JOURNAL OF BACTERIOLOGY, Aug. 2008, p. 5256–5264 Vol. 190, No. 15
0021-9193/08/$08.00+0 doi:10.1128/JB.01536-07

Modulation of Hexa-Acyl Pyrophosphate Lipid A Population under *Escherichia coli* Phosphate (Pho) Regulon Activation▽

Martin G. Lamarche,[1] Sang-Hyun Kim,[2,3] Sébastien Crépin,[1] Michael Mourez,[1] Nicolas Bertrand,[1] Russell E. Bishop,[2] J. Daniel Dubreuil,[1] and Josée Harel[1]*

Groupe de Recherche sur les Maladies Infectieuses du Porc, Université de Montréal, Faculté de Médecine Vétérinaire, C.P. 5000, Saint-Hyacinthe, Québec, Canada J2S 7C6[1]; Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Ontario, Canada L8N 3Z5[2]; and The National Primate Research Center, KRIBB, OChang, Chungbuk 363-883, Republic of Korea[3]

Received 24 September 2007/Accepted 22 May 2008

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 extraintestinal 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.

Under circumstances such as drastic environmental changes, the phenotypic diversity occurring within a bacterial cell population due to noise in gene expression favors the natural selection of subpopulations best fit to survive (37, 39, 40, 54). In addition, bacteria possess more specialized machineries, the two-component regulatory systems, which permit sensing and response to environmental changes (20). Environmental stimuli are translated into signaling 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 (P_i) through interaction with the phosphate-specific transport (Pst) system. The activation signal is a P_i 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 P_i. 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 reference 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 extraintestinal pathogenic *E. coli* (ExPEC) strains. χ7122 (O78:K80) and 5131 (O115:K"V165") are ExPEC strains that share many virulence attributes and cause extraintestinal infections in poultry and swine, respectively (19, 33). Pleiotropic effects and loss of virulence were observed in *pst* mutants of extraintestinal 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). 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 diglucosamine structure (Fig. 1). The remaining one-third possess a pyrophosphoryl group at the C-1

* Corresponding author. Mailing address: Groupe de Recherche sur les Maladies Infectieuses du Porc, Université de Montréal, Faculté de Médecine Vétérinaire, C.P. 5000, Saint-Hyacinthe, Québec, Canada J2S 7C6. Phone: (450) 773 8521, ext. 8233. Fax: (450) 778 8108. E-mail: josee.harel@umontreal.ca.

▽ Published ahead of print on 30 May 2008.

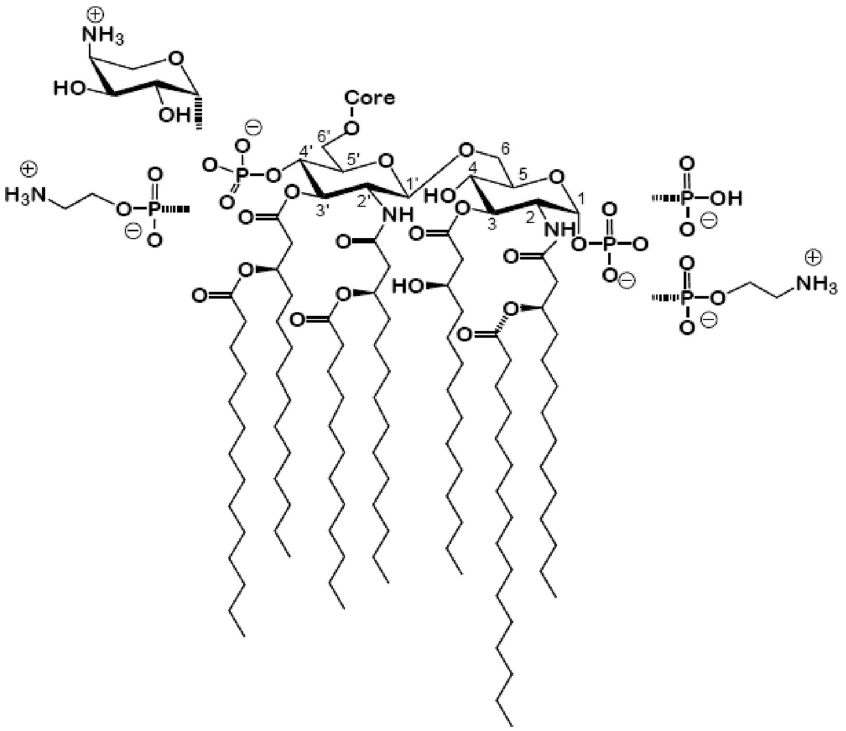

FIG. 1. *E. coli* lipid A structure. Two glucosamine units are linked by a β 1′-6 glycosidic bond to compose the lipid A backbone. Each glucosamine unit bears a phosphoryl group and two principal *R*-3-hydroxymyristoyl chains. The acyl chains of the distal glucosamine unit are further acylated with laurate 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, pEtN, and palmitate.

position (Fig. 1). Bacterial pathogens such as *S. enterica* serovar Typhimurium and some *E. coli* strains 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. The *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 medium. LP broth is a low-phosphate medium consisting 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. All experiments were 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 medium was used (data not shown). When required, antibiotics were used at the following final concentrations: tetracycline, 10 μg/ml; kanamycin, 50 μg/ml; nalidixic acid, 40 μg/ml; chloramphenicol, 10 μg/ml; and streptomycin, 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 (strains K2 and K3, respectively) were confirmed to contain a deletion in the *pst* operon as determined by PCR amplification, Southern blot hybridization, and reverse transcription-PCR (RT-PCR) (25 and data not shown). The pAN92 (23) plasmid carrying the functional *pst* operon was used to complement the Δ*pstCAB* mutants to create strains CK2 and CK3.

MIC assays using PMB, CP1, and vancomycin. Experiments were done in LB or LP medium for bacterial cell 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 10^6 CFU/ml. Microwell plates were loaded with the 10^6-CFU/ml cultures, and different concentrations of PMB or cecropin P1 (CP1) were added to each well. Plates were incubated overnight, and wells were evaluated for growth by spectrophotometry. For vancomycin assays, plates were incubated for 3 h. 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 nonexposed cells. Experiments are the mean of three biological and technical replicates. Statistical analyses were performed using the GraphPad Prism 4 package. A one-way analysis of variance followed by a multiple-comparison Tukey's test was applied.

Killing assays using PMB. 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 medium. Cells (200 μl) were mixed on a microtiter plate with various concentrations of PMB (0.125, 0.25, 0.375, and 0.5 μg/ml) and incubated at 37°C for 30 min. Then, 150 μl of PMB-treated cells was 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 survival was defined as

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Strains		
SM10λ*pir*	*thi-1 leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pir* Km^r	28a
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[F′ *proAB lacI*^{q}*Z*ΔM15 Tn*10* (Tet^r)]	Stratagene
5131	Porcine ExPEC O115:K"V165":F165	12a
K2	5131 Δ*pst*::*kan* Km^r	This study
CK2	K2 + pAN92; Km^r Cm^r	This study
χ7122	APEC O78:K80:H9 *gyrA* Nal^r	33
K3	χ7122 Δ*pstCAB*::*kan*	25
CK3	K3 + pAN92	25
Plasmids		
pCR2.1	Cloning vector	Invitrogen
pKNG101	Suicide vector; Sm^r *sacB*	23a
pKNG800K	pKNG101::*pstCAB*::*kan*; *sacB* Sm^r Km^r	25
pAN92	pACYC184::*pst* operon; Cm^r	23

[a] Resistance abbreviations: Km^r, kanamycin; Cm^r, chloramphenicol; Sm^r, streptomycin; Nal^r, nalidixic acid, and Tet^r, tetracycline.

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 analysis of variance followed by a multiple-comparison Tukey's test was applied.

Analysis of radiolabeled lipid A by TLC. Experiments were performed in LB or LP medium for bacterial cell growth. The method used for analysis of lipid A released by mild acid hydrolysis from $^{32}P_i$-labeled cells was adapted from Zhou et al. (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 3 h. The $^{32}P_i$-labeled cells were harvested by centrifugation and washed once with 5 ml of phosphate-buffered saline (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 min of 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 [vol/vol]). This pellet was then dispersed in 1.8 ml of 12.5 mM sodium acetate (pH 4.5), containing 1% sodium dodecyl sulfate (SDS), with sonic irradiation in an ultrasonic bath. The mixture was incubated at 100°C for 30 min to cleave the ketosidic linkage between the first inner core 3-deoxy-D-*manno*-oct-2-ulosonic 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 [vol/vol]). The lower-phase lipid A sample was collected and dried under a steam of nitrogen gas. The lipid A sample was dissolved in 100 μl of chloroform-methanol (4:1 [vol/vol]), and 1,000 cpm of the sample was applied to the origin of a silica gel 60 thin-layer chromatography (TLC) plate. TLC was conducted in a developing tank by using the solvent chloroform–pyridine–88% formic acid–water (50:50:16:5 [vol/vol]). 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, an overnight culture grown at 37°C was diluted 100-fold into 5 ml of LB and was allowed to grow to mid-log phase (optical density at 600 nm of 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 I 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 (1 mg/ml), the cDNAs were purified using a Microcon YM-30 centrifugal filter (Milipore, Billerica, MA). The cDNAs were fragmented with DNase I (Ambion, Austin, TX) and were 3′ biotinylated using GeneChip DNA labeling 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 additional information, see the Affymetrix website 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 for normalization, background correction, and expression value calculation (22). Expression levels obtained from three independent replicates, under each condition, were compared using FlexArray RC3 software (M. Blazejczyk, M. Miron, R. Nadon, Genome Quebec, Montreal, Canada; http://genomequebec.mcgill.ca/FlexArray/). The robustness of the data was further enhanced by EB algorithm and *P* value calculation. The microarrays results were validated by quantitative RT (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 (C_T) was normalized to the C_T obtained for the *tus* gene amplified from the corresponding sample. Fold changes were calculated using the $2^{-\Delta\Delta C_T}$ 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 $^{32}P_i$. 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 min at room temperature. Then, 30 μl of chloroform was added, and the samples were vortexed vigorously and incubated for 15 min at room temperature. The phases were separated by centrifugation at 12,000 × *g* for 10 min, and the upper phase was transferred to a new tube. This extraction procedure was repeated twice using 100 μl of water instead of chloroform, and the combined upper phases were evaporated in a 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), heated at 100°C for 1 h, and dried in a SpeedVac. 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 4 M HCl in 20 ml of 95% ethanol) and centrifuged at 2,060 × *g* for 10 min. 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 min, and lipid A was dissolved in 100 μl of chloroform-methanol-water (2:3:1 [vol/vol]). The samples were sent to the Proteomic and Mass Spectrometry Center, Molecular Medicine Research Centre, University of Toronto, for 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 [vol/vol]) 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 tempera-

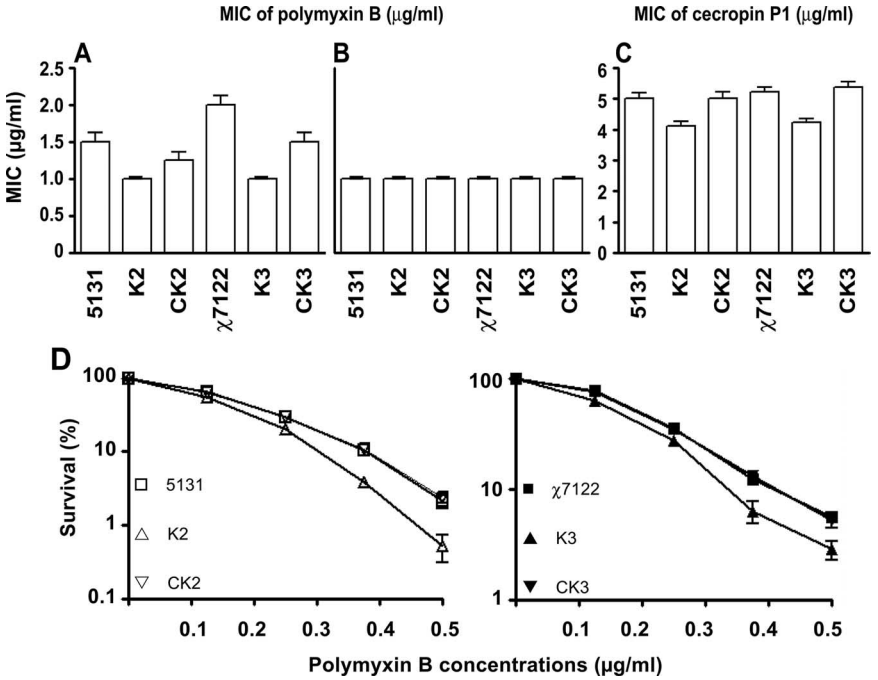

FIG. 2. CAMP assays. The graphs show the MIC of CAMPs necessary to inhibit the growth of the different strains: the ExPEC strains 5131 and χ7122, their isogenic *pst* mutants K2 and K3, and *pst*-complemented strains CK2 and CK3, respectively. The MIC was considered the lowest drug concentration that reduced growth >50% compared with growth in the control well. (A) PMB MIC for strains grown in LB medium. (B) PMB MIC for strains grown in LP medium. (C) CP1 MIC for strains grown in LB medium. (D) Killing assays using polymyxin for strains grown in LB medium. The PMB and CP1 MICs for both *pst* mutant strains (K2 and K3), as well as their survival of PMB, were significantly lower than those for their respective parent strains ($P < 0.05$). No statistical differences were observed when LP medium was used for bacterial growth.

ture. Spectra were acquired in both positive- and negative-ion linear modes. Each spectrum was the average of 50 laser shots.

RESULTS

CAMP 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 (Fig. 2A and C). 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 with 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 the wild-type resistance (Fig. 2D). Also, as observed in MIC assays, the porcine ExPEC strain 5131 is less resistant to PMB than the avian pathogenic *E. coli* (APEC) strain χ7122 (Fig. 2A and D).

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* K-12 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; Fig. 1) is reproducibly observed (Fig. 3B and C), 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 Δ*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 LP 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 LP medium. In LP medium, all strains have the same PMB MIC. Furthermore, wild-type and complemented *pst* mutant strains have a PMB MIC similar to that of *pst* mutants grown in LB medium (Fig. 2B). Strikingly, all strains exhibit a similar amount of the hexa-acylated 1-pyrophosphate form of lipid A

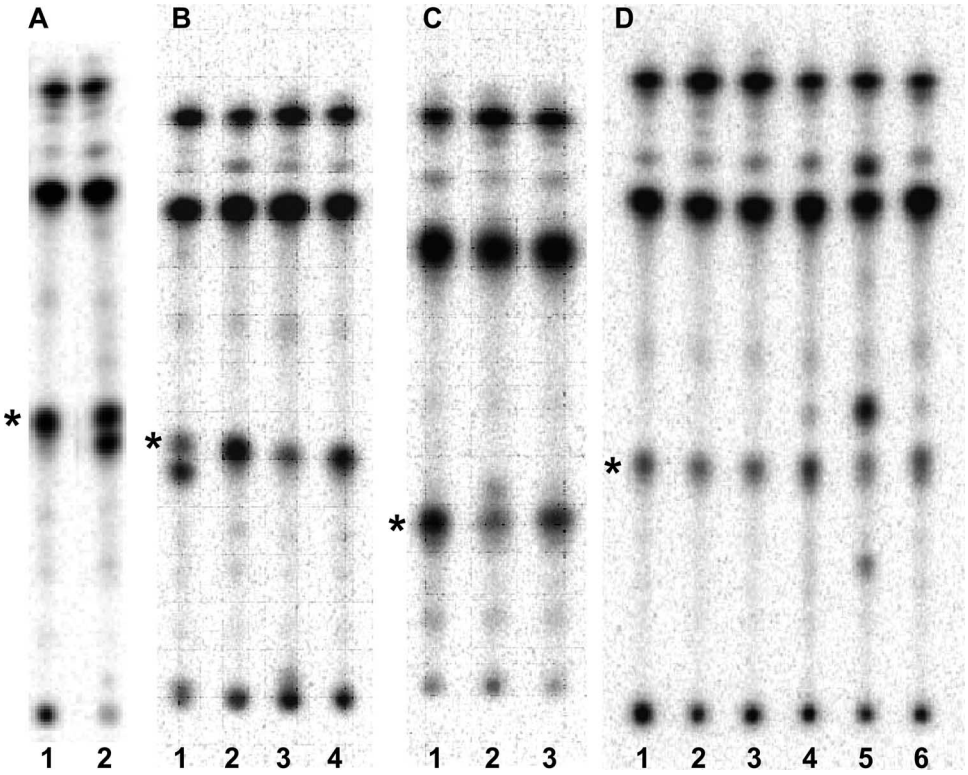

FIG. 3. Analysis of radiolabeled lipid A by TLC of strains grown in LB medium. The asterisks (*) mark the position of the hexa-acylated 1-pyrophosphate lipid A. (A) *E. coli* K-12 MC1061 (lane 1) compared to the EHEC O157:H7 strain (lane 2), as established by Kim and coworkers (24). (B) EHEC O157:H7 strain (lane 1), ExPEC strain 5131 (lane 2), the *pst* mutant K2 (lane 3), and complemented *pst* mutant strain CK2 (lane 4). (C) Avian ExPEC strain χ7122 (lane 1), the *pst* mutant K3 (lane 2), and complemented *pst* mutant strain CK3 (lane 3). (D) Analysis of radiolabeled lipid A by TLC of strains grown in LP medium. Shown are 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), and complemented *pst* mutant strain CK3 (lane 6).

(Fig. 3D). Furthermore, these amounts are similar to those obtained for *pst* mutants grown in LB medium (Fig. 3B and C). 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. enterica* serovar 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 compared to that of 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* 1,797) bearing two pEtN residues (*m*/*z* 2,043) was identified (Fig. 5B). Other ions are also present in the K3 strain (*m*/*z* 1,996 and 2,108), but these species do not correspond to known lipid A structures. MALDI-TOF spectra obtained for χ7122 and K3 lipid A, when strains were grown in LB medium, are similar to the spectrum of χ7122 lipid A when LP medium is used for growth (data not shown).

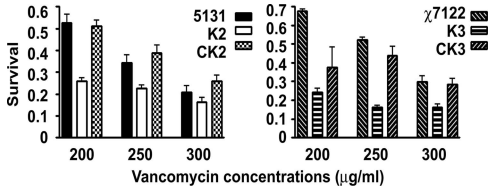

FIG. 4. Vancomycin assay. The graphs show vancomycin survival of the ExPEC strains 5131 and χ7122 and their *pst* mutant derivatives. Vancomycin survival was calculated from the ratio of vancomycin-exposed to nonexposed cells. The survival rates for both *pst* mutant strains, K2 and K3, were significantly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

Microarray analysis. A microarray study of the wild-type strain χ7122 and the *pst* mutant K3 grown in the high-phosphate (LB) medium was used to analyze LPS-related genes at the transcription level (Table 2). All LPS-related genes identified are downregulated in the *pst* mutant K3, in contrast to the wild-type strain. Many of the genes identified belong to the enterobacterial common antigen (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 of the *wzxE* and *wzyE* genes, as determined by using the Pho box prediction weight matrix elaborated by Finan and colleagues (53). These genes code, respectively, for

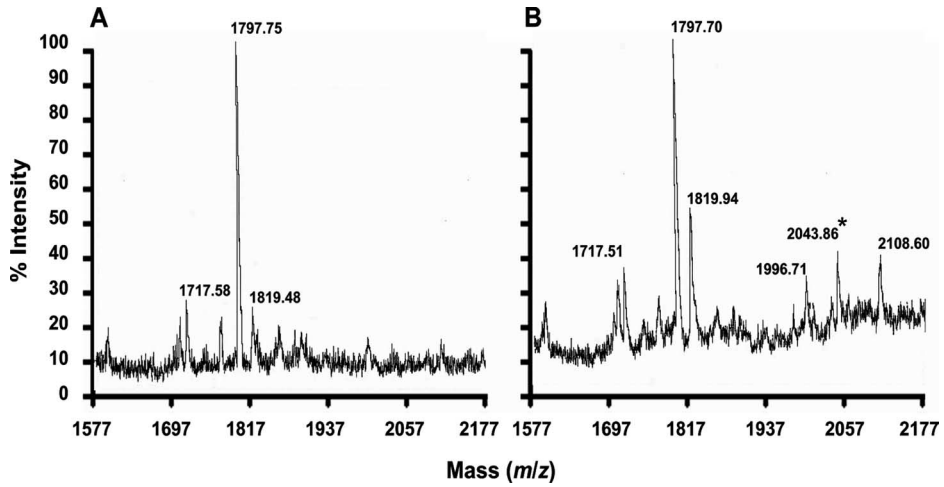

FIG. 5. MALDI-TOF analyses of lipid A from the wild type (A) and the *pst* mutant K3 (B) grown in LP medium. The prototype lipid A species (hexa-acylated 1,4′-bis-phosphate) corresponds to the major peak in both spectra, at *m/z* 1,797. One variant that is visible in the K3 spectrum (*) includes the addition of pEtN residues to each of the 1′ and 4′ lipid A phosphate groups (*m/z* 2,043).

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 *rfaJ* gene belonging to *rfaQGPSBIJYZ-waaU* operon is also downregulated. The *rfaJ* gene coding for an LPS 1,2-glucosyltransferase is involved in the LPS core biosynthesis (Table 2). A Pho box is located upstream of the *rfaJ* gene as well as just upstream of the

TABLE 2. LPS-related genes identified by microarray experiments using Affymetrix GeneChip *E. coli* genome 2.0 array[a]

Gene(s)	Gene function (or putative functions)	Genomic context[b]	Ratio variation (log_2)	*P* value
ECA biosynthesis				
wecD/*rffC*	TDP-fucosamine acetyltransferase involved in biosynthesis of ECA	*rfe-wzzE-rffEDGHCA-**wzxE**-rffT-**wzyE**-rffM* operon	−0.729	2.03×10^{-3}
rffH	dTDP-glucose pyrophosphorylase 2 involved in biosynthesis of ECA		−0.592	9.94×10^{-4}
rffG	dTDP-glucose 4,6-dehydratase involved in biosynthesis of ECA		−0.952	1.19×10^{-4}
wecC/*rffD*	UDP-*N*-acetyl-D-mannosamine dehydrogenase/UDP-*N*-acetyl-D-mannosaminuronic acid dehydrogenase involved in biosynthesis of ECA		−0.872	2.06×10^{-4}
LPS core biosynthesis				
rfaJ	LPS 1,2-glucosyltransferase; RfaJ adds 3rd glucose (GlcIII) to 2nd glucose (GlcII) of LPS outer core	*rfaQG**P**SBI**J**YZ-waaU* operon	−0.751	6.73×10^{-3}
Covalent modification of lipid A				
eptA	*S. enterica* serovar Typhimurium *pmrC* ortholog, pEtN transferase that uses phosphatidylethanolamine as donor of pEtN for covalent modification of lipid A	Likely in operon with *basRS* (*eptA-basRS*)	−0.745	1.06×10^{-2}
Potential role in C-1 pyrophosphate defect[c]				
cdh	CDP-diacylglycerol phosphotidylhydrolase	Monocistron	+2.099	1.49×10^{-5}
yeiU	Hypothetical protein/undecaprenyl pyrophosphate phosphatase	*yeiRU* operon	−0.653	7.31×10^{-3}
ydhJ	Hypothetical protein/undecaprenyl pyrophosphate phosphatase	*ydhIJK* operon	−1.038	2.91×10^{-5}

[a] Shown are the up- or down-regulated genes in the *pst* mutant K3 compared to the wild-type strain χ7122 for strains grown in LB medium.
[b] Pho boxes are present upstream of boldface genes.
[c] Gene candidates selected for a potential role in the C-1 pyrophosphate defect observed in *pst* mutants.

starting codon of the *rfaP* and *rfaY* genes, which are both kinases involved in LPS inner-core phosphorylation. Concerning the lipid A biosynthetic gene expression, only the *eptA* gene is downregulated. This gene is an ortholog of *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 *cdh* encoding a CDP-diglyceride pyrophosphatase (34) is upregulated in the *pst* mutant K3. Cdh was shown to cleave a phosphoryl group from UDP-2,3-diacylglucosamine in vitro, but a distinct enzyme, LpxH, performs this function in vivo (2, 8). The in vivo function of Cdh is still unknown (7, 9, 21, 34, 36). Additionally, genes *ydhJ* and *yeiU*, which are downregulated in the *pst* mutant K3, encode undecaprenyl pyrophosphate phosphatases that remove phosphoryl groups from inner membrane phosphate-lipid carriers. The *yeiU* (renamed *lpxT*) gene product was recently shown to be responsible for the synthesis of the pyrophosphate form of lipid A, as demonstrated by Trent and colleagues (see Discussion) (43). The *yeiU* gene is located downstream of *yeiR* and forms an operon with this gene. No Pho boxes were found in the promoter region of *cdh*, *yeiR*, and *ydhJ* genes. qRT-PCR experiments ($\log_2$ changes [fold], +1.6, −1.3, and −2.6 for *cdh*, *ydhJ*, and *yeiU*, respectively) confirmed our microarray results (Table 2). qRT-PCR on 15 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 (LB) medium. 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 could decrease affinity of PMB toward lipid A. 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 component 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 LP medium. In *pst* mutants, the Pho regulon is constitutively activated, and this mimics the induced state of wild-type strains. In LP medium, 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 LB medium (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 LP medium. In LP medium, 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*), which is downregulated 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 amounts 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-polyacrylamide gel elctrophoresis and Western blot analyses (25; unpublished results), our microarray results suggest that molecular changes could indeed occur within the LPS structure in the *pst* mutant strains. Downregulation 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* K-12, covalent modifications have been observed in PMB-resistant strains and in cells treated with ammonium

metavanadate (a nonspecific inhibitor of mammalian phosphatases) (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* K-12, we hypothesized that lipid A variants observed in the K3 strain could correspond to substitutions identified by Zhou et al. (55). 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* 2,043) (Fig. 5B), which was previously identified by Zhou and coworkers in their studies (55). 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 (M. G. Lamarche et al., unpublished data). All of 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.

ACKNOWLEDGMENTS

We are grateful to Turlough Finan and Dick Morton, McMaster University, and Ying Fong, Université d'Ottawa, for kindly providing the *E. coli* Pho box predictions. We thank François Lepine for helpful discussion and initial experimentations. We also thank Mahendrasingh Ramjeet, Faculté de Médecine Vétérinaire, Université de Montréal, for technical advice during this study. We are grateful to France Daigle for construction of the *pst* mutant K2. We are also grateful to Éliane Auger for revisions of the manuscript.

M. G. Lamarche was supported by the Fonds Québécois de la Recherche sur la Nature et les Technologies. This work was supported by grants to J. Harel from the Natural Sciences and Engineering Research Council of Canada (RGPIN-25120) and to R. E. Bishop from CIHR (MOP-84329).

REFERENCES

1. **Allen, N. E., and T. I. Nicas.** 2003. Mechanism of action of oritavancin and related glycopeptide antibiotics. FEMS Microbiol. Rev. **26:**511–532.
2. **Babinski, K. J., S. J. Kanjilal, and C. R. Raetz.** 2002. Accumulation of the lipid A precursor UDP-2,3-diacylglucosamine in an *Escherichia coli* mutant lacking the *lpxH* gene. J. Biol. Chem. **277:**25947–25956.
3. **Bishop, R. E.** 2005. Fundamentals of endotoxin structure and function. Contrib. Microbiol. **12:**1–27.
4. **Bishop, R. E., S. H. Kim, and A. El Zoeiby.** 2005. Role of lipid A palmitoylation in bacterial pathogenesis. J. Endotoxin Res. **11:**174–180.
5. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37:**911–917.
6. **Breazeale, S. D., A. A. Ribeiro, and C. R. Raetz.** 2003. Origin of lipid A species modified with 4-amino-4-deoxy-L-arabinose in polymyxin-resistant mutants of *Escherichia coli*. An aminotransferase (ArnB) that generates UDP-4-deoxyl-L-arabinose. J. Biol. Chem. **278:**24731–24739.
7. **Bulawa, C. E., J. D. Hermes, and C. R. Raetz.** 1983. Chloroform-soluble nucleotides in *Escherichia coli*. Role of CDP-diglyceride in the enzymatic cytidylylation of phosphomonoester acceptors. J. Biol. Chem. **258:**14974–14980.
8. **Bulawa, C. E., and C. R. Raetz.** 1984. The biosynthesis of gram-negative endotoxin. Identification and function of UDP-2,3-diacylglucosamine in *Escherichia coli*. J. Biol. Chem. **259:**4846–4851.
9. **Bulawa, C. E., and C. R. Raetz.** 1984. Isolation and characterization of *Escherichia coli* strains defective in CDP-diglyceride hydrolase. J. Biol. Chem. **259:**11257–11264.
10. **Daigle, F., J. M. Fairbrother, and J. Harel.** 1995. Identification of a mutation in the *pst-phoU* operon that reduces pathogenicity of an *Escherichia coli* strain causing septicemia in pigs. Infect. Immun. **63:**4924–4927.
11. **Echols, H., A. Garen, S. Garen, and A. Torriani.** 1961. Genetic control of repression of alkaline phosphatase in *E. coli*. J. Mol. Biol. **3:**425–438.
12. **El Ghachi, M., A. Derbise, A. Bouhss, and D. Mengin-Lecreulx.** 2005. Identification of multiple genes encoding membrane proteins with undecaprenyl pyrophosphate phosphatase (UppP) activity in *Escherichia coli*. J. Biol. Chem. **280:**18689–18695.

12a. **Fairbrother, J. M., A. Broes, M. Jacques, and S. Lariviere.** 1989. Pathogenicity of *Escherichia coli* O115:K"V165" strains isolated from pigs with diarrhea. Am. J. Vet. Res. **50:**1029–1036.

13. **Frirdich, E., and C. Whitfield.** 2005. Lipopolysaccharide inner core oligosaccharide structure and outer membrane stability in human pathogens belonging to the Enterobacteriaceae. J. Endotoxin. Res. **11:**133–144.
14. **Giacometti, A., O. Cirioni, R. Ghiselli, L. Goffi, F. Mocchegiani, A. Riva, G. Scalise, and V. Saba.** 2000. Polycationic peptides as prophylactic agents against methicillin-susceptible or methicillin-resistant *Staphylococcus epidermidis* vascular graft infection. Antimicrob. Agents Chemother. **44:**3306–3309.
15. **Groisman, E. A., J. Kayser, and F. C. Soncini.** 1997. Regulation of polymyxin resistance and adaptation to low-Mg^{2+} environments. J. Bacteriol. **179:**7040–7045.
16. **Gunn, J. S., K. B. Lim, J. Krueger, K. Kim, L. Guo, M. Hackett, and S. I. Miller.** 1998. PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. Mol. Microbiol. **27:**1171–1182.
17. **Gunn, J. S., and S. I. Miller.** 1996. PhoP-PhoQ activates transcription of *pmrAB*, encoding a two-component regulatory system involved in *Salmonella typhimurium* antimicrobial peptide resistance. J. Bacteriol. **178:**6857–6864.
18. **Guo, L., K. B. Lim, J. S. Gunn, B. Bainbridge, R. P. Darveau, M. Hackett, and S. I. Miller.** 1997. Regulation of lipid A modifications by *Salmonella typhimurium* virulence genes *phoP-phoQ*. Science **276:**250–253.
19. **Harel, J., C. Forget, M. Ngeleka, M. Jacques, and J. M. Fairbrother.** 1992. Isolation and characterization of adhesin-defective Tn*phoA* mutants of septicaemic porcine *Escherichia coli* of serotype O115:K−:F165. J. Gen. Microbiol. **138:**2337–2345.
20. **Hoch, J. A., and T. J. Silhavy (ed.).** 1995. Two-component signal transduction. American Society for Microbiology, Washington, DC.
21. **Icho, T., C. E. Bulawa, and C. R. Raetz.** 1985. Molecular cloning and sequencing of the gene for CDP-diglyceride hydrolase of *Escherichia coli*. J. Biol. Chem. **260:**12092–12098.
22. **Irizarry, R. A., B. Hobbs, F. Collin, Y. D. Beazer-Barclay, K. J. Antonellis, U. Scherf, and T. P. Speed.** 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics **4:**249–264.
23. **Jans, D. A., A. L. Fimmel, L. Langman, L. B. James, J. A. Downie, A. E. Senior, G. R. Ash, F. Gibson, and G. B. Cox.** 1983. Mutations in the *uncE* gene affecting assembly of the c-subunit of the adenosine triphosphatase of *Escherichia coli*. Biochem. J. **211:**717–726.

23a. **Kaniga, K., I. Delor, and G. R. Cornelis.** 1991. A wide-host-range suicide vector for improving reverse genetics in gram-negative bacteria: inactivation of the *blaA* gene of *Yersinia enterocolitica*. Gene **109:**137–141.

24. **Kim, S. H., W. Jia, V. R. Parreira, R. E. Bishop, and C. L. Gyles.** 2006. Phosphoethanolamine substitution in the lipid A of *Escherichia coli* O157:H7 and its association with PmrC. Microbiology **152:**657–666.
25. **Lamarche, M. G., C. M. Dozois, F. Daigle, M. Caza, R. Curtiss III, J. D. Dubreuil, and J. Harel.** 2005. Inactivation of the *pst* system reduces the virulence of an avian pathogenic *Escherichia coli* O78 strain. Infect. Immun. **73:**4138–4145.
26. **Lamarche, M. G., B. L. Wanner, S. Crépin, and J. Harel.** 2008. The Pho regulon and bacterial virulence: a regulatory network connecting phosphate homeostasis and pathogenesis. FEMS. Microbiol. Rev. **32:**461–473.
27. **Lee, H., F.-F. Hsu, J. Turk, and E. A. Groisman.** 2004. The PmrA-regulated *pmrC* gene mediates phosphoethanolamine modification of lipid A and polymyxin resistance in *Salmonella enterica*. J. Bacteriol. **186:**4124–4133.
28. **Livak, K. J., and T. D. Schmittgen.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods **25:**402–408.

28a. **Miller, V. L., and J. J. Mekalanos.** 1998. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane

proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170:**2575–2583.
29. **Ngeleka, M., J. Harel, M. Jacques, and J. M. Fairbrother.** 1992. Characterization of a polysaccharide capsular antigen of septicemic *Escherichia coli* O115:K"V165":F165 and evaluation of its role in pathogenicity. Infect. Immun. **60:**5048–5056.
30. **Nikaido, H.** 2003. Molecular basis of bacterial outer membrane permeability revisited. Microbiol. Mol. Biol. Rev. **67:**593–656.
31. **Nikaido, H., and M. Vaara.** 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. **49:**1–32.
32. **Nummila, K., I. Kilpelainen, U. Zahringer, M. Vaara, and I. M. Helander.** 1995. Lipopolysaccharides of polymyxin B-resistant mutants of *Escherichia coli* are extensively substituted by 2-aminoethyl pyrophosphate and contain aminoarabinose in lipid A. Mol. Microbiol. **16:**271–278.
33. **Provence, D. L., and R. Curtiss III.** 1992. Role of *crl* in avian pathogenic *Escherichia coli*: a knockout mutation of *crl* does not affect hemagglutination activity, fibronectin binding, or curli production. Infect. Immun. **60:**4460–4467.
34. **Raetz, C. R., C. B. Hirschberg, W. Dowhan, W. T. Wickner, and E. P. Kennedy.** 1972. A membrane-bound pyrophosphatase in *Escherichia coli* catalyzing the hydrolysis of cytidine diphosphate-diglyceride. J. Biol. Chem. **247:**2245–2247.
35. **Raetz, C. R., C. M. Reynolds, M. S. Trent, and R. E. Bishop.** 2007. Lipid A modification systems in gram-negative bacteria. Annu. Rev. Biochem. **76:**295–329.
36. **Raetz, C. R. H.** 1996. Bacterial lipopolysaccharides: a remarkable family of bioactive macroamphiphiles, p. 1035–1063. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed., vol. 1. American Society for Microbiology, Washington, DC.
37. **Raser, J. M., and E. K. O'Shea.** 2005. Noise in gene expression: origins, consequences, and control Science. **309:**2010–2013.
38. **Rida, S., J. Caillet, and J.-H. Alix.** 1996. Amplification of a novel gene, *sanA*, abolishes a vancomycin-sensitive defect in *Escherichia coli*. J. Bacteriol. **178:**94–102.
39. **Rosenfeld, N., J. W. Young, U. Alon, P. S. Swain, and M. B. Elowitz.** 2005. Gene regulation at the single-cell level. Science **307:**1962–1965.
40. **Smits, W. K., O. P. Kuipers, and J. W. Veening.** 2006. Phenotypic variation in bacteria: the role of feedback regulation. Nat. Rev. Microbiol. **4:**259–271.
41. **Tamayo, R., B. Choudhury, A. Septer, M. Merighi, R. Carlson, and J. S. Gunn.** 2005. Identification of *cptA*, a PmrA-regulated locus required for phosphoethanolamine modification of the *Salmonella enterica* serovar Typhimurium lipopolysaccharide core. J. Bacteriol. **187:**3391–3399.
42. **Tamayo, R., S. S. Ryan, A. J. McCoy, and J. S. Gunn.** 2002. Identification and genetic characterization of PmrA-regulated genes and genes involved in polymyxin B resistance in *Salmonella enterica* serovar Typhimurium. Infect. Immun. **70:**6770–6778.
43. **Touze, T., A. X. Tran, J. V. Hankins, D. Mengin-Lecreulx, and M. S. Trent.** 2008. Periplasmic phosphorylation of lipid A is linked to the synthesis of undecaprenyl phosphate. Mol. Microbiol. **67:**264–277.
44. **Tran, A. X., J. D. Whittimore, P. B. Wyrick, S. C. McGrath, R. J. Cotter, and M. S. Trent.** 2006. The lipid A 1-phosphatase of *Helicobacter pylori* is required for resistance to the antimicrobial peptide polymyxin. J. Bacteriol. **188:**4531–4541.
45. **Trent, M. S.** 2004. Biosynthesis, transport, and modification of lipid A. Biochem. Cell Biol. **82:**71–86.
46. **Valvano, M. A.** 2008. Undecaprenyl phosphate recycling comes out of age. Mol. Microbiol. **67:**232–235.
47. **Wanner, B. L.** 1997. Phosphate signaling and the control of gene expression in *Escherichia coli*, p. 104–128. *In* S. W. Silver and W. Walden (ed.), Metal ions in gene regulation. Chapman and Hall, Ltd., Sterling, VA.
48. **Wanner, B. L.** 1996. Phosphorus assimilation and control of the phosphate regulon, p. 1357–1381. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed., vol. 1. American Society for Microbiology, Washington, DC.
49. **Willsky, G. R., R. L. Bennett, and M. H. Malamy.** 1973. Inorganic phosphate transport in *Escherichia coli*: involvement of two genes which play a role in alkaline phosphatase regulation. J. Bacteriol. **113:**529–539.
50. **Yethon, J. A., J. S. Gunn, R. K. Ernst, S. I. Miller, L. Laroche, D. Malo, and C. Whitfield.** 2000. *Salmonella enterica* serovar Typhimurium *waaP* mutants show increased susceptibility to polymyxin and loss of virulence in vivo. Infect. Immun. **68:**4485–4491.
51. **Yethon, J. A., and C. Whitfield.** 2001. Purification and characterization of WaaP from *Escherichia coli*, a lipopolysaccharide kinase essential for outer membrane stability. J. Biol. Chem. **276:**5498–5504.
52. **Yi, E. C., and M. Hackett.** 2000. Rapid isolation method for lipopolysaccharide and lipid A from gram-negative bacteria. Analyst **125:**651–656.
53. **Yuan, Z. C., R. Zaheer, R. Morton, and T. M. Finan.** 2006. Genome prediction of PhoB regulated promoters in *Sinorhizobium meliloti* and twelve proteobacteria. Nucleic Acids Res. **34:**2686–2697.
54. **Zhou, L., G. Grégori, J. M. Blackman, J. P. Robinson, and B. L. Wanner.** 2005. Stochastic activation of the response regulator PhoB by noncognate histidine kinases. J. Integr. Bioinform. **2:**11.
55. **Zhou, Z., S. Lin, R. J. Cotter, and C. R. Raetz.** 1999. Lipid A modifications characteristic of *Salmonella typhimurium* are induced by NH_4VO_3 in *Escherichia coli* K12. Detection of 4-amino-4-deoxy-L-arabinose, phosphoethanolamine and palmitate. J. Biol. Chem. **274:**18503–18514.
56. **Zhou, Z., A. A. Ribeiro, S. Lin, R. J. Cotter, S. I. Miller, and C. R. Raetz.** 2001. Lipid A modifications in polymyxin-resistant *Salmonella typhimurium*: PMRA-dependent 4-amino-4-deoxy-L-arabinose, and phosphoethanolamine incorporation. J. Biol. Chem. **276:**43111–43121.
57. **Zhou, Z., A. A. Ribeiro, and C. R. Raetz.** 2000. High-resolution NMR spectroscopy of lipid A molecules containing 4-amino-4-deoxy-L-arabinose and phosphoethanolamine substituents. Different attachment sites on lipid A molecules from NH_4VO_3-treated *Escherichia coli* versus *kdsA* mutants of *Salmonella typhimurium*. J. Biol. Chem. **275:**13542–13551.