***Yersinia pseudotuberculosis* and *Yersinia pestis* show increased outer membrane permeability to hydrophobic agents which correlates with lipopolysaccharide acyl-chain fluidity**

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The hydrophobic probe *N*-phenyl-1-naphthylamine accumulated less in non-pathogenic *Yersinia* spp. and non-pathogenic and pathogenic *Yersinia enterocolitica* than in *Yersinia pseudotuberculosis* or *Yersinia pestis*. This was largely due to differences in the activity of efflux systems, but also to differences in outer membrane permeability because uptake of the probe in KCN/arsenate-poisoned cells was slower in the former group than in *Y. pseudotuberculosis* and *Y. pestis*. The probe accumulation rate was higher in *Y. pseudotuberculosis* and *Y. pestis* grown at 37 °C than at 26 °C and was always highest in *Y. pestis*. These yersiniae had LPSs with shorter polysaccharides than *Y. enterocolitica*, particularly when grown at 37 °C. Gel $\leftrightarrow$ liquid-crystalline phase transitions (T_c 28–31 °C) were observed in LPS aggregates of *Y. enterocolitica* grown at 26 and 37 °C, with no differences between non-pathogenic and pathogenic strains. *Y. pseudotuberculosis* and *Y. pestis* LPSs showed no phase transitions and, although the fluidity of LPSs of *Y. pseudotuberculosis* and *Y. enterocolitica* grown at 26 °C were close below the T_c of the latter, they were always in a more fluid state than *Y. enterocolitica* LPS. Comparison with previous studies of *Salmonella choleraesuis* subsp. *choleraesuis* serotype *minnesota* rough LPS showed that the increased fluidity and absence of transition of *Y. pseudotuberculosis* and *Y. pestis* LPSs cannot be explained by their shorter polysaccharides and suggested differences at the lipid A/core level. It is proposed that differences in LPS–LPS interactions and efflux activity explain the above observations and reflect the adaptation of *Yersinia* spp. to different habitats.

Keywords: *Yersinia*, outer membrane, permeability, lipopolysaccharide

INTRODUCTION

The yersiniae are members of the *Enterobacteriaceae* and include both pathogenic and non-pathogenic species and biotypes. Whereas *Yersinia intermedia*, *Yersinia mollaretii*, *Yersinia rohdei*, *Yersinia bercovieri*, *Yersinia frederiksenii* and *Yersinia kristensenii* are merely opportunistic pathogens found mostly in the environment, *Yersinia enterocolitica* includes both non-pathogenic

and pathogenic biogroups and *Yersinia pseudotuberculosis* and the closely related *Yersinia pestis* are characteristically pathogenic (Bercovier & Mollaret, 1984). *Y. enterocolitica* and *Y. pseudotuberculosis* cause intestinal infections in humans but they differ in the degree of invasiveness which, although limited in both, is greater in the latter (Brubaker, 1991). In contrast, *Y. pestis* enters the body by routes other than the gut, it is very invasive and characteristically multiplies within non-activated macrophages (Brubaker, 1991; Straley & Harmon, 1984a, b). Pathogenic yersiniae carry a virulence plasmid (pYV) and many virulence-related properties are regulated by environmental factors such as temperature, pH, Ca²⁺ availability and contact with

Abbreviations: DOC, sodium deoxycholate; FTIR, Fourier-transformed infrared spectroscopy; Kdo, 3-deoxy-o-manno-2-octulosonic acid; NPN, *N*-phenyl-1-naphthylamine; OM, outer membrane.

eukaryotic cells, at both plasmid and chromosome levels (Brubaker, 1991; Cornelis & Wolf-Watz, 1997; Straley & Perry, 1995).

Because of the importance of outer membranes (OMs) in the interaction of Gram-negative bacteria with the environment, we have hypothesized that the wide differences in habitat and pathogenicity between yersiniae should be reflected in their OM properties (Bengoechea *et al.*, 1996). In a previous study, we found that plasmid-bearing (pYV⁺) and plasmid-cured (pYV⁻) pathogenic *Y. enterocolitica* had OMs resistant to polycations compared to *Y. enterocolitica* biogroup 1A (environmental and non-pathogenic) and enteropathogenic *Escherichia coli*. In addition, when grown with Mg²⁺ but with no Ca²⁺ available, pathogenic *Y. enterocolitica* biogroups displayed a marked increase in OM permeability to hydrophobic probes which was not observed in the non-pathogenic biogroup or in *E. coli* (Bengoechea *et al.*, 1996). We have extended these studies to *Y. pseudotuberculosis* and *Y. pestis*, the more invasive of the pathogenic yersiniae, and considered the influence of both the OM barrier and possible efflux systems. We report here that there are large differences between *Yersinia* spp. in their ability to repel hydrophobic compounds and that such differences correlate with the degree of acyl-chain fluidity in their respective LPS. We also present indirect evidence suggesting differences in efflux systems between yersiniae.

METHODS

Bacterial strains and growth conditions. The strains used (Table 1) were stored in skimmed milk at -80 °C and inocula were taken directly from these frozen seeds to minimize loss of pYV for all experiments (Bengoechea *et al.*, 1996). Selection of the pYV⁺ (virulent) and plasmid-cured pYV⁻ (avirulent) isogenic strains was performed on Congo Red/magnesium/oxalate medium (Riley & Toma, 1989). For permeability studies, cells were grown in standard tryptic soy broth in sidearm flasks on an orbital shaker (250 r.p.m.) at 26 or 37 °C and growth was monitored by measuring OD₅₄₀. To test the effect of pYV expression, the cells were grown in magnesium/oxalate medium (Riley & Toma, 1989). Exponential phase cells were harvested (5000 g, 20 min, 5 °C) and resuspended immediately in the appropriate buffer or broth (see below). For LPS extraction, *Y. pseudotuberculosis* and *Y. enterocolitica* were grown in tryptic soy broth and *Y. pestis* in standard brain-heart infusion in 2 l flasks (800 ml per flask) on an orbital shaker (200 r.p.m.) for 24 h at 26 or 37 °C.

Sensitivity to hydrophobic antibiotics and dyes. Serial dilutions of the appropriate agents (Table 2) were made in sterile 96-well polystyrene plates (Nunc) with Mueller-Hinton broth as the diluent. A fresh suspension (approx. 1.25×10^6 c.f.u. ml⁻¹) of exponential phase cells grown at 37 °C was prepared in the same broth and 100 µl aliquots were pipetted into each well. Plates were incubated at 37 °C for 16 h and the lowest concentration of each agent that completely inhibited visible growth was taken as its MIC.

N-Phenyl-1-naphthylamine (NPN) uptake. NPN is a hydrophobic fluorescent dye which, unless hindered, partitions into the hydrophobic moiety of membranes with a simultaneous increase in quantum yield and which has been proven useful in

OM permeability studies (Bengoechea *et al.*, 1996; Loh *et al.*, 1984; Freer *et al.*, 1996; Martínez de Tejada & Moriyón, 1993; Träuble & Overath, 1973). Exponentially growing cells were resuspended in 2 mM HEPES (pH 7.2) with or without KCN plus sodium arsenate (1–25 mM for both inhibitors; see Results) to an OD₆₀₀ of 0.5. Cells were then transferred to 1 cm diameter fluorimetric cuvettes, NPN (500 µM in acetone) was added to a final concentration of 10 µM and changes in fluorescence were monitored with an LS-50 fluorimeter (Perkin-Elmer; excitation 350 nm, emission 420 nm, slit width 2.5 nm). Measurements were performed at the growth temperature and results were expressed as relative fluorescence units (RFU) (Bengoechea *et al.*, 1996). Measurements were repeated at least twice with each of two independently grown batches of bacteria.

Sensitivity to deoxycholate. Late exponential phase cells were resuspended to an OD₄₅₀ of 0.21 using saline with or without 1 mM sodium arsenate/1 mM KCN and 2 ml was transferred to tubes containing increasing concentrations of sodium deoxycholate (DOC) (see Results). After 1 h at the growth temperature, lysis was measured as the percentage OD₄₅₀ of bacterial suspensions incubated without DOC. All experiments were performed in duplicate with two independently grown batches of bacteria.

LPS preparations. The LPSs of *Y. enterocolitica* PR O:1,6, WA 289 O:8 and WE 245/92 O:3, *Y. pseudotuberculosis* WE 23/90 and *Y. pestis* KIM were obtained from the water phase of a water/phenol extract as described by Sprott *et al.* (1994) and fractionation of the LPSs into the water but not the phenol phase was confirmed. Crude LPSs were dispersed (10 mg ml⁻¹) in 0.8% NaCl/0.05% NaN₃/0.1 M Tris/HCl (pH 7), digested with nucleases and proteinase K as described previously (Bengoechea *et al.*, 1996) and the purified LPSs were sedimented by ultracentrifugation (100000 g, 6 h) and freeze-dried. The 3-deoxy-D-manno-2-octulosonic acid (Kdo) content was determined by the thiobarbituric acid method modified to correct interference due to deoxysugars (Díaz-Aparicio *et al.*, 1993) and protein content was determined as described by Markwell *et al.* (1978) using serum albumin as standard. SDS-PAGE and silver staining for LPS were performed as described by Tsai & Frasch (1982). As a reference, the deep R-LPS from *Salmonella choleraesuis* subsp. *choleraesuis* serotype *minnesota* (hereafter referred to as *S. minnesota* Re595 (obtained by the phenol/chloroform/petroleum ether method) was included in all gels. Absence of phospholipids was shown by analysis of chloroform/methanol extracts of the LPS preparations by conventional methods (Thiele & Schwinn, 1973).

Determination of the gel ↔ liquid-crystalline (β ↔ α) phase transition of Yersinia LPSs. The β ↔ α phase transition of the acyl chains of LPS from a well-ordered (gel) into a fluid (liquid-crystalline) state at a lipid-specific temperature, *T_c*, was measured by two methods: Fourier-transformed infrared spectroscopy (FTIR) and fluorescence polarization spectroscopy. This allows the determination of the acyl-chain fluidity – inversely proportional to the state of order – which is a measure of the mobility of the hydrocarbon chains at a given temperature. Thus, in the gel phase, well below *T_c*, the acyl chains are in a highly ordered, rigid state and in the liquid-crystalline phase, well above *T_c*, they are highly disordered. The natural salt forms of the LPSs were used in the experiments described below.

(i) FTIR. To ensure homogeneity, LPS dispersions 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 suspensions were

Table 1. Bacterial strains used

Strain	Serogroup (biogroup)	Characteristics
<i>Y. kristensenii</i> WA 396	O:11, 24	Non-pathogenic
<i>Y. intermedia</i> WA 9/91	O:40	Non-pathogenic
<i>Y. frederiksenii</i> WA 99/92	O:48	Non-pathogenic
<i>Y. bercovieri</i> WS 68/91	O:73, 58	Non-pathogenic
<i>Y. mollaretii</i> WA 19/88	O:75	Non-pathogenic
<i>Y. rohdei</i> WA 9/90	O:76	Non-pathogenic
<i>Y. enterocolitica</i> PR	O:1, 6 (1A)	Environmental (non-pathogenic)
<i>Y. enterocolitica</i> MA 279	O:5 (1A)	Environmental (non-pathogenic)
<i>Y. enterocolitica</i> MA 280	O:6 (1A)	Environmental (non-pathogenic)
<i>Y. enterocolitica</i> MA 282	O:7, 8, 13, 19 (1A)	Environmental (non-pathogenic)
<i>Y. enterocolitica</i> IP 135	O:1, 2a, 3 (3)	Pathogenic (chinchilla), pYV ⁺
<i>Y. enterocolitica</i> IP 178	O:2a, 2b, 3 (5)	Pathogenic (hare), pYV ⁺
<i>Y. enterocolitica</i> WE 245/92	O:3 (4)	Pathogenic, pYV ⁺
<i>Y. enterocolitica</i> ID	O:3 (4)	Pathogenic, pYV ⁺
<i>Y. enterocolitica</i> WA 289	O:8 (1B)	Pathogenic, pYV ⁺
<i>Y. enterocolitica</i> WA	O:8 (1B)	Pathogenic, pYV ⁺
<i>Y. enterocolitica</i> WE 517/91	O:9 (2)	Pathogenic, pYV ⁺
<i>Y. enterocolitica</i> PT	O:9 (2)	Pathogenic, pYV ⁺
<i>Y. pseudotuberculosis</i> WE 23/90	I	Pathogenic, pYV ⁺
<i>Y. pseudotuberculosis</i> WS 41/91	I	Pathogenic, pYV ⁺
<i>Y. pseudotuberculosis</i> WS 45/91	II	Pathogenic, pYV ⁺
<i>Y. pseudotuberculosis</i> WS 66/89	III	Pathogenic, pYV ⁺
<i>Y. pestis</i> KIM		Avirulent, pYV ⁻
<i>S. minnesota</i> Re595		Deep R-LPS (Re chemotype)
<i>S. minnesota</i> Rd ₁ p+		Deep R-LPS (Rd ₁ chemotype)
<i>E. coli</i>	O111 K58 H2	Enteropathogenic

Table 2. MICs of hydrophobic agents

Hydrophobic agent (partition coefficient)*	MIC ($\mu\text{g ml}^{-1}$) for:†							
	<i>Y. enterocolitica</i> WA 289 O:8	<i>Y. pseudotuberculosis</i> WE 23/90	<i>Y. pseudotuberculosis</i> WS 41/91	<i>Y. pseudotuberculosis</i> WS 45/91	<i>Y. pseudotuberculosis</i> WS 66/89	<i>Y. pestis</i> KIM	<i>S. minnesota</i> Rd ₁ p+	<i>S. minnesota</i> Re595
Crystal violet (14.4)	256	64	16	8	16	32	<2	<2
Malachite green (4.2)	32	8	16	8	8	8	<2	<2
Novobiocin (>20)	256	16	32	32	64	32	<2	8
Nafcillin (0.31)	256	32	32	32	64	64	64	64
Brilliant green	2	ND	2	2	8	ND	<2	<2

* Partition coefficient in 1-octanol/0.05 M sodium phosphate (pH 7.0) at 24 °C (taken from Nikaido, 1976).

† ND, not done.

stored at 4 °C for several hours before analysis. Measurements were performed with a Bruker IFS 55 apparatus as previously described (Brandenburg & Seydel, 1990). Briefly, LPS dispersions (water content >90%) were analysed in CaF₂ cuvettes with 12.5 μm thick Teflon spacers. For each measurement 50 interferograms were accumulated, Fourier-transformed and converted to absorbance spectra. The measurements were performed in continuous heating scans (2 °C min⁻¹) from 10 to 60 °C. The peak position of the symmetric stretching vibration of the methylene groups, $\nu_s(\text{CH}_2)$, around 2850 cm⁻¹ was taken as a measure of the state of order (fluidity) of the acyl chains (Casal & Mantsch, 1984).

(ii) Fluorescence polarization (*P*). LPS dispersions were prepared as described for the FTIR experiments and labelled by incubation for a few minutes in 1 mM diphenylhexatriene. Measurements were performed with a Kontron SFM 25 fluorescence spectrometer. The dye was excited by polarized light at 360 nm and the emission intensities (*I*) at 420 nm were measured simultaneously parallel ($\parallel$) and perpendicular ($\perp$) to the polarization plane of the incident beam. For each measurement, the emission was corrected by the grating factor, $G = I_{\perp}/I_{\parallel}$. Fluorescence polarization, $P = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + I_{\perp})$, was determined as a function of temperature from 10 to 60 °C at a heating rate of 3 °C min⁻¹.

RESULTS

Sensitivity of *Yersinia* spp. to hydrophobic antibiotics and dyes

The *Y. enterocolitica* and *Y. pseudotuberculosis* strains and *Y. pestis* KIM (Table 1) were grown in tryptic soy broth at 26 °C for 18 h and 20 µl aliquots of these fresh inocula were seeded on MacConkey, *Salmonella-Shigella* and brilliant green agars (Difco) and incubated at 26 or 37 °C for 24 h. All *Y. enterocolitica* strains grew on both MacConkey and *Salmonella-Shigella* agars, regardless of the temperature. By contrast, *Y. pestis* KIM was completely inhibited in both media at 26 and 37 °C. All *Y. pseudotuberculosis* grew on MacConkey agar at 26 and 37 °C, but they produced only pin-point colonies on *Salmonella-Shigella* agar at 26 °C and there was no growth at 37 °C. All yersiniae were completely inhibited on brilliant green agar at both 26 and 37 °C.

The MICs of hydrophobic dyes and antibiotics to representative *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis* strains are shown in Table 2. With the exception of brilliant green, *Y. pseudotuberculosis* and *Y. pestis* were more sensitive to those agents than *Y. enterocolitica*, but they were not as sensitive as the deep rough (R) *S. minnesota*.

NPN uptake

Representative results obtained with cells grown at 37 °C are presented in Fig. 1. In the absence of membrane metabolic inhibitors, pathogenic (WA 289 O:8) and non-pathogenic (MA 279 O:5) *Y. enterocolitica* took up

only a limited amount of the probe (up to 25 RFU), whereas a marked uptake was observed for *Y. pseudotuberculosis* WE 23/90 and *Y. pestis* KIM in particular. These relative differences did not change when the remaining *Y. enterocolitica* and *Y. pseudotuberculosis* strains were tested. Moreover, *Y. kristensenii* WA 396, *Y. intermedia* WA 9/91, *Y. frederiksenii* WA 99/92, *Y. bercovieri* WS 68/91, *Y. mollaretii* WA 19/88 and *Y. rohdei* WA 9/90 yielded results identical to those of *Y. enterocolitica* (not shown). As expected, *E. coli* showed NPN uptake similar to that of *Y. enterocolitica* strains, whereas *S. minnesota* Re595 took up more probe (not shown). Although the NPN uptake of *Y. pseudotuberculosis* and *Y. pestis* was slightly reduced, similar observations were made with all strains in Table 1 grown at 26 °C.

The action of efflux systems was tested using non-pathogenic *Y. enterocolitica* and pathogenic yersiniae strains shown in Table 1 (grown at 37 °C) with KCN and sodium arsenate as inhibitors (Berger, 1973), used simultaneously at 1–25 mM concentrations. Results with 10 mM KCN plus 10 mM sodium arsenate and representative strains are presented in Fig. 1(b). It can be seen that inhibitors had no detectable effect on NPN uptake by *Y. pseudotuberculosis* and *Y. pestis* and that they caused NPN to be taken up by non-pathogenic and pathogenic *Y. enterocolitica*. These effects were maximal at concentrations as low as 1 mM for pathogenic *Y. enterocolitica* and 10 mM for non-pathogenic strains, with identical kinetics of uptake and final fluorescence values for higher concentrations. Fig. 1 also shows that the rate of NPN uptake was highest for *Y. pestis* and *Y. pseudotuberculosis* (185 and 115 RFU, respectively, in

Fig. 1. OM permeability to NPN in *Y. enterocolitica* MA 279 O:5 (Y.e. O:5), *Y. enterocolitica* WA 289 O:8 (Y.e. O:8), *Y. pseudotuberculosis* WE 23/90 (Y.pstb.) and *Y. pestis* KIM (Y.p.). Cells were grown at 37 °C and fluorescence monitored with (a) or without (b) membrane metabolic inhibitors.

Fig. 2. Cell lysis induced by DOC. *Y. enterocolitica* WA 289 O:8 (Δ), *Y. pseudotuberculosis* WE 23/90 (■) and *Y. pestis* KIM (○) grown at 37 °C were incubated with DOC without (a) or with (b) membrane metabolic inhibitors and the decrease in OD₄₅₀ was recorded after 1 h incubation at 37 °C. Results are the mean of two independent experiments (coefficient of variation less than 5% for all groups of data).

5 s), intermediate for pathogenic *Y. enterocolitica* (110 RFU in 45 s) and lowest for non-pathogenic *Y. enterocolitica* (110 RFU in 90 s). *Y. pseudotuberculosis* WE 23/90 and *Y. enterocolitica* WA 289 O:8 pYV⁺ and pYV⁻ pairs yielded similar results both with and without inhibitors and at either 26 or 37 °C (not shown).

Sensitivity of *Yersinia* spp. to DOC

With cells growing exponentially at 37 °C, DOC-induced lysis in the absence of metabolic inhibitors was greater for *Y. pestis* KIM than for *Y. pseudotuberculosis* WE 23/90 and no lysis was observed for *Y. enterocolitica* WA 289 O:8 (Fig. 2a). Membrane metabolic inhibitors (at concentrations having maximal effect in the NPN experiments) rendered these three yersiniae susceptible to DOC-induced lysis, but the relative differences in susceptibility were maintained (Fig. 2b). Similar results were obtained with the remaining *Y. pseudotuberculosis* strains (not shown) and none of them was as sensitive to DOC as *Y. pestis* KIM. Although lysis was less marked, similar observations

Fig. 3. SDS-PAGE followed by silver staining of LPS preparations (10 µg) from cells grown at the indicated temperature. Lanes: 1, *Y. pestis* KIM; 2, *Y. pseudotuberculosis* WE 23/90; 3, *Y. enterocolitica* WE 245/92 O:3; 4, *Y. enterocolitica* WA 289 O:8. The deep R-LPS from *S. minnesota* Re595 (lane Sm) is included as a reference.

were made with cells grown at 26 °C (not shown). Interestingly, stationary phase cells of *Y. pseudotuberculosis* and *Y. pestis* were more sensitive to DOC than exponential phase cells; this effect was not observed with *Y. enterocolitica*.

LPS characterization

Fig. 3 shows SDS-PAGE profiles of the LPSs from *Y. enterocolitica* WA 289 O:8 and WE 245/92 O:3, *Y. pseudotuberculosis* WE 23/90 and *Y. pestis* KIM cells grown at 26 and 37 °C. The *Y. enterocolitica* LPSs showed both short and long polysaccharide molecules typical of smooth (S)-LPS and the absence (WE 245/92 O:3 LPS) or presence (WA 289 O:8 LPS) of the ladder-like pattern that relates to the number of sugars in the O-chain repetitive units (Hoffman *et al.*, 1980; Tomshich *et al.*, 1987). *Y. enterocolitica* PR O:1,6 (non-pathogenic) also had an S-LPS, but with no conspicuous ladder-like pattern (not shown). These analyses also showed enrichment of the low molecular mass components at 37 °C, with a conspicuous fast-migrating band (Fig. 3). *Y. pseudotuberculosis* WE 23/90 LPS only showed traces of long polysaccharide LPS when grown at 26 °C and also showed enrichment of a fast-migrating component at 37 °C (Fig. 3). Finally, *Y. pestis* KIM LPS was similar at 26 and 37 °C and contained a component of mobility similar to that of the fast-migrating component of *Y. pseudotuberculosis* plus a second component migrating closer to the running front of the gel (Fig. 3). The Kdo contents of the LPSs at 26 and 37 °C were, respectively, 2.28 and 2.84% for *Y. enterocolitica* WA 289 O:8, 1.95 and 2.80% for *Y. enterocolitica* WE 245/92 O:3, 1.14 and 2.03% for *Y. enterocolitica* PR O:1,6, 2.47 and 5.65% for *Y. pseudotuberculosis* WE

Fig. 4. Temperature dependence of (a) maximum of the peak position of the symmetric stretching vibration, $\nu_s(\text{CH}_2)$, and (b) fluorescence polarization, P , in LPSs of *Y. enterocolitica* WA 289 O:8 (continuous lines), *Y. pseudotuberculosis* WE 23/90 (dashed lines) and *Y. pestis* KIM (dotted lines) grown at 26 or 37 °C.

23/90, and 6.84 and 8.08% for *Y. pestis* KIM. No phospholipid was detected in these preparations and protein contents were below 1.0%.

For many biological process, e.g. interactions at cell membrane level, acyl-chain fluidity is known to play an important role. For this, we determined the $\beta \leftrightarrow \alpha$ phase transition of the hydrocarbon chains of LPS preparations from cells grown at 26 and 37 °C. The temperature-dependent shift in the maximum of the peak position of the symmetric stretching vibration, $\nu_s(\text{CH}_2)$, of LPS aggregates is presented in Fig. 4(a). For the *Y. enterocolitica* WA 289 O:8 LPS and regardless of the growth temperature, the peak positions were in the 2850.0 to 2850.5 cm⁻¹ range at temperatures below 15 °C and over 2852.0 cm⁻¹ above 35 °C. For this LPS, a clear $\beta \leftrightarrow \alpha$ phase transition in the 19–35 °C interval was observed (Fig. 4a) with a T_c of 29 °C. The *Y. enterocolitica* WE 245/92 O:3 and PR O:1,6 LPSs yielded similar results (not shown) with T_c values from 28 to 31 °C. The results obtained with the LPS of *Y. pseudotuberculosis* WE 23/90 grown at 26 °C were close to those of the *Y. enterocolitica* LPSs, but no clear phase transition was observed (Fig. 4a). With the LPS of *Y. pseudotuberculosis* WE 23/90 grown at 37 °C, the peak positions were higher over the whole temperature range, with no phase transition (Fig. 4a). The results obtained

with the *Y. pestis* KIM LPS were similar for the two growth temperatures, with peak positions constantly higher than those obtained with the LPSs of the other *Yersinia* spp. and no phase transition (Fig. 4a).

Analysis of $\beta \leftrightarrow \alpha$ phase transitions was also carried out by measuring the change in fluorescence polarization, P , of diphenylhexatriene-labelled LPS aggregates as a function of temperature with results in good agreement with those obtained by FTIR. This second method showed clear phase transitions in LPS aggregates of *Y. enterocolitica* WA 289 O:8 grown at either 26 or 37 °C with only a 2 °C difference with respect to the T_c value obtained by FTIR (Fig. 4b). The LPSs of *Y. enterocolitica* WE 245/92 O:3 and PR O:1,6 yielded similar results (not shown) to those of the WA 289 O:8 LPS, but no phase transitions were observed for the LPSs of *Y. pseudotuberculosis* WE 23/90 or *Y. pestis* KIM, irrespective of the growth temperature (Fig. 4b). For cells grown at 26 °C, and in agreement with the FTIR results, the P values of the *Y. pseudotuberculosis* WE 23/90 LPS were closer to those of the *Y. enterocolitica* LPSs at temperatures below the T_c of the latter and those of the *Y. pestis* KIM LPS indicated a fluid state over the whole temperature range (Fig. 4b). With cells grown at 37 °C, the fluidity values were similar for the LPSs of *Y. pseudotuberculosis* WE 23/90 and *Y. pestis* KIM.

DISCUSSION

This study provides coincident indirect (growth differences on selective media and MICs) and direct (NPN uptake and DOC-induced cell lysis) evidence showing that *Yersinia* spp. vary in their ability to repel hydrophobic agents and surfactants. Moreover, agreement was found between the different observations with regard to variations associated with species or growth temperature. Accordingly, *Y. pestis* KIM was inhibited on selective media on which the remaining *Yersinia* spp. grew and it also showed the highest NPN accumulation and DOC sensitivity. Also, *Y. pseudotuberculosis*, which grew on *Salmonella*-*Shigella* agar at 26 °C but not at 37 °C, showed intermediate NPN accumulation and DOC sensitivity, both of which were enhanced at 37 °C. All these data are in agreement with previous observations on the variable growth responses of *Y. pestis* and *Y. pseudotuberculosis* on MacConkey agar and, depending on the temperature, also on *Salmonella*-*Shigella* agar (Bercovier & Mollaret, 1984).

It has been shown that some Gram-negative bacteria are endowed with efflux systems that pump out hydrophobic permeants, thereby complementing the barrier function of the OM to different extents (Hancock, 1997; Nikaido, 1994, 1996). Since efflux systems use energy, increased accumulation of a given compound in the presence of membrane metabolic inhibitors is usually taken as indirect evidence for such a system (Hancock, 1997; Nikaido, 1994, 1996). In this work, KCN plus sodium arsenate were used, rather than uncouplers such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), because we have observed that this agent causes artefacts in bacteria having OMs permeable to hydrophobic agents (A. Sola, J. A. Bengoechea & I. Moriyón, unpublished) and artefacts in membrane studies have also been observed by others (Hancock & Braun, 1976; Helgersson & Cramer, 1977). Since the net entry of hydrophobic compounds into cells of *Yersinia* spp. was increased by inhibitors not having side effects, the results strongly suggest that efflux systems are more efficient in *Y. enterocolitica* and in non-pathogenic strains in particular, than in *Y. pseudotuberculosis* or *Y. pestis*. With *Y. pseudotuberculosis* and *Y. pestis*, inhibitors had no apparent effect in the NPN uptake test, but the very high rate of spontaneous uptake could mask possible efflux systems. In fact, the presence of efflux systems in *Y. pseudotuberculosis* and *Y. pestis* is suggested by the increase of DOC-induced cell lysis observed with metabolic inhibitors. Alternatively, this apparent discrepancy could reflect the specificity of the efflux systems, since DOC is chemically closer to permeants present in the host. However, differences in efflux systems cannot account for the observations by themselves. Even at optimal inhibitor concentrations, the net entry of both NPN and DOC decreased in the order *Y. pestis*-*Y. pseudotuberculosis*-pathogenic *Y. enterocolitica*-non-pathogenic *Y. enterocolitica*, suggesting that OM structural differences were also affecting the rate of partition of the permeants tested. Indeed, this is

in agreement with the idea that efficient efflux systems call for efficient OM barriers.

The increase in OM permeability to hydrophobic compounds was paralleled both by a reduction in the LPS polysaccharide moiety and by an increase in acyl-chain fluidity. LPS molecules are located only in the outer leaflet of the OM and, at least in *E. coli* and *Salmonella* S cells, they are tightly bridged by divalent cations, thereby excluding phospholipids from the outer leaflet (Hancock, 1984; Nikaido & Vaara, 1985). *S. typhimurium* Rd and Re (deep R) mutants and their equivalent *E. coli* D21f1 and D21f2 mutants, which have no or reduced heptose and phosphate in the LPS core, are hypersensitive to hydrophobic antibiotics and dyes. Since a role of the LPS O-specific polysaccharide is ruled out by the similar OM permeability of S cells and Ra, Rb and Rc mutants, the higher permeability of Rd and Re mutants can be explained by the presence of phospholipid molecules in the OM outer leaflet through which hydrophobic permeants can penetrate (Nikaido & Vaara, 1985). In addition, penetration can occur through LPS-covered areas where the overall OM structure becomes altered as a consequence of the absence of core components necessary for effective bridging (Nikaido & Vaara, 1985). Furthermore, it has been shown in synchrotron X-ray small angle diffraction studies that, under conditions under which LPS samples independent of the length of the sugar chain adopt lamellar structures (low water content and high Mg^{2+} concentration), the values for the lamellar repeat d_1 are 6.0–6.5 nm for Re and Rd LPS, but increase considerably to more than 8.0 nm for Rc LPS (Seydel *et al.*, 1993). From this, the hydration shells of Rc LPS and those LPSs with longer sugar chains can be assumed to be much larger than for the Rd and Re LPSs. Thus, in addition to the changed LPS-LPS interactions, the reduced hydration of Re or Rd LPS might explain the increased susceptibility (permeability) to hydrophobic drugs of the corresponding mutants. Phospholipid exposure at the OM surface has been clearly shown in *S. typhimurium* deep R mutants and, accordingly, direct permeation through OM phospholipid patches is the likely explanation for their increased OM permeability (Nikaido & Vaara, 1985). However, this is not always the case because *Brucella abortus* (which carries an S-type LPS) readily takes up hydrophobic agents (Martínez de Tejada & Moriyón, 1993; Freer *et al.*, 1996) and there is evidence that this uptake can be explained on the sole basis of the properties of the LPS (Freer *et al.*, 1996; Martínez de Tejada & Moriyón, 1993).

The reduced polysaccharide content of *Y. pseudotuberculosis* LPSs could suggest a situation similar to that of the deep R *S. minnesota* and *E. coli* mutants. However, although they showed a growth temperature-dependent increase in the roughness of the LPS, the SDS-PAGE analyses showed no component of mobility similar to the *S. minnesota* Re LPS in the LPS of *Y. pseudotuberculosis* WE 23/90 grown at either 26 or 37 °C. Those analyses also showed two overlapping

components in the LPS of *Y. pestis* KIM, one of which could be equivalent to the Re595 *S. minnesota* LPS, while the other was similar to the rougher component detected in *Y. pseudotuberculosis* WE 23/90 at 37 °C. Therefore, this strain cannot be considered as totally equivalent to the deep R *S. minnesota* strains. Although there are no chemical data for the LPSs of *Y. pseudotuberculosis* grown at 37 °C, the SDS-PAGE result is in agreement with the work of Samuelsson *et al.* (1973) who showed that the LPSs of *Y. pseudotuberculosis* serogroups I–V grown at 26 °C carry an oligosaccharide stub attached to the outer core. Therefore, the evidence available suggests that the *Y. pseudotuberculosis* LPS is not truly R but similar to the lipo-oligosaccharides of *Neisseria*, *Bordetella* or *Haemophilus* spp. (Hitchcock *et al.*, 1986). As discussed below, the acyl-chain fluidity analyses also showed important differences (absence or presence of transitions, respectively) between the LPSs of pathogenic yersiniae and deep R *S. minnesota*.

The acyl-chain fluidity analyses point to reduced LPS–LPS interactions as a likely reason for the increased OM permeability to hydrophobic agents. Both FTIR and fluorescence polarization spectroscopy showed that (i) *Y. pseudotuberculosis* WE 23/90 and *Y. pestis* KIM LPSs formed aggregates with more fluid acyl chains than those of other *Yersinia* spp., (ii) *Y. pestis* KIM LPS aggregates had more fluid acyl chains than those of *Y. pseudotuberculosis* WE 23/90 and (iii) the acyl-chain fluidity of the LPSs from *Y. pseudotuberculosis* WE 23/90 and *Y. enterocolitica* were closer when the cells were grown at 26 °C than when grown at 37 °C. Obviously, these three sets of data match the NPN net uptake, DOC sensitivity, inhibition on selective media and effect of the growth temperature observed for live *Yersinia* spp. cells. To the best of our knowledge, *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis* are the first bacteria in which a positive correlation between LPS acyl-chain fluidity and OM permeability is established and, although experimental conditions or interaction with proteins can alter the fluidity in the intact OM, it seems rather unlikely that such values would not remain comparatively meaningful.

The variations in LPS acyl-chain fluidity (and the concomitant variations in LPS–LPS interactions) must have a structural explanation. Since electrostatic and/or hydrophilic interactions between polysaccharide moieties of LPSs can decrease the overall LPS fluidity (Brandenburg & Seydel, 1984, 1990) and the LPS polysaccharide length decreased in the order *Y. enterocolitica*–*Y. pseudotuberculosis*–*Y. pestis*, this sort of effect could account in part for the increase in acyl-chain fluidity. Moreover, the same effects could explain the increase in acyl-chain fluidity observed for the LPS of *Y. pseudotuberculosis* grown at 37 °C. However, as phase transitions have been observed for R LPSs of enterobacteria under conditions similar to those used in this study (Brandenburg & Seydel, 1984, 1990), their absence in the LPSs of *Y. pseudotuberculosis* and *Y. pestis* is intriguing and cannot be explained on the sole basis of a reduced interaction between poly or oligo-

saccharide LPS moieties. The fluidity of the *Y. pseudotuberculosis*, *Y. pestis* and *Y. enterocolitica* LPS acyl chains were very close at temperatures above the T_c of the latter LPS. This shows that the absence of transitions in the LPSs of *Y. pseudotuberculosis* and *Y. pestis* reflects a constant liquid-crystalline state and an explanation for this is a lower intensity of the hydrophobic effect at the lipid A level. Thus, a lipid A structure different from that of better characterized enterobacteria can be predicted for *Y. pseudotuberculosis* and *Y. pestis*. In fact, Hartley *et al.* (1974) have reported an increased proportion of hydroxylated fatty acids with respect to other fatty acids in the LPS of *Y. pestis* and suggested that this should correspond to a lipid A with a structure more simple than that of other enteric bacteria.

In a previous study we have shown that the OMs of *Y. enterocolitica* pathogenic biogroups, but not those of non-pathogenic biogroups, become permeable to hydrophobic agents when grown with Mg^{2+} but no Ca^{2+} available (Bengoechea *et al.*, 1996). As invasiveness is nil in non-pathogenic yersiniae, reduced in pathogenic *Y. enterocolitica*, significant in *Y. pseudotuberculosis* and high in *Y. pestis* (Brubaker, 1991; Mäki *et al.*, 1983), OM permeability and efflux systems within the genus seem to correlate to habitat. Because of the presence of bile surfactants and enzymes, an OM impermeable to such agents is useful for bacteria colonizing the intestine or entering into the host tissues by this route and this correlates the OM impermeability of pathogenic *Y. enterocolitica* grown under standard conditions. In keeping with this and with our observations with *Y. pseudotuberculosis*, the lethal dose of *Y. enterocolitica* is over a thousand-fold lower than that of *Y. pseudotuberculosis* when they are administered orally (Pepe & Miller, 1993; Rosqvist *et al.*, 1988). However, as illustrated by the results obtained with both the environmental *Yersinia* spp. and the non-pathogenic biogroups of *Y. enterocolitica*, OMs are also impermeable in bacteria from non-enteric habitats in which surfactants or enzymes are not significant. Thus, the gradual increase in OM permeability of the pathogenic *Y. enterocolitica*–*Y. pseudotuberculosis*–*Y. pestis* series cannot be unequivocally explained as the loss of a property which becomes useless in a non-enteric environment. Although alternative explanations remain speculative, a permeable OM could be advantageous for bacteria living within host tissues and cells since exchange of hydrophobic nutrients and metabolites could be facilitated. Research with mutants showing altered OM permeability is in progress to test some of the above hypotheses.

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