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Increased Pho Regulon Activation Correlates with Decreased Virulence of an Avian Pathogenic *Escherichia coli* O78 Strain▽

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Avian pathogenic *Escherichia coli* (APEC) strains are associated with respiratory infections, septicemia, cellulitis, peritonitis, and other conditions, since colibacillosis manifests in many ways. The Pho regulon is jointly controlled by the two-component regulatory system PhoBR and by the phosphate-specific transport (Pst) system. To determine the specific roles of the PhoBR regulon and the Pst system in the pathogenesis of the APEC O78 strain χ7122, different *phoBR* and *pst* mutant strains were tested *in vivo* in chickens and *in vitro* for virulence traits. Mutations resulting in constitutive activation of the Pho regulon rendered strains more sensitive than the wild type to hydrogen peroxide and to the bactericidal effects of rabbit serum. In addition, production of type 1 fimbriae was also impaired in these strains. Using a chicken competitive infection model, all PhoB constitutive mutants were outcompeted by the wild-type parent, including strains containing a functional Pst system. Cumulative inactivation of the Pst system and the PhoB regulator resulted in a restoration of virulence. In addition, loss of the PhoB regulator alone did not affect virulence in the chicken infection model. Interestingly, the level of attenuation of the mutant strains correlated directly with the level of activation of the Pho regulon. Overall, results indicate that activation of the Pho regulon rather than phosphate transport by the Pst system plays a major role in the attenuation of the APEC O78 strain χ7122.

Avian pathogenic *Escherichia coli* (APEC) O78:K80:H9 strain χ7122 is involved in the development of colisepticemia, cellulitis, and respiratory disease, and it belongs to one of the most prevalent serogroups that cause avian colibacillosis (13). In addition, APEC is a subset of extraintestinal pathogenic *E. coli* (ExPEC) and shares virulence traits with strains isolated from human cases of neonatal meningitis, urinary tract infections, and septicemia. Thus, APEC strains represent a high risk of zoonotic infection (23), and their virulence gene pool may contribute to emergence of other ExPEC strains (16).

Bacterial pathogens use specific mechanisms to mediate adaptive responses to the different environments and stresses encountered within the host (34). As a result, this dynamic process leads to adaptation and survival in different niches during infection. Bacteria use two-component regulatory systems (TCRS) to translate environmental stimuli into expression of a highly precise response (36). The PhoBR TCRS present in many bacterial species responds to external phosphate concentrations and controls gene transcription of the Pho regulon (51). The activation signal, phosphate-limiting conditions, promotes the autophosphorylation of the sensor kinase PhoR (33). Then, the phosphoryl group is transferred to its cognate response regulator, PhoB (32). In its activated state, PhoB binds to consensus sequences in the upstream regions of Pho-dependent genes, the Pho boxes, where it regulates transcription through interaction with RNA polymerase associated with σ^{70} (2, 31).

The *pst* operon belongs to the Pho regulon. The Pst system consists of a high-affinity ABC transporter of inorganic phosphate (P_i), which includes a periplasmic P_i binding protein (PstS), two membrane permeases (PstA and PstC), an ATPase (PstB), and PhoU (41, 47). Moreover, the molecular mechanisms that lead to turning off the Pho regulon involve the Pst system. Indeed, most mutations in the Pst system result in constitutive expression of the Pho regulon, regardless of environmental phosphate concentrations (51). Furthermore, it is well recognized that inactivation of the Pst system, constitutive expression of the Pho regulon, and bacterial virulence are linked in diverse bacterial species (28). In APEC strain χ7122, deletion of the *pstCAB* genes was shown to reduce virulence in a chicken infection model and to affect multiple virulence attributes, including production of type 1 fimbriae and resistance to cationic peptides, to the bactericidal effects of rabbit serum, to acid shock, and to oxidative stress (10, 26). Transcriptional analyses demonstrated the induction of a general stress response in the *pst* mutant, including increased expression of genes involved in adaptation to acid stress, oxidative stress, and the general stress response (notably RpoS-regulated genes). In addition, genes associated with cell surface composition were modulated (10). Since the Pst system contributes both to regulation of the Pho regulon and to high-affinity uptake of P_i, it remains to be determined if the effects of Pst inactivation on APEC virulence are mediated through the PhoBR TCRS or deficiency in phosphate uptake (28).

Hence, the purpose of this investigation was to test the

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Strain or description		
DH5α	F^- λ^- ϕ80 Δ(*lacZYA-argF*) *endA1 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*	Invitrogen
JWK0389_1	K12 Δ*phoB*::*kan*	1
JWK3705_1	K12 Δ*pstC*::*kan*	1
χ7122	APEC O78:K80:H9 *gyrA* Nal^r	40
Δ*phoB*	χ7122 Δ*phoB*::*kan*	This work
Δ*pstC*	χ7122 Δ*pstC*::*FRT*	This work
Δ*pstC*::*kan*	χ7122 Δ*pstC*::*kan*	This work
Δ*pstC* Δ*phoB*	χ7122 Δ*phoB*::*kan*/Δ*pstC*::*FRT*	This work
phoR(T220N)	χ7122 *phoR*(*T220N*)	This work
Δ*pstCAB*	χ7122 Δ*pstCAB*::*kan*	26
Δ*pstCAB* + pAN92	χ7122 Δ*pstCAB*::*kan* + pAN92	26
Δ*pstC* + pAN92	χ7122 Δ*pstC*::*FRT* + pAN92	This work
MGN-617	SM10 λ*pir* derivative, *thi thr leu tonA lacY supE* λ*pir recA*::RP4-2-Tc::Mu (Kan^r) Δ*asdA1*	24
χ7279	χ7122 Δ*fim*	10
862	ExPEC O115:K, serum sensitive	11
QT51	χ7122 Δ*lacZ*	30
Plasmids		
pMEG-375	*sacRB mobRP4 oriR6K* Cm^r Ap^r	S. Tinge, Megan Health
pCP20	FLP^+ λ *c*I857$^+$ λ p_RRep(Ts) Ap^r Cm^r	9
pKM200	*Ptac-gam-bet-exo* operon from pTP806	Kenan Murphy
pGEMÒ-T	pGEMR-5Zf(+) vector with EcoRV at base 51, with a T added to both 3′ ends	Promega
pAN92	pACYC184::*pst* operon, Cm^r	21

hypothesis that the PhoBR TCRS, rather than the Pst system, is critical to the virulence of APEC. To that end, a series of mutants in the *pst* and *phoBR* operons of the APEC O78:K80:H9 strain χ7122 were generated and were tested for expression of the Pho regulon and for various attributes of virulence. We report here that constitutive activation of the Pho regulon, through the PhoBR TCRS, is responsible for attenuation of APEC χ7122 virulence. Moreover, the activation level of the Pho regulon correlates with decreased virulence in this pathogenic *E. coli* strain. In addition, we have demonstrated that the PhoB regulator, which is critical for activation of the Pho regulon, is not required for virulence of APEC in the chicken infection model.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and media. The *E. coli* strains and plasmids used for this study are listed in Table 1. Primers used for PCR amplifications are listed in Table 2. Bacteria were grown in Luria-Bertani (LB) broth at 37°C. LB broth is a high-phosphate medium. Low-P_i (LP) broth was used for alkaline phosphatase assays and to determine sensitivity to hydrogen peroxide. LP broth is composed of 50 mM Tris-HCl (pH 7.4), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.4 mM $MgSO_4$, 0.1% yeast extract, 20 mM glucose, and 1 mM methionine. For experimental infections of chickens, beef heart infusion broth and MacConkey-lactose agar plates were used. Antibiotics or supplements were used at the following final concentrations, when required: chloramphenicol (Cm), 12.5 μg/ml; kanamycin (Kan), 50 μg/ml; nalidixic acid (Nal), 40 μg/ml; and 5-bromo-4-chloro-3-indolylphosphate (XP or BCIP), 40 μg/ml.

TABLE 2. Primers used for PCR amplifications

Primer	Sequence
phoR-Fext	5′-GTGCGCGGTACAGGATATCGTTTTTCAACC-3′
phoR-Rint	5′-AGGTAACCCTGTAACACGTTCAATGGCGTAC-3′
phoR-Rext	5′-CAGTCAGTATGACAGCACCTGAAGCGCAAT-3′
phoR-Fint	5′-CGTACGCCATTGAACGTGTTACAGGGTTACC-3′
phoR-FSacI	5′-GAGCTCGGAATTTATTGCGCCTTTCATGG-3′
phoR-RSacI	5′-CTCGAGTCAGGCGACTTTCGTGATGATTC-3′
phoB-Fext	5′-ACCTGAAGATATGTGCGACGAGCTT-3′
phoB-Rext	5′-CGTTCCAGCACGTAAGATACTCCAG-3′
pstC-Fext	5′-CAGAACAAGGCACAGAAGTGCTGA-3′
pstC-Rext	5′-GGGTAGCGGGCTGTTAATTTTGTG-3′

Generation of the *phoR*(*T220N*) mutant by allelic exchange. Site-specific mutagenesis of *phoR* was used to generate a mutant in which the Pho regulon is constitutively active (51). *E. coli* strain DH5α was used for cloning experiments with pGEM-T. A first PCR fragment was amplified from the wild-type strain, χ7122, using the primers phoR-Fext and phoR-Rint (which contain the desired point mutation). A second PCR fragment was amplified from strain χ7122 using the primers phoR-Rext and phoR-Fint (which also contains the desired point mutation). The 2 PCR fragments were used as a template to amplify a full *phoR*(*T220N*) PCR fragment using the phoR-FSacI and phoR-RSacI primers, which both contain SacI restriction sites. This final PCR product was ligated into the SacI site of pGEM-T. This construct was digested with SacI, and the *phoR*(*T220N*) fragment was ligated into the SacI site of the suicide vector pMEG-375. The resulting construct was transferred to strain MGN-617 and was then mobilized in χ7122 by conjugation. Single-crossover integrants of strain χ7122 were selected on LB agar containing appropriate antibiotics (XP, Nal, and Cm). Selection for double-crossover allele replacement was obtained by *sacB* counterselection on LB agar plates without NaCl but containing 5% sucrose (25), XP, and Nal. The mutant strain was confirmed to contain the desired point mutation *phoR*(*T220N*) and no other nucleotide changes, as determined by sequencing.

Construction of mutant derivatives of APEC strain χ7122. *phoB* and *pst* knockout mutants were obtained by homologous recombination using the λ *red* recombinase method (12). Briefly, the Δ*phoB*::*kan* allele from *E. coli* K-12 strain JWK0389_1 was used to introduce a Δ*phoB* mutation into χ7122 using the phoB-Fext and phoB-Rext primers, generating strain χ7122 Δ*phoB*::*kan*. The Δ*pstC*::*kan* allele from *E. coli* K-12 strain JWK3705_1 was used to introduce the Δ*pstC* mutation into χ7122 with the pstC-Fext and pstC-Rext primers, generating the χ7122 Δ*pstC*::*kan* strain. Then, the χ7122 Δ*pstC*::FRT strain was generated by FLP-mediated excision of the kanamycin cassette from χ7122 Δ*pstC*::*kan* by using the plasmid pCP20 (12). To create the Δ*pstC* Δ*phoB* double mutant, the Δ*phoB*::*kan* allele from *E. coli* K-12 strain JWK0389_1 was used to introduce the Δ*phoB* mutation into the χ7122 Δ*pstC*::FRT background, generating χ7122 Δ*pstC* Δ*phoB*. Mutations were confirmed by PCR and sequencing, using primers flanking the specific gene region. Restoration of the Pst system in the χ7122 Δ*pstC*::FRT mutant was achieved by complementation with plasmid pAN92, which contains a functional *pst* operon.

Alkaline phosphatase assay. Alkaline phosphatase was measured as described previously (4, 26). Briefly, 4 μg/ml of *p*-nitrophenyl phosphate was added to 500 μl of mid-log-phase (optical density at 600 nm [OD_{600}] of 0.6) culture cells

permeabilized by 50 μl of 1% sodium dodecyl sulfate (SDS) and 50 μl of chloroform. Color development was monitored at 420 nm, and alkaline phosphatase activity was expressed in enzyme units per minute, calculated as follows: $1{,}000 \times [OD_{420} - (1.75 \times OD_{550})]/T$ (min) $\times V$ (ml) $\times OD_{600}$, where T stands for the length of reaction time and V stands for the culture cell volume.

Sensitivity of *E. coli* strains to hydrogen peroxide. Sensitivity to hydrogen peroxide-induced oxidative stress was determined by an agar overlay diffusion method on LB or LP plates (1.5% agar) as described previously (3, 43), with some modifications (10). Overnight cultures grown in LB or LP broth were adjusted to an OD_{600} of 0.5. One hundred microliters of each culture was suspended in 4 ml of LB or LP molten top agar (0.5% agar) and poured over the LB or LP agar plates. Sterile blank disks were added to the surfaces of the solidified overlays, and 10 μl of hydrogen peroxide (30%) was spotted onto the disks. The plates were then incubated overnight at 37°C, and following growth, the diameters of inhibition zones were measured.

Serum bactericidal assay. The serum bactericidal assay was adapted from the method of Taylor and Kroll (48) as described previously (26). Briefly, bacteria were grown overnight in LB broth at 37°C. Bacterial cultures were then resuspended in fresh medium at a 10-fold dilution, incubated at 37°C, and harvested during the mid-log phase (OD_{600} of 0.6). Bacteria were washed at room temperature with gelatin-Veronal-buffered saline (pH 7.35) and then resuspended to a concentration of 10^7 CFU/ml. A volume of 0.1 ml of the bacterial suspension was added to 0.9 ml of normal rabbit serum and then incubated at 37°C. Viable cell counts were determined at 0, 1, 2, and 3 h by spreading dilutions of the suspension on LB agar plates. The survival rate was calculated as the CFU determined at each time point divided by the initial CFU present at time zero.

Yeast cell aggregation assay. To test the production of type 1 fimbriae, mannose-sensitive yeast agglutination assays were performed. The yeast aggregation assay was derived from a microhemagglutination assay in 96-well round-bottom plates (40) as described previously (10). Briefly, cultures were grown to mid-log phase (OD_{600} of 0.6) in LB broth at 37°C without shaking 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 3×10^{10} CFU/ml. Samples were then serially diluted 2-fold in microtiter wells, and equal volumes of a 3% commercial yeast suspension were added to each of the wells. After 1 h 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 fimbria mutant strain χ7279 (10) was used as a negative control.

Experimental infection of chickens via the air sacs. A competitive coinfection model was used as described previously (43), with some modifications. Strains were prepared from a diluted 24-h beef heart infusion broth culture, and equal quantities (5×10^6 CFU) of each mutant strain and a virulent Δ*lacZYA* derivative of strain χ7122, strain QT51, were used as the 100-μl inoculum. Use of QT51 in coinfections permitted a direct evaluation of the number of colonies of QT51 (Lac^- colonies) compared to the isogenic mutants (Lac^+ colonies) on each plate. All birds were euthanized at 48 h postinfection and were then necropsied. Organs were removed aseptically. Blood samples and the right lung, liver, and spleen of each animal were weighed, suspended in PBS, and homogenized. Dilutions of homogenates were plated onto MacConkey-lactose agar plates with appropriate antibiotics for bacterial quantification. Several randomly selected colonies per organ were verified by PCR. The competitive index (CI) was calculated as the number of CFU/g of the mutant strain divided by the number of CFU/g of strain QT51 in each tissue or blood sample divided by the same ratio in the initial input inoculum.

Coculture. Coculture was performed as described previously (6, 20), with some modifications. Briefly, equal quantities (5×10^6 CFU) of the QT51 Δ*lacZYA* derivative of strain χ7122 and either mutant strain were used from overnight LB cultures to initiate the coculture in 5 ml of fresh LB medium without antibiotics. Cocultures were incubated at 37°C with shaking. Viable counts (in CFU) were determined for the input time points, 24 and 48 h, by plating dilutions onto MacConkey-lactose agar plates without antibiotics. The coculture ratio was calculated as the CFU of the QT51 mutant strain (Lac^-) divided by the CFU of the competitor mutant strain (Lac^+) divided by the same ratio in the initial input inoculum.

Statistical analyses. Statistical analyses were performed using the Prism 4.03 software package (GraphPad Software). A paired one-tailed Student t test was used to determine significant differences for alkaline phosphatase activity, sensitivity to hydrogen peroxide, and yeast cell aggregation assays. Analysis of variance followed by Tukey's multiple-comparison test was used for the serum assays. For the coinfection experiments, geometric means of the CIs were determined, and a Student t test (two-tailed) was used to determine whether the logarithmically transformed ratios differed significantly from 0 (14). P values below 0.05 were considered to be statistically significant.

TABLE 3. Alkaline phosphatase activities of χ7122, isogenic mutants, and complemented strains in high (LB)- or low (LP)-phosphate medium

Strain name or description	Alkaline phosphatase activity (arbitrary units ± SD) in[a]:	
	LB	LP
χ7122	2.4 ± 1.2	82.6 ± 6.4
Δ*pstCAB*	**134.7 ± 26.8***	**180.2 ± 9.6***
Δ*pstC*	**33.8 ± 5.3**	**119.6 ± 11.1**
phoR(*T220N*)	**19.3 ± 3.5**	70.7 ± 5.8
Δ*phoB*	1.4 ± 2.2	1.9 ± 2.1
Δ*pstC* Δ*phoB*	3.1 ± 2.1	2.8 ± 1.3
Δ*pstCAB* + pAN92	2.7 ± 1.5	75.7 ± 12.1
Δ*pstC* + pAN92	4.2 ± 3.5	76.3 ± 11.8

[a] Data presented are the means ± the standard deviations of three independent experiments. Values indicated in bold text are significantly different ($P < 0.05$) from the mean for the wild-type strain as calculated by Student's t test. Values marked with asterisks are significantly different ($P < 0.05$) from the mean for every other strain as calculated by Student's t test.

RESULTS

To distinguish between the specific contributions of the PhoBR TCRS and the Pst system for APEC virulence, we used a series of *pst* and *phoBR* mutant derivatives of APEC O78 strain χ7122 (Table 1). Both Δ*pstCAB* and Δ*pstC* mutants were complemented with the plasmid pAN92, containing a functional *pst* operon, restoring wild-type alkaline phosphatase (AP) activities. However, we were unable to restore a wild-type phenotype in the *phoR*(*T220N*) mutant, which demonstrates constitutive expression of the Pho regulon, by introducing a *phoBR*-carrying plasmid. This is likely due to dominance of *phoR*(*T220N*) over the wild-type copy of the *phoR* gene. The growth kinetics of the mutants and complemented strains under low or high concentrations of phosphate were similar to that of the wild-type strain (data not shown).

To determine the activation status of the Pho regulon in our mutants, the AP activities of strains were measured under phosphate-limiting and high-phosphate conditions (Table 3). The AP encoded by *phoA*, a member of the Pho regulon, is widely used as an indicator to monitor the level of expression of the Pho regulon (8, 17, 20, 26). Under high-phosphate conditions, the wild-type strain demonstrated a minimal baseline of AP production. On the other hand, Δ*pstCAB*, Δ*pstC*, and *phoR*(*T220N*) mutants produced AP constitutively ($P < 0.05$) compared to the wild-type parent strain, χ7122 (Table 3). Among the constitutive AP-producing mutants, there was a stronger induction of the Pho regulon in the Δ*pstCAB* mutant ($P < 0.05$) (Table 3). In low-phosphate medium, the AP production of the wild-type strain was activated, although the AP activities of Δ*pstCAB* and Δ*pstC* mutants were higher than the wild-type strain ($P < 0.05$). AP production in the Δ*pstCAB* mutant was markedly elevated compared to that in all other strains ($P < 0.05$) when grown in low-phosphate medium. In contrast, the AP activity of the *phoR*(*T220N*) mutant was similar to that of the wild-type strain. In the Δ*phoB* and Δ*pstC* Δ*phoB* mutants, no AP activity was observed under either high-phosphate or low-phosphate conditions, indicating that

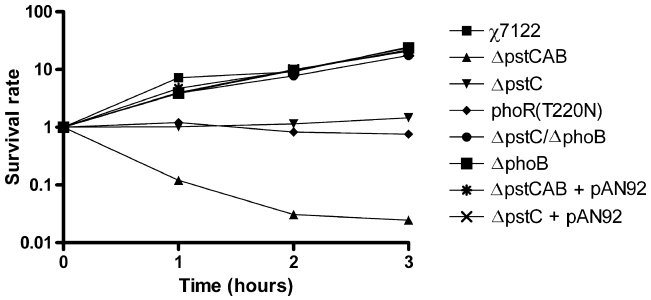

FIG. 1. Bacterial resistance to serum for APEC χ7122, isogenic mutants, and complemented strains. Data presented are the means of results from three independent experiments. The survival rates were measured in 90% rabbit serum for various periods of time. Strains tested were wild-type APEC strain χ7122 and the Δ*pstCAB*, Δ*pstC*, *phoR*(*T220N*), Δ*phoB*, Δ*pstC* Δ*phoB*, Δ*pstCAB* + pAN92, and Δ*pstC* + pAN92 strains. The survival rate of the Δ*pstCAB* mutant strain was significantly lower than the survival rates of every other strain at each time point. From 2 to 3 h of exposure to rabbit serum, the Δ*pstC* and *phoR*(*T220N*) strains were significantly more sensitive to serum than the wild-type strain. No significant differences were observed between the wild-type χ7122 strain and the Δ*pstC* Δ*phoB*, Δ*phoB*, Δ*pstCAB* + pAN92, and Δ*pstC* + pAN92 strains. The control strain, 862, did not survive after 1 h of exposure to 90% rabbit serum (data not shown). Analysis of variance followed by Tukey's multiple comparison test was used for statistical analyses ($P < 0.05$).

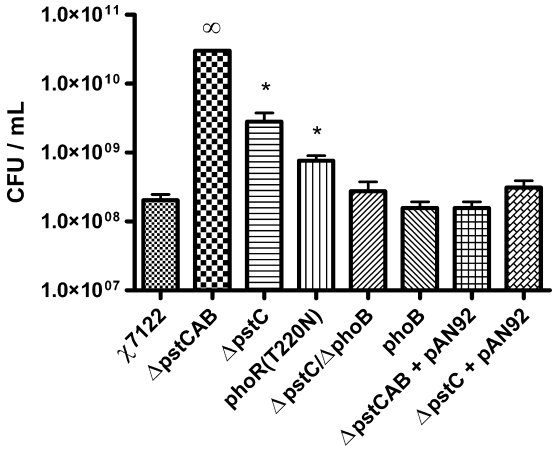

FIG. 2. Minimal bacterial titers allowing yeast agglutination of APEC χ7122, isogenic mutants, and complemented strains. Data presented are the means ± the standard deviations of results from three independent experiments. Strains tested were wild-type APEC strain χ7122 and the Δ*pstCAB*, Δ*pstC*, *phoR*(*T220N*), Δ*phoB*, Δ*pstC* Δ*phoB*, Δ*pstCAB* + pAN92, and Δ*pstC* + pAN92 strains. The χ7122 Δ*fim* (χ7279) strain was used as a negative control. The "∞" symbol shows no agglutination was observed at the highest cell titer for the Δ*pstCAB* strain, which was also observed for Δ*fim* strain χ7279 (data not shown). Asterisks indicate significant differences observed between bacterial titers of the wild-type χ7122 strain and both the Δ*pstC* and *phoR*(*T220N*) mutant strains ($P < 0.05$) as calculated by Student's *t* test.

the Pho regulon is inactivated in these strains. Consequently, the activation of the Pho regulon in the Δ*pstC* mutant is therefore *phoB* dependent. Taken together, results demonstrate that there are different degrees of Pho regulon activation due to introduction of the different *pst*- and *phoBR*-specific mutations.

Virulence determinants. To determine whether the Pst system and the Pho regulon contribute to resistance to serum, we tested the ability of the strains to survive in 90% rabbit serum (Fig. 1). The wild-type χ7122 strain multiplied in serum and was considered serum resistant. The Δ*pstCAB* mutant was rapidly killed by rabbit serum and was significantly more sensitive to serum than all other strains at each time point ($P < 0.05$). The bacterial counts of the Δ*pstC* and *phoR*(*T220N*) mutants showed a significant decrease compared to that of the wild-type strain after 2 and 3 h ($P < 0.05$). In addition, Δ*phoB*, Δ*pstC* Δ*phoB*, and complemented strains were as resistant to serum as the parent strain, χ7122. The serum-sensitive control strain, 862, did not survive after 1 h of exposure to 90% rabbit serum (data not shown).

To assess the effects of a *pst* mutation and Pho regulon activation on APEC strain χ7122 for production of type 1 fimbriae, we used a yeast cell agglutination assay (Fig. 2). Yeast cells are rich in mannose surface molecules, which are recognized by the type 1 fimbrial adhesin. The Δ*pstCAB* strain did not produce agglutination at the highest bacterial titer ($P < 0.05$). The minimal bacterial titer allowing yeast agglutination was higher for the *phoR*(*T220N*) and Δ*pstC* mutants than for the wild-type strain ($P < 0.05$). On the other hand, no differences were observed with the Δ*phoB* and Δ*phoB* Δ*pstC* mutants, which agglutinated at titers comparable to that of the wild-type parent. Complementation of the Δ*pstCAB* and Δ*pstC* mutants restored yeast agglutination titers to wild-type levels, which corresponds to a restoration in production of type 1 fimbriae. The χ7122 Δ*fim* (χ7279) strain was used as a negative control and did not show agglutination of yeast cells (data not shown).

To investigate the role of the Pst system and the Pho regulon in sensitivity to hydrogen peroxide, we used the H_2O_2 agar overlay diffusion method (Table 4). Under phosphate-sufficient conditions (LB), diameters of inhibition zones were significantly larger with Δ*pstCAB*, Δ*pstC*, and *phoR*(*T220N*) mutants than with the parent strain, χ7122 ($P < 0.05$). The PhoR mutant demonstrated a smaller diameter of sensitivity, suggesting that this strain was slightly more resistant to hydrogen peroxide than the other Pho constitutive mutants, although this difference was not statistically significant. Complementation of the Δ*pstCAB* and Δ*pstC* mutants restored the wild-type

TABLE 4. Growth inhibition zones of APEC χ7122, isogenic mutants, and complemented strains with hydrogen peroxide

Strain name or description	Growth inhibition zone (mm) in[a]:	
	LB	LP
χ7122	17.7 ± 0.6	33.5 ± 0.6
Δ*pstCAB*	**21.3 ± 0.6**	34.8 ± 0.4
Δ*pstC*	**21.0 ± 0.5**	34.2 ± 0.8
phoR(*T220N*)	**20.1 ± 0.4**	35.8 ± 1.7
Δ*phoB*	18.3 ± 0.6	**39.7 ± 0.5**
Δ*pstC* Δ*phoB*	18.6 ± 0.5	**38.8 ± 1.7**
Δ*pstCAB* + pAN92	18.7 ± 0.6	**36.5 ± 1.0**
Δ*pstC* + pAN92	18.4 ± 0.6	34.5 ± 0.8

[a] Data presented are the means ± standard deviations for six independent experiments. The compound used was 10 μl of H_2O_2 (30% [vol/vol]) on LB or LP agar plates. Values indicated in bold text are significantly different ($P < 0.05$) from the mean for the wild-type strain as calculated by Student's *t* test.

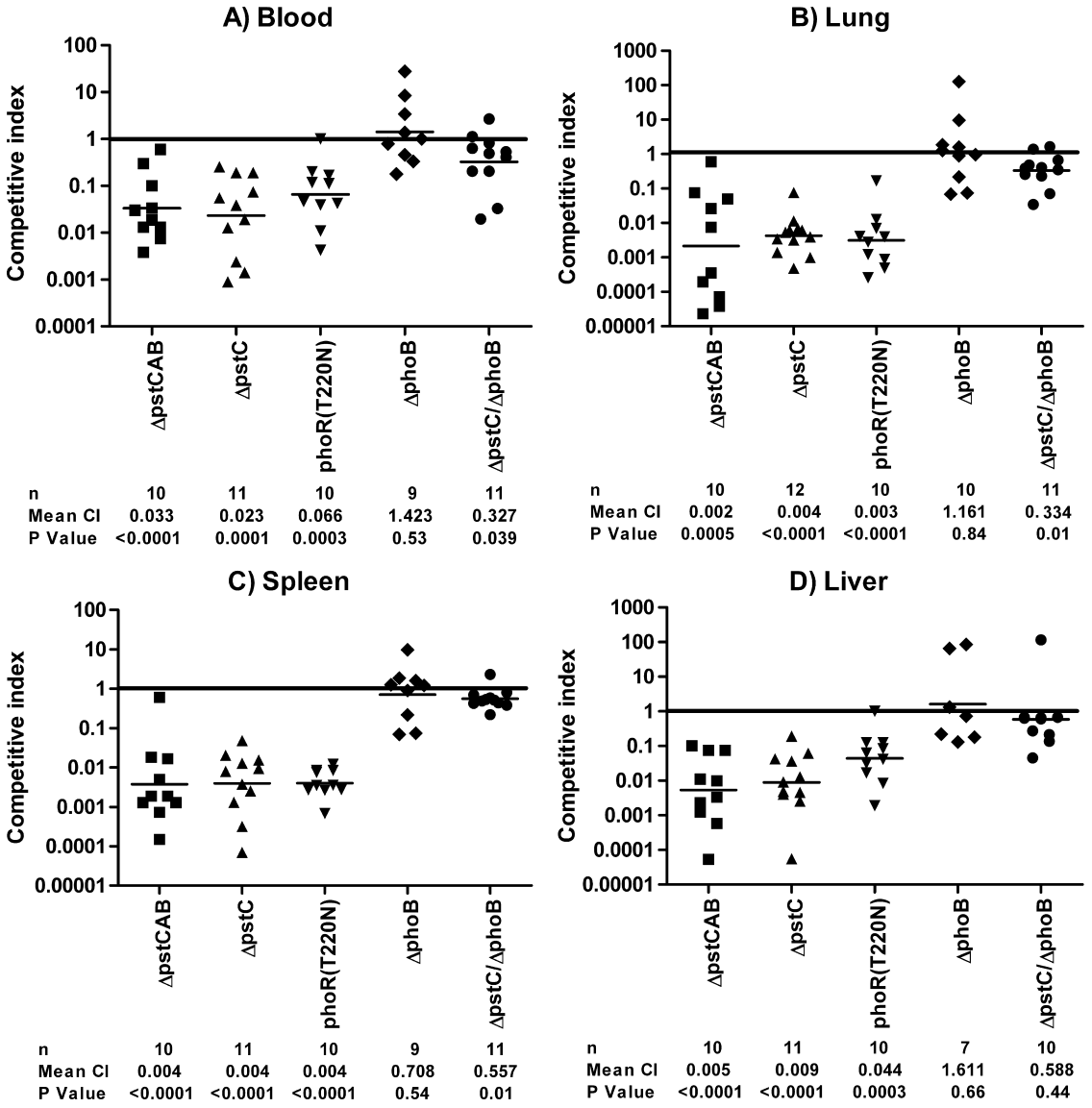

FIG. 3. Competitive indexes from different organs of chickens coinfected with APEC χ7122 isogenic mutants and virulent χ7122 Δ*lacZYA* derivative strain QT51. Mixtures of each of the χ7122 isogenic mutants and the virulent χ7122 Δ*lacZYA* derivative strain QT51 were inoculated in 3-week-old pathogen-free chickens. At 48 h postinfection, heart blood (A), lungs (B), spleens (C), or livers (D) were collected, and bacterial counts were determined. Results are shown as CI values (mutant/strain QT51) and normalized for the inoculums. CI values lower than 1 indicate a decreased capacity for the mutant to compete with the virulent test strain. Horizontal bars indicate the geometric mean CI values. Each point represents a CI value from a blood or tissue sample from an individual chicken. The table summarizes the number of animals sampled (n), the geometric mean of the CI (Mean CI), and the *P* value from a two-tailed *t* test. Asterisks indicate that logarithmically transformed CIs differed significantly from 0 ($P < 0.05$). The Δ*pstCAB*, Δ*pstC*, *phoR*(*T220N*), Δ*phoB*, and Δ*pstC* Δ*phoB* strains were tested.

phenotype. On the other hand, there was no difference in measurements of inhibition zones in response to H_2O_2 with the Δ*phoB* and Δ*pstC* Δ*phoB* mutants from that with the wild-type parent strain χ7122. The results in low-phosphate medium showed that χ7122 cultured in LP has the same sensitivity pattern as the Δ*pstCAB*, Δ*pstC*, and *phoR*(*T220N*) Pho constitutive strains. In addition, the Δ*phoB* and Δ*pstC* Δ*phoB* mutants exhibited an increased sensitivity to H_2O_2.

Competitive coinfection model for APEC virulence. To investigate the importance of a *pst* mutation, as well as Pho regulon activation, for the virulence of APEC, we used a competitive coinfection model (Fig. 3). In experimental coinfections of chickens, the bacterial counts isolated from blood samples, lung, spleen, and liver were significantly reduced for the Δ*pstCAB*, Δ*pstC*, and *phoR*(*T220N*) mutants ($P < 0.05$) 48 h postinfection. However, regarding the *phoR*(*T220N*) mutant, there was a less marked decrease in the CI for liver (0.044) and blood (0.066) compared to those of both the Δ*pstCAB* and Δ*pstC* mutants. On the other hand, the wild-type strain QT51 significantly outcompeted ($P < 0.05$) the Δ*pstC* Δ*phoB* mutant in lung, blood, and spleen samples but not in liver samples. Nevertheless, the means of the CI of the double Δ*pstC* Δ*phoB* mutant in lung (0.344), blood (0.327), and spleen (0.577) tissue were considerably higher than those of the

TABLE 5. *In vitro* competition assays of APEC χ7122 isogenic mutants versus χ7122 Δ*lacZYA* derivative strain QT51

Genotype of competitor strain	Normalized coculture ratio[a]	
	24 h	48 h
Δ*pstCAB*	4.7 ± 1.8	12.3 ± 4.3
Δ*pstC*	3.3 ± 0.7	4.1 ± 0.4
Δ*phoR*	1.6 ± 0.4	1.9 ± 0.7
Δ*phoB*	1.3 ± 1.1	0.6 ± 0.4
Δ*pstC* Δ*phoB*	1.4 ± 0.6	1.5 ± 0.2

[a] Data presented are the means ± standard deviations for three independent experiments. Equal quantities of χ7122 Δ*lacZYA* derivative strain QT51 and either mutant were grown in LB coculture. Viable counts (in CFU) were determined for the input time point, 24 or 48 h, by plating dilutions on MacConkey-lactose agar plates without antibiotics. The coculture ratio was calculated as the CFU of the QT51 mutant strain divided by the CFU of the competitor strain divided by the same ratio in the initial input inoculum.

Δ*pstCAB*, Δ*pstC*, and *phoR*(*T220N*) mutants (Fig. 3 B to D). In contrast, the Δ*phoB* mutant was as virulent as the wild-type strain in all tissues and blood. To determine whether the outcompetition observed *in vivo* was due to different growth characteristics, we performed an *in vitro* competition (Table 5). At 24 and 48 h, there was a less than 1 log difference in the amount of mutants present compared with that of the wild-type derivative QT51 strain except that at 48 h, there was slightly more than 1 log difference between the Δ*pstCAB* mutant and QT51. However, these results do not account for the dramatic decreases in recovery observed for the mutants (up to nearly 3 logs) compared with the 48-h *in vivo* cochallenge.

DISCUSSION

In *E. coli* and many other bacterial species, the Pho regulon is activated when cells face phosphate limitation, whereas its expression is inhibited when phosphate levels are replete. 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 (5, 10, 20, 26). In our study, we dissected the specific contributions of both the PhoBR two-component regulatory and Pst systems to virulence of an APEC O78 strain. Taken together, our results indicate that constitutive Pho regulon activity, through the PhoBR TCRS, is largely responsible for APEC attenuation. This statement is based on the fact that all Pho constitutive mutants were affected in every *in vivo* and *in vitro* assay. On the other hand, it is possible that a functional Pst system may provide a slight *in vivo* advantage, since the *phoR*(*T220N*) mutant is less attenuated *in vivo* than the Δ*pstCAB* and Δ*pstC* mutants. Also, a basal level of expression of the *pst* genes may explain why the Δ*pstC* Δ*phoB* mutant has a small but significant influence on virulence *in vivo* whereas the Δ*phoB* mutant is not affected.

Our findings are in conformity with previous observations. For example, a recent study reported that Pst contributes to the virulence of atypical enteropathogenic *E. coli* (EPEC) and *Citrobacter rodentium* through the response regulator PhoB (8). Indeed, they showed that adherence attenuation phenotypes in *pst* mutants were restored by deleting *phoB*. Also, a polar *pstS* insertion mutation leads to smaller plaque formation in a PhoB-dependent manner in eukaryotic cell (Henle) monolayers than is seen with the parental *Shigella flexneri* strain (42). On the other hand, the Pst system was reported to affect adherence of EPEC independently of the Pho regulon (17). There are major differences between this study on EPEC and results we obtained with APEC strain χ7122. An EPEC Δ*pstC* Δ*phoB* double mutant still displayed reduced levels of adherence. Further, a *phoR*(*T220N*) Pho constitutive EPEC mutant, where the Pst system was functional, adhered to cells as well as the EPEC parent strain. In contrast, our *phoR*(*T220N*) mutant of the APEC χ7122 strain was attenuated, whereas a Δ*pstC* Δ*phoB* double mutant regained virulence attributes. Accordingly, in our study, *phoB* is epistatic to *pst* in terms of the Pho constitutive phenotype and attenuation of virulence attributes. Taken together, our results indicate that constitutive activation of the Pho regulon rather than a loss of phosphate transport mediated by the Pst system is predominantly responsible for the attenuation of APEC χ7122. Similarly, in *Vibrio cholerae*, a recent study demonstrated that the attenuation observed in a *pst* mutant is due to induction of the Pho regulon and not loss of a functional Pst system, using a *pstA*(*R454Q*) point mutation which allows wild-type expression of genes in the Pho regulon but prevents Pst-mediated phosphate transport (39). In addition, deletion of *phoB*, and hence inactivation of the Pho regulon, did not alter APEC virulence, since the Δ*phoB* mutant retained virulence and demonstrated virulence phenotypes comparable to those of the wild-type strain. Interestingly, this is in contrast to the case with *V. cholerae*, for which a *phoB* null mutant was less able to colonize adult rabbit ligated ileal loops in a competitive model and in the infant mouse model of cholera (39, 49, 50). These contrasting results between different bacterial pathogens emphasize the various effects that alteration in the regulatory control of the Pho regulon may have on virulence.

The virulence phenotype in APEC χ7122 is dependent on the activation level of the Pho regulon. We demonstrate for the first time to our knowledge that the degree of attenuation in *E. coli* correlated with increased activity of the Pho regulon. In fact, increases in AP activity (Table 3) correlated with decreased virulence or competitive fitness in chickens and differences in sensitivity to hydrogen peroxide and serum and in yeast agglutination (Fig. 3, Table 4, and Fig. 1 and 2, respectively). The Δ*pstCAB* mutant was more affected than both the Δ*pstC* and *phoR*(*T220N*) mutants in those assays, and the Pho regulon activation level in the Δ*pstCAB* mutant was more important than those in both the Δ*pstC* and *phoR*(*T220N*) mutants. The *phoR*(*T220N*) mutant is defective in PhoR inhibitory activity (its phosphatase activity) but not in its ability to activate PhoB (7) and is known to have a modest level of constitutive Pho activity (51, 54). The AP activity differences between the Δ*pstCAB* and Δ*pstC* mutants could be due to the fact that the Δ*pstCAB* mutant lacks a functional PstA permease and PstB ATPase in addition to having a loss of the PstC permease. Indeed, it was suggested that PstB is important for inhibition of the Pho regulon by acting in concert with PhoU (52). It is also possible that differences between the Δ*pstCAB* and Δ*pstC* strains may be due to loss of an intergenic region 3′ of *pstA* in the Δ*pstCAB* mutant. Recently this region was suggested to stimulate translation of *rpoS* (44), and RpoS, an alternative sigma factor of RNA polymerase, plays a central role in stress response under many stress conditions and modulates the ex-

pression of genes such as those belonging to the Pho regulon (45). Overall, it is likely that a stronger induction of the Pho regulon leads to a higher expression of genes associated directly or indirectly with virulence.

Our results demonstrated that the resistance to serum is impaired when the Pho regulon is constitutively active and that sensitivity to serum is relative to the level of Pho regulon activity (Fig. 1). This is significant since there is a correlation between resistance to the bactericidal effects of serum and the capacity of APEC strains to cause septicemia and mortality (29, 35). Resistance to the bactericidal effect of complement is a multifactorial phenomenon. It is recognized that resistance to serum can correlate with the expression of specific capsular K antigens in combination with O polysaccharides (19, 37, 53). In fact, an O78-negative LPS mutant of strain χ7122 became serum sensitive and was unable to persist in body fluids and internal organs of infected chickens (35). Our previous work also reported that the Pho regulon is involved in modifications of lipid A, since a major decrease (66%) in the 1-pyrophosphate lipid A species is observed in *pst* mutants (27). Perhaps the Pho regulon may participate in the increased sensitivity of mutants to the bactericidal effects of serum by contributing to bacterial surface perturbations (26).

A decrease in the production of type 1 fimbriae also correlated with the level of activation of the Pho regulon (Fig. 2). Our previous work has also shown that genes involved in type 1 fimbrial biosynthesis were downregulated in a χ7122 APEC *pst* mutant (10). Additionally, type 1 fimbriae are expressed in the primary site of initial respiratory infection, namely, the air sacs of poultry (15, 38). Therefore, a decreased production of type 1 fimbriae in Pho-activated mutants may contribute to reduced APEC colonization and virulence.

Sensitivity to oxidative stress following exposure to hydrogen peroxide also correlated with increased levels of constitutive AP activity. However, no notable difference was observed between the different mutants where the Pho regulon is activated. This was probably due to the low sensitivity of the technique (Table 4). The ability to resist reactive oxygen species (ROS) is crucial for full virulence when pathogens face oxidative stress during infection of the host (10, 22). Differential expression data for genes involved in oxidative stress observed in a Pst mutant indicated that the mutant is subjected to increased oxidative stress during growth and is likely less able to cope with additional stresses incurred from exogenous ROI-generating compounds (10, 46).

Constitutive Pho activity results in reduced virulence in chickens. By using an avian experimental coinfection model, we demonstrated that activation of the Pho regulon of χ7122 leads to attenuation of virulence, since the Δ*pstCAB*, Δ*pstC*, and *phoR*(*T220N*) mutants were all outcompeted in the chicken by the wild-type strain (Fig. 3). These results indicate that during systemic infection, the Pho constitutive phenotype represents a selective disadvantage in regard to immune defenses, nutritional limitations, and other environmental stresses encountered within the host.

Taken together, our results confirm that the PhoB-mediated constitutive activity of the Pho regulon plays a major role in attenuation of APEC χ7122 virulence and associated traits. In contrast, the P_i transport function of the Pst system was shown to play a limited role in virulence since the Pst system is not expressed in a virulent PhoB mutant and the Pst system is also fully functional in the *phoR*(*T220N*) attenuated mutant, which demonstrated increased constitutive activity of the Pho regulon. Results also further emphasize the fact that PhoB has a dual function as a response regulator for phosphate homeostasis and as a modulator of virulence attributes. There is a clear need for novel approaches to prevent and control bacterial infections, including avian colibacillosis. Suitable attenuated vaccines should be sufficiently invasive and persistent to induce protective immunity and minimize susceptibility to natural infection (18). Interestingly, since the degree of attenuation in the APEC mutants described herein varied according to the degree of constitutive activation of the Pho regulon, this could be useful in the design of new attenuated vaccine strains.

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