-blooded hosts (37°C), lipid A is composed primarily of longer chain fatty acids (18 carbons in length). Recent work from our lab determined this shift in lipid A fatty acid composition was regulated at the genetic and enzymatic level utilizing two N-linked acyltransferases, LpxD1 and LpxD2 (**Fig. 1**).

lpxD is conserved in most Gram-negative organisms and is present in an operon containing the essential genes *fabZ*, *lpxB* and *lpxA*. LpxD adds N-linked fatty acids to the glucosamine backbone at the "lipid X" stage present on the inner leaflet of the inner membrane and prior to MsbB transport to the outer leaflet of the inner membrane. Activity of LpxD has been shown to be required for bacterial viability. Interestingly, sequence comparisons of *lpxD1* and *lpxD2* from Francisella show low amino acid sequence identity (34%) suggesting distinct genetic origins, as opposed to gene duplication. *lpxD1* is similar to the *lpxD*s found in enteric bacteria, whereas *lpxD2* is more closely related to *lpxD*s from anaerobic bacteria, such as Desulfovibrio and Fusobacterium. The close relationship to the anaerobic species is striking, as Francisella is a strict aerobe. *lpxD1* and *lpxD2* are highly conserved across all Francisella subspecies (> 98%) indicating a critical role in remodeling the bacterial membrane. Also, the duplication of *lpxD* in the

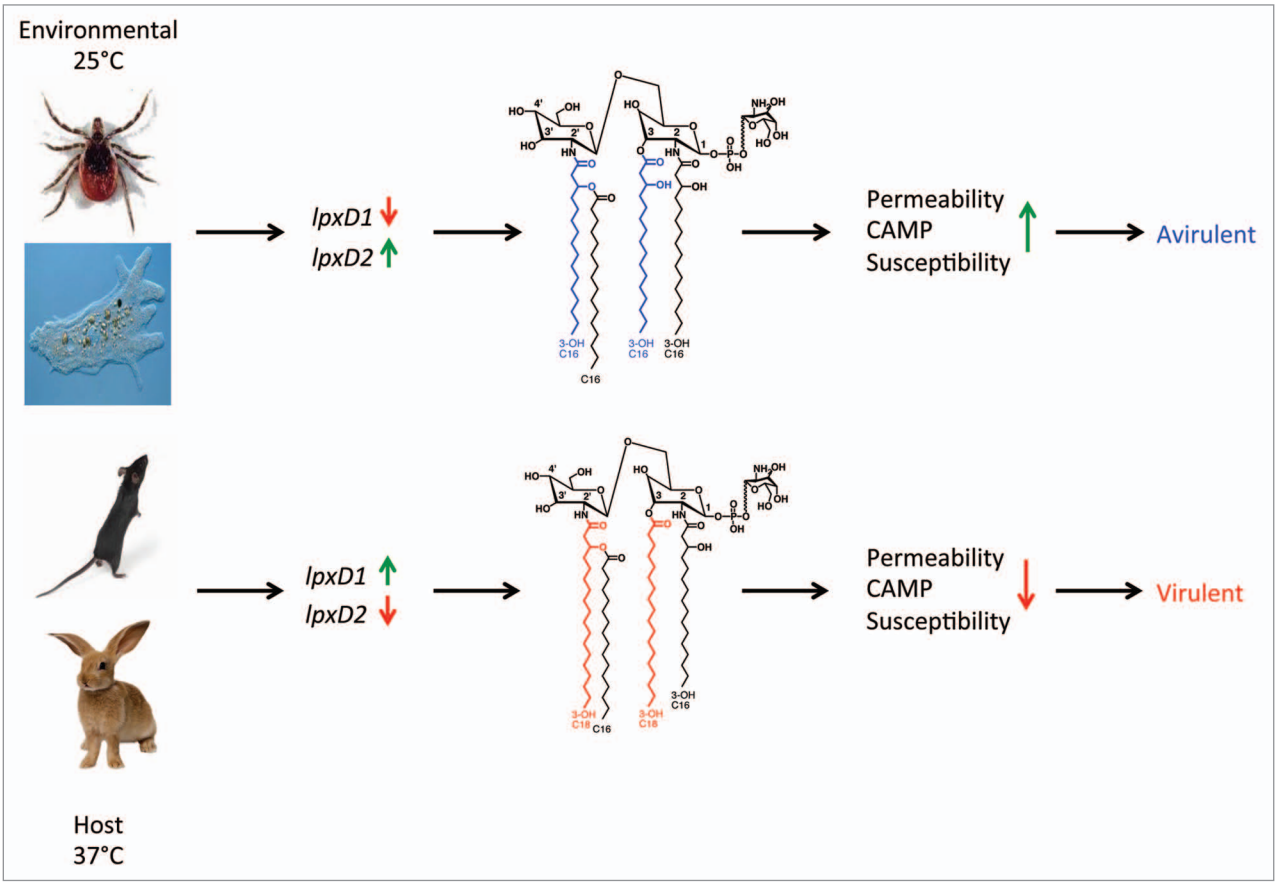

Figure 1. Overview of *lpxD* control of pathogenesis. Francisella has a unique ability to adapt upon entering various environmental niches. Of particular interest is the activity of the acyltransferases LpxD1 and LpxD2. Upon entry of warm temperatures Francisella upregulates *lpxD1* and this causes a larger lipid A structure, leading to decreased permeability as well as decreased sensitivity to killing by CAMPs. Conversely at cooler temperatures *lpxD2* is upregulated, generating a smaller lipid A giving rise to increased permeability and sensitivity to CAMPs. Mutants of *lpxD1* locked in the cooler state become avirulent while mutants of *lpxD2* locked in the warm state remain virulent.

relatively small genome of Francisella (1,782 genes) highlights a potentially critical role for both genes in bacterial virulence.

Regulation of both *lpxD1* and *lpxD2* is tightly controlled in Francisella. Microarray data showed that *lpxD1* was upregulated at 37°C, in contrast to *lpxD2* which was upregulated at 25°C. Not only was the expression of these genes temperature regulated, but their abilities to catalyze acylation was also linked to temperature. The LpxD1 enzyme catalyzed the addition of longer chain acyl groups at 37°C, while the LpxD2 enzyme added shorter chain acyl groups at 25°C. Genetic deletion of these genes allowed further insight into their role in pathogenesis, leading to distinct phenotypes. Deletion of lpxD1 resulted in a smaller lipid A structure similar to that seen after growth of wild-type Francisella at lower temperatures, whereas deletion of *lpxD2* "locked" the lipid A into a larger configuration similar to wild-type samples grown at warm temperatures.

These mutants, having different lipid A phenotypes and lacking the ability to alter their membrane composition, were tested in a variety of assays to determine their role in virulence. Francisella is lethal to mice at extremely low doses (LD_{100} ~10 CFU) regardless of infection route. To assess if the overall lethality of the mutants was affected, mice were infected with increasing amounts of each mutant, as well as WT Francisella. The Δ*lpxD2* mutant maintained full virulence at amounts similar to that of the WT strain. Interestingly, the Δ*lpxD1* mutant, which is locked in the smaller, cold phenotype, showed a total lack of virulence even at the highest doses tested (5×10^6 CFU, 500,000 × LD_{100}). As the Δ*lpxD1* mutant was completely attenuated after a single primary infection dose, we sought to determine if the Δ*lpxD1* mutant could induce protective immunity against a lethal wild-type challenge. Our results showed that protection was dependent on the primary infection dose. When mice were vaccinated using a prime-boost model they were completely protected, regardless of the immunizing dose.

To determine if specific membrane alterations were responsible for attenuation in the *lpxD* mutant strains, a variety of lipid A/membrane functions were examined. As is seen in lipid A isolated from WT strains of Francisella, neither mutant produced a lipid A that resulted in NFκB-mediated proinflammatory responses through TLR4, suggesting that the attenuation was not due to increased recognition by the host innate immune sensors. Another function of lipid A is to resist killing by host peptides by modulating both the charge and permeability of the outer membrane. The Δ*lpxD2* mutant, with the larger lipid A was shown to be more resistant to cationic antimicrobial peptides (CAMPs) presumably due to decreased permeability in the outer membrane when compared with WT Francisella. Decreased permeability was confirmed by measuring the uptake of

ethidium bromide into cells. Conversely, the Δ*lpxD1* mutant showed increased permeability and sensitivity to CAMPs.

Temperature regulation of virulence factors is common in many bacterial species. Generally, temperature regulates the production of classical virulence factors such as toxins in *C. difficile*, or master regulators of virulence, such as *prfA* in *L. monocytogenes*. Here we have shown that a single, temperature regulated, non-classical virulence gene is important for pathogenesis of Francisella. The inability to alter its lipid A and thus remodel its membrane upon infection of warm-blooded hosts is an important mechanism in the overall pathogenesis of Francisella. Though the loss of *lpxD2* showed no effect on murine pathogenesis, it may be required for growth in the environment or arthropod vectors. These questions are currently being pursued in alternative models.

The importance of maintaining proper membrane function and organization upon adaptation to varied environmental conditions is essential. In Francisella, the tight control of lipid A biosynthetic enzymes occurring at multiple levels; transcriptional and enzymatic mark a critical role in pathogenesis. Controlled regulation of the *lpxD1* gene potentially works as a "virulence switch" and alters not only the membrane permeability and resistance to host defenses but plays a critical role in turning on or off pathogenesis. The consequences of this seemingly simple acyl-chain alteration, addition of two carbons to lipid A remain to be explored. Additionally modulation of lipid A structure has a profound impact on the protein constituency in the outer membrane, suggesting a role for specific outer-membrane proteins to be present or absent during pathogenesis. This new cadre of membrane proteins may have far-reaching effects on signaling both from extracellular stimuli and other partners in the membrane. These initial observations in Francisella invite examination of the temperature controls placed on lipid A in other bacterial species that are found at multiple temperatures and how these may affect virulence.

The fate of not sensing phosphate: Downregulation of type 1 fimbriae through activation of the Pho regulon reduces *E. coli* urinary tract virulence

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Phosphate, in terms of cellular content, is the fifth most important element. Its homeostasis is controlled by the two-component system (TCS) PhoBR. The *pstSCAB-phoU* operon, encoding the phosphate specific transport system (Pst), is among the genes regulated by PhoBR. It mediates phosphate acquisition and is required for virulence in pathogenic bacteria. Recently, we demonstrated in uropathogenic *Escherichia coli* (UPEC) that inactivation of the Pst system negatively regulates expression of type 1 fimbriae and alters the ability to colonize the mouse urinary tract.

Phosphate Homeostasis

Phosphate homeostasis is controlled by the two component system (TCS) PhoBR, where PhoR encodes the inner-membrane histidine kinase and PhoB, the cytoplasmic response regulator. In phosphate starvation conditions, i.e., when the extracellular concentration falls below 4 μM, PhoBR activates genes belonging to the Pho regulon, which encodes genes involved in phosphate acquisition and metabolism, such as the Pst system, an ABC transporter (Hsieh and Wanner, Curr Opin Microbiol 2010). In addition to its role in phosphate transport, the Pst system also represses PhoBR, as its inactivation results in constitutive expression of PhoBR regulated genes, regardless of environmental phosphate availability. Thereby, inactivation of the Pst system mimics phosphate starvation conditions. Moreover, the Pst system is also required for virulence (Crepin et al., Vet Micro 2011; Lamarche et al., FEMS Micro Rev 2007). However, the mechanisms connecting the Pst system, the Pho regulon (PhoBR) and virulence are not well defined.

Mechanisms Connecting PhoBR TCS and Virulence in UPEC

Urinary tract infections (UTIs) affect, annually, millions of persons worldwide. Each year, in the US, the associated cost (medical visits and treatments) for UTIs is estimated at 2.5 billion dollars (Hannan et al., FEMS Micro Rev 2012). Uropathogenic *Escherichia coli* (UPEC) strains are responsible for up to 85% of community acquired UTIs (Griebling, Urologic Diseases in America 2007). Despite the availability of effective antibiotic therapies, episodes of resistance, recurrence and persistence are common.

Recently, we showed that the *pst* mutant of the UPEC strain CFT073 is less able to colonize the mouse urinary tract, which is directly linked to the decreased expression of type 1 fimbriae (pili), a key virulence factor in UPEC strains. These fimbriae are found at the bacterial cell surface and they mediate, notably, adhesion to host cells. In UPEC strains, they are expressed in the bladder and mediated adhesion to urothelial cells, allowing the colonization of the bladder. In addition to mediating adhesion to bladder cells, type 1 fimbriae also promote its invasion and the formation of intracellular bacterial communities (IBC) (Hannan et al., FEMS Micro Rev 2012). Not surprisingly, the *pst* mutant is impaired in its invasion capacity. As formation of IBCs allows the persistence of UPEC strains in the host, the virulence defect of the *pst* mutant could be attributed to the cumulative effects of the defect in colonization and invasion of bladder cells, leading to the inability to persist in the host.

In our study, we observed that the decreased expression of type 1 fimbriae, in the *pst* mutant, was concomitant with the altered expression of the regulators *fimB*, *ipuA* and *ipbA*. However, as *ipuA* and *ipbA* were described in a strain devoid of *fimB* and *fimE* (Bryan et al., Infect Immun 2006), the major

regulators of type 1 fimbriae, we hypothesized that the decreased expression of *fim*, in the *pst* mutant, is mainly attributed to *fimB*, while *ipuA* and *ipbA* may have an accessory role.

We also identified that one of the mechanisms by which the Pho regulon alters expression of type 1 fimbriae seems to be through the alarmone ppGpp, a second messenger produced by nutritional starvation involved in global physiological adaptation. Indeed, we observed that the *pst* mutant produces 3-fold less ppGpp than the wild-type strain, which is in agreement with what was previously observed in an *E. coli* K-12 strain (Spira et al., J Bacteriol 1995; Spira and Yagil, Mol Gen Genet 1998). As production of ppGpp activates expression of *fimB*, which mediates expression of type 1 fimbriae (Aberg et al., Mol Microbiol 2006), we hypothesized that the decrease expression of *fimB* and then, the type 1 fimbriae, is connected to the altered production of ppGpp in the *pst* mutant.

A basal level of cellular ppGpp is essential for proper Pho regulon expression. Furthermore, physiological induction of the Pho regulon, when the extracellular concentration of P_i falls below 4 μM, also induces synthesis of ppGpp (Spira et al., J Bacteriol 1995; Spira and Yagil, Mol Gen Genet 1998; Bougdour and Gottesman, Proc Natl Acad Sci U S A 2007). Its synthesis relies on the GDP pyrophosphokinase/GTP pyrophosphokinase RelA, while it is degraded by the guanosine 3'-diphosphate 5'-triphosphate 3'-diphosphatase SpoT. Furthermore, SpoT can also induce synthesis of ppGpp through its synthase activity. Indeed, phosphate starvation conditions activate ppGpp synthesis through SpoT (Spira and Yagil, Mol Gen Genet 1998; Bougdour and Gottesman, Proc Natl Acad Sci U S A 2007). As our results show that constitutive activation of the Pho regulon, through inactivation of the *pst* system, represses production of ppGpp, it suggests that constitutive activation of PhoBR has a deleterious effect on bacterial adaptation to starvation. Interestingly, *spoT* is not differentially expressed in the *pst* mutant, suggesting that the Pho regulon, PhoB, may regulate, directly or indirectly, SpoT at a post-transcriptional level. Thereby, inactivation of the Pst system could inhibit the synthase or activate the hydrolase activity of SpoT. Further experiments are required to elucidate regulatory mechanisms connecting the Pho regulon and production of ppGpp, and its role in type 1 fimbriae expression.

Role of the PhoBR Two-Component System (TCS)

In tested conditions, i.e., LB broth, human urine and in vivo (infected bladder), type 1 fimbriae were downregulated in the *pst* mutant. As for constitutive activation of PhoBR, through inactivation of *pst*, physiological activation of PhoBR, through phosphate starvation, had the same effect on *fim* expression. Interestingly, bladder and urine are phosphate-replete conditions, i.e., *phoBR* and the Pho regulon are not induced in these environments. Thereby, strains found in the bladder produce type 1 fimbriae and cause UTI. In this way, during UTI, it would be possible to modulate the expression level of *phoBR* in order to decrease the production of type 1 fimbriae and potentially decrease colonization of the urinary tract. Indeed, as TCS are absent from humans and other animals, they are choice targets for therapeutic strategies. Due to increased use of antibiotics, pathogenic strains are increasingly resistant. However, small chemical molecule libraries have been developed and have shown an inhibitory effect on these regulatory systems (Stephenson and Hoch, Curr Med Chem 2004). Furthermore, some small molecules have been shown to inhibit the expression or the activity of virulence factors (Cegelski et al., Nat Rev Microbiol 2008). In this manner, small molecules activating the Pho regulon, through inactivation of the Pst system or by direct activation of PhoBR, could be considered as therapeutic agents.

Role of Other TCS in Environmental Regulation of Adherence Factors

TCS are widely present in bacteria and regulate gene expression or protein function by responding to various environmental signals. They are thus important for bacterial adaptation and virulence. In our study, we determined that the constitutive activation of the TCRS PhoBR is detrimental to the bacteria. Indeed, constitutive activation of PhoBR inhibits its expression of the type 1 fimbriae and consequently, decreased the colonization of the mouse urinary tract.

It has previously been demonstrated that several TCS are able to regulate the expression of adherence and motility factors such as fimbriae, curli and flagella. Indeed, the QseBC TCS, which responds to quorum sensing, is involved in type 1 fimbriae, curli and flagella expression. Recently, it has been shown in the UPEC strain UTI89 that deletion of *qseC*, encoding the histidine kinase, resulted in a reduction of type 1 fimbriae and flagella expression/production as well as curli expression (Kostakioti et al., Mol Microbiol 2009; Kostakioti et al., Infect Immun 2012). The authors demonstrated that in the absence of *qseC*, the response regulator QseB remained constitutively active and led to the downregulation of type 1 fimbriae, curli and flagella. As with the deregulation of *phoBR*, constitutive activation of *qse* leads to a preferentially OFF orientation of the *fimS* promoter, the phase variable promoter of type 1 fimbriae, leading to a decreased expression of type 1 fimbriae genes (Kostakioti et al., Mol Microbiol 2009). Moreover, in enterohemorrhagic *E. coli*, decreased flagella production is the consequence of the direct binding of QseB to the promoter of *flhDC*, the master regulators of flagella (Clarke and Sperandio, Mol Microbiol 2005).

If constitutive activation of both PhoB and QseB downregulates expression of type 1 fimbriae, deletion or inactivation of other TCRSs may also inhibit expression of fimbriae or flagella. This is especially the case for BarA-UvrY, CpxAR and RcsCDB TCRSs, where CpxAR and RcsCDB respond to stresses affecting the bacterial envelope whereas the stimulus to BarA-UvrY remains to be determined. In a UPEC strain, deletion of the response regulator *uvrY* resulted in decreased invasion of uroepithelial cells, while in the avian pathogenic *E. coli* (APEC) strain χ7122, deletion of *barA* and *uvrY* decreased adhesion and invasion of chicken embryo fibroblasts. These defects are related to the decreased production of type 1 and P fimbriae (Herren et al., Infect Immun 2006; Palaniyandi et al., PLoS One 2012). Similarly to *barA-uvrY*, deletion of *cpxR* led to an assembly defect of P fimbriae in a UPEC strain. Indeed, it has been shown that CpxR binds to the *pap* promoter and favors the ON orientation of this promoter (Hung et al., EMBO J 2001). As well, deletion of *rcsB* decreased expression of *fimB*, increased expression of *fimE* and lead to the repression of type 1 fimbriae.

Several TCSs in *E. coli* are thus implicated in the regulation of adhesion and motility. Contrary to the response regulators UvrY, CpxR and RcsB, which activate expression of fimbriae genes, PhoB and QseB repress it. However, with the exception of CpxR, it remains unclear if this regulation occurs by a direct binding of these

regulators at promoter sites, or indirectly by positively regulating other genes implicated in direct regulation of fimbrial gene expression.

Concluding Remarks

In conclusion, downregulation of type 1 fimbriae was identified as a key factor connecting the Pst system, the TCS PhoBR and virulence. Indeed, low production of type 1 fimbriae is directly linked to the attenuated virulence of the *pst* mutant. These fimbriae are repressed both in vitro and in vivo in the *pst* mutant. We also began to dissect the regulatory pathway linking the Pst system, the PhoBR TCRS and expression of type 1 fimbriae. Indeed, the decreased expression of type 1 fimbriae is linked to the differential expression of their regulators, which seems to rely, at least in part, on production of ppGpp. The specific molecular mechanisms connecting the Pho regulon (the Pst system) and the type 1 fimbriae remain to be determined. Elucidation of mechanisms controlling type 1 fimbriae through activation of the Pho regulon and regulation by other TCS may open new avenues for therapeutics or prophylactics against urinary tract infections.

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