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Outer Membrane Differences between Pathogenic and Environmental *Yersinia enterocolitica* Biogroups Probed with Hydrophobic Permeants and Polycationic Peptides

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Sensitivities to polycationic peptides and EDTA were compared in *Yersinia enterocolitica* pathogenic and environmental biogroups. As shown by changes in permeability to the fluorescent hydrophobic probe *N*-phenylnaphthylamine (NPN), the outer membranes (OMs) of pathogenic and environmental strains grown at 26°C in standard broth were more resistant to poly-L-lysine, poly-L-ornithine, melittin, cecropin P1, polymyxin B, and EDTA than *Escherichia coli* OMs. At 37°C, OMs of pathogenic biogroups were resistant to EDTA and polycations and OMs of environmental strains were resistant to EDTA whereas *E. coli* OMs were sensitive to both EDTA and polycations. Similar results were found when testing deoxycholate sensitivity after polycation exposure or when isogenic pairs with or without virulence plasmid pYV were compared. With bacteria grown without Ca^{++} available, OM permeability to NPN was drastically increased in pathogenic but not in environmental strains or *E. coli*. Under these conditions, OMs of pYV^+ and pYV^- cells showed small differences in NPN permeability but differences in polycation sensitivity could not be detected by fluorimetry. O:1,6 (environmental type) lipopolysaccharide (LPS), but not O:3 or O:8 LPS, was markedly rough at 37°C, and this could explain the differences in polycation sensitivity. LPSs from serotypes O:3 and O:8 grown at 37°C were more permeable to NPN than O:1,6 LPS, and O:8 LPS was resistant to polycation-induced permeabilization. These data suggest that LPSs relate to some but not all the OM differences described. It is hypothesized that the different OM properties of environmental and pathogenic biogroups reflect the adaptation of the latter biogroups to pathogenicity.

Yersinia enterocolitica is a gram-negative bacterium that can be isolated from a variety of sources, such as water, foodstuffs, and animals. In addition, some *Y. enterocolitica* strains behave as gastrointestinal pathogens of humans causing mesenteric lymphadenitis, diarrhea, and enteritis (7). On the basis of standard bacteriological tests, five biogroups have been recognized within the species, and biogroup 1 is further divided into two subgroups (1A and 1B) (6). While pathogenic *Y. enterocolitica* strains belong to biogroups 1B, 2, 3, 4, and 5, the environmental (nonpathogenic) strains are clustered within biogroup 1A. In addition to biogroups, serotypes can be defined. The human pathogenic strains belong to a few serotypes which in general have a defined geographical distribution: most isolates from Europe, Canada, Japan, and South Africa belong to serotypes O:9 (biogroup 2), O:5,27 (biogroup 2), and O:3 (biogroup 4), whereas those from the United States belong to serotypes O:8, O:4, O:13, and O:21 (all belonging to biogroup 1B) (6).

Most studies of *Y. enterocolitica* pathogenicity have been focused on the expression of virulence markers by the pathogenic biogroups. Only virulent strains harbor the 70-kb virulence plasmid pYV, which is related to resistance to unspecific host defenses (complement and phagocytosis; for a review, see reference 5) and codes for several proteins, such as the so-called *Yersinia* outer membrane proteins (Yops) and the *Yersinia* adhesin A (YadA) (5, 6). Other virulence markers are chromosome encoded (enterotoxin [Yst], mucoid *Yersinia* factor [Myf], and the Inv and Ail proteins [5, 17, 18]). Expression of those virulence markers is modulated by growth temperatures at both the plasmid and the chromosome level (5, 40) and also by pH (17, 18, 32), oxygen tension (31), and growth phase (32). In addition, comparative studies of pYV^+ (virulent) and pYV^- (avirulent) strains of the pathogenic biogroups have shown differences in surface hydrophobicity and cell charge, which are possibly linked to the expression of Yops and to virulence (2, 19, 25). However, to the best of our knowledge, no comparative studies of the constitutive properties of the outer membranes (OMs) of environmental and pathogenic strains have been carried out. Such studies could help to find features absent from environmental yersiniae but present in pathogenic biogroups which may be necessary for pathogenicity. In this work, we have probed the OMs of representative strains of all *Y. enterocolitica* biogroups with EDTA and several polycationic peptides in the presence of compounds (*N*-phenylnaphthylamine [NPN] and deoxycholate) permeating into the hydrophobic moiety of the OM. This approach has been chosen because there is an increasing body of evidence linking OM sensitivity to polycations and virulence in enteric (9, 11, 37) and nonenteric (27) bacteria. This link exists because the bactericidal peptides of mammals and lower vertebrates (defensins and beta-defensins, proline- and arginine-rich peptides, amphipathic helical peptides, and ranalexins [for a review, see reference 28]) are amphipathic polycationic peptides that share with some other polycations (46) the initial OM binding steps which alter OM permeability, thereby gaining access to internal targets (11, 13, 21, 28, 37, 46). We report here differences in OM permeability and polycation sensitivity between environmental and pathogenic biogroups, which are manifested under different growth conditions (Ca^{++} availability and temperature). Moreover, in pathogenic but not in environmental biogroups, these properties differed from those of

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TABLE 1. Bacterial strains used in this study

Strain	Serogroup(s)	Biogroup	Virulence marker		Origin
			pYV[a]	Myf[b]	
Y. enterocolitica					
PR	O:1,6	1A	−	−	Environment
MA 257	O:7,8,13,19	1A	−	−	Environment
MA 268	O:7,8	1A	−	−	Environment
MA 279	O:5	1A	−	−	Environment
MA 280	O:6	1A	−	−	Environment
MA 282	O:7,8,13,19	1A	−	−	Environment
WA 289	O:8	1B	+	+	Clinical isolate
WA	O:8	1B	+	+	Clinical isolate
Y7P	O:8	1B	+	+	Clinical isolate
WE 517/91	O:9	2	+	+	Clinical isolate
PT	O:9	2	+	+	Clinical isolate
IP 135	O:1, O:2a, O:3	3	+	+	Chinchilla
WE 245/92	O:3	4	+	+	Clinical isolate
ID	O:3	4	+	+	Clinical isolate
IP 178	O:2a, O:2b, O:3	5	+	+	Hare
E. coli	0111K58H2				Clinical isolate

[a] Presence (+) or absence (−) of the *Yersinia* virulence plasmid.
[b] Presence (+) or absence (−) of the mucoid *Yersinia* factor.

OMs of better-characterized enteric bacteria (e.g., *Escherichia coli*), which were used as controls throughout the experiments.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used are listed in Table 1. The strains were stored in skim milk at −80°C, and for the experiments carried out with the pYV^+ isogenic pairs, inoculi were taken directly from the frozen seeds to minimize pYV^- dissociation. All were grown in tryptic soy broth (TSB; bioMérieux, Marcy l'Etoile, France) in sidearm flasks in an orbital shaker water bath (200 rpm) (New Brunswick Scientific Co., Inc., Edison, N.J.) at 37 or 26°C. By atomic absorption spectrophotometry, standard TSB contained 0.2 mM Ca^{++}. For maximal induction of the Yop regulon, bacteria were grown in TSB supplemented with 20 mM Cl_2Mg–20 mM sodium oxalate (MOX) (35). Bacterial growth was monitored by measuring the A_{540}, and cells were harvested (5,000 × *g*, 20 min, 5°C) in the appropriate phase of growth (see below) and resuspended immediately in the appropriate buffer for the OM permeability and sensitivity studies described below. For lipopolysaccharide (LPS) extraction, bacteria were grown in TSB in 2-liter flasks (500 ml per flask) in an orbital shaker (200 rpm) for 24 h at 37 or 26°C. The *Y. enterocolitica* strains of pathogenic biogroups used in LPS extraction were pYV^+ (see below).

Virulence markers. To select the virulent (pYV^+) and the isogenic plasmid-cured avirulent (pYV^-) strains, the Congo red-MOX medium was used (35). Presence or absence of pYV was confirmed by detection of protein YadA by an autoagglutination test (38). The mucoid *Yersinia* factor (Myf) was detected by coagglutination (41).

LPS preparations. The LPS of *Y. enterocolitica* PR O:1,6 (biogroup 1A), WA 289 O:8 (biogroup 1B), WE 517/91 O:9 (biogroup 2), and WE 245/92 O:3 (biogroup 4), were obtained from the water phase of a water-phenol extract as described elsewhere (39). To purify the LPSs, the preparations were dispersed (10 mg/ml) in 0.8% NaCl–0.05% NaN_3–0.1 M Tris-HCl (pH 7) and digested with nucleases (50 μg/ml of DNase II type V and RNase A [Sigma Chemical Co., St. Louis, Mo.]) for 18 h at 37°C. Proteinase K was added (50 μg/ml; E. Merck, Darmstadt, Germany), and the mixture was incubated for 1 h at 55°C and for 24 h at room temperature. The proteinase K digestion was repeated twice, and the LPSs were sedimented by ultracentrifugation (6 h, 100,000 × *g*), resuspended in distilled water, and freeze dried. The 3-deoxy-D-manno-2-octulosonic acid and protein contents were determined by the thiobarbituric acid (49) and the modified Lowry (24) methods, respectively. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining for LPS was performed as described by Tsai and Frasch (43).

Fluorimetry. NPN is a fluorescent probe whose quantum yield suddenly increases when transferred from a hydrophilic to a hydrophobic environment (42) and which has been used in LPS and OM permeability studies (3, 23, 26, 27). This probe was used in two different protocols.

(i) Viable cells. Exponentially growing cells were resuspended in 1 mM KCN–2 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.2) at an optical density at 600 nm of 0.5 and transferred immediately to 1-cm-diameter fluorimetric cuvettes. NPN (500 μM in acetone) was added to a final concentration of 10 μM. Immediately after NPN addition, peptides and EDTA were added at the following final concentrations: poly-L-lysine, 80 μg/ml; poly-L-ornithine, 40 μg/ml; melittin, 10 μg/ml; cecropin P1, 6 μg/ml; polymyxin B, 50 U/ml; EDTA, 10^1 to 10^4 μM; and Tris, 3.0 mM. In preliminary experiments, those agent concentrations were found to be the minimal concentrations showing the characteristic effects in this assay (see Results) and, in the case of polycations, were also bactericidal for the strains tested. Quenching under these conditions was not observed. Changes in fluorescence caused by the addition of the above agents were monitored with an LS-50 fluorimeter (Perkin-Elmer Ltd., Beaconsfield, England) set as follows: excitation, 350 nm; emission, 420 nm; and slit width, 2.5 nm. Results were expressed in relative fluorescence units (23, 27). Fluorescence measurements were performed at the temperature (26 or 37°C) at which a particular batch of cells had been grown. For each strain or isogenic pair, three independently grown batches of bacteria were used in the above-described fluorescence measurements and each measurement was repeated at least twice with each batch. Measurements were recorded as ASCII files and exported to a conventional personal computer for plotting. The kinetics of partition of the NPN probe was calculated as the fluorescence increase relative to the time it took for the probe to give maximal emission (relative fluorescence units per second).

(ii) LPS. The permeability to NPN of LPS aggregates from cells grown at 26 or 37°C was tested by fluorimetry in the presence or absence of EDTA and polycations. To verify that equal amounts of 26 and 37°C LPSs of each strain were being compared, silver-stained SDS-PAGE electropherograms (see above) were analyzed by densitometry with a JX-325 scanner and Image Master software (Pharmacia Fine Chemicals, Uppsala, Sweden) and the total LPS and the proportion of short (rough) and long (smooth) polysaccharide LPSs were determined (see Results). With these data, suspensions containing the same amount of 26 and 37°C LPS (approximately 400 μg) were prepared by brief sonication in 1.5 ml of 1 mM KCN–2 mM HEPES (pH 7.2) and, where appropriate, briefly incubated in the presence of either EDTA (10^3 μM), poly-L-lysine (4.5 μg/ml), or poly-L-ornithine (9 μg/ml). In preliminary experiments, those agent concentrations were found to be the minimal concentrations showing an effect in this assay. The suspension was then transferred to the cuvettes, and fluorescence was monitored as described above both before and after NPN addition.

Deoxycholate sensitivity induced by polycations. The assay described by Vaara and Vaara (47) was used. Briefly, exponentially growing cells were resuspended in saline to a final concentration of 10^9 CFU/ml in the presence of increasing concentrations (see Results) of poly-L-lysine, poly-L-ornithine, and polymyxin B. After 10 min of incubation at the growth temperature, cells were pelleted (7,000 × *g*, 15 min, 4°C) and resuspended in the same volume of 0.25% deoxycholate in saline. This cell suspension was incubated for 10 min (incubation up to 30 min did not change the results) at the growth temperature and the decrease in the optical density at 450 nm was measured. Results were expressed as percentages of the optical densities at 450 nm of controls incubated in the absence of polycations.

Polycations. Polymyxin B is a lipopeptide antibiotic which binds to a variety of LPSs and has an OM-disturbing action (46). Poly-L-lysine (molecular weight, 7,000 to 10,000) and poly-L-ornithine (molecular weight, 12,000 to 22,000) are synthetic peptides with reported bactericidal and OM-disturbing actions (46). Bee venom melittin is a bactericidal amphiphilic peptide which also binds to LPS

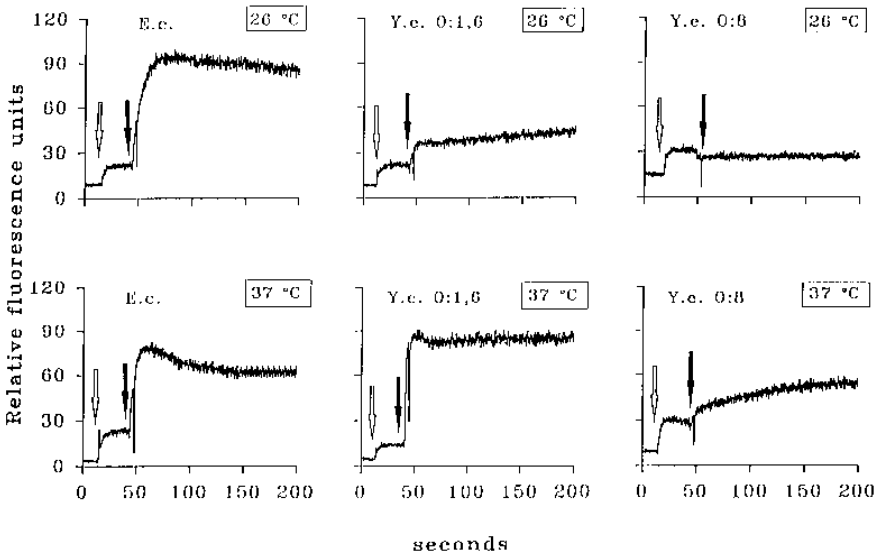

FIG. 1. Effect of poly-L-lysine on OM permeability to NPN of bacteria grown in TSB. *E. coli* 0111 (E.c.), *Y. enterocolitica* PR O:1,6 (Y.e. O:1,6), and *Y. enterocolitica* WA 289 O:8 (Y.e. O:8) were grown at either 26 or 37°C, and fluorescence was monitored at the indicated growth temperature. For each experiment, the addition of NPN is marked with an open arrow and addition of poly-L-lysine is marked with a filled arrow.

(8). Cecropin P1 is a bactericidal peptide from pig intestine (20). All the above-described agents were purchased from Sigma either as pure peptides or in the sulfate (polymyxin B) or hydrobromide (poly-L-lysine and poly-L-ornithine) forms.

Statistical analysis. Comparisons among results obtained with groups of pathogenic and environmental strains were made by the two-sample *t* test or, when the requirements were not met, by the Mann-Whitney U test.

RESULTS

Experiments with cells grown in TSB. (i) OM permeability to hydrophobic probes and effect of polycations. Figure 1 presents representative results of fluorimetric experiments carried out with cells grown in TSB in which the action of poly-L-lysine on the OM barrier to the hydrophobic NPN probe was tested. As can be seen, addition of NPN by itself (Fig. 1, open arrows) caused only small increases in fluorescence, which were similar in the cell suspensions of *E. coli* (control), *Y. enterocolitica* PR O:1,6 (environmental), and *Y. enterocolitica* WA 289 O:8 (pathogenic), regardless of the growth temperature. However, depending on the growth temperature and strain, increases in the fluorescence of the above-described NPN cell suspensions were observed after addition of poly-L-lysine (Fig. 1, filled arrows). When bacteria were grown at 26°C (Fig. 1, upper panels), poly-L-lysine caused a marked increase in the permeability of the *E. coli* OM to NPN, as shown by the sudden increase in fluorescence (Fig. 1, upper left panel). Under the same conditions, poly-L-lysine had only a reduced effect on *Y. enterocolitica* PR O:1,6 (Fig. 1 upper middle panel) and no effect on *Y. enterocolitica* WA 289 O:8 (Fig. 1, upper right panel). With cells grown at 37°C, the results obtained with *E. coli* (lower left panel) and *Y. enterocolitica* PR O:1,6 (lower middle panel) were similar to those obtained with *E. coli* at 26°C. On the other hand, poly-L-lysine had only a limited effect on *Y. enterocolitica* WA 289 O:8 (pathogenic) cells grown at 37°C, which was manifested both as a very slow increase in the kinetics of fluorescence and as a comparatively lower level of maximal fluorescence.

Similar experiments were performed with poly-L-lysine, poly-L-ornithine, melittin, cecropin P1, and polymyxin B and with cells from all the representative strains included in the study. At the growth temperature of 26°C (Table 2), no matter which strain was used, poly-L-lysine, poly-L-ornithine, and melittin caused only a very slow (from 0.01 to 1.73 relative fluorescence units [RFU/s]) partition of NPN into the OMs of *Y. enterocolitica*, with the exception of strain MA 280 O:6 (5.09 RFU/s for poly-L-lysine), whose value was close to that of *E. coli* (4.93 RFU/s for poly-L-lysine). When the data of the environmental and pathogenic strains were compared, no statistically significant differences ($P \geq 0.01$) were found for poly-L-lysine, poly-L-ornithine, or polymyxin B. Although pathogenic strains were statistically more resistant to melittin than environmental strains, the difference in permeability increase was very small (0.27 ± 0.18 versus 0.88 ± 0.43 [$P = 0.004$], respectively).

The actions of the same agents were tested on cells grown at 37°C (Table 3). The strains of biogroup 1A (environmental) showed an increase in the kinetics of NPN partition into the OMs when they were exposed to polycations, and this was also true for strain MA 280 O:6. A similar increase was not observed for the strains of the pathogenic biogroups grown at 37°C, and differences with the environmental group were statistically significant, in particular for poly-L-lysine ($P = 0.001$) and poly-L-ornithine ($P = 0.001$) but also for melittin ($P = 0.013$) and polymyxin B ($P = 0.015$). Furthermore, only little overlapping between the 95% confidence intervals of the mean was observed for melittin (0.3 to 2.0 versus 1.3 to 7.9) and none was observed for the other polycations. The data also suggested that serotype O:8 *Y. enterocolitica* strains were more sensitive to melittin than those of other pathogenic biogroups, but no statistical assessment was possible because of the limited number of strains tested. A limited number of experiments showed that the permeabilizing action of cecropin P1 was higher than that of poly-L-lysine, poly-L-ornithine, and melittin, but the similarities and differences in sensitivity between environmental and pathogenic strains grown at 26 and 37°C were similar to those described above (Tables 2 and 3).

Although the picture obtained with polymyxin B was not as clear as that with poly-L-lysine or poly-L-ornithine when considering only the kinetics of NPN uptake, the differences be-

TABLE 2. Effects of polycations on the permeability to NPN of the OMs of *E. coli* and *Y. enterocolitica* grown in TSB at 26°C

Strain	Serotype(s) (biogroup)	Result[a] of experiment with polycation:				
		Poly-L-lysine	Poly-L-ornithine	Melittin	Cecropin P1	Polymyxin B
E. coli	0111	4.93 ± 0.1	1.60 ± 0.1	3.48 ± 0.3	4.92 ± 0.2	16.70 ± 0.2
Y. enterocolitica						
PR	O:1,6 (1A)	0.20 ± 0.2	1.73 ± 0.4	0.60 ± 0.1	2.71 ± 0.2	2.32 ± 0.1
MA 257	O:7,8,13,19 (1A)	0.01 ± 0.2	0.97 ± 0.2	0.79 ± 0.1	2.01 ± 0.0	10.84 ± 0.2
MA 268	O:7,8 (1A)	0.80 ± 0.0	0.71 ± 0.1	1.51 ± 0.3	ND[b]	18.63 ± 0.4
MA 279	O:5 (1A)	0.09 ± 0.1	0.13 ± 0.1	1.26 ± 0.2	ND	2.25 ± 0.1
MA 280	O:6 (1A)	5.09 ± 0.1	2.58 ± 0.4	0.79 ± 0.0	ND	4.64 ± 0.1
MA 282	O:7,8,13,19 (1A)	0.19 ± 0.1	0.03 ± 0.1	0.33 ± 0.2	ND	2.30 ± 0.1
WA 289	O:8 (1B)	0.10 ± 0.2	0.08 ± 0.3	0.53 ± 0.0	1.83 ± 0.1	1.83 ± 0.0
WA	O:8 (1B)	0.20 ± 0.1	0.10 ± 0.1	0.44 ± 0.1	ND	1.77 ± 0.2
Y7P	O:8 (1B)	0.07 ± 0.1	0.25 ± 0.3	0.37 ± 0.1	ND	1.84 ± 0.0
WE 517/91	O:9 (2)	0.12 ± 0.2	0.13 ± 0.2	0.17 ± 0.1	2.12 ± 0.1	1.72 ± 0.1
PT	O:9 (2)	0.08 ± 0.3	0.10 ± 0.1	0.23 ± 0.0	ND	1.15 ± 0.2
IP 135	O:1, O:2a, O:3 (3)	0.14 ± 0.1	0.05 ± 0.2	0.20 ± 0.1	ND	ND
WE 245/92	O:3 (4)	0.10 ± 0.0	0.08 ± 0.1	0.14 ± 0.0	ND	3.41 ± 0.2
ID	O:3 (4)	0.09 ± 0.1	0.14 ± 0.0	0.20 ± 0.0	ND	2.59 ± 0.1
IP 178	O:2a, O:2b (5)	0.33 ± 0.1	0.09 ± 0.2	0.08 ± 0.1	ND	ND

[a] Results (means ± standard deviations of three independent experiments) are expressed as the kinetics of NPN partition into the cell envelope (relative fluorescence units per second).
[b] ND, not done.

tween environmental and pathogenic biogroups were clear when the corresponding fluorescence plots were compared. Figure 2 (upper panels) shows that the kinetics of polymyxin-induced NPN uptake were almost identical when the PR O:1,6 (biogroup 1A, environmental) strain and the WA 289 O:8 (biogroup 1B, pathogenic) strain were grown at 26°C. On the other hand, the results obtained with the environmental strain grown at 37°C (Fig. 2, lower left panel) resembled those obtained with *E. coli* and poly-L-lysine (Fig. 1, lower left panel) in which cells exposed to polymyxin B (50 U) took up the fluorescent probe at levels close to those obtained when the cells were damaged by brief heating (100°C, 3 min). In similar experiments, consistently less probe was taken up by the WA 289 O:8 strain (Fig. 2, lower right panel), although the kinetics of uptake was not very different from that obtained with the PR O:1,6 strain.

The OM-sensitizing effects of poly-L-lysine, poly-L-ornithine, and polymyxin B to the lytic action of the surfactant deoxycholate were tested first on cells grown at 37°C. With poly-L-lysine and poly-L-ornithine (Fig. 3, left and middle panels), this test confirmed the differences in OM sensitivity between environmental and pathogenic *Y. enterocolitica* biogroups, as strain PR O:1,6 (biogroup 1A, environmental) yielded results closer to those obtained with *E. coli* and both *Y. enterocolitica* PR

TABLE 3. Effects of polycations on the permeability to NPN of the OMs of *E. coli* and *Y. enterocolitica* grown in TSB at 37°C

Strain	Serotype(s) (biogroup)	Result[a] of experiment with polycation:				
		Poly-L-lysine	Poly-L-ornithine	Melittin	Cecropin P1	Polymyxin B
E. coli	0111	3.51 ± 0.1	6.41 ± 0.0	4.27 ± 0.1	4.50 ± 0.2	13.36 ± 0.3
Y. enterocolitica						
PR	O:1,6 (1A)	9.92 ± 0.2	2.89 ± 0.0	5.97 ± 0.3	19.96 ± 0.1	8.96 ± 0.2
MA 257	0:7,8,13,19 (1A)	6.18 ± 0.1	3.64 ± 0.1	4.07 ± 0.2	7.32 ± 0.1	7.32 ± 0.0
MA 268	O:7,8 (1A)	4.90 ± 0.0	2.92 ± 0.2	1.10 ± 0.0	ND[b]	3.56 ± 0.2
MA 279	O:5 (1A)	3.52 ± 0.4	3.42 ± 0.0	5.51 ± 0.2	ND	9.55 ± 0.2
MA 280	O:6 (1A)	14.79 ± 0.1	6.21 ± 0.1	9.49 ± 0.2	ND	11.2 ± 0.5
MA 282	O:7,8,13,19 (1A)	1.60 ± 0.1	1.27 ± 0.0	1.47 ± 0.3	ND	9.08 ± 0.0
WA 289	O:8 (1B)	0.27 ± 0.3	0.39 ± 0.1	2.07 ± 0.2	3.87 ± 0.1	3.25 ± 0.1
WA	O:8 (1B)	0.30 ± 0.1	0.25 ± 0.1	1.97 ± 0.0	ND	3.40 ± 0.1
Y7P	O:8 (1B)	0.20 ± 0.1	0.49 ± 0.1	2.20 ± 0.1	ND	3.01 ± 0.2
WE 517/91	O:9 (2)	0.68 ± 0.2	0.93 ± 0.0	0.48 ± 0.1	3.46 ± 0.1	3.71 ± 0.0
PT	O:9 (2)	0.54 ± 0.1	0.88 ± 0.1	0.39 ± 0.2	ND	3.58 ± 0.1
IP 135	O:1, O:2a, O:3 (3)	0.33 ± 0.2	0.26 ± 0.1	0.28 ± 0.1	ND	ND
WE 245/92	O:3 (4)	0.45 ± 0.2	0.36 ± 0.2	0.51 ± 0.0	ND	4.01 ± 0.3
ID	O:3 (4)	0.34 ± 0.1	0.47 ± 0.01	0.35 ± 0.2	ND	3.76 ± 0.0
IP 178	O:2a, O:2b (5)	0.02 ± 0.1	0.01 ± 0.2	0.33 ± 0.2	ND	ND

[a] Results (means ± standard deviations of three independent experiments) are expressed as the kinetics of NPN partition into the cell envelope (relative fluorescence units per second).
[b] ND, not done.

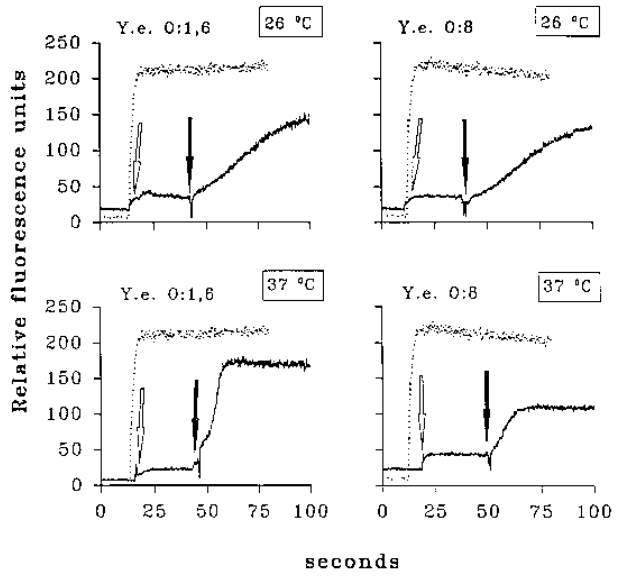

FIG. 2. Effect of polymyxin B on OM permeability to NPN of bacteria grown in TSB. *Y. enterocolitica* PR O:1,6 (Y.e. O:1,6) and *Y. enterocolitica* WA 289 O:8 (Y.e. O:8) were grown at either 26 or 37°C, and fluorescence was monitored at the indicated growth temperature. For each experiment, the addition of NPN is marked with an open arrow and addition of polymyxin B is marked with a filled arrow. Dotted lines are the results obtained with cells damaged by brief heating.

O:1,6 and *E. coli* were more sensitive than strains WA 289 O:8, WE 517/91 O:9, and WE 245/92 O:3. Moreover, this test showed differences among the pathogenic biogroups which were not revealed by the above-described fluorimetric studies. Both with poly-L-lysine and with poly-L-ornithine, strain WE 245/92 O:3 was more sensitive (Fig. 3, left and middle panels) than strains WA 289 O:8 and WE 517/91 O:9. The above effects were not detectable when the more potent polycation (polymyxin B) was used (Fig. 3, right panel). However, when the cells were grown at 26°C, the differences between environmental and pathogenic biogroups were detectable not only with poly-L-lysine and poly-L-ornithine but also with polymyxin B (not shown). As reported for other members of the family *Enterobacteriaceae* (47), deoxycholate by itself had no action on either *E. coli* or *Y. enterocolitica.*

All the above-described experiments were repeated to compare the virulent (pYV^+) and the plasmid-cured (pYV^-) avirulent isogenic pairs of strains WA 289 O:8, WE 245/92 O:3, and WE 517/91 O:9. No differences between the corresponding isogenic pairs were observed (not shown).

(ii) Effects of EDTA and Tris. The effect of EDTA (from 10^1 to 10^4 μM) was tested with *Y. enterocolitica* PR O:1,6, and WA 289 O:8 and with *E. coli* as a control. Regardless of the growth temperature, EDTA had a permeabilizing effect on the *E. coli* OM (Fig. 4, left panels) which was detectable even at the lowest EDTA concentration tested (not shown). In contrast, EDTA had no apparent effect on either the environmental or the pathogenic *Y. enterocolitica* strains (Fig. 4, middle and right panels), even at the highest (10^4 μM) concentration tested. These experiments were repeated with all the environmental strains and with all the strains belonging to serotypes O:8, O:9, and O:3 listed in Table 1, and the differences in EDTA sensitivity between *Y. enterocolitica* and *E. coli* were confirmed. Although EDTA by itself did not alter the entry of NPN into the OMs, it sensitized the OMs of environmental and pathogenic strains (grown at either 26 or 37°C) to polycations since NPN uptake with polymyxin B alone was slower than with polymyxin B plus 10^2 to 10^4 μM EDTA (not shown). Likewise, although Tris did not increase permeability to NPN, it sensitized the cells to polymyxin B (not shown). No differences were observed between pYV^+ and pYV^- isogenic cells of strains WA 289 O:8, WE 245/92 O:3, and WE 517/91 O:9.

OM permeability to NPN and polycation sensitivity of cells grown in MOX. When bacteria were grown in MOX at 37°C, the properties of the OMs of the environmental PR O:1,6 strain were similar to those obtained in standard TSB: they did not take up NPN spontaneously and were permeabilized to NPN by exposure to polycations (Fig. 5, upper panel). Permeabilization was maximal (equivalent to that obtained when the cell membranes were damaged by brief heating) when the polycation was added before the probe (Fig. 5, upper panel, discontinuous line). On the other hand, the OMs of pathogenic strains were partially permeable to NPN. Figure 5 (middle and lower panels) shows that strains WA 289 O:8 and WE 517/91 O:9 quickly took up NPN and also that the fluorescence obtained with viable cells was less than that obtained with cells damaged by heating. Moreover, these experiments revealed a small but consistently lower level of OM permeability in pYV^+ cells compared with isogenic pYV^- cells of serotypes O:8 (Fig. 5, middle left and right panels, respectively), O:9 (Fig. 5, lower left and right panels, respectively), and O:3 (not shown).

Although OM permeability to NPN did not allow us to observe the action of polycations on pathogenic strains in a manner similar to that used with cells grown in TSB, addition of polymyxin B (50 U) after NPN did not boost the level of

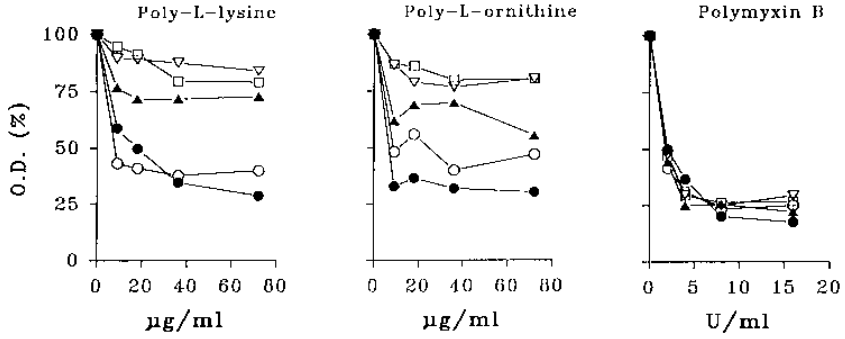

FIG. 3. Cell lysis induced by deoxycholate after exposure to poly-L-lysine, poly-L-ornithine, and polymyxin B. *E. coli* (○), *Y. enterocolitica* PR O:1,6 (●), WE 245/92 O:3 (▲), WE 517/91 O:9 (▽), and WA 289 O:8 (□) were grown in TSB at 37°C. Results are the averages of two independent experiments (the coefficient of variation was less than 6% for all groups of data). O.D., optical density.

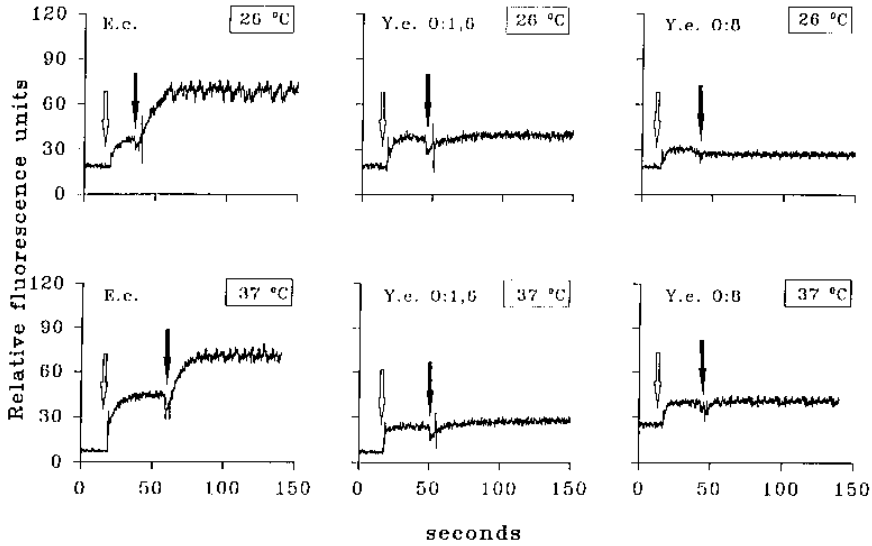

FIG. 4. Effect of EDTA on OM permeability to NPN of bacteria grown in TSB. *E. coli* 0111 (E.c.), *Y. enterocolitica* PR O:1,6 (Y.e. O:1,6), and WA 289 O:8 (Y.e. O:8) were grown at either 26 or 37°C, and fluorescence was monitored at the indicated growth temperature. For each experiment, the addition of NPN is marked with an open arrow and addition of EDTA (final concentration, 10^3 μM) is marked with a filled arrow.

fluorescence in either pYV^+ or pYV^- cells to the levels reached when cells were damaged by heating (Fig. 5, middle and lower left panels, continuous lines), as was observed in the environmental strain (Fig. 5 upper panel). The action of polymyxin B was noticed only as an increase in the kinetics of NPN uptake when the cells were previously exposed to polymyxin B (Fig. 5, middle and lower panels, discontinuous lines). Similar results to those described for polymyxin B were obtained when poly-L-ornithine was used (not shown).

When strains WA 289 O:8, WE 517/91 O:9, and WE 245/92 O:3 were grown in MOX at 26°C, their OMs were less permeable to NPN than at 37°C. However, under these growth conditions, the OMs of strains WA 289 O:8, WE 517/91 O:9, and WE 245/92 O:3 were more permeable than those of the same strains grown in TSB at 26°C. The action of polycations was similar to that observed with cells grown at 37°C. All the results obtained at 26°C with the environmental strain were similar to those obtained at 37°C, and in both cases they were similar to those obtained with *E. coli.*

Partition of NPN into LPS aggregates. By SDS-PAGE followed by periodate silver staining, the LPS preparations tested showed the bimodal distribution into short- and long-polysaccharide LPS molecules which has been observed previously in the LPSs of *Y. enterocolitica* O:3 (1, 4). Densitometric analysis showed that the proportions of short- and long-polysaccharide LPS molecules were similar at 26 and 37°C for strain WA 289 O:8 (64 and 68% short-polysaccharide LPS molecules, respectively). A small increase in the proportion of the short-polysaccharide LPS was observed at 37°C for strain WE 245/92 O:3 (from 44% at 26°C to 53% at 37°C). In contrast, the environmental strain PR O:1,6 showed 30% short-polysaccharide LPS at 26°C and 58% short-polysaccharide LPS at 37°C. The increases in 3-deoxy-D-manno-2-octulosonic acid in LPSs from cells grown at 26 and 37°C were in agreement with the densitometric analysis: from 2.28 to 2.84% for strain WA 289 O:8, from 1.95 to 2.80% for strain WE 245/92 O:3, and from 1.14 to 2.03% for strain PR O:1,6. Protein content was less than 1% for all preparations.

When the properties of the LPSs aggregates were compared by fluorimetry, it was observed that fluorescence in the absence

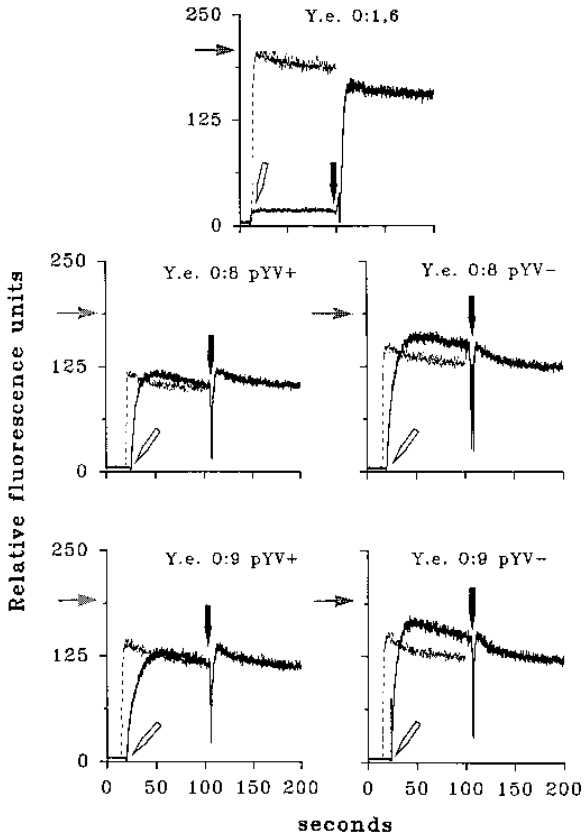

FIG. 5. OM permeability to NPN and sensitivity to polymyxin in environmental and pathogenic *Y. enterocolitica* grown in MOX at 37°C. Upper panel, strain PR O:1,6 (Y.e. O:1,6); middle panels, strain WA 289 O:8 (Y.e. O:8) pYV^+ (left) and pYV^- (right); lower panels, strain WE 517/91 O:9 (Y.e. O:9) pYV^+ (left) and pYV^- (right). Addition of NPN is marked with an open arrow, and addition of polymyxin B (final concentration, 50 U/ml) is marked with a filled arrow. The maximal fluorescence obtained with cells damaged by heating is marked on the relative fluoresce units axis with a shaded arrow. Discontinuous lines are the results of experiments in which polymyxin B was added before NPN. Results of two independent experiments (the coefficient of variation was less than 3%) are presented.

TABLE 4. Effects of EDTA and polycations on the partition of NPN into LPS aggregates of representative strains of *Y. enterocolitica* grown at the indicated temperature

LPS source	Serotype (biogroup)	Growth temperature (°C)	Result[a] of experiment with:			
			No agent	EDTA[b]	Poly-L-lysine[c]	Poly-L-ornithine[d]
Y. enterocolitica PR	O:1,6 (1A)	26	88 ± 0.10	115 ± 0.03[e]	93 ± 0.22[e]	104 ± 0.17[e]
		37	90 ± 0.22	100 ± 0.10[e]	106 ± 0.19[e]	153 ± 0.06[e]
Y. enterocolitica WE 245/92	O:3 (4)	26	91 ± 0.16	118 ± 0.27[e]	91 ± 0.17	93 ± 0.08
		37	116 ± 0.06	141 ± 0.21[e]	122 ± 0.14[e]	119 ± 0.02[f]
Y. enterocolitica WA 289	O:8 (1B)	26	105 ± 0.21	132 ± 0.07[e]	128 ± 0.19[e]	113 ± 0.16[e]
		37	182 ± 0.16	182 ± 0.02	182 ± 0.13	182 ± 0.14

[a] Results (means ± standard deviations of six experiments) are expressed as the final NPN fluorescence (relative fluorescence units).
[b] Final concentration, 10^3 μM.
[c] Final concentration, 4.5 μg/ml.
[d] Final concentration, 9 μg/ml.
[e] Significantly different from the results for the controls with no agent ($P \leq 0.001$).
[f] Significantly different from the results for the controls with no agent ($P \leq 0.02$).

of the probe was negligible (1 to 2 RFU). Upon addition of NPN, fluorescence levels increased suddenly, no matter which strain and growth temperature were used. Although the O:1,6 LPS was clearly affected by the growth temperature, the corresponding LPS aggregates yielded similar fluorescence values (Table 4). On the other hand, the fluorescence levels obtained with LPSs from strain WE 245/92 O:3 and, in particular, strain WA 289 O:8 grown at 37°C were higher than those obtained with the LPSs of the same strains grown at 26°C. The LPS of the serotype O:9 strain (WE 517/91) gave irregular results (not shown), presumably because of the high level of free-lipid contamination of this phenolic S-LPS (16).

Addition of EDTA (before NPN) increased the permeability to NPN of the LPSs from cells grown at either 26 or 37°C, with the exception of strain WA 289 O:8 grown at 37°C (Table 4). The results obtained with poly-L-lysine and poly-L-ornithine resembled those obtained with EDTA in that, at 37°C, the LPS of WA 289 O:8 was resistant to the destabilizing action of those polycations (Table 4). The values obtained did not represent the saturation of the LPS aggregates with the probe, because treatment with an excess (100 U) of polymyxin B increased the fluorescence levels to 220 RFU.

DISCUSSION

It is known that the OMs of a variety of gram-negative bacteria act as barriers to hydrophobic permeants (29). This property is thought to be due to electrostatic effects, tight packing of the lipid A hydrocarbon chains, and exclusion of phospholipids from the outer leaflet caused by divalent cation bridging of negatively charged groups in the LPS core and lipid A (29, 46). Moreover, it is generally acknowledged that those negatively charged groups play an important role in the interaction of polycations with the OMs and in the sensitivity to divalent cation chelators (46). Experiments carried out with organic solvents (42) have demonstrated that the quantum yield (fluorescence) of NPN increases when it is transferred from a hydrophilic to a hydrophobic environment. Therefore, the fluorimetric experiments with cell suspensions in the presence of NPN and in the absence of OM-disturbing agents have to be interpreted in terms of the presence or absence of a barrier to hydrophobic permeants (23, 26, 27, 42, 46). Accordingly, such a barrier was present in *Y. enterocolitica* cells grown in TSB, no matter which growth temperature and biogroup were used. However, the *Y. enterocolitica* OM barrier was different from that described for *E. coli* and *Salmonella* spp. (29), because its resistance to EDTA by itself shows that it does not depend merely on divalent cation stabilization of the OM (see also below). An additional difference was that the *Y. enterocolitica* OM barrier was not a constant feature, since NPN's ability to permeate the barrier increased when pathogenic strains were grown in MOX medium. Although MOX contains a chelator (oxalate), three different reasons make it unlikely that this increased NPN permeability could result from an artifact like those induced on some gram-negative bacteria by several chelators. First, as pointed out above, EDTA by itself had no effect on the permeability to NPN of cells grown in TSB; second, growth in MOX had no effect on the OM barrier of either *E. coli* or environmental *Y. enterocolitica*; third, although MOX has no Ca^{++} available, it contains a large amount of Mg^{++}, which as illustrated by the normal behavior of *E. coli* in this medium, acts as an OM stabilizer (29, 46). Therefore, since a general artifact is not possible, the different OM permeabilities to NPN should reflect structural properties by which pathogenic *Y. enterocolitica* biogroups differ from the environmental biogroup and *E. coli*. On the basis of our present knowledge of the structures of the OMs of *E. coli* and *Salmonella* spp. (29), it could be hypothesized that those differences relate, at least in part, to the LPS (see also below).

Although the absence of a barrier to hydrophobic permeants has been observed in some mucosal (13, 27) and intracellular pathogens (26, 27), to the best of our knowledge there are no reports of bacteria in which OM permeability to hydrophobic probes shifts dramatically depending on the medium. Since the hydrophobic barrier was observed in TSB (0.2 mM Ca^{++}) but not in MOX, the OM permeability of pathogenic *Y. enterocolitica* could be linked specifically to the availability of Ca^{++}. MOX is thought to represent the Ca^{++} restriction and Mg^{++} availability within the host cells (6), and although there are contradictory reports on the intracellular location of *Y. enterocolitica* in vivo (14, 15, 22, 33, 44, 45, 48), these bacteria probably enter the host through M cells, from which they are delivered to macrophages (12, 36). Thus, the changes in OM permeability could also happen in vivo and perhaps represent a physiological adaptation to environments differing in Ca^{++} and Mg^{++}. Moreover, the change in OM properties could be a factor related to the release of virulence-related Yops within those environments.

Expression of pYV-encoded virulence genes in vitro is modulated by Ca^{++} (5, 6), but pYV does not seem to play a major role in conferring the OM properties described here for virulent biogroups because pYV^- and pYV^+ cells were very similar in all tests. A minor role of Yops or other plasmid-encoded factors could, however, relate to the small but consistently

higher level of NPN permeability shown by pYV$^-$ cells grown in MOX. By testing the partition in xylene-water (19) and the attachment to nitrocellulose (19) and to octyl-Sepharose (25), it has been reported that, under Ca^{++} restriction, pYV$^+$ cells have surfaces that are more hydrophobic than those of pYV$^-$ cells, and a link between this property and cell attachment has been proposed (25). Although this is in apparent contradiction with our results, surface hydrophobicity is not necessarily related to permeability to hydrophobic probes, as the latter condition can be brought about by subtle changes in LPS structure or phospholipid distribution (29) without disturbance of overall surface properties.

The stability of the OM of *Y. enterocolitica* grown in TSB was tested with polycations by two different protocols. In all *Y. enterocolitica* biogroups grown at 26°C, the entry of NPN (kinetics and final fluorescence) into the OM was not affected to the same extent as in *E. coli* when poly-L-lysine, poly-L-ornithine, melittin, cecropin P1, and polymyxin B were added. At this growth temperature, however, environmental strains were more affected by deoxycholate in the presence of polycations than pathogenic strains. These OM differences were enhanced in cells grown at 37°C, since the fluorimetric protocol showed that the OM of *E. coli* was sensitive to both EDTA and polycations, those of environmental strains were sensitive to polycations, and those of the pathogenic biogroups were resistant to both EDTA and polycations, whether pYV was present or not. These results question a general and clear-cut relationship between resistance to EDTA and resistance to polycations (29, 46) and show that the OMs of *E. coli* and *Y. enterocolitica* do not differ merely in OM negatively charged groups and counterions but in an alternative structural property which affects the interaction with polycations. Moreover, such a structural property is kept at 37°C by pathogenic but not by environmental *Y. enterocolitica*, which at 37°C was similar to *E. coli*. The similarities between *E. coli* and environmental *Y. enterocolitica* were also manifested in MOX, since no matter which growth temperature was used, both were quite sensitive to polycations.

Some of the LPS properties might relate to the OM properties described here. The only LPS whose O-chain was strongly reduced at 37°C was that of strain PR O:1,6, and in this and other environmental strains, sensitivity to polycations was manifested at 37°C. This could correspond to a decreased steric hindrance of polycations by the short-polysaccharide LPS dominant in the environmental strain at 37°C, since it has been shown that the length of the LPS polysaccharide significantly affects the binding of polycations to inner LPS targets (34). Obviously, since the LPSs of the pathogenic strains were not affected by the growth temperature to the same extent as that of strain PR O:1,6, this could account in part for the corresponding differences in polycation sensitivity. However, it is important that changes in the length of LPS polysaccharide cannot explain the differences between *Y. enterocolitica* and *E. coli* at 26°C. Therefore, there must be differences in the inner sections of LPS or in other OM components. Since NPN is taken up into the LPS hydrophobic moiety, this interpretation is also suggested by the fact that, whereas LPS aggregates from PR O:1,6 were equally permeable to NPN whether the cells were grown at 26 or 37°C, the pathogenic biogroups produced LPSs that took up more probe when the cells were grown at 37°C. These results cannot be explained either by an increased roughness of the pathogenic biogroups, because the analyses revealed only minimal differences between LPSs tested at 26 and 37°C from strain WA 289 O:8, which on the other hand, showed maximal differences in fluorescence with NPN alone. Therefore, it is likely that LPSs from pathogenic cells grown at 37°C are either more hydrophobic, form aggregates with less effective barriers to NPN, or both. Because of the paucity of data on the structure of the core and lipid A of *Y. enterocolitica*, it is not possible at present to relate those observations to chemical changes.

No clear relationship could be established between the LPS properties and the comparative resistance to polycations shown by pathogenic biogroups. In smooth *E. coli* and *Salmonella typhimurium*, divalent cation bridging of the LPS negatively charged groups is critical for OM barrier function and those negatively charged groups are, at the same time, the targets for polycations (27, 29, 46). The results of the fluorimetric experiments with LPSs do not grant such a clear relationship for *Y. enterocolitica*, since with the exception of the LPS of serotype O:8 cells grown at 37°C, all LPSs were affected by EDTA and no clear pattern of polycation sensitivity was observed. In addition to divalent cation bridging, direct LPS-LPS interactions or LPS-OM protein interactions are important in modulating OM properties (29, 46). It might be that such interactions are more important in the OMs of pathogenic biogroups, and this would be partially consistent with the reduced effect of EDTA on whole cells.

Resistance to polycations has been linked to virulence in some bacteria (9, 11, 27, 37). *Salmonella* Pho$^-$ mutants are hypersensitive to mastoparan, magainins, melittin, and cecropins and are also hypersensitive to defensin NP-1 and cryptdins and unable to survive intracellularly (10, 11, 37). This is so because, regardless of their origin, all these antimicrobial peptides begin their action by electrostatic interaction with bacterial surfaces (28), a step which is common to all bactericidal polycations (46). Because Paneth cells excrete large amounts of bactericidal peptides (lysozyme and cryptdins) (30, 37), it has been postulated that resistance to cationic peptides should be a virulence requisite for enteric pathogens (37). Obviously, both the comparative resistance to cationic peptides of pathogenic biogroups and the fact that, in contrast to environmental biogroups, this property was not lost at the body temperatures of the hosts support a relevant role for polycation resistance in *Y. enterocolitica* pathogenicity. Comparative studies with polycation-sensitive mutants and with other pathogenic *Yersinia* spp. are necessary to test such a hypothesis.

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