Yersinia pseudotuberculosis* and *Yersinia pestis* are more resistant to bactericidal cationic peptides than *Yersinia enterocolitica

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The action of bactericidal polycationic peptides was compared in *Yersinia* spp. by testing peptide binding to live cells and changes in outer membrane (OM) morphology and permeability. Moreover, polycation interaction with LPS was studied by measuring the dependence of dansylcadaverine displacement and zeta potential on polycation concentration. When grown at 37 °C, *Yersinia pestis* and *Yersinia pseudotuberculosis* bound less polymyxin B (PMB) than pathogenic or non-pathogenic *Yersinia enterocolitica*, regardless of virulence plasmid expression. *Y. pseudotuberculosis* OM's were unharmed by PMB concentrations causing extensive OM blebbing in *Y. enterocolitica*. The permeability to lysozyme caused by PMB was greater in *Y. enterocolitica* than in *Y. pseudotuberculosis* or *Y. pestis* and differences increased at 37 °C. Similar observations were made with other polycations using a polymyxin/novobiocin permeability assay. With LPS of cells grown at 26 °C, polycation binding was highest for *Y. pseudotuberculosis* and lowest for *Y. pestis*, with *Y. enterocolitica* yielding intermediate results which were lower for pathogenic than for non-pathogenic strains. With LPS of cells grown at 37 °C, polycation binding remained unchanged for *Y. pestis* and pathogenic *Y. enterocolitica*, increased for non-pathogenic *Y. enterocolitica* and decreased for *Y. pseudotuberculosis* to *Y. pestis* levels. Polycation binding related in part to differences in charge density (zeta potential) of LPS aggregates, suggesting similar effects at bacterial surfaces. It is suggested that species and temperature differences in polycation resistance relate to infection route, invasiveness and intracellular multiplication of *Yersinia* spp.

Keywords: *Yersinia*, polycations, lipopolysaccharide

INTRODUCTION

The yersiniae are Gram-negative bacteria distributed into several species which show wide differences in pathogenicity and invasiveness. *Yersinia enterocolitica* includes both non-pathogenic and pathogenic biogroups, and *Yersinia pseudotuberculosis* and *Yersinia pestis* are characteristically pathogenic (Brubaker, 1991; Straley & Perry, 1995). In humans, pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* cause intestinal infections but the latter is more invasive (Brubaker,

1991). In contrast, *Y. pestis* enters the body by non-enteric routes and it is characteristically able to multiply intracellularly (Brubaker, 1991; Straley & Harmon, 1984a, b). Pathogenic yersiniae carry a virulence plasmid (pYV) and many virulence-related properties regulated at plasmid and chromosome levels are manifested only under certain conditions such as growth at 37 °C and divalent cation restriction (Brubaker, 1991; Cornelis *et al.*, 1995; Straley & Perry, 1995).

It has been shown that, independently from pYV, pathogenic *Y. enterocolitica* is more resistant to polycations than non-pathogenic *Y. enterocolitica* or enteropathogenic *Escherichia coli* (Bengoechea *et al.*, 1996). This could reflect an adaptation to environments where bactericidal peptides act (Eisenhauer *et al.*, 1992;

Abbreviations: DC, dansylcadaverine; Kdo, 3-deoxy-D-manno-2-octulosonic acid; OM, outer membrane; PMB, polymyxin B; PMBN, deacylated nonapeptide derivative of polymyxin B.

Groissman, 1994; Selsted *et al.*, 1992) and, if so, the hypothesis can be formulated that the more invasive yersiniae should have an increased resistance. We report here that live cells of *Y. pseudotuberculosis* and *Y. pestis* are more resistant to polycations than those of pathogenic *Y. enterocolitica*. Moreover, since LPS is a main polycation target (Vaara, 1992) and an assessment of its role necessarily precedes and could make chemical characterization relevant (Helander *et al.*, 1994, 1996; Nummila *et al.*, 1995; Sidorchuk *et al.*, 1983), we have analysed LPS-polycation affinity and the effects at supramolecular (i.e. aggregate) level.

METHODS

Bacterial strains and growth conditions. The strains used were *Y. enterocolitica* PR serotype O:1,6 (non-pathogenic) and WE 245/92 serotype O:3 (pathogenic), *Y. enterocolitica* WA 289 serotype O:8 (pathogenic), *Y. pseudotuberculosis* WE 23/90 serogroup I, WS 41/91 serogroup I, WS 45/91 serogroup II and WS 66/89 serogroup III, and *Y. pestis* KIM. All pathogenic strains and species, except *Y. pestis* KIM, carried pYV, as shown by detection of the YadA protein by autoagglutination (Skurnik & Toivanen, 1992). Selection of pYV⁺ and plasmid-cured pYV⁻ isogenic pairs was performed on Congo Red/magnesium/oxalate medium (Riley & Toma, 1989). These pairs were stored in skimmed milk at -80 °C and, where appropriate, inocula were taken directly from these frozen seeds to minimize pYV⁺ dissociation. For polycation binding and sensitivity studies, bacteria were grown in sidearm flasks containing tryptic soy broth or, for pYV expression, magnesium/oxalate broth (Riley & Toma, 1989) on an orbital shaker at 26 or 37 °C. Growth was monitored by measuring OD₅₄₀ and exponentially growing cells were harvested (5000 g, 20 min, 5 °C), resuspended in the appropriate buffer (see below) and tested immediately. For LPS extraction, *Y. enterocolitica* and *Y. pseudotuberculosis* were grown in tryptic soy broth and *Y. pestis* in brain-heart infusion at 26 and 37 °C.

Polymyxin B (PMB) binding by viable cells. Bacteria (1.25×10^{10} c.f.u. ml⁻¹) were incubated with PMB (12.5 µg ml⁻¹) in 2 mM HEPES (pH 7.5) for 5 min at the growth temperature and sedimented (12000 g, 10 min). Unbound PMB was measured in the supernatants. For this, Petri dishes (10 cm diam.) were layered with 12 ml of a solution containing

0.1% glucose, 0.1% peptone, 0.05% yeast extract, 1% agarose [type II-A (medium electroendosmosis); Sigma] previously inoculated with 6.1×10^5 c.f.u. *E. coli* K-12 ml⁻¹, and wells (5 mm diam.) were punched and filled with 30 µl of the supernatants. After overnight incubation in a wet chamber at 37 °C, inhibition haloes were measured and the amount of antibiotic calculated using PMB dilutions tested on the same plate. Direct binding was assessed by taking advantage of the fact that the fluorescent group of dansylated PMB increases its quantum yield when the peptide moiety becomes attached to a target. Fresh cells at an OD₆₀₀ of 0.5 in 1 mM KCN/2 mM HEPES (pH 7.2) were supplemented with dansyl-PMB (3.8 µM final concentration) (Schindler & Tauber, 1975). The fluorescence was measured at the growth temperature in 1 cm diameter cuvettes with a LS-50 fluorimeter (Perkin-Elmer; excitation 340 nm, emission 485 nm, slit width 4.0 nm) and results were expressed in relative fluorescence units (RFU). All the above measurements were performed twice with each of two independently grown batches of cells.

Assessment of OM damage. Cells (10^{10} c.f.u. ml⁻¹) were incubated with PMB (10 µg ml⁻¹) in 2 mM HEPES (pH 7.1) for 20 min at the growth temperature, sedimented (12000 g, 5 min), fixed with glutaraldehyde/cacodylate and OsO₄/cacodylate and embedded in agarose. Thin sections were examined by electron microscopy (Martínez de Tejada *et al.*, 1995).

Changes in OM permeability were tested using lysozyme and novobiocin as hydrophilic and hydrophobic probes, respectively. Cell suspensions (OD₅₀₀ of 0.8) were incubated with lysozyme (50 µg ml⁻¹) and PMB (12.5 µg ml⁻¹) in 2 mM HEPES (pH 7.1) and lysis was assessed as the decrease in OD₅₀₀ after 1 h (time at which lysis was maximal for sensitive strains) at the growth temperature. Lysozyme or PMB alone did not produce any decrease in OD under these conditions. For novobiocin, antibiotic dilutions were made in Mueller-Hinton broth in 96-well polystyrene plates which were then inoculated with 1.25×10^6 exponentially growing c.f.u. (37 °C) in 100 µl broth containing appropriate amounts of polycations (Table 1) (Vaara & Vaara, 1983). After incubation at 37 °C for 18 h, the lowest novobiocin concentration that inhibited growth was taken as the MIC. Polycations by themselves had no inhibitory effect under those conditions. All measurements were performed two times with each of two independently grown batches of cells.

LPS preparations. The LPSs of *Y. enterocolitica* PR O:16 (non-pathogenic), WA 289 O:8 (pathogenic) and WE 245/92

Table 1. Effect of subinhibitory concentrations of polycations on the permeability of *Yersinia* spp. to novobiocin

Strain	Novobiocin MIC (µg ml ⁻¹)				
	Polycation (µg ml ⁻¹)				
	No polycation	PMB (0.01)	Poly-L-lysine (25)	Poly-L-ornithine (25)	Melittin (2)
<i>Y. enterocolitica</i> WA 289 O:8	256	32	16	4	32
<i>Y. pestis</i> KIM	32	32	32	32	32
<i>Y. pseudotuberculosis</i> WE 23/90	16	8	16	16	16
<i>Y. pseudotuberculosis</i> WS 41/91	32	16	32	32	32
<i>Y. pseudotuberculosis</i> WS 45/91	32	32	32	32	16
<i>Y. pseudotuberculosis</i> WS 66/89	64	16	64	64	64

O:3 (pathogenic), *Y. pseudotuberculosis* WE 23/90 and *Y. pestis* KIM were extracted, purified, characterized as described in the accompanying article [Bengoechea *et al.*, 1998; protein, phospholipid and 3-deoxy-D-manno-2-octulosonic acid (Kdo) contents, and SDS-PAGE] and used as the natural salt forms.

Affinity of polycations for LPS molecules. LPS–polycation affinity was assessed using dansylcadaverine (DC) as probe and estimated as the amount of PMB (in nM) displacing 50% of the DC bound by a given LPS (David *et al.*, 1992). To obtain DC–LPS complexes, LPS stocks (3 mg ml⁻¹) were diluted in 1.5 ml 2 mM HEPES (pH 7.5) in fluorimetric cuvettes at a concentration equivalent to 4 nmol Kdo ml⁻¹ and 12 nmol DC was added. This DC concentration yielded 90% of the maximal fluorescence possible and did not produce detectable inner-filter effects. Fluorescence was recorded with a SPEX F1T11 apparatus (excitation 340 nm, emission scan from 400 to 600 nm, slit width for both windows 1.5 nm) and the DC occupancies were calculated as $(F-F_0)/(F_{\max}-F_0)$ where fluorescence values are that of DC alone (F_0), DC–LPS ($F_{\max}$) and DC–LPS/PMB mixtures (F). The 50% occupancy was calculated from occupancy versus PMB concentration plots. For each LPS, all experiments were performed twice with two different LPS suspensions. PMB did not produce any decrease in the fluorescence intensity of free DC.

Effect of polycations on zeta potentials of LPS aggregates.

The surface charge density of LPS aggregates and the effect of polycations was measured as the electrophoretically effective potential (zeta potential or ζ_{sm}) of the aggregates (Cafiso *et al.*, 1983) with varying concentrations of PMB or its deacylated nonapeptide (PMBN). Particle size and ζ_{sm} were measured in a ZetaSizer 4 apparatus (Malvern Instruments) using a 5 mW He/Ne laser and a temperature-controlled electrophoresis cell to measure the electrophoretic mobility by laser Doppler anemometry and the size distribution by photon correlation spectroscopy at a scatter angle of 90°. The cell was frequently checked for instrumental drifts and, if necessary, cleaned and recalibrated. The electrokinetic mobility (μ_{cl}) of the aggregates was measured at 22 °C in a driving electric field of 19.2 V cm⁻¹ from which, independent of the particle size, ζ_{sm} can be calculated according to the Helmholtz–Smoluchowsky equation, $\zeta_{sm} = (\mu_{cl} \times \eta) / (\epsilon_r \times \epsilon_0)$, where μ_{cl} is the electrokinetic mobility, η is the viscosity (0.97 mPa), ϵ_r is the dielectric constant of the buffer (79) and ϵ_0 is the permittivity of free space (Hunter, 1981). LPS aggregates were prepared in 1 mM CsCl/2.5 mM Tris/HCl (pH 7.0) by sonication for 20 min at 40 °C in an ultrasonic bath (Sonorex RK100, Bandelin Electronics) and allowed to equilibrate at 4 °C overnight before measurements. The aggregates thus formed had similar broad size distributions with mean diameters between 65 and 118 nm. The suspensions were adjusted to the same Kdo concentration, approximately 40.5 µM, and for each LPS two different suspensions were examined, with six independent measurements for each PMB or PMBN concentration.

Chemicals. PMB (8000 units mg⁻¹) and PMBN, poly-L-lysine (molecular mass 7000–10000 Da), poly-L-ornithine (12000–22000), melittin, lysozyme, novobiocin, dansylcadaverine and dansyl chloride were all purchased from Sigma.

RESULTS

Action of polycations on *Yersinia* spp.

As shown in Fig. 1(a), the amount of PMB absorbed by bacteria grown at 37 °C progressively decreased in the order non-pathogenic *Y. enterocolitica* PR O:1,6–pathogenic *Y. enterocolitica* WA 289 O:8–*Y. pseudo-*

Fig. 1. PMB binding to *Yersinia* spp. live cells and influence of pYV. (a) Amounts of PMB absorbed by *Y. enterocolitica* (Y.e.), *Y. pseudotuberculosis* (Y.pstb.) and *Y. pestis* (Y.p.) grown at 26 °C (□) or 37 °C (■). (b) Dansyl-PMB binding to *Y. enterocolitica* WA 289 O:8 (Δ, ▲) and *Y. pseudotuberculosis* WE 23/90 (□, ■) live cells lacking (▲, ■) or carrying (Δ, □) pYV, grown at 37 °C in magnesium/oxalate medium. In both sets of experiments, each value is the mean of two independent measurements performed with two independently grown batches of cells (coefficient of variation was less than 5% for all groups of data).

tuberculosis WS 66/89–WS 45/91–WE 23/90–WS 41/91, with *Y. pestis* KIM giving results similar to those of *Y. pseudotuberculosis* WS 45/91. The differences were not so conspicuous with cells grown at 26 °C and, whereas *Y. pestis* and all *Y. pseudotuberculosis* strains except WS 66/89 absorbed more polycation than when grown at 37 °C, *Y. enterocolitica* absorbed less at 26 °C than at 37 °C. These results were confirmed by fluorimetry with dansyl-PMB (not shown). After exposure to PMB, electron microscopy of *Y. enterocolitica* WA 289 O:8 and *Y. pseudotuberculosis* WE 23/90 grown at 26 or 37 °C showed OM blebbing only in the former (not shown).

The role of pYV-encoded factors on polycation binding was examined in all pathogenic *Y. enterocolitica* and *Y.*

Table 2. LPS–polycation affinities determined as the concentration of PMB and PMBN causing a 50% displacement of bound DCValues are means $\pm$ SD, $n = 4$.

Source of LPS	PMB (nM)		PMBN (nM)	
	26 °C	37 °C	26 °C	37 °C
<i>Y. enterocolitica</i> PR O:1,6	940 $\pm$ 20	1300 $\pm$ 80	900 $\pm$ 30	1560 $\pm$ 60
<i>Y. enterocolitica</i> WE 245/92 O:3	640 $\pm$ 10	690 $\pm$ 20	630 $\pm$ 30	630 $\pm$ 40
<i>Y. enterocolitica</i> WA 289 O:8	800 $\pm$ 10	750 $\pm$ 25	740 $\pm$ 30	710 $\pm$ 35
<i>Y. pseudotuberculosis</i> WE 23/90	1220 $\pm$ 45	440 $\pm$ 20	1190 $\pm$ 35	440 $\pm$ 25
<i>Y. pestis</i> KIM	430 $\pm$ 35	400 $\pm$ 20	400 $\pm$ 30	420 $\pm$ 30

pseudotuberculosis strains using dansyl-PMB. For all concentrations tested, the levels of fluorescence (proportional to the bound probe) were similar in pYV⁺ and pYV⁻ isogenic pairs grown in magnesium/oxalate medium at 37 °C (Fig. 1b shows representative results), thus ruling out a role of pYV in polycation resistance.

Functional OM damage was assessed by testing the effect of subinhibitory concentrations of polycations on OM permeability to lysozyme or novobiocin. With cells grown at 37 °C, there was an 80% decrease in turbidity (cell lysis) for pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* WS 66/89 but no lysis was observed for the other *Y. pseudotuberculosis* strains or for *Y. pestis* KIM. With cells grown at 26 °C, incubation with PMB–lysozyme caused decreases in turbidity of 70% for pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* WS 66/89, 55% for the remaining *Y. pseudotuberculosis* strains and 30% for *Y. pestis* KIM. The experiments were extended to other polycations using novobiocin as probe. With no polycations, *Y. pseudotuberculosis* and *Y. pestis* MICs were lower than those of *Y. enterocolitica* (Table 1), a result in keeping with their different OM permeability to hydrophobic drugs (Bengoechea *et al.*, 1998). Polycations increased the *Y. enterocolitica* novobiocin MIC 8 to 64-fold, produced no effect on the *Y. pestis* MIC, and, depending on the polycation and strain, only increased the MIC of *Y. pseudotuberculosis* 2 to 4-fold (Table 1).

LPS–polycation affinities

To test whether the observations made with live bacteria correlated with LPS affinities, LPSs were incubated with DC and the DC 50% occupancy (affinity) was assessed by displacement with PMB or PMBN (Table 2). With LPSs from bacteria grown at 37 °C, the affinity was highest for non-pathogenic *Y. enterocolitica* PR O:1,6, intermediate for pathogenic *Y. enterocolitica* WA 289 O:8 (greater than WE 245/92 O:3) and lowest for *Y. pseudotuberculosis* WE 23/90 and *Y. pestis* KIM ($P < 0.01$; unpaired *t*-test), thus reproducing the results obtained with live cells. This parallelism was not so clear with LPSs from bacteria grown at 26 °C because

Fig. 2. Effect of PMB on the ζ_{sm} of LPS aggregates of *Y. pestis* KIM (○), *Y. pseudotuberculosis* WE 23/90 (■), *Y. enterocolitica* WA 289 O:8 (pathogenic) (△) and *Y. enterocolitica* PR O:1,6 (non-pathogenic) (●) grown at 26 (a) or 37 °C (b). Each point corresponds to six measurements; the SD was so small as to be hidden by the symbols.

DC affinity decreased in the order *Y. pseudotuberculosis* WE 23/90–non-pathogenic *Y. enterocolitica* PR O:1,6–pathogenic *Y. enterocolitica* WA 289 O:8 (greater than WE 245/92 O:3)–*Y. pestis* KIM. Likewise, the shifts in polycation binding by live cells grown at 26 or 37 °C were not paralleled by clear LPS affinity shifts in pathogenic *Y. enterocolitica* and *Y. pestis* (compare Fig. 1 and Table 2).

ζ_{sm} of LPS aggregates and effect of polycations

The ζ_{sm} values with respect to PMB concentration are presented in Fig. 2. For the native LPS aggregates (zero PMB) and independent of growth temperature, ζ_{sm} was

always negative with no marked differences or clear correlation with the amounts of polycation bound by live cells (Fig. 1) or LPSs (Table 2). Despite this, very different amounts of PMB were necessary to saturate ($\zeta_{sm} = 0$ mV) these LPS aggregates. With LPSs from cells grown at 26 °C (Fig. 2a), saturation was achieved in the order *Y. pestis* KIM–*Y. enterocolitica* WA 289 O:8 (and WE 245/92 O:3; not shown)–*Y. pseudotuberculosis* WE 23/90, with no saturation for the LPS of non-pathogenic *Y. enterocolitica* PR O:1,6 at the highest PMB concentration tested. With LPSs from cells grown at 37 °C, the ζ_{sm} vs. PMB plot of *Y. pestis* LPS was similar to that observed for LPSs from cells grown at 26 °C, that of *Y. pseudotuberculosis* LPS became close to that of *Y. pestis* LPS and a less marked shift was observed for the LPS of *Y. enterocolitica* WA 289 O:8 (and WE 245/92 O:3; not shown). In contrast, the LPSs of non-pathogenic *Y. enterocolitica* PR O:1,6 grown at 37 °C needed larger amounts of PMB than its 26 °C-grown counterpart to reach similar ζ_{sm} values. Finally, the PMB results were confirmed with PMBN, although it had a measurable reduced effect on the LPSs of pathogenic strains and species (not shown). PMBN and PMB had similar measurable effects on non-pathogenic *Y. enterocolitica* LPS.

DISCUSSION

Despite their variety of structures and origin (Nicolas & Mor, 1995; Vaara, 1992), bactericidal cationic peptides start their action by electrostatic interaction with negatively charged groups of the surface of target cells (Nicolas & Mor, 1995; Stivanage *et al.*, 1989; Vaara, 1992). Thus, melittin, PMB, poly-L-lysine and poly-L-ornithine mimic in part the bactericidal peptides of vertebrates and, therefore, the progressive increase in polycation resistance observed from the less invasive (*Y. enterocolitica*) to the more intracellular (*Y. pestis*) yersiniae strongly suggests that this property helps to overcome the increasing host defences from mucosal to phagocytic levels. This interpretation is consistent with the previous observation that whereas polycation resistance is lost at 37 °C in non-pathogenic *Y. enterocolitica*, it is only reduced in pathogenic *Y. enterocolitica* (Bengoechea *et al.*, 1996). Moreover, *Y. pseudotuberculosis* WE 23/90, WS 41/91 and WS 45/91, and *Y. pestis* KIM polycation resistance was more marked than that of pathogenic *Y. enterocolitica* and increased at 37 °C. Thus, the relevance of polycation resistance in the biology of yersiniae is also suggested by the fact that it is lost, kept or enhanced at the host growth temperature in accordance with the transition from non-pathogenic biogroups to the most invasive spp. It has been shown that introduction of pYV in non-pathogenic *Y. enterocolitica* does not result in virulence, despite expression of pYV-encoded factors (Heesemann *et al.*, 1984). Since non-pathogenic *Y. enterocolitica* differs clearly from pathogenic yersiniae in polycation resistance (Bengoechea *et al.*, 1996; this work), it can be

speculated that this trait provides a background on which pYV acquisition leads to full virulence.

Although *Y. pseudotuberculosis* WS 66/89 was more resistant than pathogenic *Y. enterocolitica*, both exhibited diminished resistance at 37 °C. Compared to other *Y. pseudotuberculosis* strains, WS 66/89 had a higher novobiocin MIC, showing that it is closer to pathogenic *Y. enterocolitica* in terms of OM permeability. Thus, consistent with the transition in OM properties shown for the genus (Bengoechea *et al.*, 1998), WS 66/89 OMs display intermediate properties. Since WS 66/89 was the only serotype III strain tested, further studies are necessary to find out whether enhanced resistance at 37 °C is a specific property of *Y. pseudotuberculosis* or whether there is serogroup or strain variability.

In a previous study (Bengoechea *et al.*, 1996), no differences were observed when LPS aggregates of pathogenic and non-pathogenic *Y. enterocolitica* were tested for polycation-induced permeability changes to hydrophobic probes. The more accurate DC measurements, however, showed affinity differences between the probe and the LPSs of pathogenic and non-pathogenic *Y. enterocolitica*, thereby explaining the observations made with viable cells of this species. Negatively charged LPS sites are located at the core and lipid A and, so far, polycation resistance in enteric bacteria has been related to O-chain steric hindrance (Peterson *et al.*, 1986) and to a reduction of charge due to substitution of phosphates by arabinosamine and/or ethanolamine in lipid A (Guo *et al.*, 1997; Helander *et al.*, 1994, 1996; Nummala *et al.*, 1995; Sidorchuk *et al.*, 1983; Stivanage *et al.*, 1989). Thus, it could be that there are variations in lipid A substitutions of *Y. enterocolitica* which explain the effects on live cells. However, the DC affinity measurements were not always in full agreement with the resistance of live cells for the LPSs of *Y. pseudotuberculosis* and *Y. pestis*. Although at 37 and 26 °C, *Y. pestis* cells bound more PMB than cells of *Y. pseudotuberculosis* WE 23/90, the DC affinity for the 26 °C *Y. pestis* LPS was much less than for the 26 °C WE 23/90 LPS and similar DC affinities were observed for both 37 °C LPSs. Also, the DC affinities were higher for the 26 °C *Y. pseudotuberculosis* WE 23/90 LPS than for the 26 °C *Y. enterocolitica* LPS and this also did not correlate with the lower binding shown by WE 23/90 cells. This suggests that although LPS anionic binding sites should play an important role, quantitative differences in such sites cannot account by themselves for the effects on live cells and this is consistent with the ζ_{sm} measurements. Despite the fact that neutralization of the ζ_{sm} (0 mV) of the 26 °C LPS aggregates required amounts of PMB and PMBN roughly correlating with the DC affinities, all LPSs had a similar ζ_{sm} (–26 to –31 mV) in the absence of polycations. This shows that, in addition to charge, accessibility or other properties relating to the supramolecular structure of the aggregates are important in LPS–polycation interactions. It is noteworthy that reduced accessibility to internal targets caused by the O-chain (Peterson *et al.*,

1986) cannot account for the increased resistance of *Y. pseudotuberculosis* and *Y. pestis* because these bacteria have LPSs lacking long-chain polysaccharides (Bengoechea *et al.*, 1998). Thus, peculiar core structures and interactions between LPS molecules or with other OM elements should also play a role in the resistance of live cells.

When pathogenic *Y. enterocolitica* is grown in the absence of Ca^{2+} , the cells become permeable to hydrophobic agents (Bengoechea *et al.*, 1996) and in the accompanying article (Bengoechea *et al.*, 1998) we described that, in normal medium, *Y. pseudotuberculosis* and *Y. pestis* are readily permeable to such agents. In this study, *Y. pseudotuberculosis* and *Y. pestis* were found to be more resistant to polycations than *Y. enterocolitica*. Whether there is a structural connection in the OM between those two sets of properties is not known. The yersiniae offer the possibility of carrying out chemical and genetic studies with closely related bacteria which could help to understand the bases of those phenomena and their role in the variable environments in which they are manifested.

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REFERENCES

- Bengoechea, J. A., Díaz, R. & Moriyón, I. (1996). Outer membrane differences between pathogenic and environmental *Yersinia enterocolitica* biogroups probed with hydrophobic permeants and polycationic peptides. *Infect Immun* **64**, 4891–4899.
- Bengoechea, J.-A., Brandenburg, K., Seydel, U., Díaz, R. & Moriyón, I. (1998). *Yersinia pseudotuberculosis* and *Yersinia pestis* show increased outer membrane permeability to hydrophobic agents which correlates with lipopolysaccharide acyl-chain fluidity. *Microbiology* **144**, 1517–1526.
- Brubaker, R. R. (1991). Factors promoting acute and chronic diseases caused by yersiniae. *Clin Microbiol Rev* **4**, 309–324.
- Cafiso, D., McLaughlin, A., McLaughlin, S. & Wimshi, A. (1983). Measuring electrostatic potentials adjacent to membranes. *Methods Enzymol* **171**, 342–364.
- Cornelis, G., Iriarte, M. & Sory, M.-P. (1995). Environmental control of virulence functions and signal transduction in *Yersinia enterocolitica*. In *Signal Transduction and Bacterial Virulence*, pp. 95–110. Edited by R. Rappouli, V. Scarlato & B. Aricò. Austin, TX: R. G. Landes.
- David, S. A., Balasubramanian, K. A., Mathan, V. I. & Balaran, P. (1992). Analysis of the binding of polymyxin B to endotoxic lipid A and core glycolipid using a fluorescent displacement probe. *Biochim Biophys Acta* **1165**, 147–152.
- Eisenhauer, P. B., Harwig, S. S. S. L. & Lehrer, R. I. (1992). Cryptdins: antimicrobial defensins of the murine small intestine. *Infect Immun* **60**, 3556–3565.
- Groisman, E. A. (1994). How bacteria resist killing by host-defence peptides. *Trends Microbiol* **2**, 444–449.
- Guo, L., Lim, K. B., Gunn, J. S., Bainbridge, B., Darveau, R. P., Hackett, M. & Miller, S. I. (1997). Regulation of lipid A modifications by *Salmonella typhimurium* virulence genes *phoP-phoQ*. *Science* **276**, 250–253.
- Heesemann, J., Algermissen, B. & Laufs, R. (1984). Genetically manipulated virulence of *Yersinia enterocolitica*. *Infect Immun* **46**, 105–110.
- Helander, I. M., Kilepeläinen, I. & Vaara, M. (1994). Increased substitution of phosphate groups in lipopolysaccharides and lipid A of the polymyxin-resistant *pmrA* mutants of *Salmonella typhimurium*: a ^{31}P -NMR study. *Mol Microbiol* **11**, 481–487.
- Helander, I. M., Kato, Y., Kilepeläinen, Y., Kostianen, R., Lindner, B., Nummila, K., Sugiyama, T. & Yokochi, Y. (1996). Characterization of lipopolysaccharides of polymyxin-resistant and polymyxin-sensitive *Klebsiella pneumoniae* O3. *Eur J Biochem* **237**, 272–278.
- Hunter, R. J. (1981). *Zeta Potential in Colloid Science*. London: Academic Press.
- Martínez de Tejada, G., Pizarro-Cerdá, J., Moreno, E. & Moriyón, I. (1995). The outer membranes of *Brucella* spp. are resistant to bactericidal cationic peptides. *Infect Immun* **63**, 3054–3061.
- Nicolas, P. & Mor, A. (1995). Peptides as weapons against microorganisms in the chemical defence system of vertebrates. *Annu Rev Microbiol* **49**, 277–304.
- Nummila, K., Kilepeläinen, I., Zähringer, U., Vaara, M. & Helander, I. M. (1995). Lipopolysaccharides of polymyxin B-resistant mutants of *Escherichia coli* are extensively substituted by 2-aminoethyl pyrophosphate and contain aminoarabinose in lipid A. *Mol Microbiol* **16**, 271–278.
- Peterson, A. A., Haug, A. & McGroarty, E. J. (1986). Physical properties of short- and long-O-antigen containing fractions of lipopolysaccharide from *Escherichia coli* O111:B4. *J Bacteriol* **165**, 116–122.
- Riley, G. & Toma, S. (1989). Detection of pathogenic *Yersinia enterocolitica* by using Congo red-magnesium oxalate agar medium. *J Clin Microbiol* **27**, 213–214.
- Schindler, P. R. G. & Tauber, M. (1975). Action of polymyxin B on bacterial membranes: morphological changes in the cytoplasm and in the outer membrane of *Salmonella typhimurium* and *Escherichia coli* B. *Antimicrob Agents Chemother* **8**, 94–104.
- Selsted, M. E., Miller, S. I., Henschen, A. H. & Oullette, A. J. (1992). Enteric defensins: antibiotic peptide components of intestinal defence. *J Cell Biol* **118**, 929–936.
- Sidorchuk, Z., Zähringer, U. & Rietschel, E. T. (1983). Chemical structure of the lipid A components of the lipopolysaccharide from a *Proteus mirabilis* Re mutant. *Eur J Biochem* **137**, 15–22.
- Skurnik, M. & Toivanen, P. (1992). LcrF is the temperature regulated activator of the *yadA* gene of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. *J Bacteriol* **174**, 2047–2051.
- Stivanage, P., Martin, L. E. & Spitznagel, J. (1989). O antigen and lipid A phosphoryl groups in resistance of *Salmonella typhimurium* LT-2 to nonoxidative killing in human polymorphonuclear neutrophils. *Infect Immun* **57**, 3894–3900.
- Straley, S. C. & Harmon, P. A. (1984a). Growth in mouse peritoneal

macrophages of *Yersinia pestis* lacking established virulence determinants. *Infect Immun* **45**, 649–654.

Straley, S. C. & Harmon, P. A. (1984b). *Yersinia pestis* grows within phagolysosomes in mouse peritoneal macrophages. *Infect Immun* **45**, 655–659.

Straley, S. C. & Perry, R. (1995). Environmental modulation of gene expression and pathogenesis in *Yersinia*. *Trends Microbiol* **3**, 310–317.

Vaara, M. (1992). Agents that increase the permeability of the outer membrane. *Microbiol Rev* **56**, 395–411.

Vaara, M. & Vaara, T. (1983). Polycations sensitize enteric bacteria to antibiotics. *Antimicrob Agents Chemother* **24**, 107–113.

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