

Permeabilizing action of polyethyleneimine on *Salmonella typhimurium* involves disruption of the outer membrane and interactions with lipopolysaccharide

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Polyethyleneimine (PEI), a polycationic polymer substance used in various bioprocesses as a flocculating agent and to immobilize enzymes, was recently shown to make Gram-negative bacteria permeable to hydrophobic antibiotics and to detergents. Because this suggests impairment of the protective function of the outer membrane (OM), the effect of PEI on the ultrastructure of *Salmonella typhimurium* was investigated. Massive alterations in the OM of PEI-treated and thin-sectioned bacteria were observed by electron microscopy. Vesicular structures were seen on the surface of the OM, but no liberation of the membrane or its fragments was evident. Since a potential mechanism for the action of PEI could be its binding to anionic LPSs on the OM surface, the interaction of PEI with isolated LPSs was assayed *in vitro*. The solubility of smooth-type LPSs of *Salmonella*, regardless of the sugar composition of their O-specific chains, was not affected by PEI, nor was that of Ra-LPS (lacking O-specific chains but having a complete core oligosaccharide). PEI strongly decreased the solubility of rough-type LPSs of the chemotypes Rb2 and Re, whereas it had only a weak effect on the abnormally cationic Rb2-type *pmrA* mutant LPS, suggesting that the negative charge to mass ratio of LPS plays a critical role in the interaction.

Keywords: polyethyleneimine, Gram-negative bacteria, outer membrane, lipopolysaccharide, *Salmonella typhimurium*

INTRODUCTION

It was shown recently that polyethyleneimine (PEI), a non-toxic aliphatic and cationic polymeric substance used in various bioprocesses as a flocculating agent and to immobilize enzymes, possesses strong permeabilizing activity on Gram-negative bacteria (Helander *et al.*, 1997). Bacterial species such as *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhimurium* were effectively sensitized to the detergent SDS and to hydrophobic antibiotics by PEI in p.p.m. concentrations. However, PEI did not exert any direct bactericidal effect on Gram-negative bacteria when applied alone, even in concentrations far above those required for sensitization. These findings strongly suggest that the outer

membrane (OM) is functionally disintegrated by PEI, since the OM normally acts as a permeability barrier towards external hydrophobic solutes and detergents (Vaara, 1992). Permeabilizing agents that lack intrinsic toxicity are useful in sensitizing harmful bacteria towards bactericidal substances in, for example, disinfection or food protection.

Many polycationic substances act as permeabilizers by virtue of their ability to release LPS from the OM, the function of which as a permeability barrier is largely attributed to the LPS layer forming its outer leaflet. Accordingly, the sensitizing action of protamine and certain polylysines to antibiotics involves liberation of considerable proportions of LPS from the OM of *S. typhimurium*; the consequent disruption of the OM has been revealed by electron microscopy (Vaara & Vaara, 1983a, b). Various other polycations, such as polymyxin B nonapeptide (PMBN), permeabilize the OM without

Abbreviations: OM, outer membrane; PEI, polyethyleneimine; PMBN, polymyxin B nonapeptide.

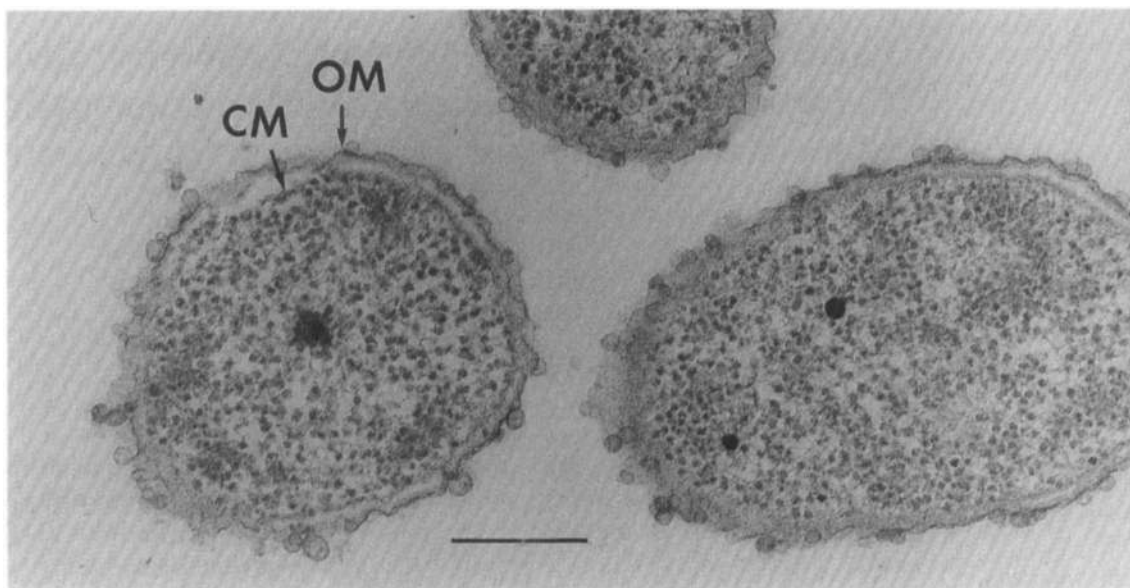


Fig. 1. Cells of *S. typhimurium* ATCC 13311 (smooth-type strain) treated with PEI ($10 \mu\text{g ml}^{-1}$). CM, cytoplasmic membrane. Bar, $0.2 \mu\text{m}$.

significant liberation of LPS (Vaara & Vaara, 1983b). In this respect, PEI and PMBN proved to be similar, as no liberation of LPS was detected in our assays in which PEI acted on *S. typhimurium*; however, the possibility of PEI forming poorly soluble complexes with LPS and hence preventing the detection of released LPS remained open (Helander *et al.*, 1997). In the present study, we have explored the mechanism of action of PEI, looking for direct evidence of its effects on the OM. We have investigated the effect of PEI treatment on the ultrastructure of *S. typhimurium* and assayed the interactions of various purified LPSs of *S. typhimurium* with PEI.

METHODS

Chemicals. PEI (mean molecular mass 50 000 Da) and n-heptadecanoic acid methyl ester were obtained from Sigma. Glutaraldehyde was purchased from Merck.

Bacterial strains, their cultivation and preparation for electron microscopy. *Salmonella typhimurium* strains ATCC 13311 (LPS chemotype S, O:4,5,12; for LPS chemotypes, see Helander *et al.*, 1996) and SH5014 (Stocker *et al.*, 1979; LPS chemotype Rb2) were used in the electron microscopy studies. They were grown in LB broth (10 g Difco tryptone, 5 g Difco yeast extract and 5 g NaCl l^{-1} , pH 7.0) at 37°C into the exponential phase of growth (OD_{630} 0.4; Multiskan MCC/340 spectrophotometer), and the cultures were centrifuged at $3000 g$ (10 min, 21°C). The deposited cells were then suspended in a similar volume of 10 mM Tris/HCl buffer (pH 7.2), centrifuged as above, and again suspended in Tris buffer, to which PEI or EDTA was added to yield concentrations of $10 \mu\text{g ml}^{-1}$ and 1 mM, respectively. After the cell suspensions had been kept for 10 min at 37°C , portions were centrifuged at 11 000 r.p.m. (1 min, 21°C ; Biofuge 13, Heraeus Instruments) and the cell pellets were resuspended in 5 mM HEPES buffer (pH 7.2) containing 2.5% (v/v) glutaraldehyde.

After 1 h at 21°C , the cells were recovered by centrifugation and stored at 4°C in 5 mM HEPES (pH 7.2).

Electron microscopy. After the pre-fixation with glutaraldehyde described above, the samples for thin-sectioning were prepared as described by Lounatmaa (1985). Electron micrographs were taken with a JEM-1200EX transmission electron microscope (JEOL) at 60 kV.

LPSs. Extraction of LPS from the smooth *S. typhimurium* strains SH2183 (Valtonen, 1970) and SH5770 (Valtonen, 1977) by the aqueous 45% phenol method has been described previously (Helander *et al.*, 1992). SH5770 is a recombinant strain carrying O antigen 6,7 instead of the normal O:4,5,12 antigen present in SH2183 (Valtonen, 1977). LPSs from the rough-type strains *his*-515 (LPS chemotype Ra; Nikaido *et al.*, 1967), SH5014 (Rb2), SH7426 (Rb2, *pmrA* mutant elaborating exceptionally cationic LPS; Vaara, 1981; Helander *et al.*, 1994) and SL1102 (Re; Wilkinson *et al.*, 1972) were extracted by the phenol/chloroform/light petroleum (boiling point range $40\text{--}60^\circ\text{C}$) method (Galanos *et al.*, 1969). Each LPS was converted to its triethylammonium salt for maximal water solubility (Galanos & Lüderitz, 1975), and stock solutions (2 mg ml^{-1}) in water were prepared.

LPS solubility assay. Pairs of LPS solutions containing 0.6 mg LPS in 1 ml water were prepared in microfuge vials, and one vial of each pair was supplemented with PEI at $20 \mu\text{g ml}^{-1}$. The other served as a control. After the solutions had been mixed at 21°C , the vials were centrifuged at 11 000 r.p.m. for 4 min at 21°C ; 800 μl supernatant was removed and freeze-dried in 20 ml headspace vials. To the dry samples, 105 μg n-heptadecanoic acid methyl ester was added, and the samples were processed for fatty acid analysis by saponification and methylation as described by Moore *et al.* (1994). The fatty acid methyl esters were analysed by GLC as described previously (Helander *et al.*, 1997). The amounts of LPS-specific fatty acids (dodecanoic acid, tetradecanoic acid, hexadecanoic acid and 3-hydroxytetradecanoic acid for *Salmonella* LPS; see Zähringer *et al.*, 1994) in the supernatants of PEI-containing

and control samples were compared to determine the LPS-precipitating activity of PEI (100% = total precipitation of LPS). Each assay was performed twice. It was shown separately that mere centrifugation of aqueous solutions of LPS deposited less than 10% of the total LPS.

RESULTS

Effect of PEI on bacterial ultrastructure

The effect of PEI treatment on the ultrastructure of *S. typhimurium* strains ATCC 13311 and SH5014 was investigated by thin-section electron microscopy. The general effect of PEI on bacterial ultrastructure is seen in Fig. 1, where the OM of *S. typhimurium* ATCC 13311 is abnormal. Strong undulation of the membrane is evident, and membrane vesicles are prominently present. The cytoplasmic membrane appears to be unaffected, at least at the resolution of thin-section electron microscopy. The effect of PEI on the smooth *S. typhimurium* strain can be seen in more detail in Fig. 2, in which the

OM of untreated cells (Fig. 2a) is rather even with minor undulation. In the cells treated with PEI (Fig. 2b), prominent undulation and folding of the OM are evident. Cells treated with EDTA (Fig. 2c) looked similar to the control cells.

In a rough strain of *S. typhimurium* (SH5014, LPS chemotype Rb2; Fig. 3), the effects of PEI were similar to those observed for the smooth strain, although vesiculation of PEI-treated cells was less prominent. Control cells of the rough (Fig. 3a) and smooth (Fig. 2a) strains were indistinguishable.

Effect of PEI on the solubility of isolated LPS

LPS molecules are normally polyanionic due to phosphate and carboxyl groups in the lipid A and inner core oligosaccharide (Helander *et al.*, 1996). For this reason, they tend to bind cations, among them metals, oligo- and polyamines (Galanos & Lüderitz, 1975), polymyxin

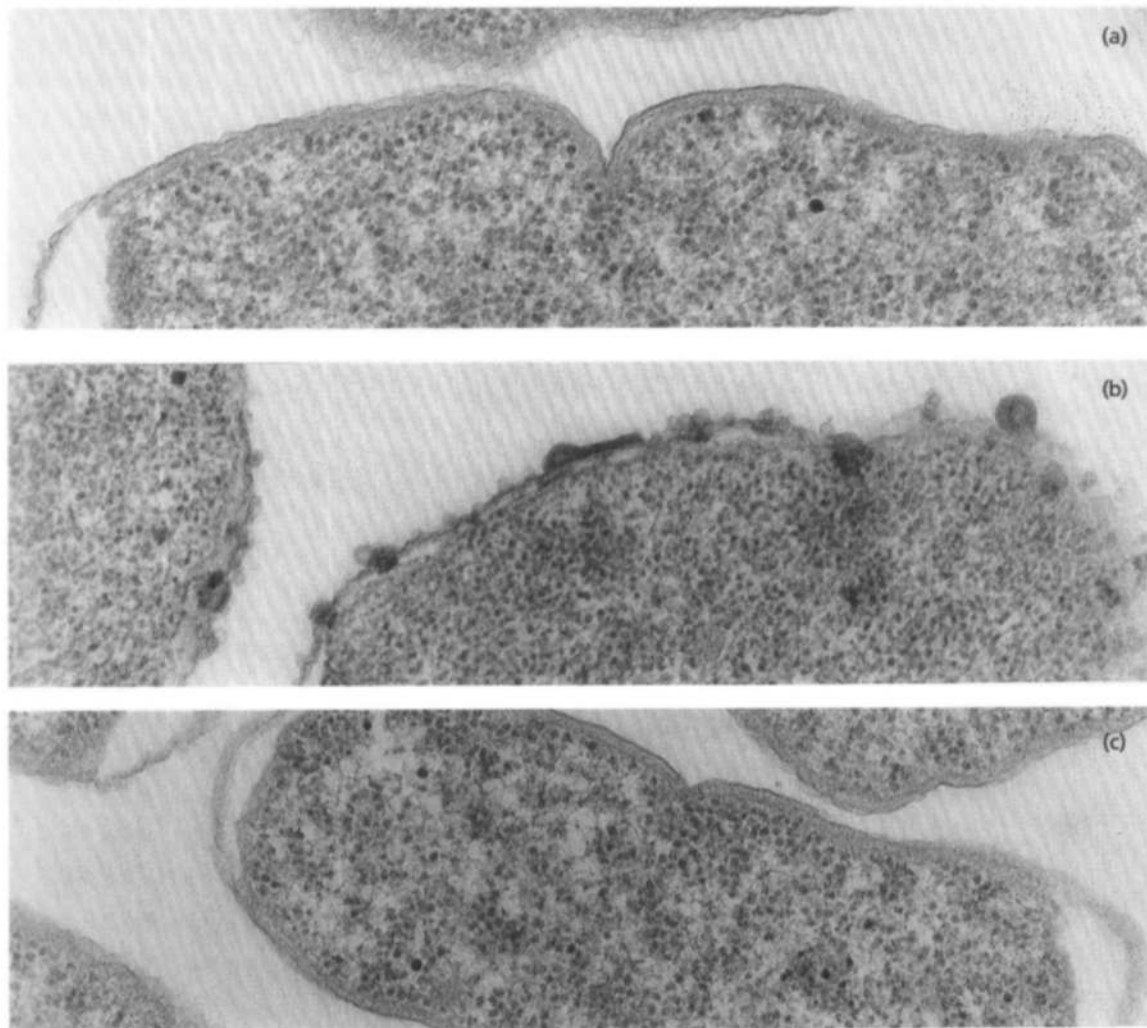


Fig. 2. Cell envelope of (a) untreated, (b) PEI-treated ($10 \mu\text{g ml}^{-1}$) and (c) 1 mM EDTA-treated *S. typhimurium* ATCC 13311. Scale as in Fig. 1.

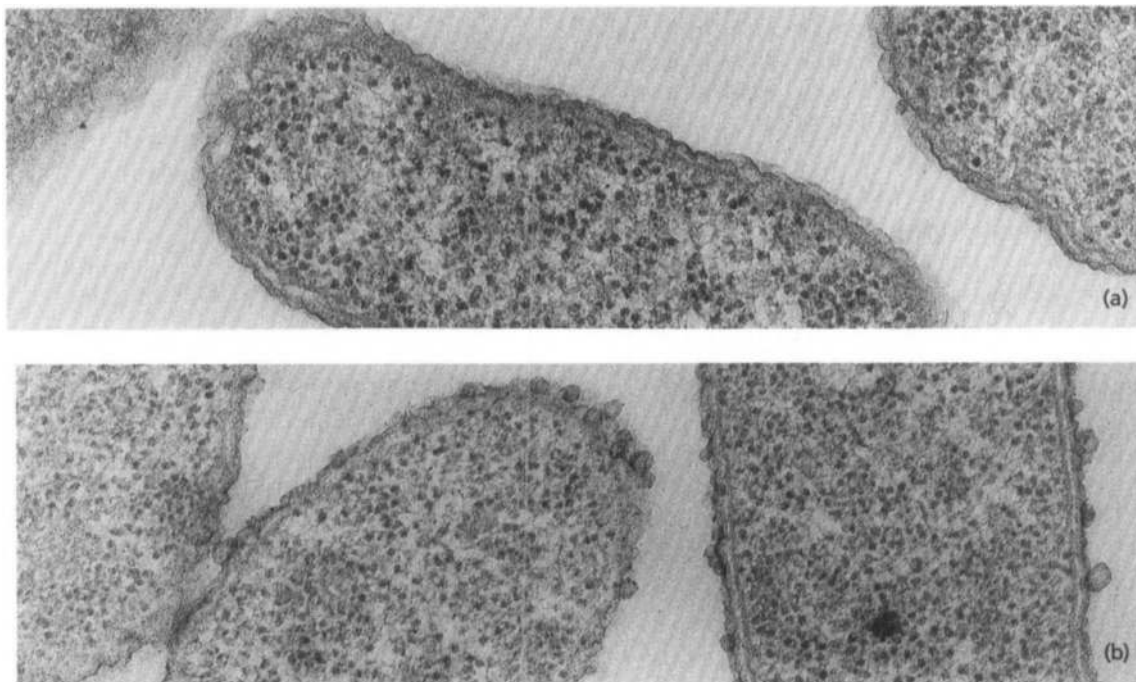


Fig. 3. Cell envelope of (a) untreated and (b) PEI-treated ($10 \mu\text{g ml}^{-1}$) rough strain SH5014 of *S. typhimurium*. Scale as in Fig. 1.

B and deacylated polymyxin B (Vaara, 1983). Since the polycationic PEI functionally and ultrastructurally altered the OM, its action could be mediated by binding to LPS, especially since PEI binds to and flocculates biological materials (Milburn *et al.*, 1990; Salt *et al.*, 1995). We tested whether isolated and purified LPS interacts with PEI in aqueous solutions. Highly soluble triethylammonium salt forms of different *S. typhimurium* LPS were mixed with PEI ($20 \mu\text{g ml}^{-1}$) and centrifuged to deposit any poorly soluble products. After centrifugation, the LPS remaining in solution was quantified by analysing the amount of LPS-specific fatty acids in the supernatant. Similarly centrifuged LPS solutions without PEI were assayed as controls. The results are shown in Table 1.

The solubility of the LPS of *S. typhimurium* SH2183 and SH5770 was not affected by PEI. These LPSs are of the smooth chemotype, containing dozens of repeating oligosaccharide units (O-specific chains), and they were chosen because their O-specific chains are different. The repeating units of SH2183 consist of D-mannose, L-rhamnose, D-galactose, 3,6-dideoxy-D-xylo-hexose and, partially, D-glucose (O specificity 4,5,12; Hellerqvist *et al.*, 1969) whereas in SH5770 the repeating units are genetically replaced by the O:6,7-specific type consisting of four molecules of D-mannose and one N-acetylglucosamine, with one D-mannose unit being partially substituted by D-glucose (Lindberg *et al.*, 1988). These O-chains differ in their content of deoxy-sugars and hence in their relative hydrophilicity, and they were

Table 1. Effect of PEI on the solubility of isolated LPS of *S. typhimurium* strains

Strain	Chemotype (O-antigenic type, relevant genotype)	Proportion of insoluble LPS after PEI treatment*
SH2183	S (O:4,5,12)	2 ± 2
SH5770	S (O:6,7)	0
<i>his-515</i>	Ra	7 ± 7
SH5014	Rb2	79 ± 13
SH7426	Rb2 (<i>pmrA</i>)	19 ± 4
SL1102	Re	77 ± 15

* Determined as percentage (mean \pm SEM) of LPS deposited from PEI-containing LPS solutions (PEI, $20 \mu\text{g ml}^{-1}$; LPS, 0.6 mg ml^{-1}) by a 4 min centrifugation at 11000 r.p.m., as compared to LPS solutions without PEI. Quantification of LPS in the supernatants was performed by analysis of LPS-specific fatty acids.

previously shown to differ in their physico-chemical properties (Helander *et al.*, 1992). Both LPS types remained fully soluble in water in the presence of PEI.

Similarly, the solubility of Ra-type LPS (*his-515*, lacking O-specific chains but possessing a complete core oligosaccharide) was unaffected by PEI. More incomplete types of LPS (Rb2 and Re) did, however, form insoluble complexes with PEI. The Rb2 LPS variant isolated from strain SH7426 is, due to the *pmrA* mutation, abnormally

cationic as a result of extensive substitution of phosphate groups by cationic compounds (Helander *et al.*, 1994). This LPS was only poorly deposited from PEI-containing solution by the centrifugation procedure.

DISCUSSION

Our results demonstrate that PEI causes massive alterations in the OM. This is consistent with our previous report that PEI-induced sensitization of Gram-negative species to detergents and hydrophobic substances indicates disruption of the permeability barrier function of the OM (Helander *et al.*, 1997). Furthermore, electron microscopy supports the previous finding based on chemical analysis that PEI did not release LPS from the OM. The possibility that liberation of LPS by PEI was obscured by the LPS-precipitating activity of PEI was excluded by our present finding that smooth-type LPS and Ra-LPS were not precipitated by PEI. We conclude that PEI intercalates in the OM and increases the membrane surface area to an extent that can be visualized by electron microscopy.

The action of PEI on the OM resembles that of the deacylated polymyxin derivative PMBN. Without releasing LPS or being directly bactericidal, both substances make the OM permeable to hydrophobic antibiotics, and they cause similar ultrastructural alterations in the OM (Vaara & Vaara, 1983b). Furthermore, PMBN was reported not to sensitize *S. typhimurium* to SDS (Vaara & Vaara, 1983b), and this was also noted in experiments where the detergent was present with PEI (Helander *et al.*, 1997). However, PEI strongly sensitized *E. coli*, *P. aeruginosa* and *S. typhimurium* to the lytic action of SDS if the bacteria were pretreated with PEI and subsequently challenged with SDS.

The interaction of PEI with purified LPS differed greatly, depending on the macromolecular nature of the LPS. Smooth-type LPS, regardless of the sugar composition of the O-specific chains, and Ra-LPS did not form poorly soluble products with PEI, whereas deeper rough-type LPS did. Precipitation of chemotype Rb2 and Re LPS with PEI seems to be associated with their much higher negative charge to mass ratio compared to that of smooth-type LPS. This is supported by our finding that abnormally cationic *pmrA* mutant Rb2 LPS was only slightly precipitated by PEI. It should be noted here that the LPS deposition assay measured only the effect of PEI on the solubility of LPS and not the binding itself; thus the results do not exclude the possibility that PEI also binds to smooth LPS without yielding insoluble complexes. To conclude, although PEI evidently does bind to the OM, and has a tendency to bind to LPS, it is possible that its binding to the OM involves interactions with other membrane components, such as proteins and phospholipids. Phospholipids are normally not present in the outer leaflet of the OM, but they can be accessible after primary disruption of the OM (Vaara, 1992). Bacterial lipids are known to be flocculated by PEI from cellular homogenates (Salt *et al.*, 1995), and it is likely

that outer-membrane proteins, many of which possess anionic character (Schmitgess & Henning, 1976; Tokunaga *et al.*, 1979), also bind to PEI.

ACKNOWLEDGEMENTS

We thank Päivi Lepistö, Anna-Liisa Ruskeepää and Arja Strandell for excellent technical assistance. Pertti Koski is thanked for valuable discussions. This work was financially supported by the Academy of Finland (to K.L.) and by the NISIN^{PLUS} project (FAIR-CT96-1148).

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Received 9 July 1997; revised 19 September 1997; accepted 20 October 1997.