

Presence of exposed phospholipids in the outer membrane of *Vibrio cholerae*

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Vibrio cholerae 569B was found to be highly sensitive to a wide range of chemicals, particularly hydrophobic compounds and neutral and anionic detergents. The phospholipid profile of the outer membrane was similar to that reported for other Gram-negative bacteria. The lipopolysaccharide (LPS) contained O-antigenic sugars and exhibited heterogeneity. In addition, the LPS moiety was characterized by a relatively low negative charge. Analysis by topological probes revealed the presence of a significant amount of exposed phospholipids in the outer membrane. The reduced negative charge of LPS molecules and the exposed phospholipids present in the outer membrane could be important in the increased permeation of exogenous compounds in *V. cholerae*.

Introduction

Normally Gram-negative bacteria with smooth-type LPS in the outer membrane are resistant to hydrophobic compounds and detergents (Nikaido & Vaara, 1985). This resistance is primarily due to the absence of exposed phospholipids in the outer membrane, which restricts the entry of non-polar compounds by diffusion (Nikaido & Vaara, 1985; Sukupolvi & Vaara, 1989). In deep rough mutants, and in mutants with deletion of major outer-membrane proteins, this structural asymmetry is lost, and exposed phospholipids appear on the cell surface (Lugtenberg & van Alphen, 1983; Nikaido & Vaara, 1985). As expected, such mutant strains exhibit higher sensitivity to hydrophobic compounds owing to the increased rate of their permeation across the outer membrane (Nikaido & Vaara, 1985; Sukupolvi & Vaara, 1989).

Several studies indicate the atypical nature of the cell surface of *Vibrio cholerae* 569B. While cholera toxin synthesized by *Escherichia coli* remained intracellular, the *E. coli* enterotoxin synthesized in *V. cholerae* was excreted (Neill *et al.*, 1983). Furthermore, *Vibrio* cells are highly sensitive to protein denaturants, and it is possible to isolate outer membranes directly from whole cells by treatment with urea (Lohia *et al.*, 1984). The present paper reports that the sensitivity of *V. cholerae* to a wide variety of chemicals might be due to the presence of

exposed phospholipids and the relatively low net negative charge of LPS molecules present in its outer membrane.

Methods

Organisms and growth conditions. *V. cholerae* strain 569B and its detergent-resistant mutant, lpa-1 (Paul *et al.*, 1990) were grown in complex media with shaking at 37 °C as described previously (Lohia *et al.*, 1984). A low phosphate medium was used for the induction of alkaline phosphatase (Roy *et al.*, 1982). *Escherichia coli* C-600 was grown with shaking at 37 °C in Luria broth.

Determination of minimum inhibitory concentration (MIC). The MIC of various compounds was determined both by broth dilution and plate assay. For broth dilution assay, about 5×10^4 cells were incubated in growth medium containing different concentrations of the compound, shaken at 37 °C for 16 h. The lowest concentration of the compound at which there was no visible growth was taken as the MIC. For plate assay, around 300–400 c.f.u. were spread on nutrient agar containing different concentrations of the test compound. After incubation for 24 h at 37 °C, the lowest concentration of the compound at which no c.f.u. were observed was taken as the MIC. The results of the two assays were comparable.

Treatment with poly-L-lysine and polymyxin binding. Cells ($4\text{--}5 \times 10^8$ c.f.u. ml⁻¹) suspended in 10 mM-phosphate buffer (pH 7.5) were incubated with poly-L-lysine (100 µg ml⁻¹) for 30 min at 37 °C, centrifuged and the supernatant and pellet were assayed for the periplasmic enzyme cyclic phosphodiesterase. To determine the deoxycholate sensitivity of the cationic-peptide-treated cells, the cell pellet was suspended in 70 mM-potassium phosphate buffer (pH 7.2) containing 0.05% deoxycholate, and the A_{585} was recorded after 10 min at 37 °C. Polymyxin binding to purified LPS was assayed according to Vaara *et al.* (1981). About 2 mg purified LPS and 1.2 mg

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polymyxin B sulphate were used for each assay. The amount of bound polymyxin was calculated from the number of free amino groups present in the LPS pellet normalized against the number of free amino groups present in the LPS sample not treated with polymyxin B.

Membrane preparation. Isolation of crude cell envelope and its fractionation into outer and inner membrane with 4 M-urea for the analysis of phospholipids and LPS, and with 1% Sarkosyl for the assay of membrane proteins, were carried out as described previously (Lohia *et al.*, 1984, 1985; Filip *et al.*, 1973). The sucrose density gradient centrifugation method for separation of the two membrane systems of Gram-negative bacteria did not work satisfactorily in *V. cholerae* (Lohia *et al.*, 1984). The inner membrane contamination in the outer membranes never exceeded 8% as detected by the cytochrome assay (Osborn *et al.*, 1972). Contamination by outer membrane in the inner membrane preparation was less than 5% as estimated by heptose assay (Wright & Rebers, 1972). Yields of crude cell envelope, and outer and inner membranes were around 14, 8 and 6 per cent respectively of the cellular dry weight.

Isolation and analysis of LPS, lipid A and phospholipids. Isolation of LPS from crude cell envelopes, and subsequent separation of the lipid A and polysaccharide moieties were carried out as described previously (Paul *et al.*, 1990). LPS was labelled by growing cells in the presence of [¹⁴C]acetate [specific activity: 56.7 mCi (2.1 GBq) mmol⁻¹, 0.2 µCi (7.4 kBq) ml⁻¹] for 3 generations. Since [¹⁴C]acetate could also label phospholipids, the radioactive LPS was freed from phospholipids by repeated extraction with chloroform/methanol (2:1, v/v) till no further counts were extractable. Finally, the labelled LPS was solubilized in Laemmli sample buffer and analysed by 14% (w/v) SDS-PAGE and fluorography (Ray *et al.*, 1984). Phospholipids were extracted from whole cells and isolated membranes by chloroform/methanol (2:1, v/v) and quantitatively analysed after their separation by TLC as described previously (Lohia *et al.*, 1985). The phospholipids were essentially free of LPS, as no 3-hydroxytetradecanoic acid could be detected by GLC (Paul *et al.*, 1990).

CNBr-activated dextran coupling. Preparation of CNBr-activated dextran T-10 (molecular mass 10 kDa) and its coupling to whole cells were done as described by Kamio & Nikaido (1976). Cells were labelled by growing them for 3 generations in media containing [2-³H]glycerol [specific activity: 1 Ci (37 GBq) mmol⁻¹, 2 µCi (74 kBq) ml⁻¹] followed by suspension and shaking for 30 min in fresh growth media containing 0.05% glycerol. The cell suspension in 50 mM-borate buffer (pH 8.5) was divided into two parts, 0.1 M-ethanolamine was added to the control tube at 0 min and the coupling reaction was initiated in the other tube with freshly prepared CNBr-dextran complex, continued for 1 h at 25 °C. After termination of the reaction with excess ethanolamine, phospholipids were isolated from both control and experimental cells and separated on TLC plates. In some cases the samples were fractionated into outer and inner membranes prior to phospholipid extraction. The separated phospholipids were visualized by iodine vapour and ninhydrin, and appropriate spots from a parallel lane were scraped into scintillation vials and assayed for radioactivity.

Dansylation of whole cells. Dansyl chloride-cyclodextrin complex (20 mg) prepared following the method of Kinoshita *et al.* (1974) was added to 10 ml of cells (1.5 × 10⁹ c.f.u. ml⁻¹) suspended in 50 mM-borate buffer (pH 8.5) containing 1 mM-MgCl₂. After stirring in the dark for 60 min at room temperature, the unreacted complex was removed by low speed centrifugation (1000 g, 5 min) and the cells were washed repeatedly in the above suspension buffer. Finally, phospholipids from isolated membranes of the treated cells were separated by TLC, visualized and photographed under UV light. All operations were carried out in the dark.

Other methods. Standard methods were used for assay of dye uptake (Wolf-Watz *et al.*, 1975). Free amino groups were assayed by 2,4-dinitrofluorobenzene using D-glutamic acid as a standard (Ghuysen & Strominger, 1963). Glucosamine in lipid A was assayed as described earlier (Paul *et al.*, 1990). Protein was estimated by the method of Markwell *et al.* (1978). Alkaline phosphatase and cyclic phosphodiesterase were assayed by standard methods using *p*-nitrophenyl phosphate and bis-*p*-nitrophenyl phosphate as substrates respectively (Roy *et al.*, 1982; Neu & Heppel, 1965). Enzyme activity in cell pellet was assayed after sonication for 1 min at 4 °C.

Results

Atypical permeability features of the outer membrane

V. cholerae cells were found to be highly sensitive to a wide variety of compounds (Table 1). Since the MIC values may vary with the experimental conditions, the sensitivity of *E. coli* strain C-600 (chemotype Ra) was determined under identical conditions and significant differences between these two strains were observed (Table 1, column 'b/a'). The sensitivity of *V. cholerae* was most marked towards hydrophobic antibiotics like novobiocin and rifampicin and neutral and anionic detergents (Triton X-100 and SDS). One exception to the above was the hydrophobic compound phenol (Table 1). Interestingly, sensitivity of *V. cholerae* towards cationic

Table 1. MIC of various compounds for *V. cholerae* 569B and *E. coli* C-600

Agent	MIC		
	<i>V. cholerae</i> (a)	<i>E. coli</i> (b)	(b/a)
Antibiotics*			
Penicillin G	2	20	10
Ampicillin	2	5	2.5
Tetracycline	2	50	25
Chloramphenicol	5	5	1.0
Neomycin	2	20	10
Novobiocin	2	750	375
Rifampicin	0.1	40	400
Nalidixic acid	1	25	25
Polymyxin B	0.2	0.05	0.25
Detergents			
Sodium dodecyl sulphate†	0.01	2.0	200
Sodium deoxycholate†	0.05	2.5	50
Triton X-100†	0.02	> 5	> 250
Cetyltrimethylammonium bromide*	20	50	2.5
Other compounds			
Crystal violet*	2	20	10
Phenol†	0.1	0.1	1.0
Stearic acid‡	0.4	> 10	> 25
Protamine sulphate*	50	50	1.0

* MIC in µg ml⁻¹.

† MIC as % (w/v).

‡ MIC as mM.

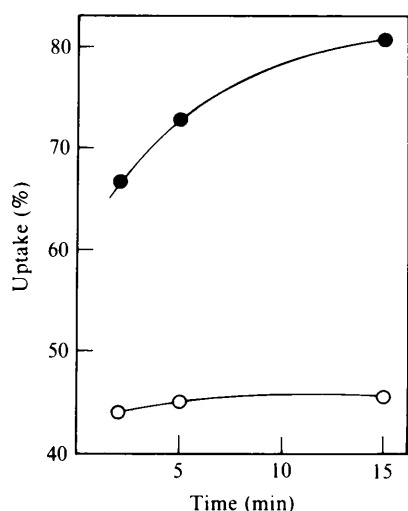


Fig. 1. Uptake of crystal violet by *V. cholerae* (●) and *E. coli* (○). Cells in the exponential phase of growth in NB (about 5×10^8 c.f.u. ml⁻¹) were treated with 10 µg crystal violet per ml. At different times during growth at 37 °C, samples were withdrawn and the amount of dye in the supernatant was estimated from the absorbance at 590 nm.

agents like cetyltrimethylammonium bromide and protamine sulphate was much less marked and the only compound found so far to which *V. cholerae* was more resistant than *E. coli* was the cationic amphipathic antibiotic polymyxin B ($b/a = 0.25$, Table 1).

Sensitivity to hydrophobic compounds and detergents suggested that the outer membrane of *V. cholerae* might not be an effective barrier to such compounds. This was confirmed by the extensive lysis (about 80%) of these cells following treatment with 0.01% SDS, sodium deoxycholate and Triton X-100. There was no detectable lysis of *E. coli* cells even at ten times the concentrations of these detergents. When crystal violet was added to *V. cholerae* cells in the exponential phase of growth, the uptake of dye continued during the entire 15 min of incubation resulting in incorporation of about 80% of the added dye (Fig. 1). In contrast, uptake of crystal violet in *E. coli* was essentially complete within 2 min of addition of the dye and not more than 45% of the dye was finally incorporated. The incorporated dye in *V. cholerae* cells was divided almost equally between the cell envelope and the cytoplasm. On the other hand, in *E. coli*, more than 95% of the dye was confined to the envelope fraction. This clearly indicated that the *V. cholerae* envelope did not constitute an effective barrier to the entry of crystal violet.

Leakage of periplasmic proteins indicated instability of the outer membrane. We have found that 15–20% of two periplasmic enzymes, cyclic phosphodiesterase and alkaline phosphatase, were consistently released into the culture medium by exponential phase cells. The unstable nature of the *V. cholerae* cell surface was further

Table 2. Distribution of bound phosphate and free amino groups in LPS of *V. cholerae* and *E. coli*

Macromolecule analysed	<i>V. cholerae</i>			<i>E. coli</i>		
	PO ₄ (a)	-NH ₂ (b)	(a/b)	PO ₄ (a)	-NH ₂ (b)	(a/b)
LPS	0.80	0.15	5.3	1.27	0.10	12.7
PS*	0.55	0.12	4.6	0.98	0.07	14.0

* PS of LPS was obtained after splitting off the lipid A by treatment with 1% acetic acid at 100 °C for 2.5 h. Results are expressed as µmol (mg LPS)⁻¹.

documented by the observation that any perturbation likely to cause cellular damage, such as penicillin treatment, resulted in a rapid release of periplasmic proteins preceding other effects of cell damage, e.g. loss of viability or cellular rigidity. Since divalent cations are known to play a significant role in the repair of outer membrane defects such as enhanced sensitivity to exogenous chemicals and leakage of periplasmic proteins (Stan-Lotter *et al.*, 1979), the leakage of periplasmic proteins and the sensitivity to exogenous compounds were compared in *V. cholerae* grown in the presence and absence of 1 mM-MgCl₂. No significant difference in any of these parameters was observed.

Surface charge

The fact that *V. cholerae* was relatively resistant to basic compounds (Table 1) prompted us to analyse the distribution of fixed charged groups present in the polysaccharide (PS). The total phosphate and free amino groups in the LPS and PS of *V. cholerae* were estimated and compared with those of *E. coli* cells. The ratio of phosphate to amino groups in the LPS and PS of *E. coli* were 2.4 and 3.0 times higher, respectively, than the corresponding values for *V. cholerae* (Table 2). Furthermore, there was a significant difference in the ability of LPS molecules isolated from *E. coli* and *V. cholerae* to bind the cationic antibiotic polymyxin B. The amount of polymyxin B bound expressed as µmol of free amino groups of bound polymyxin per mg LPS was 0.23 for *E. coli* C-600 and 0.13 for *V. cholerae* 569B. Such a low distribution of anionic groups in the outer membrane could be responsible for the ability of poly-L-lysine to destabilize the outer membrane of *V. cholerae* cells. Pretreatment of cells in the exponential phase of growth (5×10^8 c.f.u. ml⁻¹) suspended in 20 mM-phosphate buffer (pH 7.4) with poly-L-lysine (100 µg ml⁻¹) for 30 min at 37 °C did not sensitize them to deoxycholate induced lysis nor lead to any detectable increase in the release of the periplasmic enzyme cyclic phosphodiesterase.

Table 3. Composition of outer and inner membrane of *V. cholerae*

Each value is the mean of at least three sets of independent experiments.

Sample	Protein (a) [ug (mg membrane) ⁻¹]	LPS (b)	Phospholipid (c)					Distribution of phospholipid* (%)				µg phospholipid (mg membrane protein) ⁻¹	
				a	:	b	:	c	Lyso-PE	PE	PG		DPG
Crude envelope	670	138	198	3.3	:	0.66	:	1.0	—	—	—	—	295
Outer membrane	618	223	157	3.86	:	1.43	:	1.0	7	69	21	3	256
Inner membrane	728	12	256	2.84	:	0.06	:	1.0	4	76	16	4	350

* Expressed in terms of lipid phosphate taking total lipid phosphate content as 100. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol.

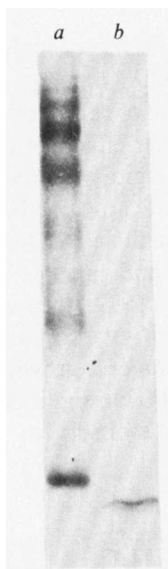


Fig. 2. LPS of *V. cholerae*. LPS was purified from cells grown in presence of [¹⁴C]acetate and examined by 14% (w/v) SDS-PAGE and autoradiography. Lanes: a, LPS of *V. cholerae*; b, phospholipids of *V. cholerae* isolated from the same culture.

Outer membrane composition

The relative proportions of proteins, phospholipids and LPS in the outer membrane of *V. cholerae*, and its phospholipid composition (Table 3) were similar to those reported for other Gram-negative bacteria (Lugtenberg & van Alphen, 1983). The ratio of phospholipids to protein was similar to that reported for *E. coli* K12 and different from deep rough or protein-deficient mutants of *E. coli* and *Salmonella* (Koplow & Goldfine, 1974; Smit *et al.*, 1975). *V. cholerae* 569B has been reported to have smooth-type LPS (Chakrabarti & Chatterjee, 1984; Gustafsson & Holme, 1985) and this has also been confirmed in our laboratory. Purified LPS from cells grown in the presence of [¹⁴C]acetate showed microheterogeneity when analysed by SDS-PAGE and fluorography (Fig. 2). Such a banding pattern is characteristic of LPS molecules varying in the degree of polymerization

of the O-antigenic side chain (Hitchcock *et al.*, 1986). Furthermore, the glucosamine content of isolated lipid A was 0.26 µmol (mg LPS)⁻¹. Assuming one glucosamine disaccharide unit in lipid A, the average molecular mass of LPS molecules of *V. cholerae* can be estimated as around 8000 Da. This agrees with the reported value of 9000 Da for the LPS of *V. cholerae* (Gustafsson & Holme, 1985) and indicates a significant contribution of O-antigenic sugars in the make-up of *V. cholerae* 569B LPS. Thus the overall composition of the outer membrane does not offer any obvious explanation of its unusual permeability to hydrophobic compounds.

V. cholerae was found to be highly sensitive to the antibiotic globomycin (MIC = 5 µg ml⁻¹), which specifically inhibits cleavage of the signal peptide from the lipoprotein, thereby preventing its linkage with the murein sacculus of Gram-negative bacteria (Inukai *et al.*, 1978). Within 2 h of globomycin treatment (5 µg ml⁻¹) the viability of *V. cholerae* cells decreased by about 50% and lysis of such globomycin-treated cells suspended in 0.05 M-phosphate buffer was about 60% greater than that of control cells. These data suggest a role for murein-bound lipoprotein in maintaining the stability of the outer membrane. Preliminary studies have shown the presence of multiple species of lipoproteins in the outer membrane of *V. cholerae*.

Presence of exposed phospholipids

The high sensitivity of *V. cholerae* to hydrophobic compounds suggested domains of lipid bilayer in its outer membrane. To check this, [2-³H]glycerol-labelled cells were treated with CNBr-activated dextran, an impermeant probe which has been used to demonstrate presence of exposed phospholipids in the outer membrane of *Salmonella* (Kamio & Nikaido, 1976). Following treatment with activated dextran, phospholipids from whole cells and outer and inner membranes were isolated and the distribution of label in the various species of phospholipids was analysed quantitatively by TLC. The only significant difference in the amounts of

Table 4. [$2\text{-}^3\text{H}$]Glycerol-labelled phospholipids from *V. cholerae* lpa-1 mutant and *E. coli* C-600 cells treated with CNBr-activated dextran

Treatment of [$2\text{-}^3\text{H}$]glycerol-labelled cells (2.5–4 mg dry weight, $2\text{--}3 \times 10^6$ c.p.m.) with CNBr-activated dextran and subsequent fractionation of control and experimental cells were done as described in Methods. The total amount of radioactivity in each sample was about 2×10^4 c.p.m. and recovery was about 84%.

Strains	Sample analysed	Radioactivity recovered (%) from					
		Origin	Lyso PE	PE	PG	DPG	Solvent front
<i>V. cholerae</i> strain 569B	Whole cell lipids – control cells	3	9	65	16	3	2
	Whole cell lipids – experimental cells	3	14	54	20	6	3
	Outer membrane lipids – control cells	5	7	64	17	2	4
	Outer membrane lipids – experimental cells	7	10	47	23	3	5
	Inner membrane lipids – control cells	5	3	72	13	3	4
	Inner membrane lipids – experimental cells	6	3	71	11	5	4
<i>V. cholerae</i> lpa-1	Whole cell lipids – control cells	3	9	62	19	3	3
	Whole cell lipids – experimental cells	3	10	61	18	3	4
<i>E. coli</i> C-600	Whole cell lipids – control cells	2	8	75	11	0	4
	Whole cell lipids – experimental cells	2	9	73	11	0	4

^3H -label recovered in the different phospholipids from whole cells was a reduction in the phosphatidylethanolamine (PE)-associated radioactivity in the experimental sample (Table 4). When isolated membranes from control and experimental samples were similarly analysed, the reduction in PE-associated label was found to be strictly restricted to lipids isolated from the outer membrane of experimental cells. PE which bound to activated dextran was not extractable by solvents, presumably owing to its attachment to a large hydrophilic molecule. The ability of the probe to react selectively with exposed amino groups of PE is shown by its failure to bind PE of the inner membrane of *V. cholerae* 569B and membrane lipids (both outer and inner) of *E. coli* C-600 (Table 4). It is important to note that membrane phospholipids of the mutant strain lpa-1 which is resistant to hydrophobic compounds and detergents (Paul *et al.*, 1990) were not labelled by CNBr-activated dextran under identical conditions (Table 4). When whole cells of *V. cholerae* 569B ([$2\text{-}^3\text{H}$]glycerol labelled) treated with activated dextran were lysed with SDS (2% w/v, 2 min, 100°C) and directly spotted on TLC plates, radioactive material could be recovered from the origin. This was not seen in control cells of *V. cholerae* when analysed under identical conditions. The amount of such radioactive material was comparable to the selective decrease in PE-associated radioactivity observed between control and experimental samples (Table 4). The origin material was eluted with water containing 2% (w/v) SDS and after extensive dialysis hydrolysed with HCl (4 M, 100°C , 4 h) and analysed by TLC. All radioactivity present in the hydrolysate could be recovered as free glycerol, and the only ninhydrin reactive spot had the same mobility as ethanolamine.

This indicated that the radioactive material at the origin was PE-complexed with activated dextran. Similar conclusions were reached using dansyl-chloride-cyclodextrin complex which has been used to analyse the topology of phospholipids in red blood cells (Schmidt-Ullrich *et al.*, 1973). When phospholipids of *V. cholerae* 569B cells treated with this probe were analysed, the PE present in the outer membrane was selectively labelled. No phospholipid of *E. coli* or *V. cholerae* lpa-1 was labelled by this probe under similar experimental conditions.

Discussion

Extensive studies have demonstrated that the hydrophobic pathway for permeation of exogenous compounds is normally non-operative in Gram-negative bacteria due to the asymmetric organization of the outer membrane (Vaara & Nikaido, 1984). This is particularly true for species which colonize segments of intestinal tissues in the presence of bile salts and other potential noxious agents (Vaara & Nikaido, 1984). It is only when this asymmetry is lost with appearance of phospholipids in the outer leaflet of the outer membrane that cells become permeable to hydrophobic compounds and detergents (Vaara & Nikaido, 1984).

The fact that about 30% of the PE in the outer membrane of *V. cholerae* was accessible to exogenous CNBr-dextran (Table 4), compared to about 10% as reported in deep rough mutants of *Salmonella* (Kamio & Nikaido, 1976) suggests significant phospholipid bilayers in the outer membrane of *Vibrio* cells. Presence of exposed phospholipids was confirmed with the use of another impermeant probe (dansyl chloride; lipid separ-

ation by TLC; data not shown). Due to lack of adequate information on the surface area of *Vibrio* cells, we have not been able to estimate the number of lipid molecules in the outer membrane which could participate in bilayer formation. Phospholipid constituted about 250 µg per mg protein in the outer membrane of *V. cholerae* (Table 3). This value is significantly higher than the corresponding values of 133 reported for wild-type *Neisseria gonorrhoeae* and 190 for that of the supersensitive *env* mutant derived from *N. gonorrhoeae* (Lysko & Morse, 1981). *N. gonorrhoeae* resembles *V. cholerae* in being sensitive to hydrophobic compounds and detergents (Cannon & Sparling, 1984). However, exogenous phospholipase C failed to release phospholipids from parental *Neisseria* cells and it was only the supersensitive *env* mutant whose phospholipids were accessible to phospholipase C (Lysko & Morse, 1981). Moreover, without other supporting evidence, accessibility to phospholipase C does not necessarily indicate presence of exposed lipids in the outer membrane (Kamio & Nikaido, 1976). Loss of structural asymmetry of the outer membrane is normally due to two kinds of defect – inability to synthesize either a major portion of the core polysaccharide moiety of LPS, or major outer-membrane proteins (Lugtenberg & van Alphen, 1983; Vaara & Nikaido, 1984). The relative proportion of the three major macromolecules constituting the outer membrane of *V. cholerae* (Table 3) suggest that such alterations are probably not a causative factor in the case of *V. cholerae*. The presence of murein-bound lipoprotein in *V. cholerae* outer membrane suggests that leakiness of periplasmic protein is probably not due to lack of stabilization of the outer membrane by this structural protein. The extensive blebbing of the outer membrane of *V. cholerae* 569B cells demonstrated by electron microscopy (Chatterjee & Das, 1967) is also indicative of the unstable nature of the outer membrane.

The strain (569B) of *V. cholerae* used in this study was shown to have retained its smooth-type LPS (Chakrabarti & Chatterjee, 1984; Gustafsson & Holme, 1985) thereby ruling out its inadvertent conversion to a rough form due to continued growth under laboratory conditions. Incidentally, previous studies had demonstrated only two classes of LPS molecules (rough and smooth) in *V. cholerae* (Chakrabarti & Chatterjee, 1984; Gustafsson & Holme, 1985) and the present study demonstrates microheterogeneity in its LPS structure (Fig. 2).

A significant feature of LPS molecules of *V. cholerae* 569B was their reduced net negative charge. Results presented here (Table 2) indicate that the PS moiety of LPS is primarily responsible for the low fixed negative charge of LPS molecules. The reduced content of anionic groups in the LPS molecules of *V. cholerae* compared to *E. coli* is also indicated by the binding of isolated LPS molecules with polymyxin B. The importance of inter-

action of divalent cations with the proximal anionic groups in the PS moiety for stabilization of the outer membrane has been amply documented (Vaara & Nikaido, 1984; Nikaido & Vaara, 1985). It is conceivable that a reduced interaction of this kind could cause leakage of periplasmic enzymes as seen in *V. cholerae*. It could also be a factor in the permeation and consequent sensitivity of *V. cholerae* to hydrophilic compounds like tetracycline and neomycin (Table 1). In this context it is worth noting the dramatic changes in permeability of rough *Salmonella* strains with further deletion of phosphate groups from LPS (Schlecht & Westphal, 1970).

We cannot at this stage offer a reason for the presence of phospholipid bilayers in the outer membrane of *V. cholerae* 569B. Removal of divalent cations results in increased electrostatic repulsion between neighbouring LPS molecules. This is known to result in the release of a fraction of LPS molecules and might also lead to a reorganization in the architecture of the outer membrane (Nikaido & Vaara, 1985). Tris has been shown to render an Rc mutant of *Salmonella* susceptible to labelling by dansyl chloride (Schindler & Teuber, 1978). Whether such a reorganization occurs in *V. cholerae* due to the demonstrable low content of anionic groups in its LPS, with consequent reduced binding and stabilization by divalent cations, remains to be established. A previous study which showed rapid lysis of *V. cholerae* 569B cells in the presence of EDTA and Tris (Lohia *et al.*, 1984, 1985) emphasized the importance of divalent cations in its structural integrity.

It is difficult to visualize how *V. cholerae* 569B colonizing the small intestine would survive in such an environment when its outer membrane contains exposed phospholipids. Changes in the composition of the outer membrane due to environmental stresses are well known (Lugtenberg & van Alphen, 1983). It would be interesting to examine the process by which the exposed phospholipids of *V. cholerae* become shielded or masked during the process of infection and colonization. The major structural change in the mutant lpa-1, which is resistant to hydrophobic compounds and does not have exposed phospholipids has been traced to defective acylation of lipid A (Paul *et al.*, 1990). We are currently investigating the basis for the nonavailability of its outer membrane phospholipids to impermeant probes.

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