Electron Microscopic Study of the Polymyxin Treated *Vibrio cholerae* Cells

T. K. Mandal

Biophysics Division, Saha Institute of Nuclear Physics, 37, Belgachia Road, Calcutta 700037, India

Z. Naturforsch. **45c**, 902–910 (1990); received October 31, 1989/January 3, 1990

Polymyxin B, Vibrios, Sensitivities, Lysis and Viability, Surface Topography, Blebs and Crenations

Polymyxin B produces dose dependent changes in the surface topography of pathogenic *Vibrio cholerae* cells. The susceptibilities of various vibrio strains to PB are also studied through analytical techniques. Statistical analysis shows significant differences among the four vibrios with regard to their sensitivities to PB, the classical strains being the most sensitive. Treating the classical strain with subinhibitory concentration of PB, we observed with both SEM and TEM that the normal smooth surface of the cell envelope develops some protruded structures (blebs and crenations). Further the TEM study of the ultrathin sections reveal that the rod like projections are formed by protrusions of the outer-membrane of the cell wall.

Introduction

Infections by *Vibrio cholerae* (a gram-negative bacterial organism) in the Indian subcontinent are frequently encountered [1, 2]. The polypeptide antibiotic polymyxin B (PB) is a clinically important antibiotic which is used to treat infections caused by a number of gram-negative bacteria [3, 4]. It has been observed that PB causes disruption of the ultrastructure of OM, as revealed by the electron microscopic studies for a number of bacteria, through the formation of blebs and crenations (finger-like structures) on the OM [5–15]. In this context, it is relevant to look specifically at the morphological changes induced by PB on *Vibrio* cells.

Earlier we have reported some preliminary electron microscopic studies on the action of PB on erythrocytes [16] and *Vibrio cholerae* [17] organisms. In this paper, we shall present a detailed study on the action of PB on different strains of *Vibrios* (four biotypes), namely, Classical, El Tor, NAG, and parahaemolyticus. It was found that as far as the surface disruption is concerned, the classical strains were most sensitive to PB.

Composition of phospholipids of whole cells, % survival (*i.e.*, % viabilities) and % lysis under PB treatment for all vibrios have been determined. Composition of phospholipids of outer membrane and inner membrane as well as the amount of total lipopolysaccharides (LPS) of OM have been estimated only for the most sensitive strains of classical vibrio. These analytical data together with the SEM and TEM pictures of the morphological changes of the OM of the cells give some indications regarding the mode of action of PB on the OM of the gram-negative *Vibrios*. Furthermore TEM studies have also revealed that blebs and crenations are the outfoldings of the outer membrane (OM).

Reprint requests to Dr. T. K. Mandal.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/90/0700–0902 $ 01.30/0

Materials and Methods

Chemicals

Polymyxin B, Deoxy ribonuclease type-1, Ribonuclease 1, Phosphatidyl ethanolamine, Phosphatidyl glycerol, cardiolipin, Phosphatidyl serine, Phospholipids kit, Bovine Serum Albumine, D-glucose, HEPES, Heptose, 2-keto-3deoxy octanate were obtained from Sigma Chemical Co., U.S.A.; Glutaraldehyde from Fluka Co., Switzerland; Osmium tetroxide, Araldite/Spurr kit embedding materials from Polysciences, U.S.A.; Urea, uranyl acetate, lead nitrate, sodium citrate from BDH, England and silica gel G from Merck, Darmstadt, F.R.G. All chemicals used were of analytical reagent grade.

Bacterial strains and growth conditions

The bacterial strains used were (i) *V. cholerae* (Classical) strains OGAWA 154, INABA 569B, 447/70, (ii) *V. parahaemolyticus* strains 2977, 2821,

975, (iii) *V. cholerae* (El Tor) strains V21/2, V125/1, MAK 757, and (iv) NAG Vibrio strains 2175, 2540, 1976. The media used for the cultivation of vibrios were (a) nutrient broth (NB) medium containing 1% (W/V) bactopeptone (Difco), 1% (W/V) lablemco powder (Oxoid), and 1% (W/V) NaCl (pH 8.0) for the preparation of bacterial suspensions and (b) Nutrient agar plates (NA plates) medium containing NB medium supplemented with 1.5% (W/V) bacto-agar (Difco) for assaying the cell viability as colony forming units (c.f.u./ml). Cells were grown at 37 °C on a gyrotory shaker (180–200 r.pm.) and bacterial growth was monitored by following the increase in culture turbidity at $OD_{650\,nm}$.

Preparation of bacterial suspensions for PB treatment

Vibrios were grown in 100-ml Erlenmeyer flasks containing 20 ml of culture media (NB) for overnight (18–20 h) at 37 °C so as to provide enough surface for satisfactory aeration. For all purposes, routinely, 0.5 ml of an overnight culture in NB medium was inoculated into 20 ml fresh broth and cells were allowed to grow with shaking at 37 °C for 120–180 min (mid logarithmic phase culture of bacterial growth) to yield $4-6 \times 10^8$ c.f.u./ml^{-1} (A_{650}, 0.6–0.7). Cells were harvested by low speed centrifugation at $6000 \times g$ (10 °C) for 5–10 min and immediately resuspended in 50 mM-Tris-HCl buffer containing 0.145 M NaCl, pH 7.4 (TSB) after two changes in the same buffer solution. The volume of bacterial (mid log phase culture) stock suspension was adjusted by TSB diluent to maintain $\sim 4 \times 10^8$ c.f.u./ml (initial OD at 650 nm = 0.30). This stock bacterial suspension was used throughout the study [18–20].

Sensitivity determination and MIC of Polymyxin B

The minimum inhibitory concentration (MIC) of PB was measured by the tube dilution method as described [21]. Briefly 0.2 ml of a 1-fold dilution of log phase bacteria ($\sim 10^8$ cell/ml) in NB media was inoculated into 3 ml of a solution of PB prepared with TSB solution (pH 7.4) at 37 °C for 15 min keeping the incubation mixture in a rotary shaker bath and then examined for growth after 18–20 h (overnight) incubation at 37 °C. The concentration of PB tested were ranged from 0.17 to 100 μg/ml. Subinhibitory dose (SID) of PB is the concentration of PB (in μg/ml) below the MIC value of PB which was expressed by the lowest concentration of the antibiotic that completely inhibited visible growth (*i.e.*, no turbidity at all) occurred after 18–20 h incubation at 37 °C. Any dose of PB lower than the MIC value is the subinhibitory dose (SID).

Turbidimetry and viability

The desired amount of PB was added to the stock bacterial suspension of mid log phase culture (initial OD being adjusted to about 0.30) kept at 37 °C as described above and lysis of cells was monitored, as change in optical density of bacterial suspension turbidimetrically at 650 nm. The percentage lysis was calculated by considering the initial OD to represent 0% lysis [18–20].

After a 15 min treatment of the stock bacterial suspension of mid log phase culture with polymyxin B at 37 °C (kept in a rotary shaker), the viable counts (the number of viable bacteria) of treated and untreated (control) bacterial suspension was determined by plating appropriate dilutions in 0.9% NaCl onto NA agar plates (kept at 37 °C for overnight incubation), following the conventional pour plate method [7].

Preparation of samples for SEM and TEM

For scanning electron microscopy (SEM), bacterial cells were fixed with 6% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) containing 10% sucrose (SCS) for 16–20 h and then with 1% Osmium tetroxide in Kellenburger buffer for 2 h at room temperature in dark. The fixed cells were dehydrated in graded ethanol (50% to 100% v/v) with final washing in amyl acetate, critical point dried in CO_2 in a Polaron series model E 3000 CPD apparatus, then coated under vacuum with a thin layer of gold in polaron E 500 sputter coating apparatus (with rotary vacuum pump Edward ED 50), and examined in a Philips PSEM 500 model scanning electron microscope operated at 25 kV [20, 22–23].

For thin section electron microscopy (TEM), the bacterial cells were fixed in the same way as in SEM preparation. The fixed cells were washed for 2 h in 0.5% uranyl acetate in the Kellenburger buffer, dehydrated in graded ethanol (50% to 100%

v/v) and embedded in Araldite/Spurr medium by routine procedures [16, 20, 24–25]. Sections were cut with glass knives and occasionally with a Du point diamond knife in an LKB ultramicrotome, stained with uranyl acetate and lead citrate and examined under a JEOL 200 CX transmission electron microscope operating at 80 kV [16, 22, 25].

Preparation of outer and inner membranes

Outer and inner membranes were isolated from the crude cell envelopes of whole cells of *V. cholerae* (classical) organisms OGAWA 154, INABA 569 B and were treated with 4 M urea as described [27].

Analysis of phospholipids, protein content and lipopolysaccharides

Phospholipids were extracted and estimated both from whole cells of *Vibrio* organisms (in mid logarithmic phase of growth) and outer and inner membrane preparations of the same *V. cholerae* (classical) organisms by two-dimensional thin-layer chromatography on silica gel G plates (Merck) using the solvent system as described previously [18].

Protein was measured by the method of Markwell *et al.* [26] with bovine serum albumin as the standard.

LPS was isolated from crude cell envelope and also from outer cell membrane preparation by 45% (w/v) aqueous phenol at 68 °C [27]. Total carbohydrates was estimated by phenol-H_2So_4 acid [28] with D-glucose as the standard. Heptose was estimated by cysteine-H_2So_4 method [29]. KDO, 2 keto 3-deoxyoctanate (a measure of LPS) was determined by the method of Karkhanis *et al.* [30].

Results

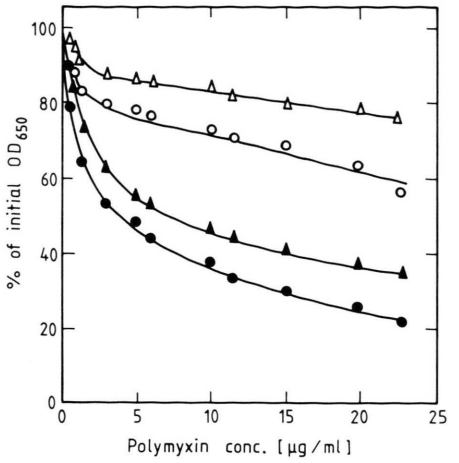
Sensitivities of Vibrios to polymyxin B

Administration of subinhibitory doses of PB (*i.e.*, less than minimum inhibitory concentration or MIC) (Table I) to different *Vibrio strains* shows rapid decrease in viability and loss of turbidity (exhibited by the absorbance at 650 nm) of varying degrees as shown in Fig. 1 and 2. Percentage of lysis was calculated by considering the initial OD to represent 0% lysis and the lysis was monitored by the decrease in OD_{650}. It may be noted that the antibacterial polycations are generally bacteriostatic at the MIC but exhibit bactericidal action at

Table I. MIC values of polymyxin and polymyxin doses required for 70% survivals of the different *Vibrio* strains after 15 min treatment at 37 °C.

No. of *Vibrios*	Strains of different *Vibrios*	MIC[a] [μg/ml]	Dose for 70% survivals Polymyxin Conc. [μg/ml]	Average conc. of PB ± SD [μg/ml]
1.	*V. cholerae* (classical)			
	OGAWA 154	6	0.70	0.723 ± 0.032
	INABA 569 B	8	0.72	
	447/70	10	0.75	
2.	*V. parahaemolyticus*			
	2977	15	1.40	1.46 ± 0.032
	2821	20	1.48	
	975	25	1.50	
3.	*V. cholerae* (El Tor)			
	V 21/2	60	2.50	2.84 ± 0.34
	V 125/1	63	2.84	
	MAK 757	65	3.20	
4.	NAG *Vibrio*			
	2175	75	15.0	16.33 ± 1.29
	2540	78	16.0	
	1977	80	18.0	

[a] The MIC was defined as the lowest concentration of Polymyxin B that prevented visible growth of *Vibrio* cells incubated for 18 h at 37 °C.



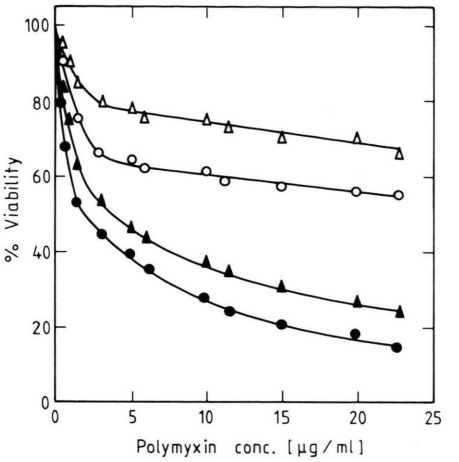


Fig. 1–2. % of initial OD and % viability of *V. cholerae* cells treated with various concentration of Polymyxin B (µg/ml) for 15 min at 37 °C. Percentage of lysis was calculated by considering the initial OD to represent 0% lysis. ●—●, *V. cholerae* (classical) strain OGAWA 154; ▲—▲, *V. parahaemolyticus* strain 2977; ○—○, *V. cholerae* (El Tor) stain V21/2; and △—△, NAG *Vibrio* strain 2175. Each experimental point was obtained as the average of three independent measurements.

a much higher dose [31]. While the classical strain is most sensitive (78% lysis resulting after 15 min, incubation by the action of 22.8 µg/ml of PB), NAG *Vibrio* is least sensitive (only 25% lysis-resulting under identical condition) as shown in Fig. 1. Sensitivities of different strains towards PB is given in Table I. The average polymyxin dose required for 70% survival varied from 0.72 ± 0.03 to 16.33 ± 1.29. We have chosen 70% survival as the indicator instead of say *e.g.* 50% so that we may get a data point for each *Vibrio* at a low PB dose. Significant differences between four *Vibrios* with respect to their sensitivities to PB have been observed through the Student t-test (Table II). Moreover, the F-value in the variance ratio test is estimated to be 137.27, which for 3 degrees of freedom convincingly demonstrate that there exist significant differences between the sensitivities of the four vibrios *i.e.*, the different vibrio biotypes are not drawn from the same parental population.

Chemical analyses of phospholipid composition, proteins and LPS content

Quantitative thin-layer chromatographic data on the phospholipid composition of whole cells as well as of outer and inner membrane are shown in Tables III and IV. Total yield of protein and LPS in the outer and inner membrane structures are shown in Table IV, where only the most sensitive strain is included for brevity.

SEM of PB-treated Vibrio cells

Scanning electron micrographs (Fig. 3–6) show that the *Vibrios*, when treated with SID of PB, develop numerous long and short finger-like projections (crenations and blebs) on the cell-surface, though the degree of such deformations varies with the different strains of the *Vibrios*. The un-

Table II. Statistical differences observed in the survivals of different vibrios (t-tests) after treatment with polymyxin B for 15 min. The probability values (P) correspond to the test of differences between the organisms in the row and column to which the particular value belongs.

Strains	*V. cholerae* (classical)	*V. parahaemolyticus*	*V. cholerae* (El Tor)	NAG *Vibrios*
V. cholerae (classical)	$P = 1$	$0.025 < P < 0.05$	$P < 0.001$	$P < 0.001$
V. parahaemolyticus	$0.025 < P < 0.05$	$P = 1$	$0.001 < P < 0.005$	$P < 0.001$
V. cholerae (El Tor)	$P < 0.001$	$0.001 < P < 0.005$	$P = 1$	$0.001 < P < 0.005$
NAG *Vibrio*	$P < 0.001$	$P < 0.001$	$0.001 < P < 0.005$	$P = 1$

treated cells however, show smooth surfaces and are devoid of any such projections (Fig. 3). The critical point drying technique has been used to prepare the artifact-free samples. SEM study has shown that the surface topographical changes, as mentioned above, are most prominent for strains from classical *Vibrio* (Fig. 4–6) and least perceptible for *Vibrio parahaemolyticus*, NAG vibrio or El Tor *Vibrio*, SEM micrograph of which is not included for brevity. A maximum of six to eight

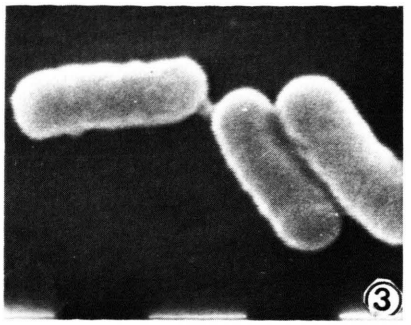
Table III. Phospholipid compositions of different Vibrio strains. The figures indicate the amounts of the different phospholipids as percentage of the respective total phospholipid. Standard deviations were calculated from measurements on four different batches of phospholipid preparation.

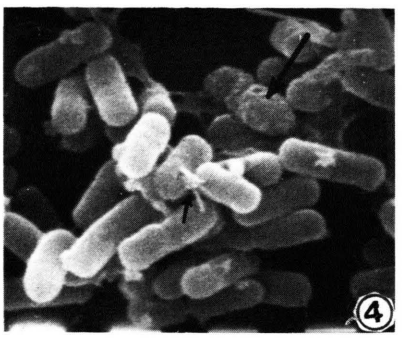
Vibrio strains	Phospholipids (%, w/w, total lipid-P)				
	Phosphatidyl ethanolamine (PE)	Phosphatidyl glycerol (PG)	Cardiolipin (CL)	Lysophosphatidyl ethanolamine (LPE)	Phosphatidyl serine (PS)
V. cholerae (classical)					
OGAWA 154	80.50 ± 1.5	12.53 ± 0.44	4.23 ± 0.04	2.00 ± 0.05	–
INABA 569 B	72.50 ± 1.0	16.50 ± 0.35	5.00 ± 0.02	4.25 ± 0.03	1.30 ± 0.01
V. parahaemolyticus					
2977	63.0 ± 1.3	18.1 ± 0.50	9.0 ± 0.32	5.0 ± 0.04	–
2821	62.0 ± 0.90	20.0 ± 0.60	12.0 ± 0.20	4.0 ± 0.02	–
V. cholerae (El Tor)					
V 21/2	50.20 ± 0.69	22.07 ± 0.70	17.85 ± 0.20	5.00 ± 0.50	3.00 ± 0.05
V 125/1	51.00 ± 0.65	22.00 ± 0.46	18.00 ± 0.39	4.00 ± 0.03	2.00 ± 0.03
NAG *Vibrio*					
2175	40.5 ± 0.50	23.0 ± 0.50	22.0 ± 0.10	7.5 ± 0.06	4.0 ± 0.02
2540	41.6 ± 0.80	22.0 ± 0.40	20.0 ± 0.40	7.0 ± 0.58	3.7 ± 0.04

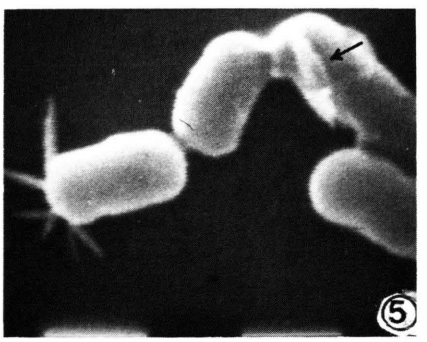
Table IV. Total yield and composition of different membrane preparations of *V. cholerae* (classical) strain. Standard Deviation (s.d.) were calculated from measurements on three independent sets of preparations. Protein, LPS and total phospholipid were expressed as percentage of dry weight of the preparation.

Strains of *V. cholerae* (classical)	Membrane preparation	% of cell dry weight ± s.d.	Protein ± s.d.	LPS[1] ± s.d.	Phospholipid Composition					
					Total yield[2] ± s.d.	Phospholipid type[3]				
						PE	PG	CL	LPE	PS[4]
V. cholerae OGAWA 154	a) crude cell envelope	28 ± 0.25	43 ± 0.50	15 ± 0.40	30 ± 0.50					
	b) outer membrane	9.0 ± 0.50	44 ± 0.60	20 ± 0.50	14 ± 0.92	82 ± 0.30	12 ± 0.10	4 ± 0.18	1.5 ± 0.20	ND
	c) inner membrane	9.8 ± 0.20	53 ± 0.90	–	18 ± 0.30	75 ± 0.50	14 ± 0.60	5 ± 0.20	3 ± 0.15	ND
V. cholerae INABA 569 B	a) crude cell envelope	23 ± 0.90	44 ± 0.90	17 ± 0.39	30 ± 0.50					
	b) outer membrane	9.8 ± 0.50	50 ± 0.95	22 ± 0.50	14 ± 0.92	71 ± 0.60	22 ± 0.40	2.5 ± 0.30	4 ± 0.02	1 ± 0.01
	c) inner membrane	9.2 ± 0.10	46 ± 0.50	–	18 ± 0.30	68 ± 0.28	20 ± 0.70	5 ± 0.10	3 ± 0.15	1.8 ± 0.05

[1] LPS was quantified by determing the heptose content of the various membrane preparations and from the known amount of heptose present in purified LPS isolated from crude cell envelope and also the estimation of KDO (a measure of LPS) as described in the text.
[2] Total phospholipids were quantified by multiplying the amount of extractable lipid phosphate (as described in the text) by 700, which was taken to be the average molecular weight of phospholipids.
[3] Phospholipid type, as % W/W of total lipid-P (as described in the text).
[4] ND: Not detected.







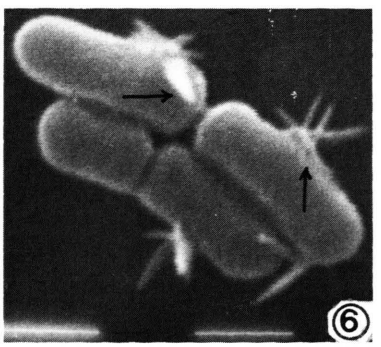


Fig. 3–6. Scanning Electron micrographs of *Vibrio* cells after treatment with PB for 15 min at 37 °C. Fig. 3–6 show *V. cholerae* (classical strain OGAWA 154 treated with PB concentrations, 0, 3.0, 4.5 and 6.0 μg/ml, respectively. Fig. 3 shows the untreated cells with smooth surface structures. The blebs (arrow heads) become more numerous with higher concentrations of PB (Fig. 5 and 6). Fig. 4 shows the surface of the bacterial cell densely covered by numerous projections (long thick arrow). At the higher concentration of 6.0 μg/ml PB (Fig. 6), protruded structures as crenations are observed from various surface points (arrows). White bar represents 1 μm.

crenations per cell was observed corresponding to a dose of 6–10 μg/ml of PB for the classical strains. Frequency distributions of a number of crenations for varying doses of PB (μg/ml) for the classical strains are shown in the Fig. 7. The surface of the bacterial cells seem to be densly covered by projections (long thick arrow in Fig. 4–6).

TEM of PB-treated Vibrio cells

Transmission electron microscopy of ultrathin sections of treated and untreated cells also reveal noticeable changes on the cell surfaces. For the sake of brevity, we have presented here the pictures of only the most sensitive strain, namely OGAWA 154 classical *Vibrio* (Fig. 8–10). Un-

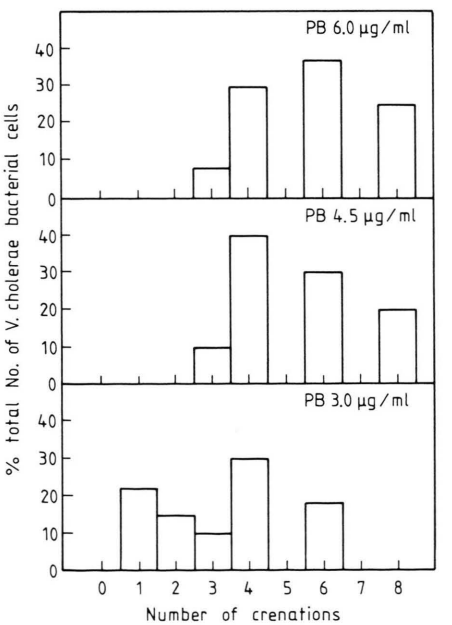
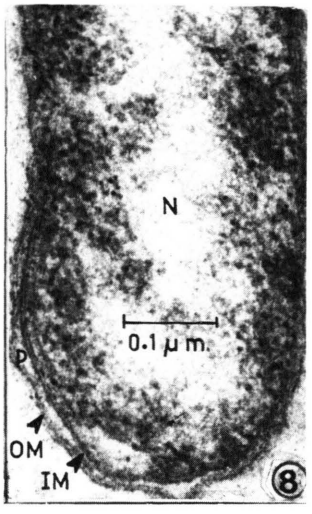
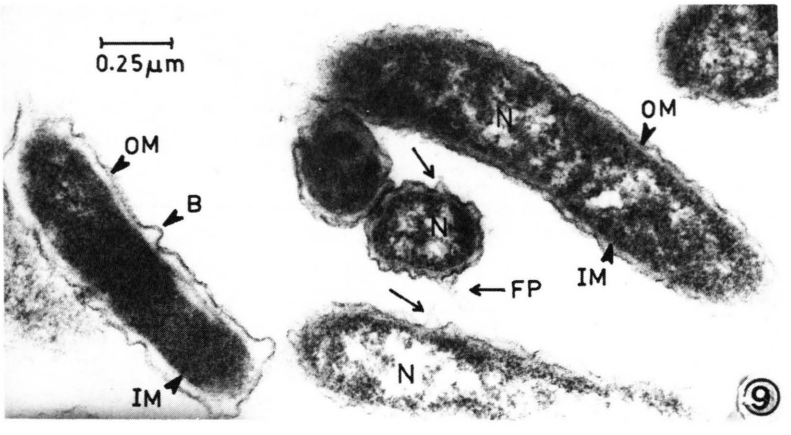


Fig. 7. Frequency distribution of the number of crenations per cell for *V. cholerae* (classical strains OGAWA 154 treated with PB.





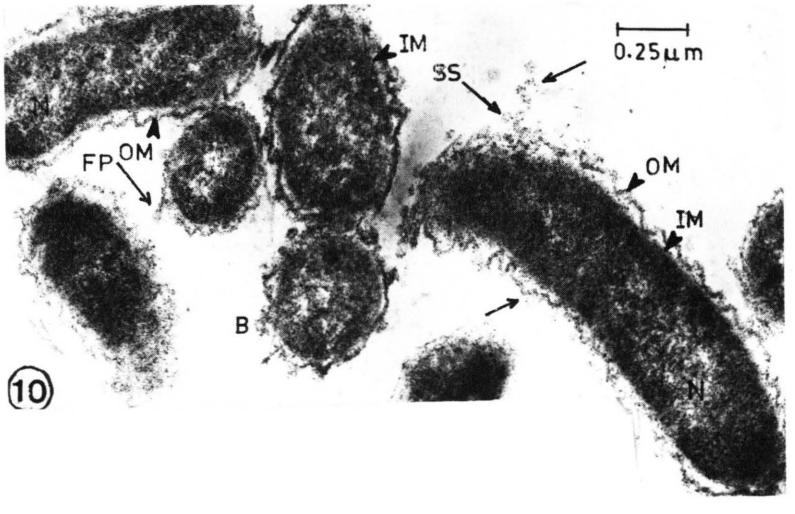


Fig. 8–10. Transmission Electron Micrographs of ultra-thin section of *V. cholerae* (classical) OGAWA 154. Fig. 8 is of the untreated cell with higher magnification. The outer and inner membrane of the cell envelope of the untreated cell can be distinctly observed. Fig. 9–10 are of the cells after treatment for 15 min at 37 °C with PB concentrations 3.0 and 4.5 μg/ml respectively. Fig. 9 (with PB concentration 3.0 μg/ml) shows that the origin of finger-like projection (FP, arrow) is at the outer membrane (OM) of the cell envelope. Fig. 10 (PB conc. 4.5 μg/ml) shows blebs (B), finger like projections (FP, arrows) and saclike structures (SS, arrows), which seem to be peeled off from the OM. All TEM photographs contain a micron bar. OM = Outer Membrane; IM = Inner Membrane; CW = Cell Wall; SS = Saclike Structure; N = Nuclear Material; P = Periplasm.

treated bacterial cells always show smooth surfaces (Fig. 8) whereas numerous projections as blebs and crenations on the OM of the cells are observed when the cells are treated with subinhibitory doses of PB (Fig. 9–10). These projections become more numerous and longer with the increasing concentrations of PB. Also small sac-like structures (LPS aggregation at OM) appear after PB treatment (Fig. 10). At the concentrations above MIC (*e.g.* 7, 30 μg/ml) of PB, numerous projections appear in the OM while cytoplasmic contents are liberated through the cracks in the cell envelope and the leakiness of the IM becomes clearly visible. No significant lysis of cells or leakage of cellular contents can be detected at the subinhibitory doses of PB.

Discussion

On the basis of these results, we may draw the following conclusions.

(1) Percentage survival and percentage lysis of the cells after the treatment with PB can be directly correlated with the acidic phospholipid content of the whole cells and that of the outer membrane structure. The same feature is confirmed also from the morphological studies through SEM and TEM. This shows that the receptor sites for PB-binding are most likely the acidic Phospholipids of the outer membrane structure.

(2) Treating the cells with varying doses of subinhibitory concentrations of PB, appearance of blebs and crenations are observed on outer-membrane surface of the cell. However, the response of all vibrios are not the same. In order of their sensitivities to PB, vibrios (four biotypes) can be arranged as follows: Classical, *Parahaemolyticus,* El Tor, NAG *Vibrio*. The most sensitive was the OGAWA 154 Classical Strain and the least sensitive was the NAG *Vibrio* 2175 strain. Moreover the number of blebs and crenations on the cell surfaces increases with the increasing dose of PB.

(3) It has been observed in the species like *Escherichia coli* that PB binds with the lipid A of the OM which contains the "O" antigens leading to reduction of the lethal endotoxic activity [6, 32–35]. It has been shown previously [12, 33] that LPS vesicles are broken down by the action of PB. Sheet-like structures of LPS are also found to be fragmented after PB-treatment. Though the toxicity of PB is well known and its therapeutic doses are not yet systematically worked out [36] still from the present study one may observe that PB is a good anti-*Vibrio* antibiotic. We may therefore hope, that even for the *Vibrio cholerae* cells, PB might bind with the lipid A and inactivate the antigens to reduce the choleragenic effects, which are induced by the cholera toxins of *Vibrios* [2, 37, 38] as also in higher doses the administration of PB can be effective in killing the bacterial cells through lysis.

Acknowledgement

Thanks are due to the Regional Sophisticated Instrumental Centre, Bose Institute, Calcutta for providing facilities for both scanning and transmission electron microscopy and also for ultramicrotomy. I am indebted to Dr. G. C. Majumder, Dr. Mukul Basu, Dr. J. Das and their colleagues as well as the staff of the Instruments Section of Indian Institute of Chemical Biology, Jadavpur, Calcutta. Lastly author is grateful to Prof. Binayak Duttaroy and Dr. K. Bhaumick of Saha Institute of Nuclear Physics in giving valuable suggestions.

[1] J. C. Feeley, World Health Organization Public Health Papers, no. 40, Geneva, 23–27 (1970).
[2] D. Barua and W. Burrows, The Cholera, pp. 1–500, W. B. Saunders Company, Philadelphia 1974.
[3] M. Teuber and R. I. Miller, Biochim. Biophys. Acta **467,** 280–289 (1977).
[4] B. R. Srinivasa and L. K. Ramachandran, J. Scient. Ind. Res. **38,** 695–709 (1979).
[5] D. R. Storm, K. S. Rosenthal, and P. E. Swanson, Ann. Rev. Biochem. **46,** 723–763 (1977).
[6] B. Lugtenberg, L. V. Alphen, Biochim. Biophys. Acta **737,** 51–115 (1983).
[7] M. Vaara and T. Vaara, Antimicrob. Agents Chemother. **24,** 114–122 (1983).
[8] H. E. Gilleland, F. R. Champlin, and R. S. Conrad, Can. J. Microb. **30,** 869–873 (1984).
[9] P. Kubesch, J. Boggs, L. Luciano, G. Maass, and B. Tummler, Biochemistry **26,** 2139–2149 (1987).
[10] M. Koike, K. Iida, and T. Matsuo, J. Bacteriol. **97,** 443–452 (1969).
[11] K. Koike and K. Iida, J. Bacteriol. **108,** 1402–1411 (1971).
[12] K. Launatmaa, P. H. Makela, and M. Sarvas, J. Bacteriol. **127,** 1402–1407 (1976).
[13] K. Launatmaa and N. Naninga, J. Bacteriol. **128,** 665–667 (1976).
[14] D. A. Weber, M. J. Nadakavukaren, and J. C. Tsang, J. Antibiotics **31,** 732–735 (1978).
[15] M. Vaara, T. Vaara, and M. Sarvas, J. Bacteriol. **139,** 664–667 (1979).
[16] T. K. Mandal and S. N. Chatterjee, Z. Naturforsch. **39c,** 776–781 (1984).
[17] T. K. Mandal, Electron microscopic study of the polymyxin treated *Vibrio cholerae* cells. Presented at Ann. Conf. Electron Microscopic Society of India, 18th, Oct. 4–6, Shillong, India, Abstract, 1988.
[18] T. K. Mandal and S. N. Chatterjee, J. Antibiotics **33,** 328–333 (1980).
[19] A. Lohia, A. N. Chatterjee, and J. Das, J. Gen. Microb. **130,** 2027–2033 (1984).
[20] A. Lohia, S. Mazumdar, A. N. Chatterjee, and J. Das, J. Bacteriol. **163,** 1158–1166 (1985).
[21] T. Kasai and J. Y. Homma, J. Antibiotics **35,** 343–348 (1962).
[22] J. H. Watson and C. N. Sun, Scanning electron microscopy of human – jejunum in whipple's disease and the evaluation of its treatment SEM, **Vol. 2,** pp. 623–632, SEM Inc., AMF O'Hare IL 60666, 1978.
[23] L. P. Watson, A. E. McKee, and R. R. Merrel, SEM, **Vol. 2,** pp. 45–56, SEM Inc., AMF O'Hare IL 60666, 1980.
[24] A. R. Spurr, J. Ultrastruct. Res. **26,** 31–43 (1969).
[25] F. S. Sjostrand, The Electron Microscopy of Cells and Tissues (F. S. Sjostrand, ed.), **Vol. 1,** pp. 138–187, Academic Press, New York 1967.
[26] M. A. K. Markwell, S. M. Hass, L. L. Bieber, and N. E. Tolbert, Anal. Biochem. **87,** 206–210 (1978).
[27] C. Galanos, O. Luderity, and O. Westphal, Eur. J. Biochem. **9,** 245–249 (1969).
[28] M. Dubois, K. A. Gillies, J. K. Hamilton, P. A. Robers, and F. Smith, Anal. Chem. **28,** 350–356 (1956).
[29] B. E. Wright and P. A. Robers, Anal. Biochem. **49,** 307–319 (1972).
[30] Y. D. Karkhanis, Y. J. Zeittner, J. J. Jackson, and D. J. Carlo, Anal. Biochem. **85,** 595–601 (1978).
[31] M. Vaara and T. Vaara, Antimicrob. Agents Chemother. **24,** 107–113 (1983).
[32] M. Teuber and J. Bader, Arch. Microb. **109,** 51–58 (1976).
[33] J. Lopes and W. E. Inniss, J. Bacteriol. **100,** 1128–1130 (1969).
[34] D. Rifkind, J. Bacteriol. **93,** 1463–1464 (1967).
[35] M. Schindler and M. J. Osborn, Biochemistry **18,** 4425–4430 (1979).
[36] K. Bartman, Antimikrobielle Chemotherapie, pp. 143–148, Springer Verlag, Berlin 1974.
[37] W. Burrows, Ann. Rev. Microb. **5,** 181–189 (1981).
[38] J. F. Miller, J. J. McKalanos, and S. Falkow, Science **243,** 916–922 (1989).