Modulation of hexa-acyl pyrophosphate lipid A population under *Escherichia coli* phosphate (Pho) regulon activation

Running title: Lipid A Structure Modification induced by a *pst* mutation

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Abstract.
Environmental phosphate is an important signal for microorganism gene regulation and it has recently been shown to trigger some key bacterial virulence mechanisms. In many bacteria, the Pho regulon is the major circuit involved in adaptation to phosphate limitation. The Pho regulon is controlled jointly by the two-component regulatory system PhoR/PhoB and by the phosphate specific transport (Pst) system, which both belong to the Pho regulon. We showed that a pst mutation results in virulence attenuation in extra-intestinal pathogenic Escherichia coli (ExPEC) strains. Our results indicate that the bacterial cell surface of the pst mutants is altered. In this study, we show that pst mutants of ExPEC strains display an increased sensitivity to different cationic antimicrobial peptides and vancomycin. Remarkably, the hexa-acylated 1-pyrophosphate form of lipid A is significantly less abundant in pst mutants. Among differentially expressed genes in the pst mutant, lpxT coding for an enzyme that transfers a phosphoryl group to lipid A, forming the 1-diphosphate species, was found to be downregulated. Our results strongly suggest that the Pho regulon is involved in lipid A modifications, which could contribute to bacterial surface perturbations. Since the Pho regulon and the Pst system are conserved in many bacteria, such a lipid A modification mechanism could be widely distributed among Gram-negative bacterial species.

Introduction
Under circumstances such as drastic environmental changes, the phenotypic diversity occurring within a bacterial cell population due to noise in gene expression favours the natural selection of subpopulations best fit to survive (37, 39, 40, 54). In addition, bacteria possess more specialised machineries, the two-component regulatory systems, which permit sensing and response to environmental changes (20). Environmental stimuli are translated into signalling events leading to the expression of a highly precise response. One such system is
PhoR/PhoB (47, 48), which responds to environmental phosphate concentration variations and controls expression of at least 47 genes in *Escherichia coli* (Pho regulon) (26). PhoR is an inner membrane sensor protein that responds to periplasmic orthophosphate (Pi) through interaction with the phosphate specific transport (Pst) system. The activation signal is a Pi concentration below 4 µM. Under these conditions, PhoR is autophosphorylated on a conserved histidine residue and transfers this phosphoryl group to a conserved aspartate residue on its cognate response regulator PhoB. To initiate or repress gene transcription, Phospho-PhoB binds specific DNA sequences known as Pho-Boxes, which are located upstream of Pho-dependent genes. The Pst system, which belongs to the Pho regulon, encodes an ATP-binding cassette transporter involved in high affinity acquisition of Pi. In many bacterial species, mutations in the Pst system result in constitutive expression of the Pho regulon regardless of environmental phosphate availabilities (11, 26, 48, 49).

Many studies reported an association between the Pst system, the Pho regulon, and bacterial virulence (for a comprehensive overview see (26)). However, molecular mechanisms that link the Pho regulon and the virulence of bacteria have not been fully established. For this research, we focused our work on extra-intestinal pathogenic *E. coli* (ExPEC) strains. χ7122 (O78:K80) (33) and 5131 (O115:K"V165") (19) are ExPEC strains that share many virulence attributes and cause extra-intestinal infections in poultry and swine, respectively. Pleiotropic effects and loss of virulence was observed in *pst* mutants of extra-intestinal strains χ7122 and 5131 (10, 25, 29). Many phenotypes of *pst* mutants, such as sensitivity to acid stress and the bacteriolytic effect of serum, are suggestive of important changes in cell surface properties (25, 26).
The outer leaflet of the outer membrane (OM) of Gram-negative bacteria is composed, in most part, of approximately two million copies of a complex glycolipid named lipid A (35). The lipid A is the anchor for the core oligosaccharide, which is usually linked to the O antigen polysaccharide that is exposed on the bacterial cell surface (3, 35, 45). The entire molecule, the lipopolysaccharide (LPS), protects bacteria from various environmental stresses that can be encountered outside and inside the host (13, 30). In *E. coli* and *Salmonella enterica* serovar Typhimurium, two-thirds of the total membrane lipid A molecules consist of the 1-monophosphate form of the phosphorylated and acylated di-glucosamine structure (Fig. 1). The remaining one-third possesses a pyrophosphoryl group at the C1 position (Fig. 1).

Bacterial pathogens such as *S. enterica* serovar Typhimurium and some *E. coli* stains have an ability to modify their lipid A structure to resist some adverse environmental cues (Fig 1) (15, 16, 18, 35, 42).

As described above, we believed that cell surface perturbations occur in *pst* mutants. Since LPS is a major component of the OM, which can be subjected to certain modifications in bacterial pathogens and plays an important protective role against bactericidal molecules, we decided to investigate LPS structural properties in *pst* mutants. Moreover, since the Pho regulon is constitutively activated in *pst* mutants, it was also possible that phosphate-rich LPS molecules could be subject to specific modifications. Remarkably, the hexa-acylated 1-pyrophosphate form of lipid A is significantly less abundant in *pst* mutants. This modification occurs concomitantly to an increased sensitivity of the bacteria to cationic antimicrobial peptides (CAMPs) such as polymyxin B (PMB). We believe that this state of lipid A could participate in the observed membrane perturbation of * pst* mutants.
Materials and methods.

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) or in LP broth at 37°C. LB is a high phosphate containing medium. LP is a low phosphate containing medium (LP: 50 mM Tris-HCl (pH 7.4), 10 mM KCl, 10 mM (NH₄)₂SO₄, 0.4 mM MgSO₄, 0.1% yeast extract, 20 mM glucose, and 1 mM methionine). All experiments have been performed using cells in the exponential phase of growth. No differences in growth rate were observed between ExPEC strains and their isogenic pst mutants, either when LB or LP was used (data not shown). When required, antibiotics were used at the following final concentrations: tetracycline 10 µg/ml; kanamycin (Kan), 50 µg/ml; nalidixic acid (NA), 40 µg/ml; chloramphenicol (Cm), 10 µg/ml; streptomycin (Sm), 100 µg/ml.

Construction of plasmids and mutant strains. The mutation of the pst operon was obtained by allelic exchange as described previously (25). 5131 and χ7122 derivatives, strain K2 and K3 respectively, were confirmed to contain a deletion in the pst operon as determined by PCR amplification, Southern blot hybridization and RT-PCR (Lamarche et al. 2005 and data not shown). The pAN92 (23) plasmid carrying the functional pst operon was used to complement the ΔpstCAB mutants to create strain CK2 and CK3.

Minimal inhibition concentration (MIC) assays using polymyxin B (PMB), cecropin P1 (CP1) and vancomycin. Experiments were done in LB or LP for bacterial cells growth. Strains were grown overnight and cultures were diluted in fresh medium to obtain cultures in exponential phase. Strains were grown to optical density of 0.7 (λ = 600 nm). Cultures were then diluted to obtain 10⁶ CFU/ml. Microwell plates were loaded with the 10⁶ CFU/ml cultures and different concentrations of PMB or CP1 were added to each well. Plates were
incubated overnight and wells were evaluated for growth by spectrophotometry. For vancomycin assays, plates were incubated 3 hours. The MIC was considered the lowest drug concentration that reduced growth by more than 50% compared with growth in the control well (14). Vancomycin survival was calculated from the ratio of vancomycin-exposed to non-exposed cells. Experiments are the mean of three biological and technical replicates. Statistical analyses were performed using the GraphPad Prism 4® package. A One-way ANOVA followed by a multiple comparison Tuckey test was applied.

**Killing assays using polymyxin B.** Experiments were performed as described previously with slight modifications (17, 44). To evaluate ExPEC strains and their isogenic Pst mutants for PMB resistance, strains were grown to mid-log phase and diluted to a concentration of 2,500 CFU/ml in LB. Cells (200 µl) were mixed on a microtiter plate with various concentrations of PMB (0.125, 0.25, 0.375, 0.5 µg/ml) and incubated at 37°C for 30 minutes. Then, 150 µl of PMB-treated cells were directly plated on LB medium. After overnight growth at 37°C, colony counts of cells incubated with the various concentrations of PMB were compared to those not exposed to PMB. The percent survival was defined as follows: survival (%) = (CFU of peptide-exposed culture/CFU of nonexposed culture) × 100. Statistical analyses were performed using the GraphPad Prism 4® package. A One-way ANOVA followed by a multiple comparison Tuckey test was applied.

**Analysis of radiolabeled lipid A by Thin Layer Chromatography (TLC).** Experiments were performed in LB or LP for bacterial cell growth. Analysis of lipid A released by mild acid hydrolysis from $^{32}$P$_i$ labelled cells was adapted from Zhou et al. (1999)(55). An overnight culture grown at 37°C was diluted 100-fold in 5 ml of fresh medium containing appropriate antibiotics and 5 µCi/ml $^{32}$P$_i$, and allowed to grow for 3h. The $^{32}$P$_i$ labeled cells were
harvested by centrifugation and washed once with 5 ml of PBS. The pellet was resuspended in 0.8 ml of PBS and converted into a single-phase Bligh/Dyer mixture by adding 2 ml of methanol and 1 ml of chloroform. After 10 minutes incubation at room temperature, the insoluble material was collected by centrifugation in a clinical centrifuge. The pellet was washed once with 5 ml of a fresh single-phase Bligh/Dyer mixture, consisting of chloroform/methanol/water (1:2:0.8, v/v). This pellet was then dispersed in 1.8 ml of 12.5 mM sodium acetate, pH 4.5, containing 1% SDS, with sonic irradiation in an ultrasonic bath. The mixture was incubated at 100°C for 30 minutes to cleave the ketosidic linkage between the first inner core 3-deoxy-d-manno-oct-2ulosonic acid unit and the distal glucosamine sugar of lipid A. After cooling, the boiled mixture was converted to a two-phase Bligh/Dyer mixture (5) by adding 2 ml of chloroform and 2 ml of methanol. Partitioning was made by centrifugation and the lower phase material was collected and washed once with 4 ml of the upper phase derived from a fresh neutral two-phase Bligh/Dyer mixture, consisting of chloroform/methanol/water (2:2:1.8, v/v). The lower phase lipid A sample was collected and dried under a steam of nitrogen gas. The lipid A sample was dissolved in 100µl of chloroform/methanol (4:1, v/v), and 1000 cpm of the sample was applied to the origin of a Silica Gel 60 TLC plate. TLC was conducted in a developing tank in the solvent chloroform/pyridine/88% formic acid/water (50:50:16:5, v/v). The plate was dried and visualized with a PhosphorImager (Storm 840 from Molecular Dynamics). Experiments were repeated three times.

**Microarray experiments.** Microarray experiments were done using Affymetrix GeneChip® *E. coli* Genome 2.0 Array. Briefly, overnight culture grown at 37 °C was diluted 100-fold into 5 ml of LB and was allowed to grow to mid-log phase (OD₆₀₀ 0.6). RNA was isolated using the RiboPure™-Bacteria Kit (Ambion, Austin, TX), according to the manufacturer’s
recommendations, with the exception that the DNAse 1 treatment was performed twice. For cDNA synthesis and biotinylation, two aliquots of 5 µg of RNA (10 µg total) were supplemented with 2 µL of 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. After treatment with RNAse A (1mg/ml), the cDNA were purified using Microcon YM-30 centrifugal filter (Milipore, Billerica, MA). The cDNA were fragmented with DNase 1 (Ambion, Austin, TX) and were 3’ biotinylated using GeneChip DNA Labelling Reagent (7.5 mM) (Affymetrix, Santa Clara, CA) and 60 U of Terminal Deoxynucleotidyl Transferase (Promega, Madison, WI). The Affymetrix GeneChip® E. coli Genome 2.0 Array (Affymetrix, Santa Clara, CA) contains 20,366 genes present in four strains of E. coli (MG1655, EDL933, SAKAI and CFT073) and includes over 700 intergenic regions. For supplemental information, see Affymetrix web site: at www.affymetrix.com. Biotinylated cDNAs were hybridized onto Affymetrix GeneChip® E. coli Genome 2.0 Array (Affymetrix, Santa Clara, CA) as recommended by the manufacturer. Hybridizations were performed at Genome Québec Innovation Centre (McGill University, Montreal, Canada). Data were processed using the robust multiarray average algorithm (RMA) for normalization, background correction and expression value calculation (22). Expression levels obtained from three independent replicates, in each condition, were compared using the FlexArray RC3 software (Michal Blazejczyk, Mathieu Miron, Robert Nadon (2007). FlexArray: A statistical data analysis software for gene expression microarrays. Genome Quebec, Montreal, Canada, URL: http://genomequebec.mcgill.ca/FlexArray/index.html). The robustness of the data was further enhanced by EB algorithm and P-value calculation. The microarrays results were validated by qRT-PCR using the QIAGEN QuantiTect® SYBR® Green RT-PCR kit, according to the manufacturer’s instructions. 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 (Ct) was normalised to the Ct obtained for the tus gene amplified from the corresponding sample. Fold change were calculated using the $2^{-\Delta\Delta Ct}$ method (28).

Lipid A extraction and mass spectrometry analysis. Cell cultures were prepared exactly as for the analysis of radiolabeled lipid A in the TLC experiments, but without the addition of $^{32}$P orthophosphate. A pool of six 5 ml cultures was used for each strain and experiment. Lipid A samples were prepared as described previously, with slight modifications (27, 52). PBS washed cells were resuspended in 300 µL of Tri-Reagent (Ambion) and incubated for 20 minutes at room temperature. Then, 30 µL of chloroform was added, and the samples were vortexed vigorously and incubated for 15 minutes at room temperature. The phases were separated by centrifugation at 12,000Xg for 10 minutes, and the upper phase was transferred to a new tube. This extraction procedure was repeated twice using 100µL of water instead of chloroform, and the combined upper phases were evaporated in a speed-vac apparatus (DNA120 SpeedVac® from ThermoSavant). The lipid A was released from the LPS by mild-acid hydrolysis. The pellet was resuspended in 500 µL of hydrolysis buffer (12.5 mM sodium acetate pH 4.5; 1% SDS) and heated at 100ºC for 1 hour, and dried in a speed-vac. To remove SDS from the samples, washing steps were performed. The pellet was resuspended in a mixture of 100 µL of water and 500 µL of acidified ethanol (100 µL of 4M HCl in 20 ml of 95% ethanol), and centrifuge at 2,060 X g for 10 minutes. The pellet was washed again in 95% ethanol using the same procedure. Washing steps were repeated once. The pellet was dried at room temperature for 5 minutes, and lipid A was dissolved in 100 µL of chloroform: methanol: water (2:3:1 v/v). The samples were sent to the Proteomic and Mass Spectrometry Center (PMSC), Molecular Medicine Research Centre, University of Toronto, for the matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry analysis.
Preparations for MALDI/TOF analysis were performed by depositing 0.3 µL of the sample dissolved in chloroform: methanol (4:1 v/v), followed by 0.3 µL of a saturated solution of 2,5-dihydroxybenzoic acid in 50% acetonitrile as the matrix. The sample was dried at room temperature. Spectra were acquired in both positive and negative ion linear modes. Each spectrum was the average of 50 laser shots.

Results

CAMPs assays in phosphate-rich medium

The ExPEC χ7122 and 5131 pst mutants are acid and serum sensitive (10, 25). This could be indicative of a bacterial cell surface modification. To further investigate this possibility, we submitted the χ7122 and 5131 ExPEC strains and their isogenic pst mutants to CAMP challenges. In LB medium using MIC assays, the sensitivity of pst mutants to PMB and to CP1 is significantly increased in contrast to that of wild-type strains (Figure 2A and 2C). The sensitivity of pst mutants to PMB was further assessed using killing assays which give precise kinetics of CFU counts for cells exposed to different antimicrobial concentrations. In accordance to MIC assays, both ExPEC Pst mutants were more sensitive to PMB in killing assays than wild-type strains while complementation of the pst mutation restores to the wild-type resistance (Figure 2D). Also, as observed in MIC assays, the porcine ExPEC strain 5131 is less resistant to PMB than the APEC strain χ7122 (Figure 2A and 2D).

Radiolabeled lipid A analyses by TLC for strains grown in phosphate-rich medium

Since the pst mutants display PMB sensitivity, which could be caused by lipid A modifications (18, 35, 45), we undertook radiolabeled lipid A analyses by TLC. Because of its known TLC profile, an E. coli O157:H7 strain was used as a control (24). Its profile is similar to that of E. coli K12 MC1061 except for an additional phosphoethanolamine (pEtN) lipid A
variant (24) (Fig. 3A). In *pst* mutants grown in LB, a reduction of 66% of the pyrophosphate form of lipid A (hexa-acylated 1-pyrophosphate lipid A; see Fig. 1) is reproducibly observed (Figure 3B and 3C), as calculated by densitometry (ImageQuant 5.0).

Vancomycin assays in phosphate-rich medium

From these results, we hypothesized that *pst* mutants should also be sensitive to vancomycin as a consequence of a generalized outer membrane perturbation. Vancomycin is a glycopeptide antibiotic that interferes with peptidoglycan biosynthesis (1). High concentrations of vancomycin are needed to kill *E. coli* cells unless the outer membrane is perturbed (38). A mutation in *pst* results in an increased vancomycin sensitivity (Fig. 4). Complementation of the *pst* mutation restores the wild-type CAMP and vancomycin resistance level, indicating that the *pst* mutant phenotypes result from the ∆*pst::kan* mutation and not from some polar effects. These results indicate that a bacterial cell surface alteration might exist in *pst* mutants allowing vancomycin to reach periplasm.

CAMP assays and TLC quantification of 1-pyrophosphate lipid A for strains grown in low-phosphate medium

To evaluate if the Pho regulon activation-state is indeed involved in the *pst* mutants lipid A phenotype, we evaluated properties of the wild-type, mutant and complemented *pst* mutant strains grown in low phosphate medium (LP). In LP, all strains have the same PMB MIC value. Furthermore, wild-type and complemented *pst* mutant strains have a PMB MIC similar to that of *pst* mutants grown in LB (Fig. 2B). Strikingly, all strains exhibit a similar amount of the hexa-acylated 1-pyrophosphate form of the lipid A (Fig. 3D). Furthermore, these amounts are similar to those obtained for *pst* mutants grown in LB (Fig. 3B and 3C). This observation confirms our hypothesis that the bacterial cell surface is altered in *pst* mutants. Furthermore, it
strongly suggests that the Pho regulon influences the biosynthesis of hexa-acylated 1-pyrophosphate lipid A.

MALDI-TOF analysis of APEC strains grown in low-phosphate medium

For avian strains grown in LP medium, TLC analyses reveal the presence of lipid A variants (Fig. 3D) which are more predominant in the pst mutant strain K3 (Fig. 3D). This observation is interesting since lipid A substitutions (Fig. 1) are infrequently observed in E. coli in comparison to S. typhimurium, in which lipid A variants are often expressed (6, 35, 56). In accordance, lipid A MALDI-TOF analyses of the pst mutant K3 grown in LP medium reveal some variations in the lipid A profile when compared to the wild-type strain χ7122 (Fig. 5). By comparison with previously known lipid A variant m/z values, a molecule corresponding to the hexa-acyl bis-phosphorylated lipid A prototype (m/z: 1797) bearing two pEtN residues (m/z: 2043) was identified (Fig. 5B). Other ions are also present in the K3 strain (m/z: 1996 and 2108), but these species do not correspond with known lipid A structures. MALDI-TOF spectra obtained for χ7122 and K3 lipid A, when strains were grown in LB, are similar to the spectrum of χ7122 lipid A when LP is used for growth (data not shown).

Microarray analysis

A microarray study of the wild-type strain χ7122 and the pst mutant K3 grown in high phosphate (LB) medium was used to analyse LPS-related genes at the transcription level (Table 2). All LPS-related genes identified are down-regulated in the pst mutant K3 in contrast to the wild-type strain. Many of the genes identified belong to the ECA biosynthesis pathway, which is encoded by a large operon that constitutes 12 genes (rfe-wzzE-rffEDGHCA-wzxE-rffT-wzyE-rffM). Pho boxes are present upstream the wzxE and wzxE genes as determined by using the Pho box prediction weight matrix elaborated by Finan and
colleagues (53). These genes code respectively for a flippase involved in the movement of the ECA trimeric sugar lipid III (Fuc4NAc-ManNacA-GlcNAc-P-P-undecaprenol) across the inner membrane and for a polymerase likely involved in ECA chain elongation. The \textit{rfaJ} gene belonging to \textit{rfaQGPSBIJYZ-waaU} operon is also down-regulated. The \textit{rfaJ} gene coding for a lipopolysaccharide 1,2-glucosyltransferase is involved in the LPS-core biosynthesis (Table 2). A Pho box is located upstream de \textit{rfaJ} gene as well as just upstream the starting codon of the \textit{rfaP} and \textit{rfaY} genes, which are both kinases involved in LPS inner-core phosphorylation. Concerning the lipid A biosynthetic gene expression, only the \textit{eptA} gene is down-regulated. This gene is an ortholog of \textit{Salmonella pmrC} involved in pEtN covalent modification of the lipid A (27). Gene candidates coding for putative factors that could be involved in the loss of the pyrophosphate lipid A species also caught our attention (Table 2). Indeed, the monocistronic gene \textit{cdh} encoding a CDP-diglyceride pyrophosphatase (34) is up-regulated in the \textit{pst} mutant K3. Cdh was shown to cleave a phosphoryl group from UDP-2,3-diacylglicosamine \textit{in vitro}, but a distinct enzyme, LpxH, performs this function \textit{in vivo} (2, 8). The \textit{in vivo} function of Cdh is still unknown (7, 9, 21, 34, 36). Additionally, genes \textit{ydhJ} and \textit{yeiU}, which are down-regulated in the \textit{pst} mutant K3, encode undecaprenyl pyrophosphate phosphatases that remove phosphoryl groups from inner membrane phosphate-lipid carriers. The \textit{yeiU} (renamed \textit{lpxT}) gene product was recently shown to be responsible for the synthesis of the pyrophosphate form of the lipid A, as demonstrated by Trent and colleagues (see discussion) (43). The \textit{yeiU} gene is located downstream of \textit{yeiR} and forms an operon with this gene. No Pho boxes were found in the promoter region of \textit{cdh}, \textit{yeiR} and \textit{ydhJ} genes. qRT-PCR experiments (fold change in log 2: +1.6, -1.3, -2.6 for \textit{cdh}, \textit{ydhJ} and \textit{yeiU} respectively) confirmed our microarray results (Table. 2). qRT-PCR on fifteen other genes, chosen randomly, further validates our microarray results (data not shown).
Discussion.

In this study we show that a mutation in *pst* causes a significant decrease (66%) of the 1-pyrophosphate lipid A species in high phosphate medium (LB). This phenotype indicates that OM composition is altered in *pst* mutants. Furthermore, it occurs concomitantly to an increased sensitivity to CAMPs. Thus, we propose that the 1-pyrophosphate of lipid A contributes to membrane cohesion. Our vancomycin sensitivity results are in accordance with the presence of cell surface perturbations in *pst* mutants. LPS structural features, such as the phosphorylation status of lipid A and the inner core, are essential for maintenance of OM integrity (3, 30, 31). Divalent cations such as Mg$^{2+}$ bridge the phosphoryl negative charges together acting as major cohesive factors for bacterial cell surface components. Thus, alteration of the cross-bridging between LPS molecules in the *pst* mutants might diminish the overall integrity of the cell surface. This could overcome the reduction of the negative charge due to loss of phosphoryl groups (50, 51). In addition to effects on LPS cross-bridging, pyrophosphoryl substitution in position 1 of the lipid A core that could decrease affinity of polymyxin B toward lipid A cannot be rule out. This phenomenon would result from structural changes within the lipid A molecule. However, to our knowledge, it has never been reported. Also, since a *pst* mutation causes pleiotropic effects, it is possible that complex phenomena, including synergetic effects of some cell surface components modifications, contribute to the increased sensitivity of *pst* mutants to CAMPs and vancomycin.

Nevertheless, the PMB resistance phenotype is likely dependent in part on the phosphorylation status of the lipid A, and the Pho regulon might play a key role in that phenomenon. Therefore, we conducted CAMP assays as well as lipid A structural analyses by TLC using bacteria from the low phosphate medium LP. In *pst* mutants, the Pho regulon is constitutively activated to mimick the induced state of wild-type strains. In LP, PhoA is
induced in both wild type strains but not at the same level as Pst mutants. Indeed, Pst mutants still demonstrate an exacerbated induction of the Pho regulon (data not shown) compared to wild types as observed in the phosphate-rich medium LB (25). However, preliminary results of microarray analysis show that wild-type χ7122 and Pst mutant K3 share similar patterns of differentially expressed genes (data not shown). In accordance, phenotypes displayed in pst mutants were also observed in wild-type strains grown in low phosphate medium. In LP, both pst mutants and wild-type strains express sensitivity to CAMPs, and the amount of 1-pyrophosphate lipid A species is similar to that of the pst mutants grown in rich medium. This observation strongly suggests that the Pho regulon influences the biogenesis of this lipid A species as well as the resistance to CAMPs.

Among gene candidates that could be involved in 1-pyrophosphate lipid A biosynthesis, the gene yeiU (lpxT) that is down-regulated in the pst mutant, as analyzed by microarray, was recently identified by Trent and colleagues to be involved in the biosynthetic origin of the lipid A 1-pyrophosphate moiety (43). Indeed, the lpxT gene product is identical to an inner membrane protein previously shown to have undecaprenyl pyrophosphate phosphatase activity (12), but also appears to catalyze the transfer of a phosphoryl group from the C_{55}-undecaprenyl phosphate lipid carrier to the C-1 phosphate of the lipid A molecule (43). This step is linked to polyisoprenyl lipid phosphate carriers recycling during peptidoglycan synthesis (43, 46). However, no Pho-boxes were found in the promoter region of the yeiRU operon. It is also possible that other candidates under the control of PhoR/PhoB participate in this modulation. As the induction of the Pho regulon controls phosphate scavenging in phosphate-limiting environments, the cell’s richer sources of phosphate, such as lipid A pyrophosphate, could provide P_{i} to ensure its availability for metabolism and essential phosphate-containing structures.
By microarray analyses, we also identified differentially expressed genes involved in LPS biosynthesis in the *pst* mutant K3. Strikingly, two genes belonging to the identified *rfaJ* operon, *rfaP* (*waaP*) and *rfaY*, possess Pho boxes in their promoter region and encode enzymes involved in the phosphorylation of the LPS inner-core (Table 2). As discussed above, reduced amount of phosphoryl groups in the inner core of LPS can result in substantial cell surface perturbations. Thus, it is possible that Pho-dependent dephosphorylation of the LPS inner-core could influence cell surface properties. Although we did not observe any changes in O-antigen and/or ECA profiles by SDS-PAGE and Western blot analyses (unpublished results), our microarray results suggest that molecular changes could indeed occur within the LPS structure in the *pst* mutant strains. Down-regulation of some of the ECA biosynthetic genes, such as the putative ECA polymerase *wzyE* gene, which also possesses a Pho box, is in accordance with such a hypothesis.

It is well known that covalent modifications of the lipid A are involved in resistance to CAMPs such as PMB (3, 4, 35, 45). In *E. coli* K12, covalent modifications have been observed in PMB resistant strains and in ammonium metavanadate (a non-specific inhibitor of mammalian phosphatases)–treated cells (32, 55, 57). Also, pEtN substitution was observed in an enterohemorrhagic *E. coli* strain (EHEC; see Fig. 3A) (24). In our study, lipid A variants were observed in APEC strains but not in porcine ExPEC strains under low phosphate conditions. By comparison to lipid A TLC profiles obtained with metavanadate-treated *E. coli* K12, we hypothesized that lipid A variants observed in the K3 strain could correspond to substitutions identified by Zhou *et al.* (1999). The MALDI-TOF analyses confirmed the presence of covalent substitutions in the K3 prototype lipid A. One variant proved to be the species twice modified by pEtN on the 1 and 4’-phosphate substituents (*m/z* 2043) (Fig. 5B), which was previously identified by Zhou and co-workers in their studies (1999). However, in
pst mutants, perturbation of the OM might require more than just the presence of lipid A variants with pEtN substitutions, because, in contrast to lipid A modification by 4-amino-4-deoxy-L-arabinose (L-Ara4N), pEtN modification alone appears to have only minor effects on CAMP resistance (27, 41).

In conclusion, we demonstrate that the pst mutants K2 and K3 are characterized by multiple defects in virulence attributes. We demonstrate that one consequence of the pst mutation is a significant reduction of the lipid A 1-pyrophosphate species and that this could participate in the sensitivity of the bacteria to PMB. However, other membrane modifications occur in pst mutants (Lamarche et al., manuscript in preparation). All these alterations most likely contribute to an increased permeability to compounds, such as antibiotics or protons. This correlates with our previous report on a pst mutant being sensitive to acid shock and exhibiting multiple virulence defect phenotypes (25). All together, our results strongly suggest that some pst phenotypes are due to the constitutive activity of the Pho regulon and that the biosynthetic pathway of the lipid A 1-pyrophosphate is influenced by the Pho regulon.

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References


Figure 1. *E. coli* lipid A structure. Two glucosamine units are linked by a β 1’-6 glycosidic bond to compose the lipid A backbone. Each glucosamine unit bears a phosphoryl group and two principal \( R \)-3-hydroxymyristoyl chains. The acyl chains of the distal glucosamine unit are further acylated with laurate and myristate. The entire structure forms a hexa-acylated 1,4’-bis-phosphate lipid A molecule. In approximately one third of the lipid A population in *E. coli*, a pyrophosphoryl group is found in position 1 of the carbon backbone instead of the single phosphoryl group. Additional covalent modifications of lipid A that can be observed under certain conditions are indicated by the dashed lines: 4-amino-4-deoxy-L-arabinose (L-Ara4N), phosphoethanolamine (pEtN), and palmitate.

Figure 2. Cationic antimicrobial peptides (CAMPs) assay. The graphs show the minimal inhibitory concentration (MICs) of CAMPs necessary to inhibit the growth of the different strains: the ExPEC strains 5131 and \( \chi \)7122, their isogenic \( \text{pst} \) mutants K2/K3 and \( \text{pst} \) complemented strains CK2/CK3 respectively. The MIC was considered the lowest drug concentration that reduced growth >50% compared with growth in the control well. (A) polymyxin B MIC for strains grown in LB; (B) polymyxin B MIC for strains grown in LP; (C) cecropin P1 MIC for strains grown in LB; (D) killing assays using polymyxin for strains grown in LB. The polymyxin B and cecropin P1 MICs, for both \( \text{pst} \) mutant strains (K2 and K3) as well as their survival to polymyxin B, was significantly lower than for their respective parent strains (\( p < 0.05 \)). No statistical differences were observed when the LP medium was used for bacterial growth.

Figure 3. Analysis of radiolabeled lipid A by thin layer chromatography (TLC) of strains grown in high phosphate (LB) medium. The asterisks (*) mark the position of the hexa-acylated 1-pyrophosphate lipid A. (A) *E. coli* K12 MC1061 (lane 1) compared to the EHEC
O157:H7 strain (lane 2) as established by Kim and co-workers (2006) (24). (B) EHEC O157:H7 strain (lane 1), ExPEC strain 5131 (lane 2), the pst mutant K2 (lane 3), complemented pst mutant strain CK2 (lane 4). (C) Avian ExPEC strain χ7122 (lane 1), the pst mutant K3 (lane 2), complemented pst mutant strain CK3 (lane 3). (D) Analysis of radiolabeled lipid A by TLC of strains grown in low phosphate (LP) medium. ExPEC strain 5131 (lane 1), the pst mutant K2 (lane 2), complemented pst mutant strain CK2 (lane 3), avian ExPEC strain χ7122 (lane 4), the pst mutant K3 (lane 5), complemented pst mutant strain CK3 (lane 6).

Figure 4. Vancomycin assay. The graphs show vancomycin survival of the ExPEC strains 5131 and χ7122 and their pst mutant derivatives. Vancomycin survival was calculated from the ratio of vancomycin-exposed to non-exposed cells. The survival for both pst mutant strains, K2 and K3, was significantly lower than for their respective parent strains (p < 0.05). Complementation of the pst mutation restores the wild-type phenotype.

Figure 5. MALDI-TOF analyses of lipid A from the wild-type (A) and the pst mutant K3 (B) grown in LP medium. The prototype lipid A species (hexa-acylated 1,4'-bis-phosphate) corresponds to the major peak in both spectra, at a m/z of 1797. One variant that is visible in the K3 spectrum (*) includes the addition of pEtN residues to each of the 1 and 4' lipid A phosphate groups A (m/z: 2043).
<table>
<thead>
<tr>
<th>Bacterial strains or plasmids</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>Bacterial strains</strong></td>
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<tr>
<td>SM10λpir</td>
<td><em>thi-1, leu, tonA, lacY, supE, recA::RP4-2-Tc::Mu, λpir, Km</em></td>
<td>Miller and Mekalanos, 1988</td>
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<td>XL1-Blue</td>
<td>*recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac[F proAB lacI'ZΔM15 Tn10 (Tet')]</td>
<td>Stratagene</td>
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<td>5131</td>
<td>Porcine ExPEC</td>
<td>Fairbrother <em>et al.</em>, 1988</td>
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<td>K2</td>
<td>5131 Δpst::kan, Km*</td>
<td>This study</td>
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<td>CK2</td>
<td>K2 + pAN92, Km*, Cm*</td>
<td>This study</td>
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<tr>
<td>χ7122</td>
<td>APEC</td>
<td>Provence and Curtiss, 1992</td>
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<td>K3</td>
<td>χ7122 ΔpstCAB::kan</td>
<td>Lamarche <em>et al.</em>, 2005</td>
</tr>
<tr>
<td>CK3</td>
<td>K3 + pAN92</td>
<td>Lamarche <em>et al.</em>, 2005</td>
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<td><strong>Plasmids</strong></td>
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<td>pCR2.1</td>
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<tr>
<td>pKNG101</td>
<td>Suicide vector, Sm*, sacB</td>
<td>Kaniga <em>et al.</em>, 1991</td>
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<td>pKNG800K</td>
<td>pKNG101 - ΔpstCAB::kan, sacB, Sm*, Km*</td>
<td>Lamarche <em>et al.</em>, 2005</td>
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<tr>
<td>pAN92</td>
<td>pACYC184::pst operon, Cm*</td>
<td>Jans <em>et al.</em>, 1983</td>
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</table>

*Kanamycin, Km\*; Chloramphenicol, Cm\*; Streptomycin, Sm\*; Nalidixic acid, Nal\*; Tetracyclin, Tet\*. 
Table 2: LPS-related genes identified by microarray experiments using Affymetrix GeneChip® E. coli Genome 2.0 array. Also represented: some of the gene candidates selected for a potential role in the C-1 pyrophosphate defect observed in *pst* mutants. Shown are the up- or down-regulated genes in the *pst* mutant K3 compared to the wild-type strain χ7122 for strains grown in high phosphate (LB) medium.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Gene functions (or putative functions)</th>
<th>Genomic context</th>
<th>Ratio variation (log 2)</th>
<th>P-value</th>
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<tr>
<td><strong>ECA biosynthesis</strong></td>
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<td><em>wecD</em> / <em>rffC</em></td>
<td>TDP-fucosamine acetyltransferase involved in the biosynthesis of Enterobacterial Common Antigen (ECA).</td>
<td><em>rfe-wzzE-rffEDGHCA-wzzE-SBI operon</em></td>
<td>-0.729 2.03 X 10⁻³</td>
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<td></td>
<td>Enterobacterial Common Antigen (ECA).</td>
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<td><em>rffH</em></td>
<td>dTDP-glucose pyrophosphorylase 2 involved in the biosynthesis of Enterobacterial Common Antigen (ECA).</td>
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<td>-0.592 9.94 X 10⁻⁴</td>
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<td><em>rffG</em></td>
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<td>-0.952 1.19 X 10⁻⁴</td>
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<tr>
<td><em>wecC</em> / <em>rffD</em></td>
<td>UDP-N-acetyl-D-mannosamine dehydrogenase / UDP-N-acetyl-D-mannosaminuronic acid dehydrogenase involved</td>
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<td>-0.872 2.06 X 10⁻⁴</td>
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<td>in the biosynthesis of Enterobacterial Common Antigen (ECA).</td>
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<td><strong>LPS-core biosynthesis</strong></td>
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<td><em>rfaJ</em></td>
<td>Lipopolysaccharide 1,2-glucosyltransferase. <em>RfaJ</em> adds the third glucose (GlcIII) to the second glucose</td>
<td><em>rfaQGQPSBIJYZ-waaU operon</em></td>
<td>-0.751 6.73 X 10⁻³</td>
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<td></td>
<td>(GlcII) of the outer core of LPS.</td>
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<tr>
<td><strong>Covalent modification of the lipid A</strong></td>
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<tr>
<td><em>eptA</em></td>
<td>S. typhymurium pmrC Ortholog: phosphoethanolamine (pEtN) transferase that use phosphatidylethanolamine as a donor of pEtN for covalent modification of the lipid A.</td>
<td>Likely in operon with <em>basRS</em> (<em>eptA-basRS</em>)</td>
<td>-0.745 1.06 X 10⁻²</td>
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<tr>
<td><strong>Gene candidates selected for a potential role in the C-1 pyrophosphate defect observed in <em>pst</em> mutants</strong></td>
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<tr>
<td><em>cdh</em></td>
<td>CDP-diacylglycerol phosphatidylhydrodrolase</td>
<td>monocistron</td>
<td>+ 2.099 1.49 X 10⁻⁶⁵</td>
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<td><em>yeiU</em></td>
<td>Hypothetical protein / Undecaprenyl pyrophosphate phosphatase</td>
<td><em>yeiRU operon</em></td>
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<tr>
<td><em>ydhJ</em></td>
<td>Hypothetical protein / Undecaprenyl pyrophosphate phosphatase</td>
<td><em>ydhIJK operon</em></td>
<td>-1.038 2.91 X 10⁻⁶⁵</td>
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</table>

*Pho box(es) are present in the promoter region of bolded genes.