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Pathogenic *Yersinia enterocolitica* Strains Increase the Outer Membrane Permeability in Response to Environmental Stimuli by Modulating Lipopolysaccharide Fluidity and Lipid A Structure

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Pathogenic biotypes of *Yersinia enterocolitica* (serotypes O:3, O:8, O:9, and O:13), but not environmental biotypes (serotypes O:5, O:6, O:7,8, and O:7,8,13,19), increased their permeability to hydrophobic probes when they were grown at pH 5.5 or in EGTA-supplemented (Ca^{2+}-restricted) media at 37°C. A similar observation was also made when representative strains of serotypes O:8 and O:5 were tested after brief contact with human monocytes. The increase in permeability was independent of the virulence plasmid. The role of lipopolysaccharide (LPS) in this phenomenon was examined by using *Y. enterocolitica* serotype O:8. LPS aggregates of bacteria grown in acidic or EGTA-supplemented broth took up more *N*-phenylnaphthylamine than LPS aggregates of bacteria grown in standard broth and also showed a marked increase in acyl chain fluidity which correlated with permeability, as determined by measurements obtained in the presence of hydrophobic dyes. No significant changes in O-antigen polymerization were observed, but lipid A acylation changed depending on the growth conditions. In standard medium at 37°C, there were hexa-, penta-, and tetraacyl lipid A forms, and the pentaacyl form was dominant. The amount of tetraacyl lipid A increased in EGTA-supplemented and acidic media, and hexaacyl lipid A almost disappeared under the latter conditions. Our results suggest that pathogenic *Y. enterocolitica* strains modulate lipid A acylation coordinately with expression of virulence proteins, thus reducing LPS packing and increasing outer membrane permeability. The changes in permeability, LPS acyl chain fluidity, and lipid A acylation in pathogenic *Y. enterocolitica* strains approximate the characteristics in *Yersinia pseudotuberculosis* and *Yersinia pestis* and suggest that there is a common outer membrane pattern associated with pathogenicity.

The yersiniae are gram-negative bacteria that belong to the family *Enterobacteriaceae*. The genus *Yersinia* encompasses nonpathogenic species found mostly in the environment, two pathogenic species (*Yersinia pseudotuberculosis* and *Yersinia pestis*), and *Yersinia enterocolitica*, which includes typically environmental strains as well as strains that, although they are able to multiply in the environment, cause mesenteric lymphadenitis, diarrhea, and enteritis in humans. The two kinds of *Y. enterocolitica* strains can be distinguished bacteriologically and serologically. Five biogroups (and two subgroups of biogroup 1) and several serotypes are recognized; the environmental strains cluster in biogroup 1A, and the pathogenic strains are members of biogroups 1B, 2, 3, 4, and 5 and belong to a relatively few serotypes (serotypes O:3, O:4, O:5,27, O:8, O:9, O:13, and O:21) (8). However, the most conspicuous difference is that the pathogenic strains carry a plasmid (pYV) coding for virulence proteins, such as the *Yersinia* outer proteins (Yops) and the *Yersinia* adhesin A (YadA), that block phagocytosis, opsonization, and complement activation (13, 16, 23). The pathogenic strains also express chromosomally encoded virulence factors, including the *Yersinia* enterotoxin (Yst), mucoid *Yersinia* factor (Myf), invasin (Inv), and attachment-invasion (Ail) proteins related to invasion and serum resistance (22, 44). Since these bacteria move from the environment to the intestine and then move through M cells into adjacent tissues, it is not surprising that expression of the virulence genes is sequentially turned on and off by one or several signals acting as landmarks for the milieus which they encounter (44). Accordingly, pYV-encoded proteins are expressed at 37°C (the body temperature of the host), and expression of Yops also requires bacterium-eukaryotic cell contact or, in vitro, restricted Ca^{2+} availability (9, 11, 44). Expression of Yst and Inv in vitro is higher at 23°C and in the stationary phase, but both proteins are also expressed at 37°C under adequate osmolarity and pH conditions. Ail and Myf are expressed at 37°C and are also regulated by oxygen tension and pH, respectively (23).

In addition to these classical virulence factors, *Y. enterocolitica* pathogenic strains differ from environmental strains in some outer membrane (OM) properties. When these bacteria grow at 37°C, the OMs of the pathogenic strains are more resistant to bactericidal peptides than the OMs of the environmental strains (4). Moreover, the OMs of the pathogenic strains become permeable to hydrophobic probes in Mg^{2+} oxalate medium (used to generate the low-Ca^{2+} conditions that induce pYV expression in vitro), whereas the OMs of the

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environmental strains do not (4). A complementary fact is that in the same Mg^{2+} oxalate medium, an increase in permeability is also observed in pYV-cured pathogenic strains (4), suggesting that the factors triggering pYV expression modulate OM structure and physiology at the chromosomal level. These observations become more intriguing when the fact that *Y. pseudotuberculosis* and *Y. pestis* OMs are permeable to hydrophobic compounds in standard media is considered (3), and therefore, it may be that permeability to hydrophobic compounds is a trait shared by all pathogenic *Yersinia* spp. which is regulated in pathogenic *Y. enterocolitica*. In the present study, we examined this hypothesis by testing the effects of acid pH, Ca^{2+} restriction, and contact with human monocytes on the permeability of *Y. enterocolitica* to hydrophobic probes. Moreover, since it is known that the permeability barrier is related at least in part to the properties of the OM lipopolysaccharide (LPS) (46, 47), we also examined this molecule for chemical and physicochemical changes linked to changes in permeability. We report here that pathogenic *Y. enterocolitica* strains change the OM permeability and lipid A composition in response to factors known to regulate the expression of *Yersinia* virulence proteins that are either pYV or chromosomally encoded.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The pathogenic strains (all harboring pYV) used were *Y. enterocolitica* WE 245 O:3, WA 289 O:8, WE 517/91 O:9, and WAT 288 O:13, and the environmental strains used were *Y. enterocolitica* MA 279 O:5, MA 289 O:6, MA 281 O:7,8, and MA 257 O:7,8,13,19. In addition, *Y. pseudotuberculosis* WE 2390, *Y. pestis* KIM, and *Escherichia coli* O111 K58 H2 (enteropathogenic) and O:8 K27 were used (3). The pYV^+ strains were stored in skim milk at −80°C, and inocula were taken directly from these frozen seed stock preparations to minimize pYV^- dissociation. The pYV^- isogenic strains were selected on Congo red-MOX medium (38), and the absence of pYV was confirmed by using the autoagglutination test (43).

To test cell permeability (see below), bacteria were grown in sidearm flasks at 37°C in an orbital water bath by using the following media: (i) standard tryptic soy broth (TSB) (bioMérieux, Marcy l'Etoile, France); (ii) TSB supplemented with 5 mM EGTA (TSB-EGTA); and (iii) medium containing tryptone (17 g/liter) and soy extract (3 g/liter) buffered to pH 5.5 with 2-(*N*-morpholino)ethanesulfonic acid (MES) (TSB-pH 5.5). Cultures were harvested by centrifugation (5,000 × *g*, 20 min, 5°C) in the middle of the exponential phase, and the bacteria were resuspended immediately in 2 mM HEPES (pH 7.2) with or without potassium cyanide and sodium arsenate (1 to 20 mM for both inhibitors) at an optical density at 600 nm (OD_{600}) of 0.5 (4). For the antibiotic sensitivity assays (see below), the broth media were supplemented with agar (15 g/liter; Difco Laboratories, Detroit, Mich.) to obtain the corresponding solid media (TSA, TSA-EGTA, and TSA-pH 5.5). For LPS extraction, bacteria were grown in 2-liter flasks (800 ml per flask), and the cultures were incubated in an orbital shaker (200 rpm) for 24 h at 37°C.

Assessment of permeability to hydrophobic compounds. (i) Novobiocin sensitivity. Sterile paper disks (6.5-mm-diameter concentration disks; Difco) that were previously loaded with 50 μg of novobiocin dissolved in 20 μl of distilled water and dried overnight at 37°C were placed on the appropriate agar medium for overnight diffusion of the antibiotic at 26°C. Exponential-phase bacteria grown in the corresponding broth were resuspended in saline at a concentration of 10^8 CFU/ml, and a lawn was inoculated onto the plates in which the antibiotic had diffused. The plates were then incubated overnight at 37°C, and inhibition halos were measured.

(ii) Permeability of bacteria and LPS aggregates to NPN. Bacterial suspensions (OD_{600}, 0.5) in 2 mM HEPES (pH 7.2)–1 mM potassium cyanide–1 mM sodium arsenate were placed in 1-cm fluorimetric cuvettes, *N*-phenylnaphthylamine (NPN) was added (10 μM in the cuvette), and partitioning into the cell envelope was assessed by determining the increase in fluorescence (expressed in relative fluorescence units [RFU]) by using an LS-50 fluorimeter (Perkin-Elmer Ltd., Beaconsfield, England) with excitation set at 350 nm and emission at 420 nm; the slit width for both windows was 2.5 nm or, for bacteria grown in the presence of monocytes, 3 nm (4). For each strain three independently grown batches of bacteria were tested, and each measurement was repeated at least three times.

The protocol described above for the NPN permeability test was also used to test the permeability of LPS aggregates. LPS aggregates were prepared in double-distilled water (1 mg/ml for single LPSs or 1 mg of each type of LPS in 2 ml for hybrid aggregates). The suspensions were first dispersed thoroughly by sonication, supplemented with an equal volume of 2% sodium deoxycholate in 0.1 M Tris-HCl (pH 8.5), incubated for 15 min at room temperature, and precipitated with 6 volumes of ethanol at −20°C for 18 h. The precipitate was sedimented by centrifugation (15 min, 10,000 × *g*), washed twice with ethanol, resuspended in water, dialyzed, and adjusted to a concentration of 400 μg/ml (40). Measurements were obtained in triplicate, and the results were analyzed by the Mann-Whitney U test.

Incubation of bacteria in contact with human monocytes. Peripheral blood monocytes were isolated from human healthy donors by standard Ficoll (Pharmacia, Uppsala, Sweden) gradient centrifugation by following the instructions of the manufacturer. They were resuspended in RPMI and incubated for 4 h in 12-well plastic culture plates (Costar, Cambridge, Mass.). Nonadherent cells were then washed off to obtain a monocyte culture that was more than 90% pure as determined with anti-CD14 monoclonal antibody. To prepare the inocula, bacteria were grown in TSB at 37°C, harvested in the exponential phase of growth, and resuspended in RPMI at a concentration of 10^9 CFU/ml. Monocyte cultures were infected by removing the culture medium and adding 250 μl of inoculum (multiplicity of infection, 100:1), and contact between bacteria and monocytes was ensured by centrifuging the plates for 5 min at 400 × *g*. After 1 h of incubation at 37°C with 5% CO_2 in a humidified atmosphere, the medium was carefully removed, and the bacteria remaining on the cells were recovered by washing the wells with RPMI and were sedimented by centrifugation (7,000 × *g*, 15 min). To assess NPN permeability, the cells were immediately resuspended in 2 mM HEPES (pH 7.2) with or without 1 mM potassium cyanide–1 mM sodium arsenate at an OD_{540} of 0.3. The controls were bacterial suspensions which were processed in exactly the same way except that the corresponding wells contained only fresh RPMI, spent RPMI taken from monocyte cultures not exposed to bacteria, or spent RPMI taken from wells in which bacteria had been in contact with monocytes. The viability of the monocytes after the experiment was more than 90% as determined by the trypan blue exclusion method and did not differ from that of controls without bacteria.

LPS preparations. Crude LPS preparations obtained by the phenol-water method (30) were dispersed (10 mg/ml) in 0.8% NaCl–0.05% NaN_3–0.1 M Tris-HCl (pH 7), digested with nucleases and proteinase K, sedimented by ultracentrifugation (6 h, 100,000 × *g*), and freeze-dried. The 3-deoxy-D-*manno*-octulosonic acid contents (determined colorimetrically by standard methods with the modifications described previously [12]) were 3.7, 4.3, and 4.3% for the LPSs of bacteria grown in TSB, TSB-EGTA, and TSB-pH 5.5, respectively. The Ca^{2+} and Mg^{2+} contents (determined by flame ionization) ranged from 1.0 to 1.1 ng/mg (dry weight) of LPS and from 0.57 to 0.67 ng/mg (dry weight) of LPS, respectively, and there were no significant differences among the LPSs of bacteria grown in the different media.

LPS analyses. (i) Determination of the transition from the gel phase to the liquid crystalline phase of LPSs. The transition of the acyl chains of LPS from a well-ordered state (gel phase) to a fluid state (liquid crystalline phase) at a lipid-specific temperature (T_c) was measured by Fourier transformed infrared spectroscopy. This allowed determination of the acyl chain fluidity, which is a measure of the mobility of the hydrocarbon chains at a given temperature. The natural salt forms of the LPSs were used, and to ensure homogeneity, LPS suspensions (10 mM) were prepared in 2.5 mM HEPES (pH 7.0) at room temperature, incubated at 56°C for 15 min, stirred, and cooled to 4°C. This heating-cooling step was repeated three times, and the suspensions were stored at 4°C for several hours before analysis. Measurements were obtained with a Bruker IFS 55 apparatus (Bruker, Karlsruhe, Germany) as described previously (5). Briefly, LPS suspensions (water content, >90%) were analyzed in CaF_2 cuvettes with 12.5-μm-thick Teflon spacers, and for each measurement 50 interferograms were accumulated, Fourier transformed, and converted to absorbance spectra. The measurements were obtained in continuous heating scans (2°C/min) at temperatures from 10 to 60°C. The peak position of the symmetric stretching vibration of the methylene groups [$\nu_s(CH_2)$] around 2,850 cm^{-1} was considered a measure of the state of order (fluidity) of the acyl chains (5). To test the effects of hydrophobic dyes, the experiments were also performed in the presence of brilliant green or crystal violet at an equimolar ratio of LPS to dye.

(ii) Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis

TABLE 1. Sensitivity to novobiocin of pathogenic and environmental *Y. enterocolitica* strains grown under different conditions[a]

Strain	Relevant characteristic(s)	Inhibition halo diameter (mm) on:		
		TSA	TSA-EGTA	TSA-pH 5.5
Y. enterocolitica				
WA 289 O:8	Pathogenic	0	28	30
WE 517/91 O:9	Pathogenic	0	10	30
WE 245/92 O:3	Pathogenic	0	23	30
MA 280 O:6	Environmental	0	0	15
MA 281 O:7,8	Environmental	0	5	21
MA 257 O:7,8,13,19	Environmental	0	4	22
E. coli O111	Enteropathogenic	23	22	26

[a] All experiments were performed with triplicate samples from two independently grown batches of bacteria. The standard error was less than 5% in all cases.

(PAGE). LPSs were analyzed in 7-cm-long 15% polyacrylamide gels (acrylamide to methylene bisacrylamide ratio, 37.5:1) as described by Laemmli (27), and the gels were stained for LPS by the periodate-alkaline silver method (45).

(iii) Degree of lipid acylation. Purified LPS was hydrolyzed in 10 mM sodium acetate (pH 4.5)–1% SDS for 1 h at 100°C and freeze-dried. The product was then washed six times with ethanol and two times with ethanol acidified with traces of HCl and freeze-dried (18, 19). Lipid A samples were dissolved in chloroform-methanol-ammonium-water (25:14:1:2), spotted on high-performance thin-layer chromatography (HPTLC) silica gel plates (E. Merck, Darmstadt, Germany), and chromatographed by using the same solvent mixture. The plates were soaked in ethanol-sulfuric acid (instead of the standard methanol-sulfuric acid mixture), and the image that developed immediately after soaking was captured with a video camera on a dark background, inverted, and contrasted by using standard software. Lipid A preparations of *E. coli* W3110 MLK3 (W3110 *htrB1*::Tn*10*; hexaacylated), W3110 MLK1067 (W3110 *msbB*::Ωcam; pentaacylated), and W3110 MLK986 (MLK53 *msbB*::Ωcam; tetraacylated) (7) were used as standards. Densitometry was performed by using the Imagemaster system (Pharmacia Biotech, Uppsala, Sweden).

RESULTS

Permeability to hydrophobic probes under different growth conditions. Novobiocin is a useful probe to test changes in OM permeability to hydrophobic compounds, and it has been used previously to determine differences in permeability among *Yersinia* species (3). Thus, our first approach was to measure changes in sensitivity to this antibiotic under different growth conditions (Table 1). As expected, all *Y. enterocolitica* strains examined were resistant to novobiocin when they were grown on standard TSA. However, on TSA-EGTA, the pathogenic strains clearly became more sensitive than the environmental strains (Table 1), and a similar differential increase in sensitivity was observed on TSA supplemented with oxalate (data not shown). Although the differences between the pathogenic and environmental strains were not so marked, the dichotomy was also observed on TSA-pH 5.5 (Table 1). Under the same conditions, the enteropathogenic *E. coli* control strain showed no changes in novobiocin sensitivity (Table 1).

We then directly assessed the OM permeability by using NPN as a probe. When grown in TSB, all *Y. enterocolitica* strains were equally impermeable to NPN (Fig. 1). However, in TSB-EGTA or in TSB-pH 5.5, the pathogenic strains showed a marked increase in permeability to NPN which was not shown by the environmental strains. No matter what growth conditions and strain were tested, metabolic inhibitors that block energy-dependent efflux pumps (potassium cyanide plus sodium arsenate) facilitated the net uptake of NPN. However, pathogenic strains still took up more NPN when they were grown in TSB-EGTA and TSB-pH 5.5 than when they were grown in TSB. In contrast, these growth conditions did not affect NPN uptake by the environmental strains. We also tested the influence of pYV using isogenic pYV$^-$ derivatives of pYV$^+$ strains WA 289 O:8, WE 517/91 O:9, and WE 245/92 O:3. These experiments showed that like the pYV$^+$ bacteria, the pYV$^-$ isogenic derivatives became permeable to NPN when they were grown in TSB-EGTA or TSB-pH 5.5. However, the fluorescence values were slightly higher (about 30 RFU) for the pYV$^-$ derivatives (data not shown). These results are in agreement with the increases in novobiocin sensitivity observed under the same conditions, and therefore, we concluded that pathogenic *Y. enterocolitica* strains increase their permeability to hydrophobic probes when the organisms are grown at pH 5.5 or in EGTA-supplemented (Ca^{2+}-restricted) media at 37°C and that this ability is not displayed by environmental strains.

Permeability to hydrophobic probes after contact with human monocytes. To test whether cell contact also modulates OM permeability, we measured the uptake of NPN by *Y. enterocolitica* WA 289 O:8 (a pathogenic strain) and MA 279 O:5 (an environmental strain) after contact with human monocytes for 1 h and compared it with the uptake of NPN by bacteria grown in RPMI. For the pathogenic strain, regardless of the presence of pYV, the fluorescence values were higher after contact with the monocytes, and this effect was not observed with the environmental strain (MA 279 O:5) (Fig. 2). As expected, metabolic inhibitors enhanced NPN uptake, but higher fluorescence values were obtained with the bacteria that had been in contact with monocytes (Fig. 2). We also considered the possibility that factors released by the bacteria and/or the monocytes could account for the permeability changes. To examine this possibility, we resuspended fresh bacteria in spent RPMI from the wells containing bacteria and monocytes or only monocytes and measured the NPN uptake after incubation for 1 h. The fluorescence values were similar to those obtained with bacteria that had not been in contact with monocytes (Fig. 2 and data not shown). Taken together, these results strongly suggest that like Ca^{2+} restriction, contact with eukaryotic cells causes OM permeability changes in pathogenic *Y. enterocolitica* strains in a pYV-independent manner.

LPS analyses. (i) Permeability. We isolated the LPS from pathogenic strain WA 289 O:8 (pYV$^-$) grown in TSB, TSB-EGTA, and TSB-pH 5.5 (referred to as LPS-TSB, LPS-EGTA, and LPS-pH 5.5, respectively, below) and analyzed possible changes in permeability to NPN. As shown in Fig. 3, the LPS-EGTA and LPS-pH 5.5 aggregates took up significantly more NPN than the LPS-TSB aggregates took up. Moreover, the hybrid aggregates consisting of LPS-TSB plus LPS-EGTA or of LPS-TSB plus LPS-pH 5.5 took up less NPN than the pure LPS-EGTA or LPS-pH 5.5 aggregates took up (Fig. 3). Thus, the permeability of the LPS aggregates paralleled the permeability observed with the whole bacteria.

(ii) Acyl chain fluidity. It has been shown previously that the comparatively increased OM permeability of *Y. pseudotuberculosis* and *Y. pestis* is correlated with increased LPS acyl chain

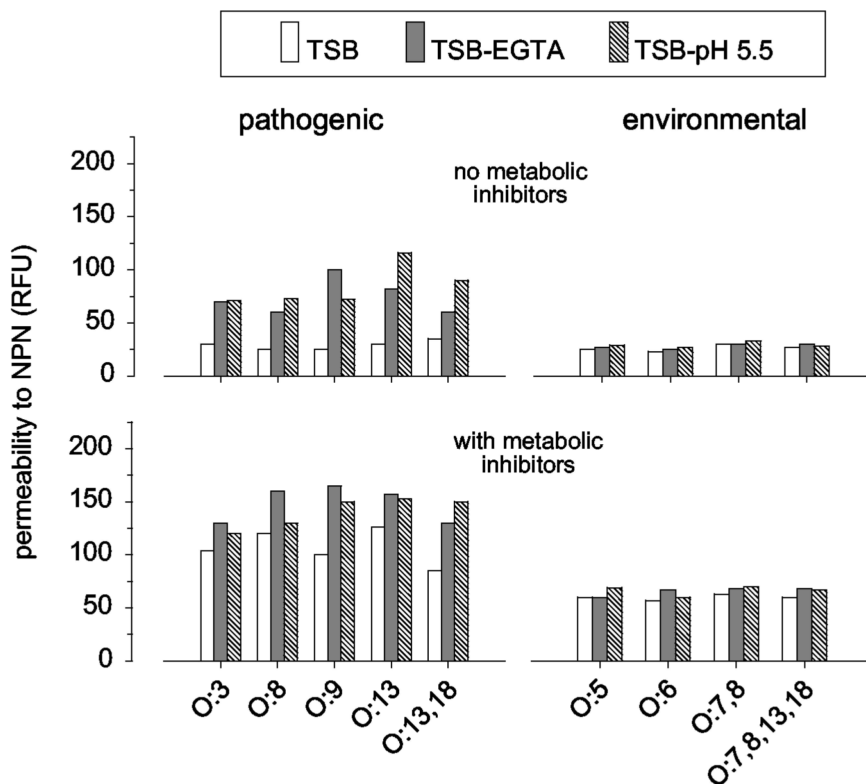

FIG. 1. Permeability to NPN (expressed in RFU) of pathogenic and environmental *Y. enterocolitica* strains grown at 37°C in TSB, in TSB-EGTA for low calcium availability, or in TSB-pH 5.5. Permeability was monitored in the absence (upper panels) or in the presence (lower panels) of membrane metabolic inhibitors.

fluidity (3). Thus, we tested whether the LPS acyl chain fluidity of pathogenic *Y. enterocolitica* strains was increased in TSB-EGTA and TSB-pH 5.5 and whether it approached that of *Y. pseudotuberculosis* and *Y. pestis*. The temperature-dependent shifts in the maximum peak positions of the $\nu_s(CH_2)$ of the acyl chains of the LPSs of pathogenic *Y. enterocolitica* strains and, as references, of the LPSs of *E. coli* O:8 K27, *Y. pseudotuberculosis* WE 2390, and *Y. pestis* KIM are shown in Fig. 4. For LPS-TSB, the peak positions were in the 2,850.0- to 2,850.5-cm^{-1} range at temperatures below 25°C and over 2,852.0 cm^{-1} at temperatures above 35°C, and there was a clear phase transition in the 25 to 35°C interval. It was noteworthy that the phase transition temperature (T_c) of the *Y. enterocolitica* LPS-TSB was 2 to 3°C lower than that of *E. coli* LPS, and both the LPS-EGTA and the LPS-pH 5.5 showed acyl chain fluidity different from that of the LPS-TSB (Fig. 4), manifested both as a much less marked or absent phase transition and as increased $\nu_s(CH_2)$ at temperatures below 30°C. Figure 4 also shows that the behavior of the *Y. enterocolitica* LPS-EGTA and LPS-pH 5.5 was more similar to that of *Y. pseudotuberculosis* and *Y. pestis* LPSs, which, as reported previously (3), showed no transitions and were in a constant fluid state throughout the temperature range tested (Fig. 4).

In *Yersinia* spp., sensitivity to the hydrophobic dye brilliant green correlates with OM permeability (3). Thus, to relate acyl chain fluidity and permeability directly, we tested whether LPS aggregates showed increased acyl chain fluidity in the presence of brilliant green (indicative of the partitioning of this bulky

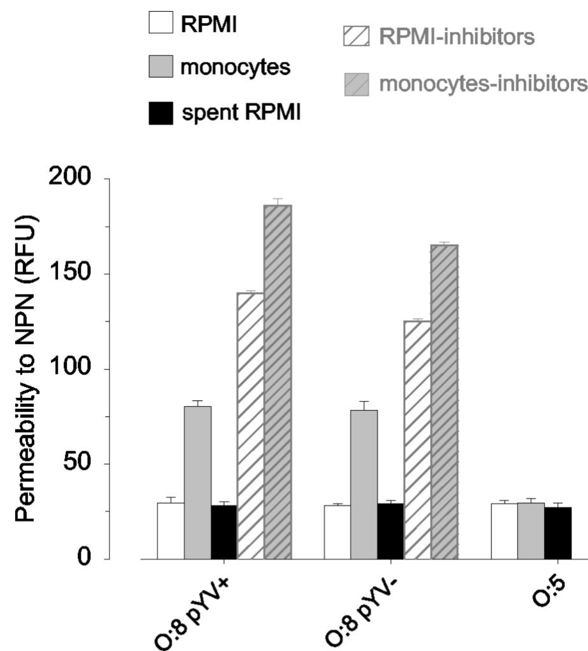

FIG. 2. Permeability to NPN (expressed in RFU) of *Y. enterocolitica* WA 289 O:8 pYV^+ and pYV^- isogenic strains (pathogenic) and of *Y. enterocolitica* MA 279 O:5 (environmental) after incubation in RPMI, in RPMI in contact with human monocytes, or in spent RPMI in which bacteria and monocytes had been incubated. Permeability was measured in the absence (solid and open bars) or in the presence (cross-hatched bars) of membrane metabolic inhibitors.

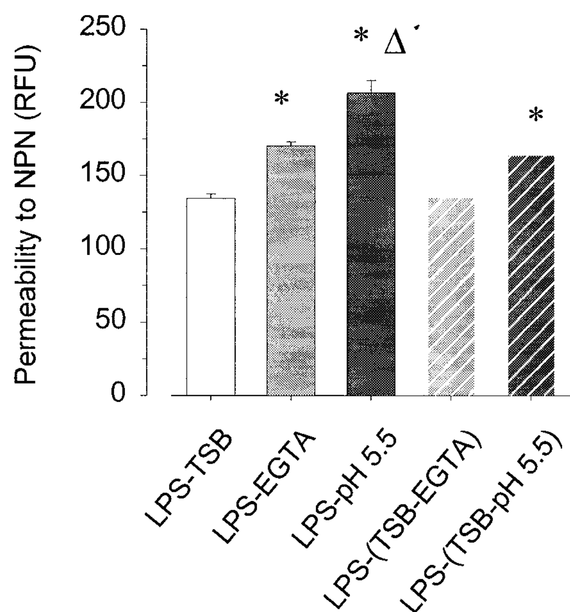

FIG. 3. Permeability to NPN (expressed in RFU) of pure LPS aggregates (LPS-TSB, LPS-EGTA, and LPS-pH 5.5) and hybrid LPS aggregates [LPS-(TSB-EGTA) and LPS-(TSB-pH 5.5)] of *Y. enterocolitica* WA 289 O:8 grown at 37°C in TSB, in TSB-EGTA for low calcium availability, or in TSB-pH 5.5. Statistically significant differences ($P < 0.05$) compared with the data for the LPS-TSB and for the LPS-(TSB-pH 5.5) aggregates are indicated by asterisks and by an open triangle, respectively.

dye into the lipid moiety). For the *E. coli* LPS, there was almost no change (Fig. 4). On the other hand, brilliant green had a marked effect on *Y. enterocolitica* O:8 LPS-TSB, which showed a displacement of T_c to lower values and higher peak positions (increased acyl chain fluidity) at temperatures above 20°C. The effect of brilliant green on the LPS-pH 5.5 of *Y. enterocolitica* O:8 (LPS-EGTA was not tested) was even more marked as it caused a conspicuous increase in the wave number of the $\nu_s(CH_2)$ of the acyl chains to values even higher than those of the *Y. pestis* and *Y. pseudotuberculosis* LPSs. As expected, the *Y. pestis* LPS was not affected by the dye because of its permanently high fluid state. Crystal violet was tested in a similar fashion, and in keeping with the inhibitory actions of the dyes on *Yersinia* sp. (3), it had a less marked effect than brilliant green (data not shown). Thus, all these measurements showed that the increase in permeability observed in vivo was directly related to the degree of acyl chain fluidity of the OM LPS.

(iii) Degree of O-sugar polymerization and lipid A acylation. The SDS-PAGE analyses did not reveal differences between LPS-TSB and LPS-EGTA in terms of the degree of polymerization of O-sugars or the mobility of the rougher LPS molecules (Fig. 5). The LPS-pH 5.5 showed a slight reduction in the amount of rougher LPS molecules and a minor increase in O-sugar polymerization (Fig. 5). On the other hand, the HPTLC analyses revealed clear differences in the *Y. enterocolitica* O:8 lipid A acylation patterns associated with the different growth conditions and also compared with the lipid A of *E. coli* (Fig. 5). As expected, the latter lipid contained mostly hexaacyl forms (over 60% of the total as judged by densitom-

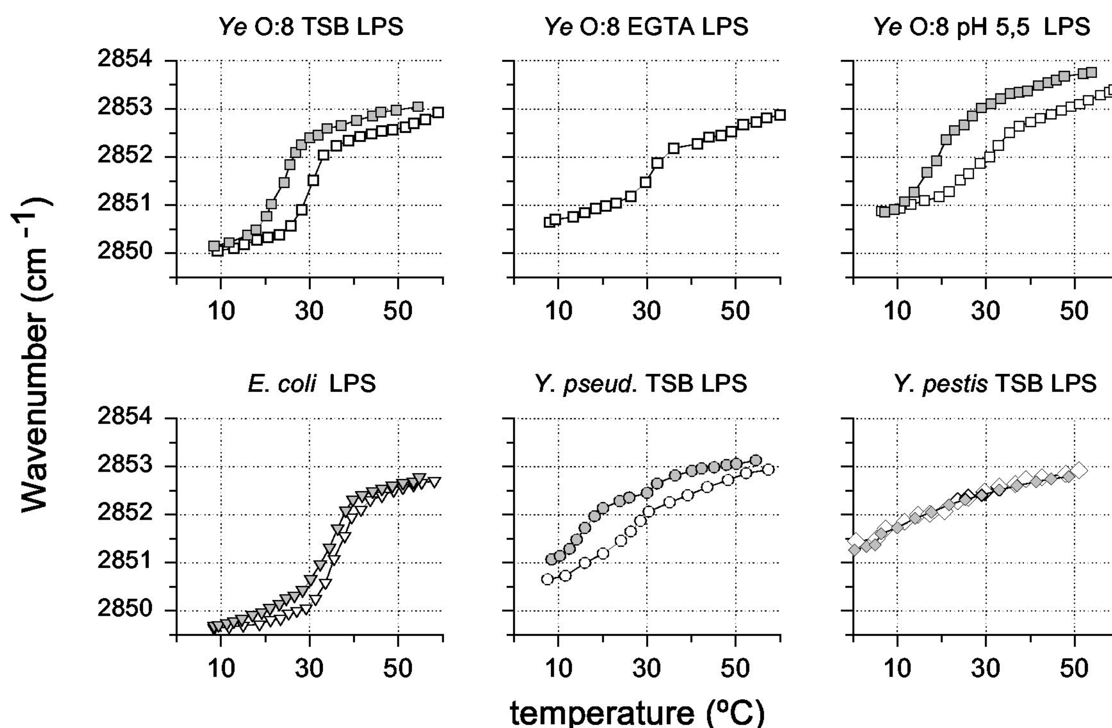

FIG. 4. Plot of the maximum values for the peak positions of $\nu_s(CH_2)$ versus temperature for the LPSs of *Y. enterocolitica* WA 289 O:8 grown at 37°C in TSB (*Ye* O8 TSB LPS), in TSB-EGTA for low calcium availability (*Ye* O8 EGTA LPS), or in TSB-pH 5.5 (*Ye* O8 pH 5,5 LPS), of *E. coli* (*E. coli* LPS), of *Y. pseudotuberculosis* (*Y. pseud* TSB LPS), and of *Y. pestis* (*Y. pestis* TSB LPS). Measurements were obtained in the absence (open symbols) or in the presence (gray symbols) of brilliant green.

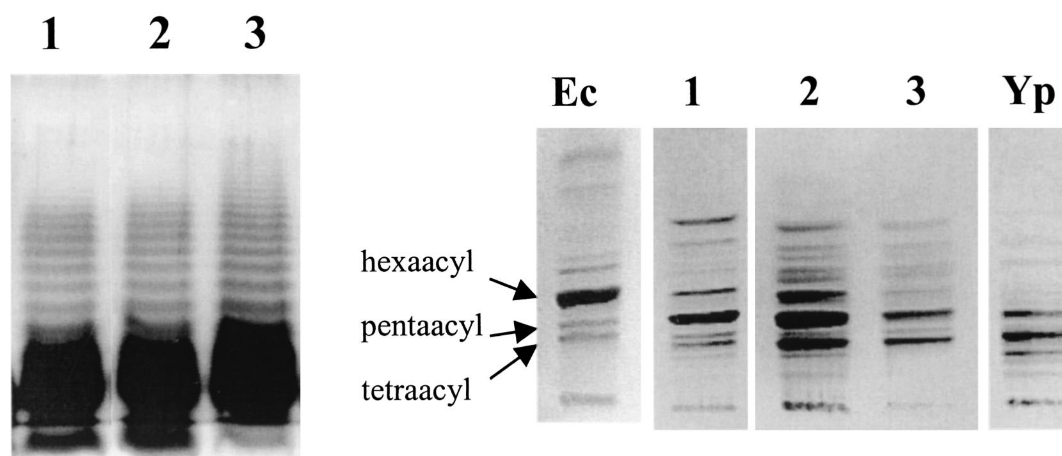

FIG. 5. Degree of O-sugar polymerization as determined by SDS-PAGE (left panel) and lipid A acylation as determined by HPTLC (right panel) of the LPS of *Y. enterocolitica* WA 289 O:8 pYV$^-$ grown in TSB (lane 1), in TSB-EGTA (lane 2), or in TSB-pH 5.5 (lane 3). The high-performance liquid chromatography standards used were lipid A of *E. coli* W3110 MLK3 (lane Ec) and lipid A of *Y. pestis* KIM (lane Yp).

etry analysis), but in *Y. enterocolitica* LPS-TSB there was less hexaacyl lipid A (15%) than pentaacyl lipid A (36%), and microheterogeneity (two close bands) was observed at the position corresponding to the tetraacyl lipid A (9 and 12%). In the LPS-EGTA, pentaacyl lipid A was also dominant (28%) over hexaacyl lipid A (17%), and the proportion of the tetraacyl form with a lower R_f was increased- (17%). The hexaacyl form was barely detected (6%) in the LPS-pH 5.5, and the proportions of the pentaacyl and tetraacyl forms were similar to those in the LPS-EGTA (31 and 21%, respectively). This pattern, in which underacylated forms were dominant, was similar to that obtained for the *Y. pestis* KIM LPS, which, consistent with recent reports, contained mostly tetraacyl lipid A (30%) and also pentaacyl (18%) and triacyl (12%) forms (2, 21, 26). Forms with high mobility (high R_f values) and microheterogeneity were also observed in the LPS-TSB and LPS-EGTA, and these forms may correspond to higher degrees of acylation reported for *Yersinia* (2). In conclusion, although the O-polysaccharide was not significantly altered, lipid A forms with lower degrees of acylation became dominant under nonstandard growth conditions.

DISCUSSION

The results of this work show that pathogenic *Y. enterocolitica* strains respond to Ca^{2+} restriction, acid pH, and contact with human monocytes by decreasing the OM permeability barrier in a pYV-independent manner. In the pathogenic serotype examined in more detail, Ca^{2+} restriction and acid pH caused changes in LPS acyl chain fluidity without significantly altering O-chain polymerization, and LPS aggregates of bacteria grown under these conditions became more permeable to the hydrophobic probe NPN. Changes in permeability were paralleled by an increase in LPS acyl chain fluidity, and the state of order of the acyl chain was more manifestly disrupted by the bulky hydrophobic dye brilliant green in the more permeable LPSs. Acyl chain fluidity is a biologically relevant property of LPS which depends on interactions between the polysaccharide moiety and acyl chain composition (reviewed in reference 42). Indeed, deacylation increases fluidity (41), and therefore, the proportions of hexa-, penta-, and tetraacyl lipid A in the LPS-TSB, LPS-EGTA, and LPS-pH 5.5 of *Y. enterocolitica* O:8 are fully consistent with the LPS acyl chain fluidity and with the increases in the permeability of cells and LPS aggregates. Although the lipid A of serotype O:8 was not examined, Aussel et al. (2) found hexa-, penta-, and tetraacyl lipid A in the LPS of *Y. enterocolitica* O:3 and O:9 grown under standard conditions. Moreover, these authors showed that there is microheterogeneity in tetraacyl forms consistent with the pattern obtained by us by HPTLC and that there are heptaacyl forms that could correspond to the bands with higher R_f values observed in our analyses. With regard to the acyl chain fluidity and the lipid A analyses, the good correspondence between the 2°C difference in the T_c of the *E. coli* LPS and the LPS-TSB, the reduced proportion of hexaacyl lipid A in the latter LPS, and the effect of brilliant green on both LPSs are worth mentioning.

It has been established that the effectiveness of the OM barrier depends on the properties of LPS. In *E. coli* and *Salmonella* (15, 33, 47, 48), this molecule is in a highly ordered state in the OM due to the combined effects of tight lateral interactions mediated by divalent bridging of negative groups in the core and lipid A, the rigidifying effect postulated for the deep core oligosaccharide domain, and the tight packing of fatty acid residues of lipid A (reviewed in reference 47). Agents that interact with LPS (such as polycationic and antimicrobial peptides) or remove divalent cations (such as EDTA) and mutations that affect lipid A or generate deeply truncated LPSs disturb this state and increase the OM permeability (6, 46, 47). Thus, tight packing of LPS is deemed essential for the OM to act as an effective barrier that complements the action of efflux pumps. Under standard growth conditions, the OMs of *Y. enterocolitica* strains are impermeable to hydrophobic probes (4; this study), and there is indirect evidence that there is efflux pump activity (3). However, despite this functional similarity, two lines of evidence demonstrate that the OMs of pathogenic *Y. enterocolitica* strains depart from the model proposed for *E. coli* and *Salmonella*. It has been shown previously that in con-

trast to *E. coli* OMs, *Y. enterocolitica* OMs are insensitive to EDTA and comparatively resistant to polycations (4), and in the present study we found that even in standard media (i.e., in TSB), there are differences between the LPSs of *E. coli* and pathogenic *Y. enterocolitica* strains manifested both as a higher wave number value for $\nu_s(CH_2)$ of the *Y. enterocolitica* LPS acyl chains at temperatures below 35°C and as a 2 to 3°C lower T_c. The relevance of these differences for permeability is shown by the fact that brilliant green had a more marked effect on *Y. enterocolitica* LPS than on *E. coli* LPS. Taken together, these observations show that the contributions of divalent cation bridging and acyl chain packing to the state of order of LPS in the OM are less important in pathogenic *Y. enterocolitica* strains grown at 37°C than in *E. coli*. Accordingly, we suggest a model for the *Y. enterocolitica* OM in which nonionic interactions at the core level compensate for both reduced divalent cation bridging and acyl chain packing and generate a degree of LPS order adequate for impermeability. If this model is correct, a further reduction in acyl chain packing not accompanied by changes in the polysaccharide moiety of LPS could alter OM permeability, and this is what was observed.

The environmental stimuli tested are known to trigger expression of chromosomally encoded and pYV-encoded factors. Myf, Inv, and Yst expression is modulated by pH (25, 44), and Ca^{2+} restriction and contact with eukaryotic cells trigger expression of Yops (11). Like genes coding for Myf, Inv, and Yst, genes involved in LPS biosynthesis are on the chromosome, and thus it is not surprising that pH modulates several chromosomally encoded characteristics simultaneously. On the other hand, Yops are pYV encoded, and pYV also carries the regulatory elements for the Yop synthesis induced by low Ca^{2+} levels (1, 10, 14, 24, 28, 43, 44). Thus, it is noteworthy that a low Ca^{2+} level caused an increase in the OM permeability of pYV^- bacteria, and our observations suggest that in addition to the pYV-encoded systems, there are chromosomal regulatory systems modulated by Ca^{2+} concentration and cell contact and that some of them act either directly or indirectly at the lipid A level. It is important to note that significant changes in lipid A structure can happen in a short time. Observations made with *Salmonella enterica* serovar Typhimurium show that 2-hydroxymiristate modification of lipid A starts within minutes after *phoP* activation and is 50% complete in 30 min (17). Thus, although they occur at a lower rate than protein expression, shifts in lipid A structure can be rapid enough to respond to changing environmental conditions in the host.

In many gram-negative bacteria efflux pumps complement the barrier function of the OM (34, 35). We observed that metabolic inhibitors further enhanced the permeability of pathogenic bacteria grown with EGTA or under acidic pH conditions, and this suggests that efflux pumps remain active. In environments with noxious hydrophobic compounds, coordination between the activity of efflux pumps and the OM barrier seems necessary as neither of these mechanisms would be completely efficient if they acted independently. However, such coordination may not be necessary in other environments, and, on the other hand, the LPS modifications linked to OM permeability may be beneficial for invading host tissues (see below). Bacteria whose OMs are not permanent barriers to hydrophobic permeants include *Brucella*, some mucosal pathogens, *Y. pseudotuberculosis*, and *Y. pestis* (3, 15, 20, 29). All these bacteria normally infect their hosts by nonenteric routes or (for *Y. pseudotuberculosis*) have much larger infectious doses when they are administered orally (36, 39). This illustrates that OM impermeability is a dispensable property for pathogens entering the body by routes other than the gut. *Y. enterocolitica* pathogenic biotypes attach to intestinal cells and, in a second step, cross the epithelial barrier to disseminate to internal organs. It could be that along with the expression of virulence factors, the OM permeability of these bacteria is increased during this second step and approaches the OM permeability of *Y. pseudotuberculosis* and *Y. pestis*. We did not observe permeability changes in environmental strains, and this suggests that modulation of OM permeability is relevant in the biology of pathogenic *Y. enterocolitica* strains. Any advantage that these bacteria could derive from this is a matter of speculation. A more permeable OM may help facilitate the exchange of hydrophobic nutrients and metabolites within host tissues. Moreover, we have found that in pathogenic *Y. enterocolitica* strains permeability is linked to qualitative and/or quantitative changes in LPS acylation, and such changes are known to influence endotoxicity (42). Since reduction of endotoxicity by modulation of lipid A acylation (18, 42) or by departure from the classical lipid A structure (32, 37) hampers detection by host innate immune systems (31), it is possible that the changes in the OM described here also enhance the ability of pathogenic *Y. enterocolitica* strains to evade host defenses during the first stages of infection. Evidence that supports this hypothesis comes from the similar lipid A patterns of the LPSs of *Y. pestis* and pathogenic *Y. enterocolitica* strains grown in EGTA-supplemented and acidic media and by the finding that compared to classical LPSs, *Y. pestis* LPS displays low endotoxic activity (26). Experiments are in progress to test the endotoxicity-related properties of the LPSs of pathogenic and nonpathogenic *Y. enterocolitica* strains grown under standard and nonstandard conditions.

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