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Absence of PmrAB-Mediated Phosphoethanolamine Modifications of *Citrobacter rodentium* Lipopolysaccharide Affects Outer Membrane Integrity▽†

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The PmrAB two-component system of enterobacteria regulates a number of genes whose protein products modify lipopolysaccharide (LPS). The LPS is modified during transport to the bacterial outer membrane (OM). A subset of PmrAB-mediated LPS modifications consists of the addition of phosphoethanolamine (pEtN) to lipid A by PmrC and to the core by CptA. In *Salmonella enterica*, pEtN modifications have been associated with resistance to polymyxin B and to excess iron. To investigate putative functions of pEtN modifications in *Citrobacter rodentium*, Δ*pmrAB*, Δ*pmrC*, Δ*cptA*, and Δ*pmrC* Δ*cptA* deletion mutants were constructed. Compared to the wild type, most mutant strains were found to be more susceptible to antibiotics that must diffuse across the LPS layer of the OM. All mutant strains also showed increased influx rates of ethidium dye across their OM, suggesting that PmrAB-regulated pEtN modifications affect OM permeability. This was confirmed by increased partitioning of the fluorescent dye 1-*N*-phenylnaphthylamine (NPN) into the OM phospholipid layer of the mutant strains. In addition, substantial release of periplasmic β-lactamase was observed for the Δ*pmrAB* and Δ*pmrC* Δ*cptA* strains, indicating a loss of OM integrity. This study attributes a new role for PmrAB-mediated pEtN LPS modifications in the maintenance of *C. rodentium* OM integrity.

The outer membrane (OM) of Gram-negative bacteria is asymmetric, with lipopolysaccharide (LPS) forming the outer leaflet (26). The LPS layer functions as a permeability barrier to antibacterial compounds, including lipophilic antibiotics and host antimicrobial peptides (AMPs). Lipophilic antibiotics must cross the OM by diffusion through the LPS layer. AMPs are typically too large to diffuse through porin channels (molecular mass cutoff, <600 Da); they penetrate the OM by perturbing the lateral interactions between neighboring LPS molecules mediated by divalent Mg^{2+} and Ca^{2+} cations. Limiting concentrations of these cations undermine the OM permeability barrier to the action of AMPs and lipophilic antibiotics (7). Enterobacteria have developed an adaptive response for remodeling the OM by modifying LPS. In response to various environmental signals, the PhoPQ and PmrAB two-component systems (TCS) control these LPS modifications at the transcriptional level (11, 15, 17). LPS modifications decrease OM permeability by adding palmitate to lipid A (encoded by the *pagP* gene) (29). They also neutralize negative charges of LPS by adding 4-aminoarabinose (encoded by the *arn* operon) or phosphoethanolamine (pEtN) (encoded by the *pmrC* and *cptA* genes) to specific phosphate groups (12, 29). Early studies have highlighted an association between PmrAB-mediated modifications of LPS and increased resistance of *Salmonella enterica* to the AMP polymyxin B (PMB) (36, 37). Later studies showed that PmrAB-mediated LPS modifications also have a role in the survival of *S. enterica* exposed to excess iron (5, 41). In *S. enterica*, the PhoPQ TCS controls the PmrAB TCS and the RcsBCD phosphorelay system (3, 10). PhoPQ controls PmrAB signaling by promoting expression of the PmrD protein, which binds the response regulator PmrA and prevents its dephosphorylation, resulting in sustained activation of PmrA-regulated genes (18, 20).

The extracellular enteric pathogen *Citrobacter rodentium* causes transmissible colonic hyperplasia in mice (32). *C. rodentium* has virulence factors similar to those of enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *Escherichia coli* (EHEC), making it an excellent model organism for the study of these human pathogens. Sequencing of the *C. rodentium* genome revealed striking differences from that of *S. enterica* (28). For example, *C. rodentium* does not appear to have the *pmrD* gene, suggesting that PhoPQ does not control PmrA-regulated genes, as observed previously in *Yersinia pestis* (40). In addition, the PmrA-regulated *arn* operon (also known as the *pmrHFIJKLM* operon) is also absent from the *C. rodentium* genome. The *arn* operon is responsible for the addition of 4-aminoarabinose to lipid A, a modification that is central to PMB resistance (16). These observations are consistent with the fact that a *C. rodentium* Δ*phoPQ* strain does not have greater susceptibility to PMB than the wild type (21). Among the other LPS-modifying PmrA-regulated genes, *C. rodentium* possesses the *pmrC* and *cptA* homologues. The *pmrC* and *cptA* genes encode pEtN transferases responsible for mediating the addition of pEtN to the 1 and 4′ phosphates of lipid A and to the phosphate of heptose I found in the LPS core, respectively (12, 29). Addition of pEtN to lipid A or the LPS core has been shown to have a modest effect on PMB resistance (13, 14, 34). Recently, it was shown that the single pEtN transferase of *Campylobacter jejuni* adds pEtN to both lipid A and the fla-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain, genotype, or plasmid	Description	Source or reference
C. rodentium strain or genotype		
DBS100	Wild-type *C. rodentium* strain (ATCC 51459)	32
Δ*phoPQ*	DBS100 Δ*phoPQ*	21
Δ*pmrAB*	DBS100 Δ*pmrAB*	This study
Δ*pmrC*	DBS100 Δ*pmrC*	This study
Δ*cptA*	DBS100 Δ*cptA*	This study
Δ*pmrC* Δ*cptA*	DBS100 Δ*pmrC* Δ*cptA*	This study
Δ*phoPQ* Δ*pmrAB*	DBS100 Δ*phoPQ* Δ*pmrAB*	This study
DBS100 *pmrC*::*lacZ*	*pmrC*::*lacZ* in DBS100, Cm^r	This study
Δ*phoPQ pmrC*::*lacZ*	*pmrC*::*lacZ* in Δ*phoPQ*, Cm^r	This study
Δ*pmrAB pmrC*::*lacZ*	*pmrC*::*lacZ* in Δ*pmrAB*, Cm^r	This study
DBS100 *cptA*::*lacZ*	*cptA*::*lacZ* in DBS100, Cm^r	This study
Δ*phoPQ cptA*::*lacZ*	*cptA*::*lacZ* in Δ*phoPQ*, Cm^r	This study
Δ*pmrAB cptA*::*lacZ*	*cptA*::*lacZ* in Δ*pmrAB*, Cm^r	This study
E. coli strain		
χ7213	*thr-1 leuB6 fhuA21 lacY1 glnV44 recA1 asdA4 thi-1* RP4-2-Tc::Mu [-*pir*] Kan^r	30
Plasmids		
pRE112	Sucrose-sensitive (*sacB1*) suicide vector, Cm^r	9
pFUSE	Suicide vector, *lacZYA*, mob^+ (RP4), ori R6K, Cm^r	2
pWSK129	Low-copy-no. cloning vector, Kan^r	38
pWSK*pmrAB*		This study
pWSK*pmrC*		This study
pWSK*cptA*		This study

gellar rod protein FlgG, affecting bacterial motility (6). This study indicated that pEtN modifications may have other roles.

C. rodentium does not have the ability to modify LPS with 4-aminoarabinose, which interferes with the addition of pEtN to the 4′ phosphate of lipid A in *S. enterica* (43). Therefore, *C. rodentium* is an organism of choice for studying the biological function of pEtN modifications. In this study, the objective was to characterize *C. rodentium pmrAB*, *pmrC*, and *cptA* deletion mutants. We found that the absence of PmrAB-mediated pEtN modifications of LPS increases OM permeability to lipophilic compounds and affects OM integrity, highlighting a new role for pEtN modifications.

MATERIALS AND METHODS

Media and reagents. Bacteria were grown at 37°C with aeration in Luria-Bertani (LB) broth, Trypticase soy broth (TSB), or N-minimal medium (pH 7.5) containing 0.2% glucose (21) and supplemented with $MgCl_2$ and $FeSO_4$, as needed. When appropriate, media were supplemented with chloramphenicol (30 μg/ml) or kanamycin (50 μg/ml). Polymyxin B (PMB), cephalothin, norfloxacin, erythromycin, novobiocin, vancomycin, ethidium bromide (EtBr), 1-*N*-phenylnaphthylamine (NPN), and carbonyl cyanide *meta*-chlorophenylhydrazone (CCCP) were purchased from Sigma. Nitrocefin was from EMD Chemicals. Restriction enzymes and *Pfx* DNA polymerase were from New England BioLabs and Invitrogen, respectively.

Construction of *C. rodentium* deletion mutants. The bacterial strains and plasmids used in this study are listed in Table 1. DNA purification, cloning, and transformation were performed according to standard procedures (31). The *C. rodentium* Δ*pmrAB*, Δ*pmrC*, Δ*cptA*, Δ*pmrC* Δ*cptA*, and Δ*phoPQ* Δ*pmrAB* deletion mutants were generated by *sacB* gene-based allelic exchange (8). The upstream and downstream sequences of the *cptA*, *pmrC*, and *pmrAB* genes were PCR amplified from *C. rodentium* genomic DNA using the primers listed in Table 2. PCR fragments were gel purified, digested with the appropriate restriction enzymes (Table 2), and ligated into pRE112 cleaved with XbaI and SacI. Resulting plasmids were verified by sequencing, transformed into the *E. coli* χ7213 donor strain, and conjugated into the appropriate *C. rodentium* strain. Chromosomal plasmid insertion was selected for by culturing on LB agar supplemented with chloramphenicol. Selected clones were cultured on peptone agar containing 5% sucrose to isolate colonies that were sucrose resistant. These resulting colonies were also tested for chloramphenicol sensitivity. Gene deletions were verified by PCR. Plasmids used for complementation were constructed by PCR amplifying the genes of interest with their promoters from *C. rodentium* genomic DNA using the primers listed in Table 2 (for *pmrAB*, CR677 and CR680; for *pmrC*, CR694 and CR697; and for *cptA*, CR756 and CR757). The resulting PCR products were cloned into the XbaI and BamHI or XbaI and SacI restriction sites of plasmid pWSK129, generating plasmids pWSK*pmrAB*, pWSK*pmrC*, and pWSK*cptA*. In pWSK*pmrAB*, *pmrAB* was under the control of the constitutive promoter located at the 3′ end of the *pmrC* coding region (33).

Construction of chromosomal *lacZ* transcriptional fusions and β-galactosidase assay. The *pmrC* or *cptA* promoter was PCR amplified using the primers listed in Table 2. The PCR fragments were digested with XbaI and SmaI and ligated upstream of the *lacZ* gene of the suicide vector pFUSE (2). The resulting constructs were transformed into *E. coli* χ7213, conjugated into the *C. rodentium* wild type, Δ*phoPQ*, and Δ*pmrAB* strains, and selected for by culturing on LB agar supplemented with chloramphenicol. Insertion of the suicide vector was verified by sequencing. β-Galactosidase activity assays were carried out in triplicate at least three times, as previously described (23).

MIC determination. MICs were determined in 96-well microtiter plates using the broth microdilution method (39). Briefly, bacterial cultures were grown in TSB, diluted to 5×10^5 CFU/ml in N-minimal medium supplemented with 10 μM $MgCl_2$ and 25 μM $FeSO_4$ and aliquoted into rows of wells. Twofold serial dilutions of the tested antimicrobial compound were added to each row of wells. The plates were incubated at 37°C for 24 h. The lowest concentration of the antimicrobial compound that did not permit any visible growth was determined to be the MIC. Determination of MIC values was repeated at least three times until no variation was observed between replicates.

OM permeability assays. N-minimal media supplemented with 1 mM $MgCl_2$ were inoculated (1:80) with LB-grown overnight cultures of the various *C. rodentium* strains. Cultures were grown to an optical density at 600 nm (OD_{600}) of 0.7 to 0.8. Cells were washed with phosphate-buffered saline (PBS) and resuspended in N-minimal medium supplemented with 10 μM $MgCl_2$ and 25 μM $FeSO_4$. At an OD_{600} of ≈1.3, cells were harvested by centrifugation and diluted in PBS to an OD_{600} of 0.4. The assay measuring ethidium influx rates was carried out as previously described (25). Briefly, cells (3 ml) were added to a quartz cuvette with stirring and incubated with 50 μM CCCP for 10 min to inactivate

TABLE 2. Primers used in this study

Primer	Sequence[a]	Usage
CR677	AGCTAGTCTAGATGTTCTCCGGAATGCCACGCAAGC	Δ*pmrAB* 5′F XbaI
CR678	AGCTAGGATATCGGTGTCGCGGGCGGTCAGGATCAG	Δ*pmrAB* 5′R EcoRV
CR679	AGCTAGGATATCGCGGTGATGGGAGTTGAGGATCAG	Δ*pmrAB* 3′F EcoRV
CR680	AGCTAGGAGCTCTTTCGCCGGTCTGCTGATGCTGGC	Δ*pmrAB* 3′R SacI
CR694	GACTAGTCTAGACCAGCAGAATCAGGCCGATAG	Δ*pmrC* 5′F XbaI
CR695	AGCCATGATATCACGGCGTAAATGCGGACTCG	Δ*pmrC* 5′R EcoRV
CR696	AGCTAGGATATCCAGCGGCATCGACAGAAAGAC	Δ*pmrC* 3′F EcoRV
CR697	CAGTGAGAGCTCCCGCTACATTCCAGAAGCCCT	Δ*pmrC* 3′R SacI
CR719	TGACATCTAGAGGAGCTGGGCAAACTACCGCTC	Δ*cptA* 5′F XbaI
CR734	TGCAGGTACCGCTAAATGCGGACGCGGGCTGG	Δ*cptA* 5′R KpnI
CR735	GGTGGGTACCTGCGCTGATTGATTATGACACTCTG	Δ*cptA* 3′F KpnI
CR722	GGCGGAGCTCTCAGGTCGTAGCCCTTCAGCAAC	Δ*cptA* 3′R SacI
CR555	AGCTAGTCTAGAGATCTTAGCCCAGCTTTCGCAGTATCCGGTCTCC	*pmrC*::*lacZ* 5′F XbaI
CR554	AGCTACCCCGGGAAACCAGCAGCAGCCAGGCGAGCAGTCTGAG	*pmrC*::*lacZ* 3′R SmaI
CR774	AGCTAGTCTAGAATGCTGGAAATGGTCTTCGCCAAAG	*cptA*::*lacZ* 5′F XbaI
CR775	AGCTACCCCGGGGCTAAATGCGGACGCGGGCTGG	*cptA*::*lacZ* 3′R SmaI
CR756	GGTATCTACAGAAATGGTCTTCGCCAAAGCGGAT	pWSK*cptA* 5′ XbaI
CR757	GCCAGAGCTCGATGTCCTCAAGTGATTATTGCCGG	pWSK*cptA* 3′ SacI

[a] Restriction sites are underlined.

efflux pumps. EtBr was added to these bacterial suspensions at a final concentration of 4 μM. The fluorescence generated from the binding of ethidium to DNA was measured using a Varian Eclipse spectrofluorometer with excitation and emission wavelengths of 545 and 600 nm, respectively. Slit widths were 5 and 10 nm, respectively. The assay monitoring partitioning of NPN into membrane phospholipids was carried out as described elsewhere (22). NPN was added at a final concentration of 5 μM to bacteria resuspended in PBS (3 ml). Fluorescence generated by partitioning of NPN into phospholipids was measured as described above with an excitation wavelength of 350 nm, an emission wavelength of 420 nm, and slit widths set at 5 nm.

β-Lactamase leakage assay. Bacterial cultures were grown as described under "OM permeability assays." Release of the chromosomally encoded *C. rodentium* β-lactamase (ROD_12321; molecular mass, 32 kDa) from the periplasm to the supernatant was compared between the various *C. rodentium* strains to assess OM integrity. The chromogenic β-lactam nitrocefin was used as a substrate (27). In brief, the released β-lactamase fractions were isolated by collecting the supernatant of 1-ml culture aliquots. The periplasmic β-lactamase fractions were obtained by osmotic lysis of the remaining cells, using the protocol supplied with the Epicentre periplasmic protein extraction kit. Enzymatic assays were performed at 22°C in N-minimal medium, and β-lactamase activity was monitored by measuring the absorbance at 482 nm for 30 min using a Powerwave X340 microplate reader (Bio-Tek Instruments). Slopes of OD_{482} as a function of time were calculated to determine β-lactamase activities in both the released and periplasmic fractions (27). Percentages of β-lactamase released into the culture supernatants were calculated by determining the ratio of β-lactamase activity in the supernatant to total β-lactamase activity (released and periplasmic fractions). Each strain was assayed in triplicate in multiple experiments.

RESULTS

Transcription of *C. rodentium pmrC* and *cptA* is regulated by PmrAB independently of PhoPQ. The PhoP-regulated *pmrD* gene that connects the *S. enterica* PhoPQ and PmrAB signaling pathways is absent from the *C. rodentium* genome (28). The *C. rodentium pmrC* and *cptA* promoters contain PmrA-binding sites found in the same context as those described for *S. enterica* (1, 35). To examine whether expression of *C. rodentium pmrC* and *cptA* is under the control of PmrAB, chromosomal β-galactosidase fusions with the *pmrC* and *cptA* genes were constructed in the wild-type, Δ*phoPQ*, and Δ*pmrAB C. rodentium* strains. The *lacZ* reporter strains were grown under PhoPQ- and PmrAB-inducing conditions (10 μM $MgCl_2$ and 25 μM $FeSO_4$). The β-galactosidase activities of the *pmrC*::*lacZ* reporter in the wild-type and Δ*phoPQ* strains were very similar (Fig. 1A). In contrast, expression from the *pmrC*::*lacZ* reporter was not observed in the Δ*pmrAB* strain, indicating that *pmrC* is regulated by PmrAB in *C. rodentium*. Similar results were obtained by using $FeCl_3$ or mild acidic pH (pH 5.5) as the PmrAB-inducing condition (data not shown). Expression from the *cptA*::*lacZ* reporter fusion was marginally affected in the Δ*phoPQ* strain and decreased by 3-fold in the Δ*pmrAB* strain, indicating that *C. rodentium cptA* is at least partly regulated by PmrAB (Fig. 1B). The residual expression from the *cptA* promoter in the Δ*pmrAB* strain suggests that other transcriptional factors regulate *cptA* expression (Fig. 1B). Transcriptional regulation of *C. rodentium pmrC* and *cptA* by PmrAB was further validated by measuring the expression of both reporter fusions in the presence of increasing concentrations of $FeSO_4$ (Fig. 1C and D). Altogether, these data confirm that PmrAB regulates expression of the *C. rodentium pmrC* and *cptA* genes, as reported previously for *S. enterica* (1, 35). They also indicate that expression of *pmrC* and *cptA* is independent of PhoPQ.

pEtN modifications protect *C. rodentium* from iron toxicity. PmrAB-regulated LPS modifications have been associated with resistance to excess iron in *S. enterica* (5, 41). The susceptibility of the *C. rodentium* wild-type, Δ*pmrAB*, Δ*pmrC*, Δ*cptA*, and Δ*pmrC* Δ*cptA* strains to $FeSO_4$ was assessed by determining MIC values. As shown in Table 3, all mutant strains were more susceptible to excess iron than the wild type. Compared to the wild-type strain, the Δ*cptA* and Δ*pmrC* strains exhibited 2- and 4-fold-lower MIC values, respectively. The Δ*pmrC* Δ*cptA* and Δ*pmrAB* strains showed much higher susceptibility to iron, with 8- and 16-fold lower MIC values, respectively. Complementation of the mutant strains with the pWSK129-derived pWSK*pmrAB*, pWSK*pmrC*, and pWSK*cptA* plasmids restored resistance to iron above the wild-type level (Table 3). Essentially similar data were obtained with $FeCl_3$ (data not shown). Altogether, the data indicate that pEtN transferases protect *C. rodentium* from excess iron and support previous observations in *S. enterica* (5, 41). Due to the higher susceptibility of the mutant strains to excess iron, all

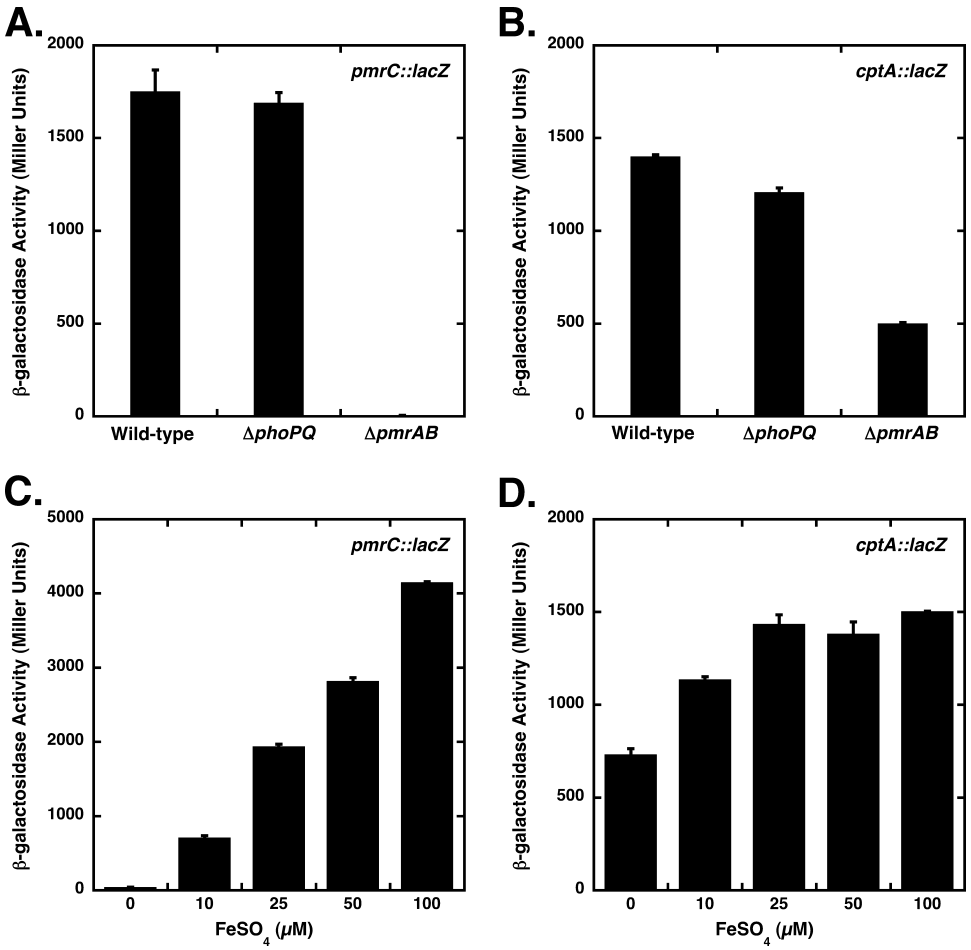

FIG. 1. Expression of *C. rodentium pmrC* and *cptA* is regulated by PmrAB independently of PhoPQ. (A) Expression of *pmrC* is strictly regulated by PmrAB. (B) Expression of *cptA* is primarily regulated by PmrAB. (C and D) β-Galactosidase activity from the *pmrC*::*lacZ* (C) or *cptA*::*lacZ* (D) reporter strains in the presence of increasing concentrations of $FeSO_4$. The various *pmrC*::*lacZ* or *cptA*::*lacZ* reporter strains were constructed as described in Materials and Methods. Strains were grown up to an OD_{600} of 0.4 in N-minimal medium containing 1 mM $MgCl_2$, washed in PBS, and resuspended in N-minimal medium supplemented with 10 μM $MgCl_2$ and $FeSO_4$, as indicated. After an additional 90-min incubation at 37°C, β-galactosidase activities were assayed using the $CHCl_3$-sodium dodecyl sulfate permeabilization procedure (23). Values are means ± standard deviations of data from three independent experiments.

subsequent experiments were performed in N-minimal medium supplemented with 25 μM $FeSO_4$, a concentration of iron that induces expression of both *pmrC* and *cptA* (Fig. 1C and D) without affecting growth or viability of the various strains as judged by OD_{600} measurements and survival assays (see Fig. S1 in the supplemental material).

TABLE 3. MICs of $FeSO_4$ for *C. rodentium* strains

Strain description	MIC of $FeSO_4$ (μM) in N-minimal medium[a]
Wild type	1,600
Δ*pmrAB*	100
Δ*pmrC*	400
Δ*cptA*	800
Δ*pmrC* Δ*cptA*	200
Δ*pmrAB*-pWSK*pmrAB*	>1,600
Δ*pmrC*-pWSK*pmrC*	>1,600
Δ*cptA*-pWSK*cptA*	>1,600

[a] N-minimal medium was supplemented with 10 μM $MgCl_2$.

pEtN modifications and PMB resistance. The role of *C. rodentium* PmrC and CptA in PMB resistance was assessed by determining MIC values. In N-minimal medium, MIC values of 2 μg/ml were obtained for all tested strains (Table 4). These results indicate that neither PmrC nor CptA is involved in resistance to PMB, at least under these experimental conditions. The data also suggest that no additional PmrAB-regulated *C. rodentium* genes play a role in PMB resistance. In LB broth, 2-fold decreases in MIC values were obtained for the Δ*pmrC*, Δ*pmrC*Δ *cptA*, and Δ*pmrAB* strains (Table 4). These slight differences in MIC values are consistent with findings of previous studies in which the marginal involvement of *S. enterica* PmrAB-mediated pEtN modifications in PMB resistance was reported (14, 34).

pEtN modifications confer resistance to lipophilic antibiotics. Since both PmrC and CptA alter the structure of the LPS layer, susceptibility to different types of antibiotics was examined. Small hydrophilic antibiotics are known to diffuse across porins, whereas lipophilic antibiotics must diffuse across the

TABLE 4. MICs of PMB for *C. rodentium* strains

Strain description	MIC (μg/ml) of PMB in:	
	N-minimal medium[a]	LB[b]
Wild type	2	0.4
Δ*pmrAB*	2	0.2
Δ*pmrC*	2	0.2
Δ*cptA*	2	0.4
Δ*pmrC* Δ*cptA*	2	0.2
Δ*pmrAB*-pWSK*pmrAB*	2	0.4
Δ*pmrC*-pWSK*pmrC*	2	0.4
Δ*cptA*-pWSK*cptA*	2	0.4

[a] N-minimal medium was supplemented with 10 μM $MgCl_2$ and 25 μM $FeSO_4$.
[b] LB was supplemented with 500 μM $FeSO_4$.

asymmetrical bilayer of the OM (7, 26). MIC values were determined for the lipophilic antibiotics novobiocin and erythromycin (Table 5). The wild-type and Δ*cptA* strains were equally susceptible to novobiocin, with MIC values of 16 μg/ml. The MIC for the Δ*pmrC* strain was decreased by 2-fold. In contrast, the MIC for the Δ*pmrC* Δ*cptA* strain showed an 8-fold decrease. Consistently, a 4-fold reduction in the MIC value was obtained for the Δ*pmrAB* strain. Complementation of the Δ*pmrAB* and Δ*pmrC* strains with pWSK*pmrAB* and pWSK*pmrC*, respectively, restored the MIC values to the wild-type level (16 μg/ml) or to a greater extent (32 μg/ml). Complementation of the Δ*cptA* strain with pWSK*cptA* did not have an observable effect on the MIC value. Interestingly, complementation of the Δ*pmrC* Δ*cptA* strain with pWSK*cptA* or pWSK*pmrC* increased the MIC values to 8 and 16 μg/ml, respectively. A similar trend was observed with erythromycin, with the fold changes being much higher. Erythromycin MIC values were decreased by 16- and 32-fold for the Δ*pmrC* and Δ*pmrC* Δ*cptA* strains, respectively. All complemented single mutant strains had their MIC values equal to the wild-type value. As for novobiocin, complementation of the Δ*pmrC* Δ*cptA* strain with pWSK*pmrC* more effectively restored resistance to erythromycin than when complemented with pWSK*cptA* (Table 5). Altogether, the data indicate that PmrC-mediated addition of pEtN to LPS may have a preponderant role compared to the CptA-mediated modification in restricting access of lipophilic drugs across the OM. In addition, the data strongly suggest that modifications introduced by both PmrC and CptA act together to reinforce the LPS permeability barrier.

MIC values were also determined for the hydrophilic antibiotics cephalothin and norfloxacin (24). For both antibiotics, MIC values for all strains were essentially similar and did not exhibit the susceptibility pattern observed for novobiocin and erythromycin (Table 5). Susceptibility to the hydrophilic antibiotic vancomycin, which is too large (molecular mass, 1,450 Da) to diffuse through porins, was also examined. Obtained MIC values reflected the pattern of susceptibility observed for novobiocin and erythromycin (Table 5). This pattern of susceptibility was also obtained for SDS, a detergent that is used to assess OM integrity (Table 5). These data further support the possibility that pEtN modifications introduced by both PmrC and CptA are involved in the control of OM permeability and/or integrity.

pEtN modifications influence OM permeability to hydrophobic dyes. In *S. enterica*, the PhoPQ-mediated modifications of LPS have been shown to strengthen the OM permeability barrier (19, 25). To determine the respective contributions of *C. rodentium* PhoPQ and PmrAB in controlling OM permeability, we compared the influx of EtBr into the wild-type, Δ*phoPQ*, Δ*pmrAB*, and Δ*phoPQ* Δ*pmrAB* strains. In this assay, diffusion of ethidium across the OM was shown to be the rate-limiting step (25). Ethidium influx rates were increased by 1.6-fold for the Δ*pmrAB* and Δ*phoPQ* Δ*pmrAB* strains compared to those for the wild-type strain (Fig. 2A). In contrast, the influx rate for the Δ*phoPQ* strain was similar to that for the wild-type strain. These observations highlight the importance of the PmrAB TCS in regulating OM permeability in *C. rodentium*. They are also consistent with the fact that transcriptional regulation of target genes by PmrAB is independent of PhoPQ in *C. rodentium* (Fig. 1). To assess whether PmrC and CptA are involved in reinforcing the OM permeability barrier, influx of ethidium into the Δ*pmrC*, Δ*cptA*, and Δ*pmrC* Δ*cptA* strains was monitored (Fig. 2B). The Δ*pmrC* Δ*cptA* and Δ*pmrAB* mutants showed similar ethidium influx rates that were 1.5-fold higher than that of the wild-type strain. Influx rates for the Δ*pmrC* and Δ*cptA* strains were intermediate and consistently higher than that of the wild type. Ethidium influx rates for the complemented strains were either similar (Δ*cptA*-pWSK*cptA*) or slightly lower (Δ*pmrC*-pWSK*pmrC* and Δ*pmrAB*-pWSK*pmrAB*) than that of the wild-type strain (data not shown). These results indicate that both pEtN mod-

TABLE 5. MICs of lipophilic and hydrophilic antibiotics and SDS for *C. rodentium* strains

Strain description	MIC (μg/ml) of:					
	Novobiocin	Erythromycin	Cephalothin	Norfloxacin	Vancomycin	SDS
Wild type	16	1,024	32	0.008	4	62,500
Δ*pmrAB*	4	32	64	0.016	1	15,625
Δ*pmrC*	8	64	32	0.016	2	31,250
Δ*cptA*	16	1,024	32	0.008	4	62,500
Δ*cptA* Δ*pmrC*	2	32	64	0.008	1	15,625
Δ*pmrAB*-pWSK*pmrAB*	32	1,024	32	0.016	8	62,500
Δ*pmrC*-pWSK*pmrC*	32	1,024	32	0.016	4	62,500
Δ*cptA*-pWSK*cptA*	16	1,024	32	0.016	4	62,500
Δ*cptA* Δ*pmrC*-pWSK*pmrC*	16	1,024	32	0.016	4	62,500
Δ*cptA* Δ*pmrC*-pWSK*cptA*	8	128	32	0.016	2	31,250

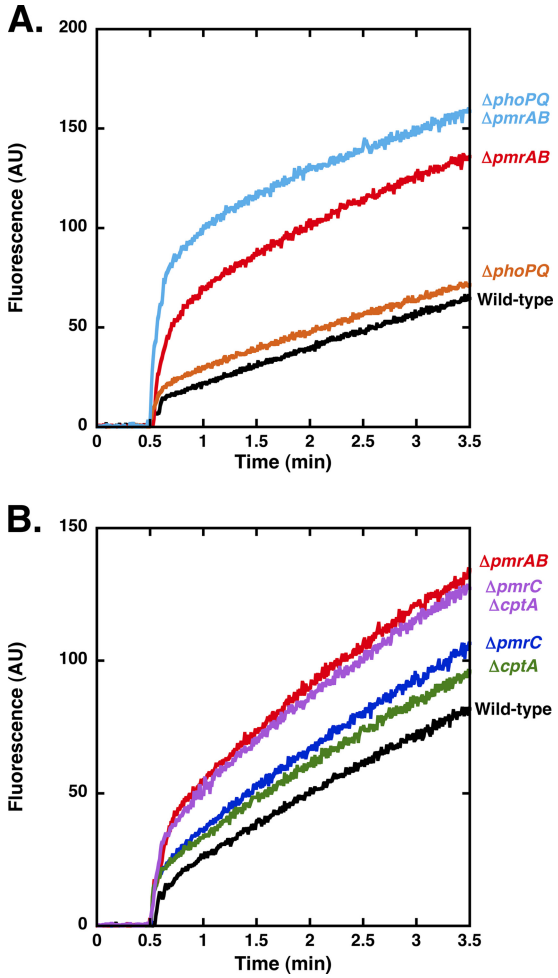

FIG. 2. Ethidium influx into *C. rodentium* mutant strains. (A) Ethidium influx into the wild-type, Δ*phoPQ*, Δ*pmrAB*, and Δ*phoPQ* Δ*pmrAB* strains. (B) Ethidium influx into the wild-type, Δ*pmrC*, Δ*cptA*, Δ*pmrC* Δ*cptA*, and Δ*pmrAB* strains. Cells were grown as described in Materials and Methods. Cultures were diluted in PBS to an OD_{600} of 0.4, transferred to a quartz cuvette, and stirred for the length of the experiment. EtBr was added at 0.5 min, and the influx of ethidium was monitored over 3 min by measuring fluorescence emission at 600 nm. Ethidium influx rates were calculated from the linear portions of the curves (1 to 3.5 min). One representative experiment of three independent experiments is shown. AU, arbitrary units.

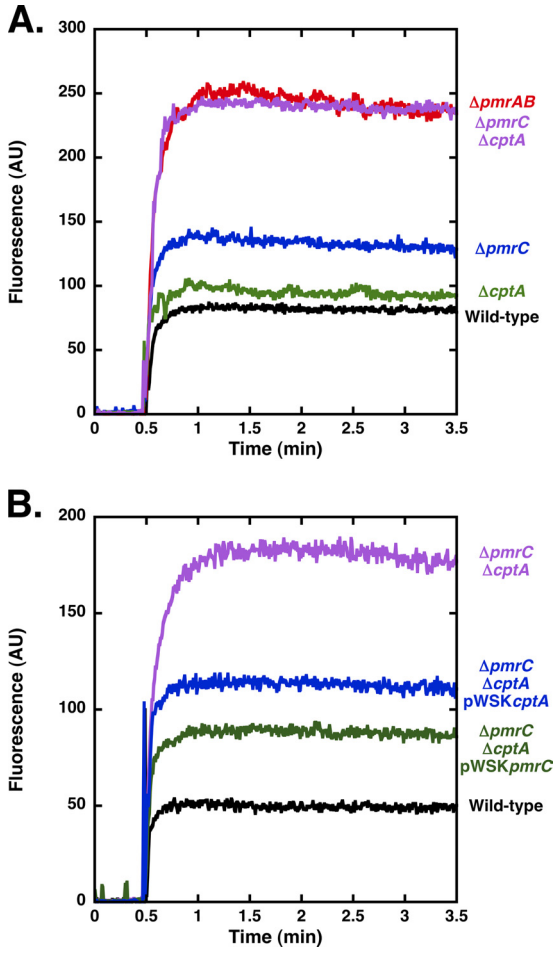

FIG. 3. Partitioning of NPN into the OM of *C. rodentium* strains. (A) Change in fluorescence due to the partitioning of NPN into the OM of the wild-type, Δ*pmrC*, Δ*cptA*, Δ*pmrC* Δ*cptA*, and Δ*pmrAB* strains. (B) Change in fluorescence due to the partitioning of NPN into the OM of the wild-type, Δ*pmrC* Δ*cptA*, Δ*pmrC* Δ*cptA*-pWSK-*pmrC*, and Δ*pmrC* Δ*cptA*-pWSK*cptA* strains. Bacterial cultures were grown as described in Materials and Methods, diluted in PBS to an OD_{600} of 0.4, and transferred to a quartz cuvette with stirring. NPN was added at 0.5 min, and NPN partitioning was monitored by measuring fluorescence emission at 420 nm over 3 min. One representative experiment of three independent experiments is shown. AU, arbitrary units.

ifications are required for the strengthening of the OM barrier and support the possibility that PmrC and CptA act in concert in this process.

To further confirm these observations, the partitioning of the uncharged fluorescent probe NPN into the asymmetric bilayer of these strains was measured. Fluorescence of NPN is weak in aqueous environments but strong in the hydrophobic environment of disrupted membranes (22). For the Δ*pmrC* Δ*cptA* and Δ*pmrAB* strains, the maximum fluorescence intensities of NPN were approximately 3-fold higher than that of the wild-type strain (Fig. 3A). Fluorescence intensities for the Δ*pmrC* and Δ*cptA* strains were consistently higher than that of the wild type, with that of the Δ*pmrC* strain consistently higher than that of the Δ*cptA* strain (Fig. 3A). These results are in agreement with the ethidium influx assays (Fig. 2). To clarify the individual contributions of PmrC and CptA in reinforcing the OM barrier, the Δ*pmrC* Δ*cptA* strain was complemented with the pWSK*pmrC* or pWSK*cptA* plasmid and assayed for NPN partitioning (Fig. 3B). In both cases, accumulation of NPN fluorescence was reduced to intermediate levels and complementation with pWSK*pmrC* consistently resulted in a larger reduction of fluorescence intensity (2.2-fold) than complementation with pWSK*cptA* (1.8-fold). These results show that both pEtN transferases are required to reach the wild-type level of OM impermeability. They also suggest that PmrC contributes to a larger extent than CptA to the strengthening of the OM barrier.

Lack of pEtN modifications affect OM integrity. To determine whether the weakened OM barrier observed in the absence of *pmrC* and *cptA* is due to strict increased permeability

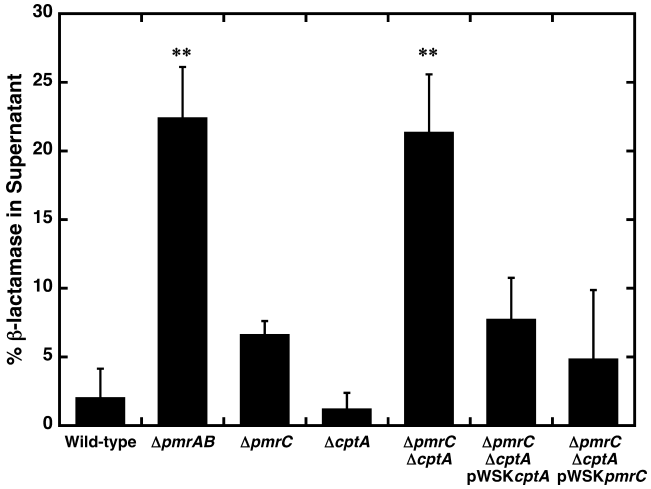

FIG. 4. Absence of PmrAB-regulated pEtN transferases affects *C. rodentium* OM integrity. Cells were grown as described in Materials and Methods. Culture supernatant and periplasmic fractions from the various *C. rodentium* strains were assayed for β-lactamase activity using nitrocefin as a substrate. The cleavage of nitrocefin was monitored by measuring the absorbance at 482 nm over 30 min. The percentage of β-lactamase in the culture supernatant was calculated by determining the ratio of β-lactamase activity in the supernatant versus total β-lactamase activity in both fractions. For each strain, total β-lactamase activity was comparable. Values are means of data from three independent experiments ± standard deviations. **, Dunnett's test, $P < 0.01$ compared to wild-type measurements.

or loss of integrity, release of the endogenous *C. rodentium* β-lactamase into culture supernatants was assessed using the chromogenic substrate nitrocefin (27). As shown in Fig. 4, minimal release of β-lactamase (2 to 3%) was obtained for the wild-type and Δ*cptA* strains. β-Lactamase release was slightly higher (6%) for the Δ*pmrC* strain, although this was statistically insignificant. More dramatic leakage of β-lactamase (~22%) was measured for the Δ*pmrC* Δ*cptA* and Δ*pmrAB* strains, which represented a 10-fold increase compared to that for wild type. These results clearly show that the lack of PmrAB-regulated pEtN transferases affects *C. rodentium* OM integrity. Complementation of the Δ*pmrC* Δ*cptA* strain with pWSK*pmrC* or pWSK*cptA* restored OM integrity to levels close to that of the wild type. However, analysis of LPS and OM protein profiles revealed that all *C. rodentium* strains exhibited a full-length LPS capped with *O*-polysaccharide and no difference in the amounts of the major OM porins (OmpA, OmpC, and OmpF) (see Fig. S2 in the supplemental material). Altogether, these data show that both PmrC- and CptA-mediated pEtN modifications are involved in maintaining OM integrity. Furthermore, they support the observation that the PmrC and CptA pEtN transferases are both required and act together in this process.

DISCUSSION

The PmrAB-regulated modifications that consist of the addition of 4-aminoarabinose (*arn* operon) and pEtN (*pmrC* and *cptA*) to LPS have been associated with PMB resistance and survival in excess iron in enterobacteria (14, 16, 34, 41). The *arn* operon, which is absent in *C. rodentium*, has been demonstrated to have a greater role in PMB resistance than the pEtN transferases encoded by *pmrC* and *cptA* (14, 34). Thus, the biological function of the pEtN transferases PmrC and CptA remains unclear. The fact that PmrC and CptA are required for maintaining OM integrity in *C. rodentium* is a novel finding. Several lines of evidence support this conclusion. First, the Δ*pmrC*, Δ*pmrC* Δ*cptA*, and Δ*pmrAB* strains had increased susceptibility to antibiotics that must cross the OM by diffusing through the LPS layer (lipophilic antibiotics and vancomycin). These increases in susceptibility were not observed for hydrophilic antibiotics that pass through porins. Second, permeability of the OM to two different fluorescent dyes (EtBr and NPN) was increased for all mutant strains, most notably for the Δ*pmrC* Δ*cptA* and Δ*pmrAB* strains. Third, β-lactamase release into culture supernatants revealed defects in OM integrity for the Δ*pmrC* Δ*cptA* and Δ*pmrAB* strains.

In MIC experiments, *C. rodentium* mutant strains showed hypersensitivity to ferrous ($FeSO_4$) and ferric ($FeCl_3$) iron compared to the wild-type strain (Table 3). Our results showed sensitivity to $FeSO_4$ in the following order (from greatest to least sensitivity): the Δ*pmrAB* strain, the Δ*pmrC* Δ*cptA* strain, the Δ*pmrC* strain, the Δ*cptA* strain, and the wild type. Restoration of MIC values above the wild-type level was observed for the Δ*pmrC*-pWSK*pmrC* and Δ*cptA*-pWSK*cptA* complemented strains, confirming the involvement of both *C. rodentium* PmrC- and CptA-mediated pEtN modifications in resistance to excess iron. In contrast, neither PmrC nor CptA was found to promote *C. rodentium* resistance to PMB in N-minimal medium (Table 4). Consistently, complementation with *pmrC*, *cptA*, or *pmrAB* from a low-copy-number plasmid did not have an effect on PMB resistance under these conditions. However, the Δ*pmrC*, Δ*pmrC* Δ*cptA*, and Δ*pmrAB* strains showed a slight increase in susceptibility to PMB in LB (Table 4). These results are in agreement with those in previous studies that had shown a minor role for pEtN modifications in PMB resistance (14, 34).

Under PmrAB-inducing conditions, PmrC appeared to be more important than CptA in maintaining a robust OM permeability barrier. The Δ*pmrC* strain was consistently more susceptible than the wild type to both lipophilic antibiotics and vancomycin, whereas no change in MIC values was observed for the Δ*cptA* strain (Table 5). Although both the Δ*pmrC* and Δ*cptA* strains showed slight increases in OM permeability to EtBr and NPN compared to the wild-type strain, the Δ*pmrC* strain consistently exhibited a slightly higher influx of ethidium and accumulation of NPN compared to the Δ*cptA* strain (Fig. 2 and 3). The greater effect of PmrC than of CptA in contributing to the OM permeability barrier may be explained by differences in transcriptional regulation of the *pmrC* and *cptA* genes. β-Galactosidase reporter assays showed that *pmrC* and *cptA* expression was upregulated 45- and 2-fold, respectively, in the presence of 25 μM $FeSO_4$ (Fig. 1C and D). Interestingly, the deletion of either *pmrC* or *cptA* did not affect OM integrity, as shown by the insignificant difference in the amounts of β-lactamase released from the periplasm compared to the wild type (Fig. 4). In contrast, the Δ*pmrC* Δ*cptA* strain exhibited more dramatic OM defects in all assays, including a large release of β-lactamase, indicative of a loss of OM integrity. These findings indicate that PmrC and CptA act together, possibly synergistically, to maintain the OM permeability bar-

rier. This observation is supported by the fact that complementation of the Δ*pmrC* Δ*cptA* strain with either pWSK*pmrC* or pWSK*cptA* restored OM integrity, although subtle OM permeability defects were still observed (Fig. 3B and 4; Table 5). Essentially similar results were obtained for the Δ*pmrAB* and Δ*pmrC* Δ*cptA* strains, suggesting that no additional PmrAB-regulated gene is involved in maintaining *C. rodentium* OM integrity. Collectively, these data show that a lack of either *pmrC* or *cptA* leads to increased OM permeability, whereas a lack of both pEtN transferases grossly affects the OM, resulting in a loss of integrity.

In a previous study, Chamnongpol et al. observed that an *S. enterica pmrA* mutant had increased uptake of vancomycin under PhoPQ- and PmrAB-inducing conditions (5). This observation is in agreement with the finding that our *C. rodentium* Δ*pmrAB* strain is highly susceptible to lipophilic antibiotics and to vancomycin under similar growth conditions (Table 5). This may suggest that the OM strengthening attributed to pEtN transferases, observed in this study, is conserved among enterobacteria that possess the *pmrC* and *cptA* genes. This possibility could be tested by characterizing similar mutations in an *S. enterica* strain deficient in the *arn* operon. The loss of OM integrity that we observed for the Δ*pmrC* Δ*cptA* and Δ*pmrAB* strains has been reported for other gene mutations. For example, a mutation in the *S. enterica waaP* gene, encoding the kinase responsible for the phosphorylation of the core heptose I, resulted in an unstable OM as well as increased susceptibility to lipophilic antibiotics (42). Interestingly, both WaaP and CptA act on the same core sugar. A loss of OM integrity was also observed in mutants of the Tol-Pal complex, which serves as a structural link between the peptidoglycan layer and other cell wall components (4).

Finally, previous reports suggested the involvement of the *S. enterica* PmrAB TCS in producing a robust OM permeability barrier under Mg^{2+}-limiting conditions (19, 25). Here, we have clearly shown the involvement of *C. rodentium* PmrAB in this process. In *S. enterica*, PmrAB is under the control of the PhoPQ TCS, which also regulates its own LPS-modifying genes (*pagP*, *pagL*, and *lpxO*). *C. rodentium* does not possess the *pagL* and *lpxO* homologues (28), the *pmrD* homologue, which bridges the PhoPQ and PmrAB signaling pathways, and a homologue to the *arn* operon. The lack of these homologues makes *C. rodentium* the appropriate model for studying the contribution of PmrAB-regulated pEtN transferases in strengthening the OM permeability barrier. The fact that *pagP* appears to be the sole *C. rodentium* LPS-modifying gene directly regulated by PhoPQ could explain the marginal contribution of this TCS to the production of a robust OM permeability barrier (Fig. 2B). The contribution of 4-aminoarabinose modifications in OM strengthening is still unclear. Kawasaki and Manabe showed that an *S. enterica* mutant of *ugd* (*pmrE*), a gene involved in the early steps of 4-aminoarabinose biosynthesis, displayed OM permeability defects similar to those of a *pmrA* mutant strain (19). This may suggest that modification of lipid A with 4-aminoarabinose also contributes to OM permeability in *S. enterica*. In conclusion, we have demonstrated that *C. rodentium pmrC* and *cptA* are the PmrAB-regulated genes responsible for maintaining a robust OM permeability barrier.

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