Isolation and Characterization of the Outer Membrane from *Vibrio parahaemolyticus*

By TETSURO KOGA AND TOMIO KAWATA

Department of Food Microbiology, Tokushima University School of Medicine, Tokushima 770, Japan

(Received 3 February 1983; revised 5 May 1983)

The outer membrane of *Vibrio parahaemolyticus* strain 3283-61 (serotype O2:K3) was isolated from blebs released upon spheroplast formation, in the presence of lysozyme and EDTA, by isopycnic sucrose density gradient centrifugation. SDS-PAGE of the outer membrane fraction prepared from cells grown in nutrient broth containing 3% (w/v) NaCl revealed five major proteins, designated a to e, with apparent approximate molecular weights: a, 44000; b, 36000; c, 33500; d, 26500; e, 22000. An increase in NaCl concentration in the growth medium resulted in an increase of proteins b and c, whereas a decrease to 0.5% (w/v) induced two additional major proteins with respective molecular weights of about 35000 and 32000. Proteins a and b appeared to be loosely associated with the peptidoglycan layer since they were largely retained after extraction with 2% (w/v) SDS at 50 °C for 30 min. Proteins c and/or e may play a role in phage VP1-receptors since phage-resistant mutants derived from strain 3283-61 had significantly diminished amounts of both proteins. The major outer membrane proteins varied in number and molecular weight in strains of *V. parahaemolyticus* belonging to different K-serotypes.

INTRODUCTION

*Vibrio parahaemolyticus* is a slightly halophilic bacterium which resides in the near-shore marine environment and causes food poisoning associated with the consumption of seafoods (Miwatani & Takeda, 1976; Blake *et al.*, 1980). It is likely that exposed surface components, lipopolysaccharide (LPS) and outer membrane proteins, play an important role in the interaction of the vibrio and the host during infection and may also affect the ecological distribution of the vibrio. Recently, Hisatsune *et al.* (1980, 1981) have comprehensively investigated the somatic O-antigen, LPS, of *V. parahaemolyticus* and concluded that 2-keto-3-deoxyoctonic acid (KDO) is absent in the LPS of the vibrio with the exception of the serotype O6. However, little is known of the outer membrane proteins of *V. parahaemolyticus* although studies with it and other members of the genus *Vibrio* have reported that cell envelopes contain several major proteins with molecular weights ranging from 25000 to 50000 (Deneke & Colwell, 1973; Manning *et al.*, 1982).

This paper describes a procedure for isolating the cytoplasmic and outer membranes of *V. parahaemolyticus*, and presents data showing chemical and morphological characterization of the outer membrane. We also report on the response of the outer membrane proteins to NaCl concentration in the growth medium and on the role of the protein species in receptor activity for bacteriophage VP1 which has been isolated from seawater (Koga *et al.*, 1982).

METHODS

*Bacteria and growth.* Strains 3283-61 (serotype O2:K3) and EB101 (O1:K1), and nine K-serotype pilot strains K-3 (O2:K3), K-11 (O4:K11), K-20 (O8:K20), K-29 (O3:K29), K-36 (O11:K36), K-42 (O4:K42), K-45 (O3:K45), K-46 (O6:K46) and K-47 (O5:K47) of *V. parahaemolyticus* were used in this study. All the strains,
except strain 3283-61 which has been kept in our laboratory for many years, were supplied from the Research Institute for Microbial Diseases, Osaka University. Bacteria were routinely grown to exponential phase in nutrient broth containing 3% (w/v) NaCl (3% NaCl broth) by incubation with vigorous shaking at 37 °C for 4 h as described previously (Koga & Kawata, 1981). Cells were also grown in nutrient broth supplemented with 0.5 and 7% (w/v) NaCl for comparative experiments.

Isolation and purification of outer and cytoplasmic membranes. Bacteria were harvested by centrifugation at 3000 g for 10 min, washed with 0.8 M sucrose in 50 mM-Tris/HCl buffer (pH 8.0), and incubated at 37 °C for 30 min in the same buffer containing 0.6 M sucrose, 100 μg lysozyme ml−1 (Seikagaku Kogyo Co., Tokyo) and 2.5 mM-EDTA (SF medium). The resulting spheroplasts were collected by centrifugation at 8000 g for 15 min. The supernatant solution which contained large amounts of blebs released from spheroplasts was centrifuged at 30000 g for 30 min, and the pellet was used as the crude outer membrane preparation. The remaining spheroplasts were disrupted osmotically in 20 mM-Tris/HCl buffer (pH 7.2) containing 5 mM-MgCl₂ and 3 μg DNAase I ml⁻¹ (Worthington Biochemical Co.) at 37 °C for 30 min with intermittent pipetting, and then cytoplasmic membrane-rich fragments were recovered by centrifugation at 30000 g for 30 min. The crude outer and cytoplasmic membrane preparations were fractionated by isopycnic centrifugation on stepwise gradients on 37 to 55% (w/w) sucrose according to the method of Mizuno & Kageyama (1978). The crude membrane preparations were suspended in 0.5 ml distilled water, layered on top of the gradients and centrifuged at 170000 g for 4 h in a Hitachi RPS-40 rotor. Fractions (six drops each) were collected from the bottom of the tube by aspiration with a thin plastic tube, and assayed for protein by measuring A280. Appropriate fractions were pooled and washed twice with distilled water by centrifugation.

Preparation of cell envelopes. Bacteria were suspended in 3% (w/v) NaCl solution, and disrupted by sonication with a Kubota insonator model 200M at 200 W for 15 min at about 4 °C. The unbroken cells were removed by centrifugation at 3000 g for 5 min, and the supernatant solution was centrifuged at 30000 g for 30 min. The pellet was washed twice with distilled water and used as the cell envelope preparation.

Extraction of cell envelopes with SDS. The cell envelope preparation was treated with 2% (w/v) SDS solution in 10% (v/v) glycerol and 10 mM-Tris/HCl buffer (pH 8.0) at various temperatures (30 to 60 °C) for 30 min. The insoluble material was then recovered by centrifugation at 100000 g for 60 min, and washed once with distilled water.

SDS-PAGE. Membrane preparations (about 20 μg protein) were solubilized by heating at 100 °C for 5 min and subjected to SDS-PAGE according to Laemmli (1970), using a separating gel of 14% (w/v) acrylamide. Proteins were detected with Coomassie brilliant blue as described by Fairbanks et al. (1971). Bovine serum albumin (Sigma), ovalbumin (ICN Pharmaceuticals), alcohol dehydrogenase from yeast, cytochrome c and chymotrypsinogen (Boehringer Mannheim) were used as molecular weight marker proteins. Protein was determined by the Lowry method with bovine serum albumin as a standard.

Enzyme assays. Activities of succinate dehydrogenase and NADH oxidase were measured as described by Mizuno & Kageyama (1978).

Preparation of phage stock. Phage stock of VP1, one of the group I phage having a hexagonal head and a tail with a contractile tail sheath, was prepared as described previously (Koga et al., 1982). Phages were propagated for 5 h at 30 °C in an exponential phase culture of strain 3283-61 in 3% NaCl broth. Unlysed cells were removed by centrifugation at 3000 g for 10 min and the supernatant containing phages was used for assay of receptor activity.

Isolation of phage-resistant mutants from strain 3283-61. Five resistant mutants P1R1, P1R2, P1R3, P1R4, and P1R5 to phage VP1 were spontaneously and independently isolated from strain 3283-61 by plating about 10⁸ cells ml⁻¹ in 3% NaCl broth with about 10⁶ p.f.u. of phage VP1 on 3% NaCl broth containing 0-6% (w/v) agar. After overnight incubation at 30 °C, phage-resistant colonies were picked up and selected by three successive single-colony isolations.

Assay of receptor activity of outer membranes for phage VP1. The outer membrane fraction (0.5 mg of protein) to be assayed was incubated with 2 × 10⁵ p.f.u. of phage VP1 at 30 °C in 1 ml 3% NaCl broth. After incubation for 20 min, a sample (0-1 ml) was withdrawn and diluted with 100 volumes of 3% NaCl broth to stop further adsorption of the phage. A portion (0-1 ml) was plated to determine the plaque-forming ability, using strain 3283-61 exponentially grown in 3% NaCl broth as an indicator. Receptor activity was expressed as the ability to inactivate the phage.

Electron microscopy. Negative staining was performed with 1% (w/v) ammonium molybdate in 0.1 M-ammonium acetate buffer (pH 7.0). Thin sections were prepared as described previously (Masuda & Kawata, 1979). Samples were prefixed with 2.5% (v/v) glutaraldehyde in 50 mM-phosphate buffer (pH 7.2), fixed with 1% (w/v) osmium tetroxide in Kellenberger and Ryter's veronal-acetate buffer (Kellenberger et al., 1958), and then treated with 0.5% (w/v) uranyl acetate in the veronal-acetate buffer. After dehydration through an alcohol series, the samples were embedded in styrene-methacrylate, cut with glass knives, and poststained with lead citrate. Specimens were examined with a Hitachi HU-11E electron microscope operating at an accelerating voltage of 75 kV.
RESULTS

Morphology of *V. parahaemolyticus*

A thin-section profile of exponentially growing cells of *V. parahaemolyticus* strain 3283-61 possessing a multilayered cell envelope typical of Gram-negative bacteria is shown in Fig. 1 (a). The outer membrane had an undulating appearance of a triple-layered structure about 80 nm in thickness. The cytoplasmic membrane also consisted of a similar triple-layered membrane and directly surrounded the cytoplasm. The peptidoglycan layer of the cell wall was not discernible in the thin section.

When a suspension of exponential phase cells which had been washed with hypertonic solution was treated with lysozyme-EDTA, the outer membrane was almost entirely peeled off from the cell surface and rolled up to form blebs (Fig. 1 b). The blebs having the triple-layered structure were subsequently released into SF medium during spheroplast formation.

Isolation and composition of outer and cytoplasmic membranes

Isolation and purification of the two crude membrane fractions obtained from spheroplasts of *V. parahaemolyticus* strain 3283-61 were achieved by isopycnic sucrose density gradient centrifugation (Fig. 2 a, b). The crude outer and cytoplasmic membrane preparations were fractionated into two (OM1 and OM2) and three (CM1, CM2 and CM3) fractions, respectively.

The protein composition of the membrane fractions was determined by SDS-PAGE (Fig. 3). The OM fractions exhibited a simpler profile containing five major bands and several minor bands. The five major proteins were referred to as proteins a, b, c, d and e with approximate molecular weights of 44000, 36000, 33500, 26500 and 22000, respectively. An additional protein band with a molecular weight of about 46000 could be resolved occasionally in the region of protein a (Fig. 3, lane 5). Proteins a, b and c were more abundant than proteins d and e. On the other hand, the CM fractions consisted of more than fifty protein bands.

Succinate dehydrogenase and NADH oxidase activities were predominantly located in the CM fractions (Table 1), whereas almost no activities of the enzymes were detected in the OM fractions, confirming the purity of the fractions since both enzymes are located in the cytoplasmic membrane (Osborn *et al.*, 1972). The CM3 fraction had the highest NADH oxidase activity, whereas the OM1 fraction the lowest. Furthermore since SDS-PAGE showed that the CM1 and CM2 fractions contained appreciable amounts of outer membrane proteins (Fig. 3), the OM1 and CM3 fractions were routinely used as the purified OM and CM fractions, respectively.

Electron microscopy of negatively stained preparations showed that the OM fraction contained smaller vesicles of relatively homogeneous size (about 0.15 to 0.2 μm in diameter; Fig. 4a), whereas the CM fraction consisted of relatively irregular vesicles of larger sizes (about 0.2 to 0.4 μm in diameter; Fig. 4b). Thin sections revealed that both the OM and CM fractions were composed of empty vesicles of various sizes bounded by a triple-layered membrane (Fig. 4c, d). However, the OM vesicles were smaller and had a more regular appearance than the CM vesicles.

Influence of NaCl concentration in the growth medium on the outer membrane protein composition

Since *V. parahaemolyticus* grows well in NaCl, the optimal concentration being about 3% NaCl (w/v), but is unable to grow in the presence of 10% (w/v) NaCl or in the absence of NaCl (Miwatani & Takeda, 1976) we examined the effect of NaCl concentration on the outer membrane protein composition of strain 3283-61. Cells grew most rapidly in 3% NaCl broth, but lower growth rates were observed in 7% NaCl broth and 0.5% NaCl broth. SDS-PAGE profiles of the OM fractions isolated from cells grown exponentially in nutrient broth containing different concentrations of NaCl are shown in Fig. 5. The major protein profile from cells grown in 7% NaCl broth was similar to that from cells grown in 3% NaCl broth except for the enrichment of proteins b and c and the reduction of protein e. However, on growth in 0.5% NaCl broth two additional major proteins with molecular weights of about 35000 and 32000 were produced and protein e was greatly diminished.
Fig. 1. Thin sections of cells of *V. parahaemolyticus* strain 3283-61. (a) Cells were grown in 3% NaCl broth at 37 °C for 4 h. The cell envelope consists of the outer membrane (OM) and cytoplasmic membrane (CM). Peptidoglycan layer is not discernible in the micrograph. (b) Cells grown similarly were washed with 0.8 M-sucrose in 50 mM-Tris/HCl buffer (pH 8.0) and incubated in SF medium at 37 °C for 20 min (see Methods). The outer membrane is peeled off from the surface of the cell to form numerous blebs and released into the medium. The bar markers represent 100 μm.

Extraction of outer membrane proteins with SDS

The presence of peptidoglycan-associated proteins in the outer membrane of strain 3283-61 was examined by extracting the cell envelope preparation with 2% (w/v) SDS solution in 10% (v/v) glycerol and 10 mM-Tris/HCl buffer (pH 8.0) at various temperatures for 30 min (Fig. 6). The SDS-PAGE profile of the untreated cell envelope preparation was quite similar to that of
Outer membrane of *V. parahaemolyticus* 3189

Fig. 2. Profiles of isopycnic sucrose density gradient centrifugation of crude membrane preparations from *V. parahaemolyticus* strain 3283-61. Fractions were combined as indicated by the bars. (a) Crude outer membrane preparation obtained from supernatant of SF medium after spheroplast formation. (b) Crude cytoplasmic membrane preparation obtained from spheroplasts by osmotic disruption. Absorbance at 280 nm (○); total activity of succinate dehydrogenase (●). The arrows correspond to fractions subjected to SDS-PAGE shown in Fig. 3.

![Fig. 2](image)

Fig. 3. SDS-PAGE of membrane fractions prepared by isopycnic sucrose density gradient centrifugation. Lanes 1 to 12 show protein composition of the membrane fractions indicated by the arrows in Fig. 2. Lanes 1 to 5, OM fractions; lanes 6 to 12, CM fractions.

![Fig. 3](image)
Table 1. Localization of enzyme activities in membrane fractions from strain 3283-61.
Membrane fractions were prepared from strain 3283-61 and assayed as described in Methods.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity [nmol min⁻¹ (mg protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OM1</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>0.001</td>
</tr>
<tr>
<td>NADH oxidase</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Fig. 4. Electron micrographs of isolated membrane fractions from V. parahaemolyticus strain 3283-61.
Negatively stained preparations: (a) OM1 fraction; (b) CM3 fraction. Thin sections of membrane fractions: (c) OM1 fraction; (d) CM3 fraction. The bar markers represent 100 nm.
Fig. 5. SDS-PAGE of outer membranes prepared from strain 3283-61 grown in nutrient broth containing different concentrations of NaCl. Lanes: 1, 3% (w/v) NaCl; 2, 0.5% (w/v) NaCl; 3, 7% (w/v) NaCl.

Fig. 6. SDS-PAGE of cell envelopes from strain 3283-61 after extraction with SDS at various temperatures. The cell envelope preparation was extracted with 2% (w/v) SDS solution for 30 min at various temperatures, and the soluble and insoluble fractions were separated as described in Methods. Before electrophoresis all samples were heated at 100 °C for 5 min. Lanes: OM fraction not treated (1), cell envelope preparation not treated (2), soluble fraction at 40 °C (3), and insoluble fractions from envelope preparations heated at 30 °C (4), 40 °C (5), 50 °C (6) and 60 °C (7).

the OM fraction. When treated at 40 °C, most of proteins d and e together with many minor proteins were solubilized, but proteins a, b and c were found in both the soluble and insoluble fractions. When extracted at 50 °C, proteins a and b were still largely retained in the insoluble fraction, but protein c was greatly reduced. At 60 °C almost all the proteins were solubilized. From these results it seems likely that both proteins a and b are loosely associated with the peptidoglycan layer as in the case of *Pseudomonas aeruginosa* (Mizuno & Kageyama, 1979).

Receptor activity of outer membranes from strain 3283-61 and its resistant mutants for phage VP1

The OM fractions isolated from the parent strain 3283-61 and mutants resistant to phage VP1 were examined for phage receptor activity. Electron microscopy revealed that many phage particles were adsorbed on the outer membrane vesicle from the parent strain and that most of their tails were contracted (Fig. 7). At the later stage of the adsorption most of the phage heads became empty, ejecting their DNA. Phage VP1 particles were completely inactivated by the OM fraction prepared from the parent strain, but were weakly inactivated by those from the resistant mutants (Table 2), indicating that the outer membrane of the resistant mutants lost receptor activity for the phage.

**SDS-PAGE of outer membranes from phage VP1-resistant mutants**

The phage VP1-resistant mutants were compared with the parent strain 3283-61 with respect to the major outer membrane proteins, by subjecting the OM fractions to SDS-PAGE (Fig. 8).
Table 2. Receptor activity of outer membrane fractions from strain 3283-61 and its phage VP1-resistant mutants

The assay of the receptor activity was carried out as described in Methods.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phage survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>34</td>
</tr>
<tr>
<td>P1R1</td>
<td>68</td>
</tr>
<tr>
<td>P1R2</td>
<td>95</td>
</tr>
<tr>
<td>P1R3</td>
<td>53</td>
</tr>
<tr>
<td>P1R4</td>
<td>100</td>
</tr>
<tr>
<td>P1R5</td>
<td>84</td>
</tr>
</tbody>
</table>

Proteins c and e were significantly diminished in all the mutants. Receptor activity for the phage appeared to vary roughly in proportion to the amount of the two proteins. These results suggest that protein c and/or e may play a role in receptor activity for phage VP1. The region of protein a band in the mutants was separated more distinctly into two protein bands, protein a and an additional protein with a molecular weight of about 46000, than that in the parent strain, probably due to the relative reduction of protein a.

Comparison of the outer membrane proteins of V. parahaemolyticus strains

Vibrio parahaemolyticus is classified into a number of serotypes including 12 O groups and 59 K types on the basis of serological identifications (Miwatani & Takeda, 1976; Blake et al., 1980). We investigated the outer membrane protein profiles of eleven strains of the vibrio belonging to ten different K types using SDS-PAGE of the OM fraction (Fig. 9). Protein a and its close-neighbouring protein with a molecular weight of about 46000 seemed to be common among all the strains with a few exceptions (strain EB101 and K-pilot strains K-42 and K-45). There was considerable heterogeneity in the region between proteins b and c. A major protein with a molecular weight ranging from 34000 to 37000 was dominant in K-pilot strains K-3, K-11, K-20
Fig. 8. SDS-PAGE of outer membranes prepared from phage VP1-resistant mutants derived from strain 3283-61. Lanes: parent strain 3283-61 (1) resistant mutants P1R1 (2), P1R2 (3), P1R3 (4), P1R4 (5) and P1R5 (6).

Fig. 9. SDS-PAGE of outer membranes prepared from various strains of V. parahaemolyticus grown in 3% NaCl broth. Lanes: strains 3283-61 (1) and EB101 (2), and K-pilot strains K-3 (3), K-29 (4), K-45 (5), K-11 (6), K-42 (7), K-47 (8), K-46 (9), K-20 (10) and K-36 (11).
and K-47 and strain EB101 instead of protein b. Protein c was rather low in all the strains except the two strains of serotype K-3, and an additional protein with a molecular weight of about 32000 was found in several K-pilot strains. Protein d with a molecular weight ranging from 25000 to 26500 gave a weak band in all the strains, whereas protein e was present only in a few strains. Thus, the major outer membrane proteins varied in number and molecular weight in strains of *V. parahaemolyticus* belonging to different K-serotypes.

**DISCUSSION**

Wolf-Watz *et al.* (1973) have found that numerous blebs consisting of fragments of the outer membrane were released from plasmolysed cells of *Escherichia coli* during spheroplast formation by treatment with lysozyme and EDTA. This phenomenon was applied to the isolation of the outer membrane from *E. coli* (Mizushima & Yamada, 1975) and *P. aeruginosa* (Mizuno & Kageyama, 1978) and in this work we have applied a similar technique to isolate the outer membrane from *V. parahaemolyticus*. Although the use of EDTA in isolating outer membranes from *P. aeruginosa* (Matsushita *et al.*, 1978), *Proteus mirabilis* (Hasin *et al.*, 1975) and *Vibrio cholerae* (Kabir, 1980) has been shown to lead to altered structure of the outer membrane and early release of some outer membrane proteins, in this study SDS-PAGE of the OM fraction obtained from blebs released upon spheroplast formation was similar to that of the cell envelope preparation obtained by sonic disruption of the cells.

In the present work, SDS-PAGE revealed that the outer membrane of *V. parahaemolyticus* consisted of four to six major proteins with molecular weights ranging from 22000 to 44000, depending on the strain. Protein a (molecular weight, 44000) occurred commonly and predominantly in the vibrio together with a close-neighbouring protein (molecular weight, 46000) with the exception of a few strains. Using cell envelopes from four strains of *V. parahaemolyticus*, Manning *et al.* (1982) also detected a major protein with a molecular weight ranging from 43000 to 46000. Furthermore, it was found that three proteins with molecular weights of 42000, 43000 and 44500 were common to most strains of *V. cholerae* but that the amounts differed vastly among the strains (Manning *et al.*, 1982). On the other hand, the common major protein of *V. cholerae* has also been reported to be a protein with a molecular weight of 48000 (Kabir, 1980) or 45000 (Kelley & Parker, 1981). These discrepancies of protein profiles in the same species probably arise from differences in methodology, cultural conditions and strains. The outer membrane of *Vibrio anguillarum* biotype I contained at least eight major proteins in which the predominant protein had an apparent molecular weight of 38000 (Buckley *et al.*, 1981). Thus, the outer membrane protein composition of *V. parahaemolyticus* appears different from that of *V. cholerae* and *V. anguillarum*.

The composition of outer membrane proteins is affected by changing nutrient constituents in the growth medium in many bacteria (Bassford *et al.*, 1977; Mizuno & Kageyama, 1978; Kelley & Parker, 1981; Crosa & Hodges, 1981). The outer membrane protein profile of *V. parahaemolyticus* was also affected by NaCl concentration in the growth medium. Increasing the NaCl concentration from 3% (w/v) to 7% (w/v) resulted in an increase of proteins b and c, whereas decreasing NaCl to 0.5% (w/v) induced two additional major protein species near proteins b and c, respectively. Although these observations might suggest an adaptive mechanism, other alterations have been associated with changes in the ionic strength of the growth medium. The ratio of saturated to unsaturated fatty acids in *V. parahaemolyticus* grown in medium containing 0-5 to 7-5% (w/v) NaCl increased with elevated incubation temperature, but the ratio was not linear with NaCl concentration (Beuchat & Worthington, 1976). Relative proportions of some protein species and phospholipids in the outer membrane of *Pseudomonas halosaccharolytica*, a moderately halophilic bacterium, were altered with NaCl concentrations in the growth medium (Hiramatsu *et al.*, 1980). Thus, the adaptive mechanisms of the outer membrane in slightly or moderately halophilic bacteria to the wide range of NaCl concentration found in the environment still remain uncertain.

The presence of peptidoglycan-associated proteins in the outer membrane has been well documented in various *Enterobacteriaceae* (Rosenbusch, 1974; Lugtenberg *et al.*, 1977; Hofstra
One or two peptidoglycan-associated proteins with molecular weights ranging from 27,000 to 40,000 remained bound to peptidoglycan after treatment with 2% (w/v) SDS at 60 °C, but were dissociated on boiling. Similarly, two or three major outer membrane proteins of *V. cholerae* are associated with the peptidoglycan (Manning et al., 1982). Two of the major proteins of *P. aeruginosa* were found to be associated with the peptidoglycan after treatment with the detergent at 35 °C but not at 60 °C (Mizuno & Kageyama, 1979). In the present work, proteins a and b of *V. parahaemolyticus* were retained in the insoluble fraction after extraction with SDS at 30 to 50 °C but were mostly solubilized at 60 °C. Therefore, it seems likely that proteins a and b are weakly associated with the peptidoglycan. Although it has been proposed that peptidoglycan-associated proteins span the outer membrane and play a role in the formation of hydrophilic channels through the outer membrane (Lugtenberg et al., 1977), a similar function for proteins a and b cannot be confirmed until these proteins have been purified to homogeneity and fully characterized.

It is clear that outer membrane proteins can act as phage receptors in other species since mutants with missing or decreased amounts of some of the major proteins have been isolated from *E. coli* (Skurray et al., 1974; Henning & Haller, 1975; Hantke, 1978) and *Salmonella typhimurium* (Nurminen et al., 1976). Furthermore, both LPS and outer membrane protein OmpC of *E. coli* K12 were required for the receptor activity for phage T4 (Mutoh et al., 1978; Henning & Jann, 1979; Yu & Mizushima, 1982). Judging from electron microscopy and assay for phage receptor activity, the isolated outer membrane from *V. parahaemolyticus* strain 3283-61 maintained the ability to act as receptor for phage VP1. Proteins c and e were significantly diminished in mutants resistant to phage VP1, suggesting that at least proteins c and/or e may play a role in receptor activity for the phage. On the other hand, LPS of the vibrio may not contribute to the phage receptor since K-pilot strain K-29, belonging to serogroup O3, as the host strain 3283-61 was shown to be completely resistant to the phage (Koga et al., 1982). However, we have not yet determined whether both the outer membrane protein(s) and LPS are necessary for receptor activity for phage VP1.

In conclusion, we have shown a method for isolating the outer membrane of *V. parahaemolyticus* from blebs released upon spheroplast formation and have characterized partially the outer membrane proteins. The outer membrane protein composition of the vibrio is different from that of other *Vibrio* species and it varies with the NaCl concentration in the growth medium. Furthermore, there is considerable heterogeneity in the region between proteins b and c among the strains belonging to different K-serotypes. Proteins a and b seem to be loosely bound with the peptidoglycan layer. However, it remains to be determined whether the proteins are responsible for the formation of permeability channels in the outer membrane. A participation of the outer membrane proteins in the phage receptor has been also inferred on the basis of the results using phage VP1-resistant mutants.

This work was supported in part by a Grant-in-Aid for Scientific Research (no. 57770330) from the Ministry of Education, Science and Culture of Japan. We wish to thank Dr T. Miwatani and Dr Y. Takeda, the Research Institute for Microbial Diseases, Osaka University, for kindly supplying the bacterial strains.

### References


