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Increased Outer Membrane Resistance to Ethylenediaminetetraacetate and Cations in Novel Lipid A Mutants

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Polymyxin-resistant *pmrA* mutants of *Salmonella typhimurium* differed from their parents in that they were resistant to tris(hydroxymethyl)aminomethane-ethylenediaminetetraacetate-lysozyme, tris(hydroxymethyl)aminomethane-ethylenediaminetetraacetate-deoxycholate, and tris(hydroxymethyl)aminomethane-ethylenediaminetetraacetate-bacitracin. Tris(hydroxymethyl)aminomethane-ethylenediaminetetraacetate released about 50% less lipopolysaccharide from the *pmrA* strains than from the parental strains when the bacteria were grown in L-broth containing 2 mM Ca^{2+}. Protamine, polylysine, octapeptin, benzalkonium chloride, cold NaCl, cold $MgCl_2$, or cold tris(hydroxymethyl)aminomethane hydrochloride (pH 7.2) caused no leakage or markedly less leakage of periplasmic β-lactamase from a *pmrA* mutant than from its parent strain. *pmrA* mutants were more resistant than their parent strains to protamine and polylysine but not to octapeptin or benzalkonium chloride, as measured by the ability of these agents to kill the bacteria or to sensitize them to deoxycholate-induced lysis. The *pmrA* strains did not differ from their parent strains in sensitivity to several antibiotics, in porin function (as measured by cephaloridine diffusion across the outer membrane), or in outer membrane-associated phospholipase A activity.

Enterobacterial outer membranes (OM) function as effective permeability barriers to hydrophobic and large hydrophilic molecules (larger than approximately 600 daltons) and are thus responsible for the unique patterns of resistance to antibiotics and other drugs in enterobacteria (32).

Several envelope mutants lack part of this OM permeability barrier function and are quite sensitive to numerous antibiotics, dyes, and detergents. In particular, these mutants include the deep rough (type Rd1, Rd2, and Re) lipopolysaccharide (LPS) mutants (37, 40), the *lpo* mutant lacking murein lipoprotein (14), and the mutants with mutations in *acrA* (4, 30) and *envA* (35). Other mutants, particularly mutants lacking the porin proteins, may have increased resistance to some drugs (9, 23). Many of these mutants have been essential tools in OM research (15, 25, 32).

In a search for novel types of mutants that would increase our understanding of the organization and function of the OM, Mäkelä et al. focused on the action of polymyxin, a polycationic antibiotic (24). The *pmrA* mutants of *Salmonella typhimurium*, which were isolated in the Central Public Health Laboratory, Helsinki, Finland, have increased resistance to polymyxin, which has been attributed to the OM (44). These mutants do not differ from their parent strains in the phospholipid or protein composition of the cell envelopes or in the LPS sugar composition (45). However, the LPSs isolated from the *pmrA* strains bind less polymyxin than the corresponding parental LPSs (45). Recently, Vaara et al. have shown that the *pmrA* mutants contain a novel type of mutation which affects the lipid A structure; the ester-linked phosphate group in the lipid A of these mutants is almost completely (60 to 70%) substituted with 4-amino-4-deoxy-L-arabinose, whereas the substitution is approximately 10 to 15% in the corresponding wild-type lipid A (44a).

In this report I show that the *pmrA* mutation leads to increased OM resistance to cations other than polymyxin and to Tris-EDTA, suggesting that 4-amino-4-deoxy-L-arabinose plays a role in the interactions among the major OM constituents.

MATERIALS AND METHODS

Bacterial strains. The relevant genotypes of the *S. typhimurium* strains used are shown in Table 1. Strains SH7518 and SH7519 are *pmrA*$^+$ and *pmrA*

TABLE 1. *S. typhimurium strains used and their sensitivities to antibiotics, as tested by the disk diffusion method*[a]

Strain	Relevant genotype	Zone diam (mm)				
		Ampicillin	Neomycin	Erythromycin	Novobiocin	Clindamycin
SH5014[b]	*rfaJ4041*	15	11	2	0	0
SH5357[b]	*rfaJ4041 pmrA19*	15	12	2	3	0
SH6482[b]	*rfaJ4041*	15	11	1	0	0
SH6497[b]	*rfaJ4041 pmrA125*	19	14	4	0	0
SH9178[c]	*rfaJ4041* (R471a)[d]	0	11	4	2	0
SH7426[c]	*rfaJ4041 pmrA163 (R471a)*	0	11	2	0	0
SH7518[e]	*rfaE543*	21	13	12	13	15
SH7519[e]	*rfaE543 pmrA168*	18	14	16	17	14

[a] Each value represents the diameter of the inhibition zone around an antibiotic-containing disk. The diameter of the disk (9 mm) has been subtracted from each value. I also tested sensitivities to fusidic acid, cephalothin, methicillin, tetracycline, lincomycin, chloramphenicol, rifamycin, vancomycin, nalidixic acid, and gentamicin. The *pmrA* strains were as sensitive as the parent strains to these agents.

[b] Described in references 24 and 45.

[c] Described in reference 44.

[d] R471a codes for β-lactamase.

[e] Isogenic derivatives of strain SL1102 (*rfaE543*) (50).

derivatives of strain SL1102 (*S. typhimurium* LT2 *rfaE*) (50), which tolerate 0.03 and 0.3 μg of colistin E sulfate per ml, respectively, as measured on nutrient agar plates (29). *Escherichia coli* strain S17 [*pldA*(Pld2$^-$)] and its parent strain S15 [*pldA*$^+$-(Pld2$^+$)] have been described previously by Doi et al. (8) and were kind gifts from C. S. Buller (University of Kansas, Lawrence).

Media and growth conditions. For all experiments, the bacteria were grown in L-broth (10 g of tryptone [Difco Laboratories, Detroit, Mich.], 5 g of yeast extract [Difco], and 5 g of NaCl per liter; pH 7.0) on a rotary shaker at 220 rpm and 37°C to the early logarithmic phase of growth (40 Klett units, as determined with a Klett-Summerson colorimeter equipped with a red filter), unless otherwise stated.

Growth inhibition tests. A 0.1-ml volume of a 1:100 dilution of bacteria was spread onto an agar plate (diameter, 15 cm) containing 60 ml of L-agar (L-broth containing 1.5% agar). Plates prepared in this way were dried at 37°C for 15 min, and then 9-mm disks containing antimicrobial agents were placed on them. The plates were incubated for 18 h at 37°C, and the diameter of growth inhibition around each disk was measured.

Inhibition by other drugs was tested in tubes containing increasing concentrations of each drug in L-broth. Each tube was inoculated with approximately 1,000 bacteria and then incubated for 24 h at 37°C. The lowest concentration of drug that completely inhibited visible growth was recorded.

In some cases growth inhibition was also tested by the method of Mäkelä et al. (24); sectors on nutrient agar plates containing different concentrations of a drug were streaked with a glass rod dipped in a tube containing a suspension of bacteria in water (approximately 10^8 cells per ml, inoculated from a fresh colony growing on L-agar).

Cephaloridine diffusion across the OM. Cephaloridine diffusion across the OM was measured by using strains SH9178 and SH7426, which produce periplasmic β-lactamase, and the method of Nikaido et al. (33). The bacteria were washed in 10 mM sodium phosphate–5 mM $MgCl_2$ (pH 7.0) and were then resuspended in the same buffer. The β-lactamase activities in these cell suspensions, in supernatants (obtained after centrifugation of the bacterial suspensions after they stood for 10 min at room temperature), and in sonic extracts (after sonication of suspensions in an ice water bath with a sonicator [Measuring & Scientific Equipment, Ltd., London, England]) were determined by incubating them with 0.8 mM cephaloridine in 10 mM sodium phosphate–5 mM $MgCl_2$ (pH 7.0) for 10 min at room temperature; then the product resulting from cephaloridine hydrolysis was measured as described by Zimmerman and Rosselet (51). In each case the intact cell β-lactamase activity was calculated as described by Nikaido et al. (33) by determining the difference between the β-lactamase activities in the cell suspension and in the supernatant. The activity in the supernatant was less than 5% of the activity in the cell suspension.

Viable counts. The viable counts of drug-treated bacterial suspensions were determined immediately by plating appropriate dilutions in L-broth onto L-agar plates.

Periplasmic β-lactamase leakage. Drug-treated bacterial suspensions were centrifuged at room temperature, and the supernatants were analyzed for β-lactamase activity by the method of Sargent (38), as described previously (44). The drugs used did not interfere with the determinations. Glucose-6-phosphate dehydrogenase activities were measured as described by Langdon (18).

Deoxycholate sensitivity. The deoxycholate sensitivity of drug-treated bacteria was measured as described previously (44) by pelleting the treated bacteria, suspending them in 0.07 M potassium phosphate

buffer (pH 7.2), and recording the decrease in absorbance of the suspension with a Klett colorimeter after a 10-min incubation at 37°C in the presence of 0.25% deoxycholate.

EDTA-released LPS. Bacterial suspensions grown in L-broth with or without 2 mM Ca^{2+} were washed twice in 0.05 M Tris-hydrochloride (pH 7.2) and then resuspended in 1-ml volumes of the same buffer to a concentration of 30 mg (dry weight) of bacteria per ml. These suspensions were prewarmed at 37°C, treated with 10 mM EDTA for 2 min at 37°C with gentle shaking, diluted 1:10 with 0.05 M Tris-hydrochloride, and centrifuged. Samples (1 ml) of the supernatants were dried in vacuo over NaOH, hydrolyzed in 0.5 M H_2SO_4 for 10 min, and analyzed for 2-keto-3-deoxyoctulosonic acid (49) as a parameter for LPS release. This release was calculated as a percentage of the total 2-keto-3-deoxyoctulosonic acid content of lyophilized cells.

OM-associated phospholipase A. The envelope material was isolated essentially as described by Ames (1) from early exponential-phase cells by using mild sonication, followed by centrifugation at 1,500 × *g* (to remove unbroken cells) and at 8,000 × *g* for 10 min. The pellet resulting from the latter centrifugation was washed twice with 10 mM Tris-hydrochloride buffer (pH 7.2) and assayed for phospholipase activity by the method of Osborn et al. (36), using [*choline-methyl*-^{14}C]phosphatidylcholine as the substrate. Protein contents were determined by the method of Lowry et al. (22), using bovine serum albumin as the standard.

Sources of chemicals. Antibiotic-containing disks were from A/S Rosco, Taastrup, Denmark. Polylysine (molecular weight, 3,800), polymyxin B sulfate, bacitracin, phosphatidylcholine, and 2-keto-3-deoxyoctulosonic acid were from Sigma Chemical Co., St. Louis, Mo. Protamine, sodium dodecyl sulfate, and sodium deoxycholate were from BDH Chemicals Ltd., Poole, England. Cetyltriammonium chloride and gentian violet B (methyl violet) were from E. Merck AG, Darmstadt, Germany. Benzalkonium chloride was from Oy Medica Ab, Helsinki, Finland. Lysozyme (from chicken egg white) was from Boehringer-Mannheim, Hannheim, Germany; benzylpenicillin was from A/S Novo Industri, Copenhagen, Denmark; and cephaloridine was from Glaxo Ltd., Middlesex, England. Octapeptin (type EM 49) was a kind gift from E. Meyers, E. R. Squibb & Sons, Princeton, N. J., [*choline-methyl*-^{14}C]phosphatidylcholine (specific activity, 53 μCi/μmol) was from New England Nuclear Corp., Boston, Mass.

RESULTS

Sensitivities to antibiotics, dyes, and detergents. I found no differences in sensitivities to antibiotics (as tested by the disk diffusion method) in corresponding *pmrA*$^+$ and *pmrA* strains (Table 1). The sensitivities of *rfaE* strains to hydrophobic antibiotics were not altered by the *pmrA* mutation. When gentamicin sensitivity was determined more exactly in tubes containing twofold serial dilutions of gentamicin in L-broth, the minimal inhibitory concentration was the same for the *pmrA* strains and their parents.

The unaltered sensitivities of the *pmrA* strains to hydrophilic antibiotics suggested unaltered penetration of these antibiotics through the OM and thus normal porin functions. To test the porin function in more detail, the methods of Zimmerman and Rosselet and Nikaido et al. (33, 51) were used. The diffusion of cephaloridine (a small hydrophilic molecule) across the OM could be measured by determining the rate of cephaloridine hydrolysis by the β-lactamase located in the periplasmic space. I found no difference between the *pmrA* strain SH7426 and its parent strain, SH9178, with respect to cephaloridine diffusion; the intact cell activity of the β-lactamase and the total β-lactamase activity in the sonic extract were 367 and 2,220 nmol of cephaloridine per min per mg (dry weight) of cells, respectively, for strain SH9178 and 300 and 1,890 nmol of cephaloridine per min per mg (dry weight) of cells, respectively, for strain SH7426. The *pmrA* mutation did not alter the sensitivities of the bacteria to the nonionic detergent TX-100 (minimal inhibitory concentration, > 20 mg/ml) and to the basic hydrophobic dye gentian violet (minimal inhibitory concentration, 30 μg/ml). The *pmrA* strains showed essentially the same sensitivities as the parent strains to sodium dodecyl sulfate and the same or slightly greater sensitivities to sodium deoxycholate (Table 2). In a separate experiment, slightly greater sensitivities were observed in four of six additional *pmrA* mutants tested. However, the sensitivity difference was small compared with the more than 100-fold sensitivity increase caused by the *rfaE* mutation.

The *pmrA* mutants were also slightly sensitized to cationic detergents (Table 2). Sensitivity to benzalkonium chloride was also tested on nutrient agar plates. The *pmrA*$^+$ strains were inhibited at a concentration of 400 μg/ml, whereas the corresponding *pmrA* strains and 12 additional, independent polymyxin-resistant mutants were inhibited at concentrations of 50 to 100 μg/ml.

Sensitivities to cationic agents. (i) General. The sensitivities of the *pmrA* strains to cationic detergents and their resistance to polymyxin suggested that these detergents acted in very different ways at the OM level. Therefore, I tested the sensitivities of the bacteria to some other cationic agents (the structurally polymyxin-related compound octapeptin and the basic polypeptides polylysine and protamine).

The growth of the wild-type strains and of the *pmrA* strains were inhibited similarly by octapeptin (Table 2). Under the conditions of the assay used, octapeptin appeared to be biologically less active than polymyxin; the minimal

inhibitory concentrations of octapeptin for wild-type *rfaJ* strains were approximately 50 times higher than the minimal inhibitory concentrations of polymyxin.

In the experiments shown in Fig. 1, increasing concentrations of different cationic agents (polymyxin, octapeptin, benzalkonium chloride, protamine, and polylysine) were incubated with the bacterial suspensions.

(ii) Viable counts. I found that a *pmrA* strain

TABLE 2. *Minimal growth inhibitory concentrations of various detergents and other inhibitory agents*[a]

Strain	Relevant genotype	Minimal inhibitory concn of:					
		Polymyxin (μg/ml)	Octapeptin (μg/ml)	Benzalkonium chloride (μg/ml)	Cetyltriammonium chloride (μg/ml)	Sodium dodecyl sulfate (mg/ml)	Sodium deoxycholate (mg/ml)
SH5014	*rfaJ*	0.012	0.8	15	15	2	>40
SH6482	*rfaJ*	0.012	0.8	18	20	2	>40
SH9178	*rfaJ*	0.012	0.8	12	30	1	>40
SH5357	*rfaJ pmrA*	0.2	0.8	6	10	1	>40
SH6497	*rfaJ pmrA*	0.8	0.4	6	10	1	40
SH7426	*rfaJ pmrA*	1.600	0.4	3	15	1	20
SH7518	*rfaE*	0.003	0.025	1	1	<0.02	0.03
SH7519	*rfaE pmrA*	0.025	0.025	0.25	0.5	<0.02	0.03

[a] Tested in tubes containing L-broth and increasing concentrations of the agents.

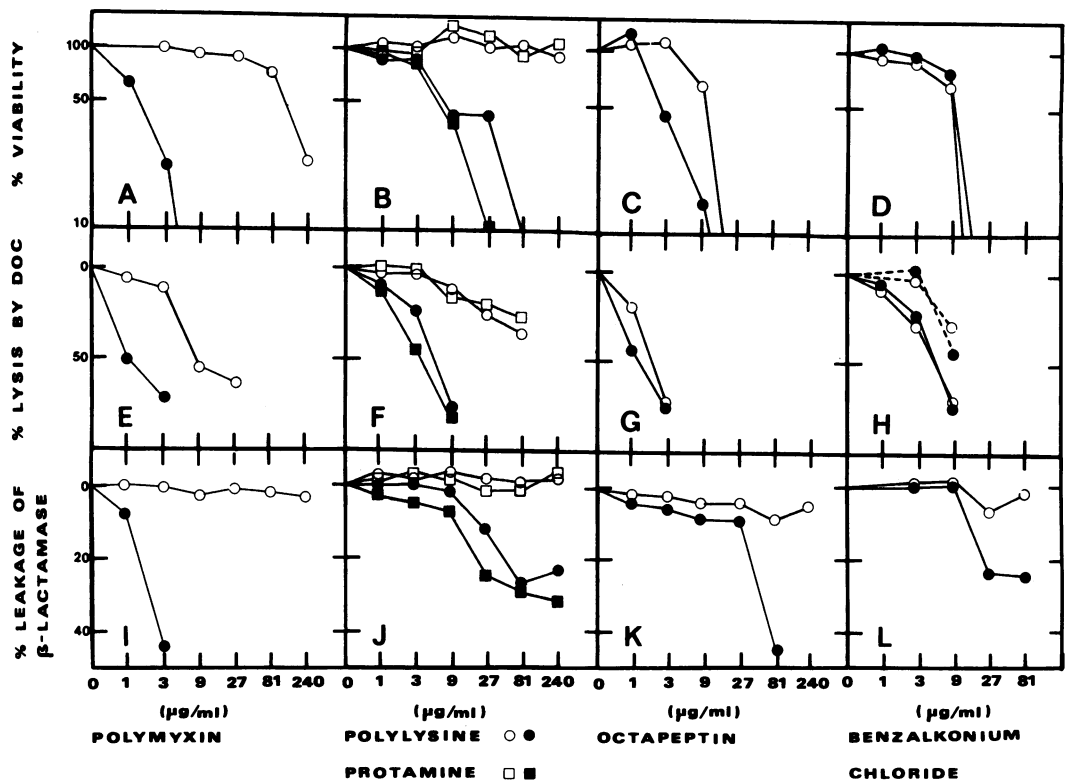

FIG. 1. *Effects of polymyxin, protamine, polylysine, octapeptin, and benzalkonium chloride on the viable count, deoxycholate (DOC) sensitivity, and periplasmic β-lactamase leakage in pmrA strain SH7426* (○ *and* □) *and in its wild-type parent, strain SH9178* (● *and* ■). *Bacteria in 0.07 M potassium phosphate buffer (pH 7.2) were incubated with each drug for 10 min at 37°C. Deoxycholate sensitivity was measured by pelleting the treated bacteria, suspending them in buffer containing 0.25% deoxycholate, and measuring the absorbance decrease of this suspension in 10 min. The dotted lines in (H) indicate the percent lysis in the absence of deoxycholate.*

differed from its parent strain in that the *pmrA* strain was resistant to polymyxin, protamine, and polylysine (Fig. 1A to D). These two strains were equally sensitive to octapeptin and benzalkonium chloride. Similar results were also obtained with another pair of strains (strains SH6497 and SH6482).

(iii) Sensitization to lysis by deoxycholate. Sublethal amounts of polymyxin sensitize bacteria to various agents, including deoxycholate. It has been thought that this indicates that polymyxin alters the OM structure and disrupts the OM permeability barrier to these agents (43, 44). As shown in Fig. 1E to H, treatment with sublethal amounts of cationic agents other than polymyxin sensitized the bacteria to deoxycholate-induced lysis. In this assay the *pmrA* strain showed increased resistance to polymyxin, protamine, and polylysine, as well as unchanged sensitivity to octapeptin and benzalkonium chloride. Similar results were obtained with strains SH6497 and SH6482.

Deoxycholate (0.25%) alone did not decrease the absorbance of either the polymyxin-sensitive bacteria or the polymyxin-resistant bacteria. In contrast to the other cationic agents used, benzalkonium chloride decreased the absorbance of the bacterial suspensions even in the absence of deoxycholate; however, this occurred at the lethal benzalkonium chloride concentration, which was approximately three times higher than the concentration required to cause lysis in the presence of deoxycholate (Fig. 1H).

(iv) Leakage of periplasmic β-lactamase. All cationic agents tested caused partial leakage of periplasmic β-lactamase from the *pmrA*$^+$ bacteria at lethal drug concentrations (Fig. 1I to L). In contrast, there was no leakage of β-lactamase from the *pmrA* bacteria.

Because all cationic agents tested caused leakage of β-lactamase from the *pmrA*$^+$ *rfaJ* strain of *S. typhimurium*, I also tested the effect of smaller cations (Table 3). NaCl, $MgCl_2$, and Tris-hydrochloride (pH 7.2) caused a remarkable release of β-lactamase from the wild-type strain when the treatment was performed in ice water. Significantly smaller amounts of β-lactamase leaked from the *pmrA* strain. There was no leakage of glucose-6-phosphate dehydrogenase (an intracytoplasmic enzyme) under any of the test conditions used.

Sensitivity to Tris-EDTA. EDTA is known to increase OM permeability, probably by removing divalent cations that link LPS molecules to each other and to proteins (21). Thus, EDTA can be used as a probe of interactions between OM components. This was done by studying the effects of EDTA on the OM permeability to lysozyme, deoxycholate, and bacitracin, as well as by measuring EDTA-induced release of LPS into the medium (Fig. 2).

TABLE 3. *Leakage of periplasmic β-lactamase in preparations containing different concentrations of NaCl, $MgCl_2$, or Tris-hydrochloride (pH 7.2)*[a]

Prepn	Concn (mM)	% Leakage of β-lactamase[b]			
		At 37°C		In ice water	
		Strain SH9178 (wild type)	Strain SH7426 (*pmrA*)	Strain SH9178 (wild type)	Strain SH7426 (*pmrA*)
Water		8	8	7	4
NaCl	100	9	9	21	12
	200	12	14	30	11
	300	24	18	41	14
$MgCl_2$	30	6	11	19	7
	100	18	15	35	10
	300	11	14	52	13
Tris	50	5	7	21	8
	90	6	8	36	13

[a] After 30 min of incubation in the test medium at 37°C or in ice water, the bacterial suspensions were centrifuged at room temperature, and the supernatants were assayed for β-lactamase activity. The activity of leaked glucose-6-phosphate dehydrogenase under any of the test conditions used was less than 3% of the total cellular activity.

[b] Total β-lactamase activities (in sonic extracts) were 68 and 60 U/10^9 cells for strains SH9178 and SH7426, respectively; 1 U hydrolyzes 1 μmol of benzylpenicillin in 1 h at 37°C.

Lysozyme (5 μg/ml) alone or deoxycholate (0.25%) alone did not have any effect on the absorbance values of the bacterial suspensions, but when 0.1 mM EDTA was added, the absorbance of *pmrA*$^+$ bacterial suspensions began to decrease, whereas all of the *pmrA* strains remained resistant. Five other polymyxin-resistant *S. typhimurium* mutants were tested for EDTA-lysozyme sensitivity; all were resistant. When the experiments were performed in 0.15 M NaCl or 0.07 M potassium phosphate buffer (pH 7.2) instead of 0.05 M Tris-hydrochloride (pH 7.2), EDTA did not sensitize even the wild-type bacteria.

To sensitize the bacteria to bacitracin by using EDTA, the bacterial suspensions were treated with EDTA and diluted into L-broth containing bacitracin (100 U/ml). This completely inhibited the growth of the *pmrA*$^+$ bacteria but had little effect on the *pmrA* bacteria (Table 4). EDTA treatment alone or bacitracin alone did not inhibit the growth of any strain.

Table 5 shows the amount of LPS released from the bacteria into the medium by EDTA treatment. EDTA released identical small

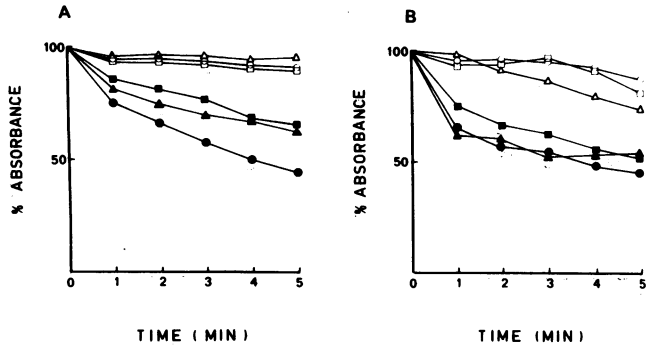

FIG. 2. *EDTA-induced sensitivity to lysozyme (A) and deoxycholate (B) in wild-type strains SH5014 (●), SH6482 (■), and SH9178 (▲) and pmrA mutant strains SH5357 (○), SH6497 (□), and SH7426 (△). To L-broth-grown bacterial suspensions in 0.05 M Tris-hydrochloride (pH 7.2) containing 5 μg of lysozyme per ml or 0.25% deoxycholate, 0.1 mM EDTA was added, and the percentage of absorbance decrease was monitored.*

TABLE 4. *EDTA-induced sensitivity to bacitracin*[a]

Strain	Turbidity increase in L-broth containing bacitracin	
	Without EDTA treatment	After EDTA treatment
SH5014	87[a]	0
SH6482	93	18
SH9178	83	1
SH5357 (*pmrA*)	87	68
SH6497 (*pmrA*)	53	45
SH7426 (*pmrA*)	88	77

[a] Some bacterial suspensions grown in L-broth were not treated with EDTA, others were treated with 0.1 mM EDTA in 0.05 M Tris-hydrochloride (pH 7.2) for 2 min with gentle shaking; then these suspensions were diluted 1:5 into L-broth containing bacitracin (100 U/ml). The optical density (in Klett units) of each preparation was measured after 1 h of incubation.

TABLE 5. *Tris-EDTA-induced LPS release*[a]

Strain	% Of LPS released	
	L-broth	L-broth containing 2 mM Ca^{2+}
SH5014	10	27
SH6482	10	31
SH9178	8	24
SH5357 (*pmrA*)	11	15
SH6497 (*pmrA*)	11	16
SH7426 (*pmrA*)	9	14

[a] Bacteria were grown in L-broth or in L-broth containing Ca^{2+}, and 30 mg (dry weight) of bacteria in 1 ml of 0.05 M Tris-hydrochloride (pH 7.2) was treated with 10 mM EDTA for 2 min. The supernatants were analyzed for 2-keto-3-deoxyoctulosonic acid (as a parameter of LPS release) and compared with the total amount of 2-keto-3-deoxyoctulosonic acid in the bacteria.

amounts of LPS (9 to 11%) from both the wild-type strains and the *pmrA* strains. These amounts corresponded to the values described for comparable rough enterobacterial strains (12, 13) and were significantly smaller than the values for smooth strains (12, 48). However, when the bacteria were grown at a higher calcium concentration (L-broth supplemented with 2 mM Ca^{2+}), which increased EDTA-induced LPS release (6, 21), EDTA released substantial amounts of LPS (24 to 31%) from the *pmrA*$^+$ bacteria and about twofold less LPS from the *pmrA* bacteria. The total concentration of LPS in all bacterial strains was the same (6 to 7 nmol/mg [dry weight] of bacteria), corresponding to the results obtained previously with comparable strains (12).

Polymyxin reportedly activates OM-associated phospholipase A (17), and the *E. coli* mutant strain S17, which lacks this phospholipase and the cytoplasmic phospholipase A (8), is resistant to EDTA-induced actinomycin sensitivity (13). However, the phospholipase A activity in the membrane preparations was not altered by the *pmrA* mutation; the activities of two *pmrA*$^+$ strains (SH5014 and SH6482) and two *pmrA* strains (SH5357 and SH6497) were 1.24 to 0.98 and 1.12 to 1.16 nmol of substrate per min per mg of envelope protein, respectively. Furthermore, strain S17 was as sensitive to polymyxin as its parent strain, as reported previously (F. H. Jahns and C. S. Buller, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, K85, p. 140).

DISCUSSION

The *pmrA* mutation does not affect the diffusion of either hydrophobic or hydrophilic molecules through the OM. This was shown by the unaltered resistance to hydrophobic antibiotics (Table 1) and by the unaltered diffusion of cephaloridine across the OM. Thus, the effect of the

pmrA mutation on the OM could be characterized as more subtle or specific than the effect of many other OM mutations.

The *pmrA* mutation slightly increased sensitivity to deoxycholate, an anionic detergent. The polymyxin-resistant *E. coli* mutants of Dame and Shapiro (7) also showed variably increased sensitivities to this detergent. However, the *E. coli* mutants must be characterized more fully before they can be regarded as equivalent to *Salmonella pmrA* mutants.

Because the LPSs from the *pmrA* strains (four tested) bind less polymyxin than the LPSs from the *pmrA*$^+$ parent strains (three tested) and because they contain 0.6 to 0.7 mol of 4-amino-4-deoxy-L-arabinose per mol of LPS as a substituent of the ester-linked lipid A phosphate (the corresponding value for the wild-type LPS is 0.1 to 0.15 mol/mol of LPS), it is probable that in the OM polymyxin binds to the anionic phosphate group(s) in lipid A (2, 28, 44a). The linkage of 4-amino-4-deoxy-L-arabinose to LPS not only eliminates one negative charge from the LPS but also introduces an additional positive charge, thus reducing the capability of LPS to bind basic molecules. The resistance of the *pmrA* mutants (Fig. 1) to the antibacterial polycationic peptides protamine (41) and polylysine (3, 16) suggests that these agents bind to the same target as polymyxin. The binding of protamine to LPS was described in 1958 (31). In addition, LPS binds other cationic agents, including mono- and divalent cations (10, 39), polyamines (10), Tris (39), and cholestyramine (34). A low-molecular-weight (15,000) basic cell surface protein with a high affinity for LPS has been described in *Salmonella minnesota,* and this protein is most probably present in other enteric bacteria as well (11).

The data give seemingly contradictory information on the effects of cationic detergents and octapeptin. On the one hand, these agents promote leakage of macromolecules (Fig. 1K and L) from *pmrA*$^+$ strains but not *pmrA* strains, suggesting an action similar to the action of polymyxin. On the other hand, *pmrA*$^+$ and *pmrA* strains are equally sensitive to these agents alone (Fig. 1C and D) or in combination with deoxycholate (Fig. 1G and H), suggesting a target separate from the target of polymyxin. These data may be interpreted by assuming there are two (or more) separate targets for octapeptin and cationic detergents, only one of which is shared by polymyxin, protamine, and polylysine; the other target(s) would be decisive for the killing action of these agents. Nevertheless, the results are rather surprising for octapeptin, whose structure is very similar to that of polymyxin (8 amino acids instead of 10, 4 amino groups instead of 5, and a somewhat longer fatty acid chain) (42). The three polymyxin-resistant variants of *E. coli* studied by Meyers et al. (27) also showed no cross-resistance with octapeptin.

The OM of the *pmrA* mutants was also resistant to EDTA-induced permeability increase (Fig. 2 and Table 4). This resistance could indicate that in these strains, in contrast to the wild-type strains (21, 47), the LPS-LPS interaction or the LPS-protein interaction or both are not mediated by divalent cations. Instead, the basic 4-amino-4-deoxy-L-arabinose residues, which are glycosidically linked to LPS, could also be ionically linked to adjacent anionic groups in neighboring molecules. Because EDTA treatment is effective only in solutions containing Tris (21; see above), it is possible that in the presence of EDTA there is an increased binding of the cationic Tris molecules to the OM. The resistance of the *pmrA* strains to Tris-EDTA could then be at least partly due to a decreased binding of Tris to LPS. EDTA-resistant mutants of both *E. coli* and *Shigella flexneri* have been described (5, 6, 48). However, the molecular mechanism for this resistance and the patterns of resistance to other agents, such as polycations, have not been elucidated.

The OM permeability barrier to macromolecules (β-lactamase) was disrupted by high concentrations of cations, such as Na^+, Mg^{2+} and $Tris^+$, in the cold (Table 3), and the *pmrA* strains were more resistant to this treatment. A probable parallel phenomenon is that cold 0.1 M Ca^{2+} renders *E. coli* and *S. typhimurium* cells capable of acting as recipients in transformation (19, 26). Although the molecular basis of the destabilizing action of cold cations on the OM remains unknown, these cations could inhibit the divalent-mediated interaction between major OM constituents. It is difficult to explain why the cations are more effective in the cold. The irreversible adsorption of bacteriophage K3 to *E. coli* K-12 OM is strongly dependent on the incubation temperature; it has been suggested that this dependence is caused by the phase transition of the LPS (46).

In conclusion, the results presented in this paper show that the *pmrA* mutation leads to increased OM resistance to several cations and Tris-EDTA. The substitution of the *pmrA* LPS by 4-amino-4-deoxy-L-arabinose could account for this resistance. Also, wild-type *Salmonella* can increase the 4-amino-4-deoxy-L-arabinose content in LPS under some growth conditions (20, 29). Thus, the LPS with 4-amino-4-deoxy-L-arabinose and the more anionic LPS without it could represent two alternative forms of LPS. Both of these LPS types form stable LPS-LPS interactions or LPS-protein interactions or both,

but it is possible that these interactions are dependent on divalent cations only in the more anionic LPS type.

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