Effect of Changes in the Osmolarity of the Growth Medium on *Vibrio cholerae* Cells

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The rate and extent of lysis of *Vibrio cholerae* cells under nongrowing conditions were dependent on the osmolarity of the growth medium. Gross alterations in cellular morphology were observed when *V. cholerae* cells were grown in media of high and low osmolarity. The rate of lysis of *V. cholerae* cells under nongrowing conditions increased after treatment with chloramphenicol. Chloramphenicol-treated *V. cholerae* 569B cells showed formation of sphaeroplast-like bodies in medium of high osmolarity, but not in low osmolarity. Changes in the osmolarity of the growth medium also regulated the expression of the outer membrane proteins. This regulation was abolished if *V. cholerae* cells were grown in P_i-depleted medium. Analysis of the lytic behavior and composition of outer membrane proteins of an osmotically fragile mutant strain revealed a similar dependence on the osmolarity of the growth medium.

Vibrio cholerae, the etiological agent of cholera, is a noninvasive, gram-negative pathogen which colonizes the small intestine, presumably by the interaction of specific bacterial cell surface components as well as nonspecific hydrophobic interactions with the intestinal mucosa (12). Excretion of cholera toxin from its cellular location has been shown to be unique to *V. cholerae*, since cholera toxin synthesized in *Escherichia coli* cells was not excreted and accumulated in the periplasmic space as is the heat-labile enterotoxin (LT) of *E. coli* (26). In contrast, when the *E. coli* LT was synthesized in *V. cholerae* cells, the product was exported out (24). It has been postulated that this behavior of *V. cholerae* may be due to an unusual organization of its envelope components (15). Studies with mutants have suggested that changes in the cell surface may also play a role in the virulence of *V. cholerae* (2).

Studies on the composition of the outer membrane proteins (11, 15), lipopolysaccharide (LPS) (29, 30), phospholipids (28), and various surface properties, such as hydrophobicity and charge density (12), of *V. cholerae* cells have revealed differences with other gram-negative enteric bacteria. It has been reported that *V. cholerae* cells are extremely sensitive to protein denaturants (19) and that it is possible to isolate the outer membrane directly from whole cells of this organism, unlike other gram-negative enteric bacteria, by treatment with urea (19). Furthermore, *V. cholerae* cells lyse rapidly in hypotonic medium outside the growth medium (19). The stability of bacteria in a hypotonic medium depends on a mechanically resistant cell wall that gives osmotic protection (9). Various factors, such as growth conditions, osmotic environment, and pH, have been recognized as being particularly important in regulating autolysis (33).

In *E. coli*, three outer membrane proteins—OmpF, OmpC, and PhoE—which are biochemically and genetically similar (20, 23), have been identified as being responsible for the passage of solutes across the outer membrane. Their expression is, however, regulated by growth conditions. The osmolarity of the growth medium is a major factor in determining the relative levels of expression of OmpF and

OmpC proteins (13, 36). The synthesis of the PhoE protein is derepressed upon phosphate starvation (25). It has also been suggested that the rigidity of the outer membrane-peptidoglycan layer might also depend upon the specific major outer membrane protein present (13).

It is in this context that the present report describes the changes in the lytic phenotype, cellular morphology, and composition of the outer membrane of a hypertoxinogenic strain (569B) of *V. cholerae* grown under various osmolarities and in phosphate-depleted (LP) growth medium. The lytic phenotype and cellular morphology were also affected drastically after treatment with chloramphenicol (CAM).

MATERIALS AND METHODS

Bacteria. The wild-type strain *V. cholerae* 569B (prototroph, hypertoxinogenic) of Inaba serotype was obtained from the National Institute of Cholera, Calcutta, India. *E. coli* C600 (SuII) and *Salmonella typhimurium* LT2 were obtained from S. Ghosh, Bose Institute, Calcutta, India. The phage-resistant mutant DC15 of *V. cholerae* 569B was obtained from D. Chakraborti, Bose Institute, Calcutta, India. The mutant strain was highly sensitive to sodium deoxycholate compared with the parent 569B strain.

Media. The *V. cholerae* strains 569B and DC15 were grown in nutrient medium containing 1% (wt/vol) Bacto-peptone (Difco), 1% (wt/vol) Lablemco powder (Oxoid), and 0.18 M NaCl at pH 8.50. This medium will be referred to as plus medium. The "minus" medium is the same as the plus medium, except that no NaCl is added. *E. coli* C600 and *S. typhimurium* LT2 cells were grown in plus and minus media at pH 7.20. LP medium was prepared as described previously (32). The defined medium used was minimal medium A (22) supplemented with 1 mM $MgSO_4$–0.01 mM $CaCl_2$–0.001% vitamin B_1–0.2% glucose–0.01% casein hydrolysate. The NaCl concentration of LP medium and minimal medium A was adjusted as in plus and minus media. Cell viability was assayed as CFU on nutrient agar plates containing 1.5% agar (Difco) with or without 0.18 M NaCl.

Lysis of whole cells. *V. cholerae* 569B and DC15 cells in the logarithmic phase of growth (5×10^8 to 6×10^8 CFU ml^{-1}; absorbance at 585 nm = 0.70) were harvested by centrifu-

gation at 6,000 × *g* and 10°C and suspended in either 0.18 M NaCl–0.36 M sucrose–0.36 M KCl–50 mM sodium phosphate buffer (pH 7.2)–0.36 M glycerol or distilled water. The temperature of incubation in distilled water and 0.18 M NaCl was either 10 or 37°C as described below. Preincubation in NaCl (0.18 or 0.5 M) or MgCl$_2$ (5 mM) was carried out at 37 or 10°C when necessary. *E. coli* C600 and *S. typhimurium* LT2 cells in the logarithmic phase of growth were similarly harvested and suspended in distilled water at 37°C. The volume of the final resuspending buffer or distilled water was adjusted to maintain 5 × 10^8 CFU ml^{-1} (absorbance at 585 nm = 0.70). Lysis was monitored turbidimetrically at 585 nm in a Sicospec 100 spectrophotometer (Scientific Instrument Co., Calcutta India).

Release of UV-absorbing material from whole cells. To measure the release of intracellular acid-soluble material upon suspension of log-phase cells in 0.18 M NaCl, samples were removed at various times during incubation at 10 or 37°C. The cells were centrifuged, and an equal volume of ice-cold 10% trichloroacetic acid was added to the supernatant and centrifuged. The absorbance of the trichloroacetic acid-soluble material in the supernatant was then measured at 260 and 280 nm. An equivalent number of cells was boiled at 100°C for 5 min, and the absorbance at 260 and 280 nm of the supernatant was taken as 100%.

Measurement of osmolarity. The osmolarity of the plus and minus media was measured with the Osmette S automatic osmometer (Precision Systems Inc., Sudbury, Mass.) and expressed as milliosmoles per liter per kilogram.

Isolation of the outer membrane. The outer membrane of *V. cholerae* cells was isolated by treatment with 4 M urea as described previously (19).

Isolation of the peptidoglycan. The peptidoglycan, along with the bound lipoprotein, was isolated after the treatment of whole cells with 4% sodium dodecyl sulfate at 100°C (4).

Analytical methods. Protein was measured by the method of Markwell et al. (21), with bovine serum albumin as the standard. LPS was isolated from crude cell envelope by 45% (wt/vol) aqueous phenol at 68°C (37). Total carbohydrate was assayed by phenol-H$_2$SO$_4$ (6), with D-glucose as the standard. Heptose was estimated by the cysteine-H$_2$SO$_4$ method (38). Rhamnose (5), fructose (31), and phosphate (1) were assayed as previously described. Glucose was assayed after the hydrolysis of LPS with 2 N HCl at 100°C for 2 h by a coupled glucose oxidase-peroxidase reaction (Sigma Glucose Assay Bulletin 510-4). Glucosamine was assayed after the hydrolysis of LPS with 4 N HCl for 4 h at 100°C (34). Phospholipids were extracted by chloroform-methanol-water (1:2:0.8 [vol/vol/vol]) (3) and quantified from the lipid phosphate. Phospholipids were identified by thin-layer chromatography on Silica Gel G (Merck) plates with the solvents chloroform-methanol-ammonia (65:25:4 [vol/vol/vol]) and chloroform-methanol-acetone-acetic acid-water (8:2:2:2:1, [vol/vol/vol/vol/vol/]). The chromatogram was developed in iodine vapor. The individual phospholipids were quantified from the lipid phosphate after elution from the silica gel by chloroform-methanol (2:1 [vol/vol]). All standard sugars and phospholipids were from Sigma Chemical Co.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Outer membrane proteins were analyzed on a 10% sodium dodecyl sulfate-polyacrylamide gel by the method of Laemmli (16).

Electron microscopy. For thin-section electron microscopy, cells were fixed first with 6% glutaraldehyde (BDH, Poole, England) in 0.125 M phosphate buffer (pH 7.20) for 14 to 16 h and then with 1% osmium tetroxide (Ted Pella, Inc.,

Tustin, Calif.) in Kellenburger buffer for 16 to 20 h at room temperature. The fixed cells were washed for 2 h in 0.5% uranyl acetate in the above buffer (14), dehydrated with ascending concentrations of ethanol, and embedded in Spurr medium (Polysciences, Inc., Warrington, Pa.) (35) at 70°C for 48 h. Sections were cut with glass knives on a JEOL ultramicrotome, stained with uranyl acetate and lead citrate, and examined under a JEOL 100C transmission electron microscope at 60 kV.

RESULTS

Lysis of *V. cholerae* 569B cells grown in media of high and low osmolarity. *V. cholerae* 569B cells grown in medium of high osmolarity (390 milliosmoles · liter · kg^{-1}) or plus medium lysed rapidly when harvested and suspended in distilled water at 37°C (Fig. 1a). The presence of impermeable solutes, such as NaCl (0.18 M) (Fig. 1a) or sucrose (0.36 M)–KCl (0.36 M)–50 mM sodium phosphate buffer (pH 7.20), in the suspending medium prevented lysis, whereas permeable solutes such as glycerol (0.36 M) were totally ineffective. The rate of lysis was reduced when cells in the stationary phase of growth were used compared with cells in the exponential phase of growth and when the incubation temperature for both exponential- and stationary-phase cells was lowered to 10°C from 37°C (Fig. 1a).

Under conditions in which the cell viability remained unaltered (cells suspended in 0.18 M NaCl solution and incubated at 37°C), a significant amount of low-molecular-weight, 260-nm-absorbing intracellular material was released (Fig. 1a). The released material was primarily nucleic acid breakdown products, as evidenced from the ratio of absorbance at 260 and 280 nm of 1.9. The leakage of this material was much lower when cells were incubated at 10°C (Fig. 1a). No detectable amount of free reducing or N-terminal groups was released in the supernatant of cells suspended in distilled water containing 0.18 M NaCl, as would be expected if autolytic muramidases or amidases were active.

Preincubation of cells with 5 mM MgCl$_2$ at 0 or 37°C prevented lysis and loss of cell viability upon suspension in distilled water. The effect of Mg^{2+} was irreversible in the sense that cells pretreated with Mg^{2+} ions and then exposed to 0.5 M NaCl were resistant to lysis.

In contrast to cells grown in plus medium, cells grown in medium of low osmolarity (137 milliosmoles · liter · kg^{-1}) or minus medium were comparatively resistant to lysis when suspended in distilled water (Fig. 1b). An initial increase in the absorbance of minus medium-grown cells suspended in 0.18 M NaCl (Fig. 1b) suggested plasmolysis of cells followed by a gradual deplasmolysis. Cell viability remained unchanged under this condition for the 120 min that this reaction was examined in the present study. The susceptibility of cells to lysis was dependent on the concentration of NaCl in the growth medium up to an NaCl concentration of 0.18 M, beyond which there was no further effect. *E. coli* C600 and *S. typhimurium* LT2 cells did not show any appreciable lysis in distilled water when grown in either plus or minus medium.

Electron microscopy of cells grown in plus and minus media showed remarkable differences in the cell morphology. Relative to cells grown in plus medium (Fig. 2a), the cytoplasm of cells grown in minus medium was stained more intensely (Fig. 2b and c) under identical experimental conditions. While the outer membrane of plus medium-grown cells appeared flexible (Fig. 2a), that of minus medium-grown cells was more rigid (Fig. 2b and c). Some of the unusual features of cells grown in minus medium were the
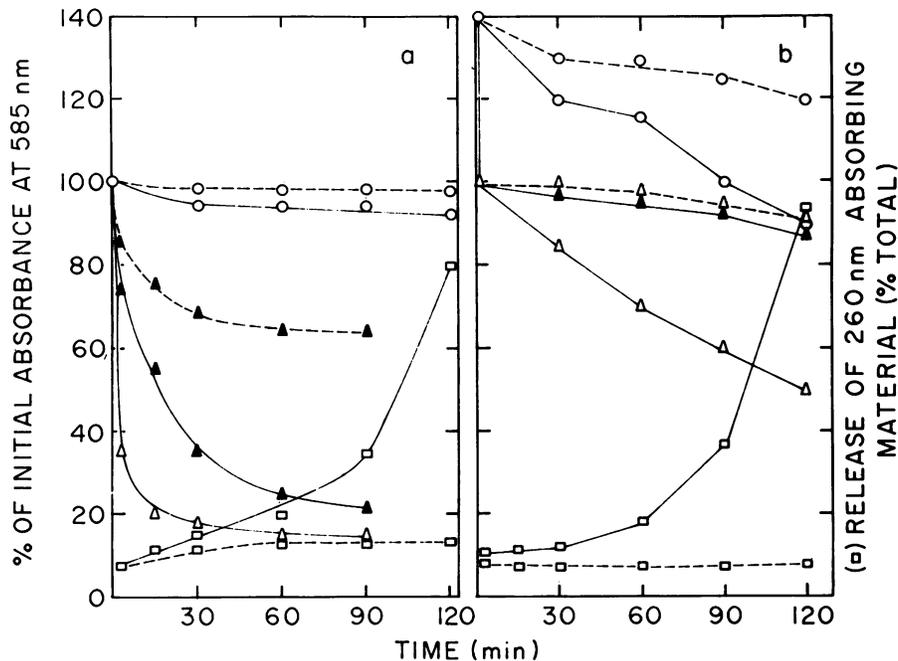
FIG. 1. Lysis of *V. cholerae* cells. *V. cholerae* 569B cells grown in plus (a) and minus (b) media were harvested, suspended in distilled water or 0.18 M NaCl, and incubated at 37 or 10°C. Lysis was monitored by the fall in absorbance at 585 nm, and the release of intracellular material from log-phase cells was measured by the increase in absorbance at 260 nm in the supernatant. The amount of 260-nm-absorbing material released after boiling an equivalent number of cells at 100°C for 5 min was taken as 100%. (O) Log-phase cells in 0.18 M NaCl; (△) log-phase cells in distilled water; (▲) stationary-phase cells in distilled water; and (□) release of 260-nm-absorbing material. Solid lines indicate incubation at 37°C, and broken lines indicate incubation at 10°C.

appearance of large nonstaining bodies in the cytosol and the presence of amorphous regions (Fig. 2c).

When cells grown in minus medium were transferred to plus medium, within 15 min of incubation at 37°C, the wavy structure of the outer membrane was restored, the staining intensity of the cytoplasm was reduced, and the nonstaining bodies disappeared (Fig. 3a). By 30 min of incubation in plus medium, the cell morphology was indistinguishable from that grown in plus medium (Fig. 3b). Cells grown in minus medium and suspended in distilled water containing 0.18 M NaCl showed no such changes in the appearance of the outer membrane and cytoplasm (Fig. 3c). On the contrary, these cells showed extensive plasmolysis.

Effect of sucrose and Mg^{2+} ions in the growth medium on the lysis of *V. cholerae* 569B cells. To investigate whether the lysis of plus medium-grown cells in distilled water was due to the high osmolarity of the growth medium alone or due to the high ionic concentration of the growth medium, *V. cholerae* 569B cells were grown in minus medium supplemented with 0.36 M sucrose, harvested, and suspended in distilled water. It was observed that the rate and extent of lysis in distilled water of *V. cholerae* 569B cells grown in minus medium supplemented with 0.36 M sucrose was identical to that of cells grown in plus medium (cf. Fig. 1a). Furthermore, the susceptibility of cells to lysis in distilled water was dependent on the concentration of sucrose in the growth medium up to a sucrose concentration of 0.4 M, beyond which there was no further effect. Similar results were obtained when cells grown in chemically defined growth medium containing various concentrations of NaCl or sucrose were examined.

It has been suggested that divalent cations bind to outer membrane components to participate in the maintenance of its structural and functional integrity. It was, therefore, necessary to examine whether the observed lysis in distilled

water of plus medium-grown cells was due to a weakening of the outer membrane structure as a result of the replacement of divalent cations such as Mg^{2+} by the preponderant Na^+ in plus medium. *V. cholerae* 569B cells were grown in plus medium, minus medium, and minus medium supplemented with 0.36 M sucrose, each of which was further supplemented with 5 mM $MgCl_2$, harvested in the log phase, and suspended in distilled water at 37°C. Although the net extent of lysis was reduced by 10 to 20% when *V. cholerae* 569B cells were grown in 5 mM $MgCl_2$-supplemented medium compared with the respective growth medium not supplemented with 5 mM $MgCl_2$, no appreciable change in the rate of lysis was observed. These results indicate that although the role of divalent cations cannot be completely ruled out, the observed lysis of *V. cholerae* 569B cells grown in plus medium was primarily due to osmotic destabilization.

Effect of CAM on the lysis of *V. cholerae* 569B cells. In gram-positive bacteria, autolysis is inhibited in the presence of CAM (33), presumably due to the continuous synthesis and thickening of the cell wall even in the absence of protein synthesis. However, no such effect of CAM was observed on the lysis of *E. coli* cells (18). To examine the effect of CAM, *V. cholerae* cells were treated with a 10 μg ml^{-1} concentration of CAM in both plus and minus media. CAM-treated cells in plus medium showed an increased rate of lysis in 50 mM phosphate buffer (pH 7.20) compared with untreated cells. The extent of lysis was dependent upon the time of CAM treatment (Fig. 4a). A similar effect was observed when minus medium-grown cells were treated with CAM (Fig. 4b). In these cells, lysis was monitored in distilled water instead of 50 mM phosphate buffer. Interestingly, CAM was seen to act as a bactericidal agent for *V. cholerae* 569B cells growing in plus medium (more than 99% killing in 120 min) but was bacteriostatic for cells growing in
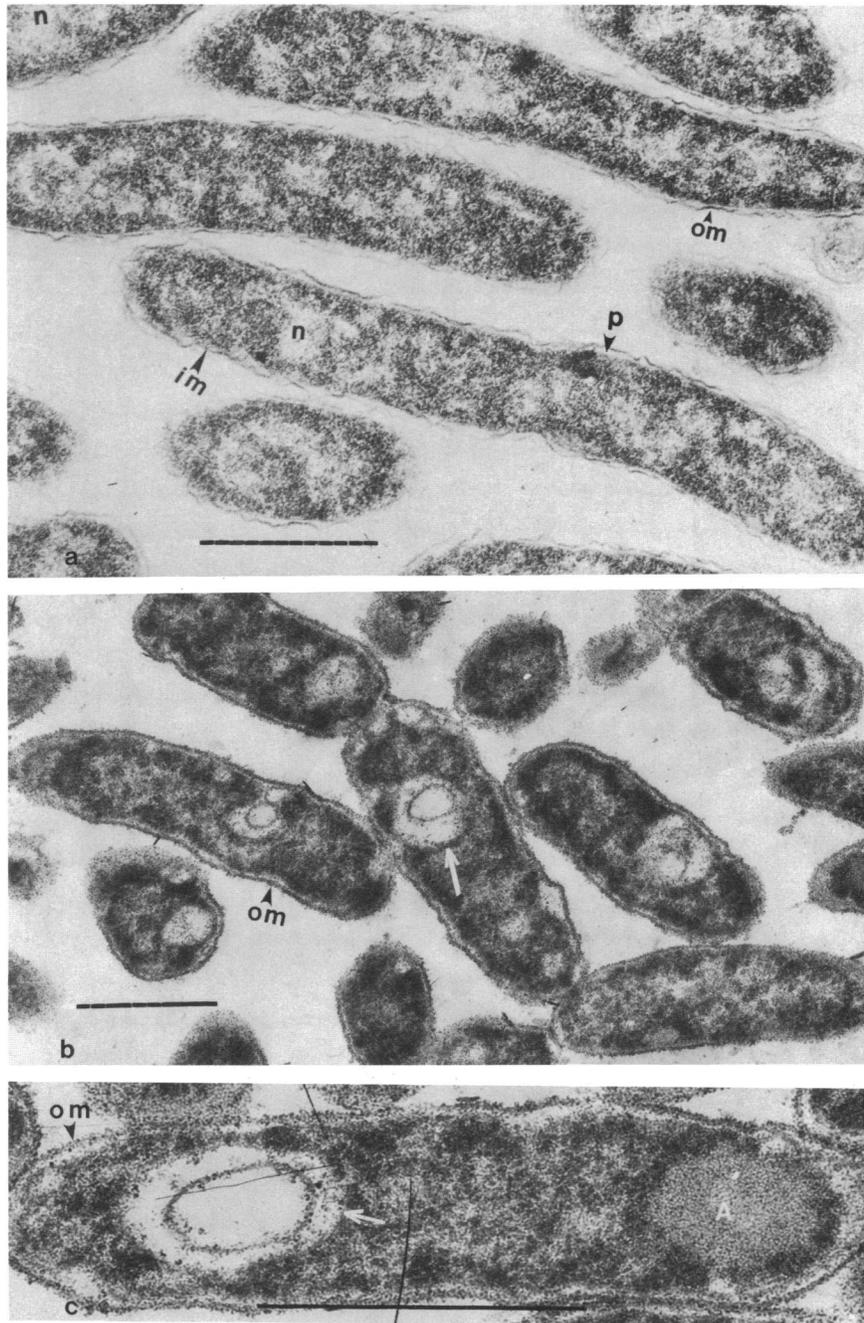
FIG. 2. Electron micrographs of thin section of *V. cholerae* 569B cells. Thin sections of *V. cholerae* 569B cells grown in plus medium (a) or minus medium (b and c). Abbreviations: im, inner membrane; om, outer membrane; p, periplasm; n, nuclear material; and A, amorphous region. Arrow shows nonstaining body. Bar, 2 μm.

minus medium. However, the absorbance of both plus and minus medium-grown cells treated with CAM remained unaltered during 120 min of treatment.

Electron microscopy of CAM-treated cells in plus medium (Fig. 5a) showed sphaeroplast-like bodies which appear to have lost most of their cytoplasmic constituents. This indicated that CAM-treated cells become osmotically fragile, hence the enhanced rate of lysis in phosphate buffer. This might also be the reason for their failure to grow and divide when plated on nutrient agar plates. Minus medium-grown cells did not show such gross alterations in cellular morphol-

ogy upon treatment with CAM (Fig. 5b). For comparison, when *E. coli* C600 cells were treated with a 50 μg ml^{-1} concentration of CAM in plus medium and were examined under an electron microscope (Fig. 5c), no such structural changes were observed (cf. Fig. 5a). It may be noted that the MIC of CAM for *V. cholerae* is ca. 5 μg ml^{-1}, whereas that for *E. coli* is 30 μg ml^{-1}.

Composition of the outer membrane of *V. cholerae* 569B cells grown in plus and minus medium. (i) Proteins. Analysis of the purified outer membrane proteins of *V. cholerae* 569B by sodium dodecyl sulfate-polyacrylamide gel electrophore-
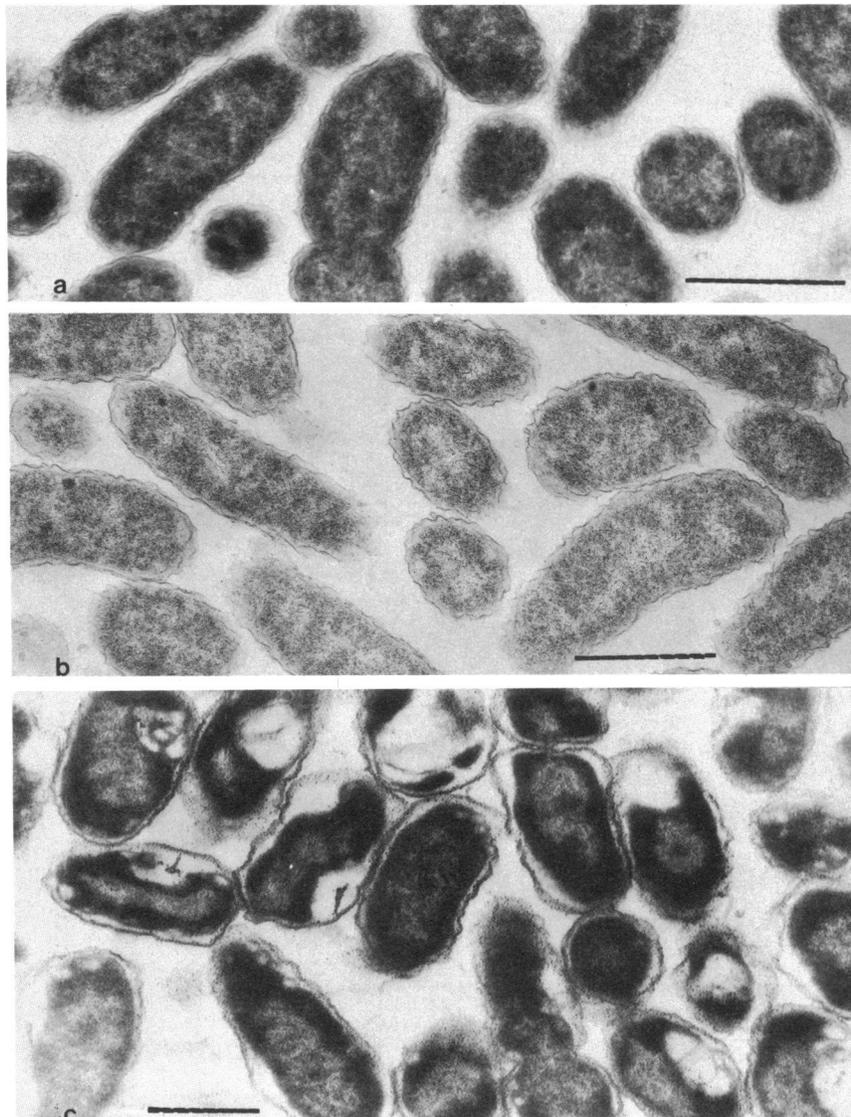
FIG. 3. Electron micrographs of thin sections of *V. cholerae* 569B cells. *V. cholerae* 569B cells grown in minus medium were suspended in plus medium for 15 min (a) and 30 min (b) at 37°C and 0.18 M NaCl for 15 min (c). Bar, 2 μm.
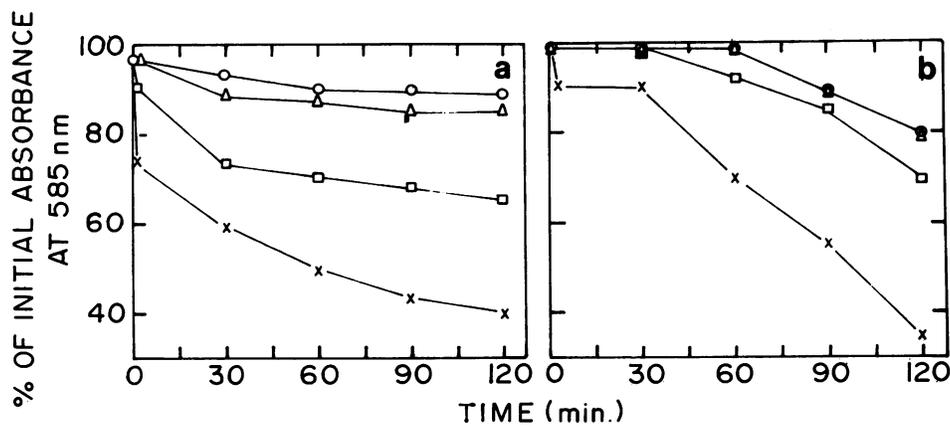

FIG. 4. Lysis of *V. cholerae* 569B cells after treatment with CAM. *V. cholerae* 569B cells were treated with CAM in plus medium (a) and minus medium (b). Cells were harvested at 30 min (△), 60 min (□), and 120 min (×) after addition of the antibiotic. (○) Untreated cells. Plus medium-grown cells were suspended in 50 mM phosphate buffer (pH 7.20) and minus medium-grown cells were suspended in distilled water. Lysis was monitored as described in the legend to Fig. 1.
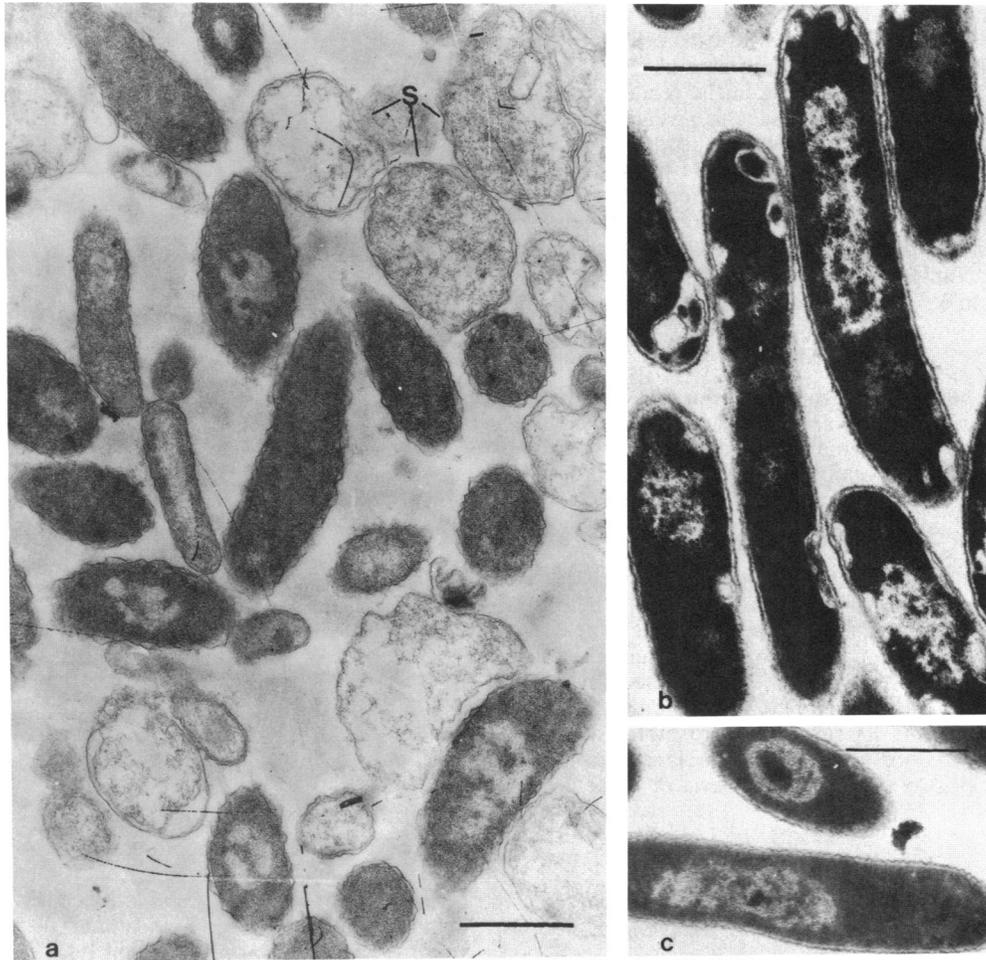
FIG. 5. Electron micrographs of thin sections of *V. cholerae* 569B and *E. coli* C600 cells treated with CAM. *V. cholerae* 569B cells growing in plus medium (a) and minus medium (b) and *E. coli* C600 cells growing in plus medium (c) were treated with CAM for 120 min each as described in the text. S, Sphaeroplast-like bodies. Bar, 2 μm.

sis has revealed that the ratio of two major outer membrane proteins of molecular weights 45,000 and 30,000 was dependent upon the osmolarity of the growth medium. In cells grown in plus medium, the amount of the 45,000-molecular-weight protein was greater than the 30,000-molecular-weight protein (Fig. 6, lane d). The relative amounts of these two proteins were reversed when 569B cells were grown in minus medium (Fig. 6, lane c). To examine the synthesis of outer membrane proteins under LP conditions, cells were grown in LP medium with or without 0.18 M NaCl. The 30,000-molecular-weight protein was not synthesized in cells grown in LP medium in the presence or absence of NaCl (Fig. 6, lanes a and b). However, the amount of the 45,000-molecular-weight protein synthesized was much higher in these cells, and its synthesis was insensitive to the osmolarity of the growth medium. Three new proteins of molecular weights 64,000, 43,000, and 33,000 were observed in the outer membrane of LP medium-grown cells.

(ii) **LPS, phospholipids, and peptidoglycan.** The carbohydrate composition of the LPS isolated from *V. cholerae* 569B cells grown in plus or minus medium did not differ significantly (Table 1). The phospholipid composition of cells grown under the two conditions was also similar (Table 1), and the 4% sodium dodecyl sulfate-insoluble residue representing the murein lipoprotein (Table 1) was synthesized in

equal amounts in plus and minus medium-grown cells. The amount of this residue was, however, less than that observed in *E. coli* cells (8). No significant difference was observed in the total yield of the outer membrane and the cell envelope with respect to the cellular dry weight under the two growth conditions.

Characterization of an osmotically fragile mutant (DC15) of *V. cholerae* **569B.** Several detergent-sensitive, phage-resistant mutants of *V. cholerae* 569B were isolated after *N*-methyl-*N*-nitro-*N*-nitrosoguanidine mutagenesis. One of these mutants, designated DC15, was found to be osmotically fragile compared with the parent *V. cholerae* 569B, although no significant difference in the composition of the O antigen was detected. The generation time of the mutant cells in plus medium was 60 min compared with 30 min for the wild-type cells, and in minus medium the values were 90 and 45 min, respectively. Cells of strain DC15 grown in plus medium showed rapid lysis in distilled water and 50 mM Tris-hydrochloride (pH 8.0) buffer, irrespective of the age of the culture and temperature of incubation (Fig. 7a). Unlike the parent strain, these mutant cells lysed in 50 mM phosphate buffer (pH 7.20). It may be noted that the minimum stabilizing concentration of NaCl for the mutant cells was 0.18 M compared with 0.09 M for the wild-type cells. The mutant showed reversion of its lytic phenotype when grown

in minus medium (Fig. 7b), although the extent of its reversion was less than that of the wild-type cells.

Analysis of the outer membrane proteins of DC15 grown in plus medium revealed a reversion in the relative expression of the 45,000- and 30,000-molecular-weight proteins (Fig. 6, lane f) compared with the wild-type cells (Fig. 6d). However, minus medium-grown DC15 cells showed almost equal amounts of the 45,000- and 30,000-molecular-weight proteins (Fig. 6, lane e). Thus, although no definite pattern can be assigned to the expression of these two major proteins, they definitely respond to osmosensory mechanisms operating in *V. cholerae* cells.

DISCUSSION

The present report demonstrates that the rate and extent of lysis of *V. cholerae* cells are largely dependent on the osmolarity of the growth medium. The degree of protection imparted by the peptidoglycan to the cell varies in different gram-negative bacteria. In *E. coli*, although the peptidoglycan consists of a thin monolayer (9), enzymatic cleavage of this macromolecule is essential before the cells become osmotically fragile. On the other hand, halophilic bacteria are capable of regular growth and division, despite their low peptidoglycan content (17). However, they need a hypertonic environment for their growth and survival. The removal of the protective ionic milieu from the growth or suspension medium causes complete disintegration of such bacteria (17). As far as its response to osmotic stress is concerned, the behavior of *V. cholerae* is atypical and resembles more closely that of halobacteria or other gram-

TABLE 1. Comparison of the LPS, phospholipids, and peptidoglycan of *V. cholerae* 569B cells grown in plus and minus medium[a]

Cell component and parameters	Values for *V. cholerae* 569B cells grown in:	
	Plus medium	Minus medium
LPS		
Total carbohydrate[b]	27.0	25.8
Phosphate[b]	2.10	1.70
Fructose[c]	1.0	1.0
Heptose[c]	2.50	3.80
Glucose[c]	1.53	1.44
Glucosamine[c]	1.76	2.28
Rhamnose[c]	0.36	0.39
Phospholipids[d]		
Phosphatidyl ethanolamine	60	67.5
Lysophosphatidyl ethanolamine	6	4.5
Phosphatidyl glycerol	29	27
Cardiolipin	ND[e]	ND
Phosphatidyl serine	ND	ND
Peptidoglycan[f]	0.52	0.48

[a] LPS, phospholipids, and peptidoglycan were isolated, purified, and quantitated as described in the text. The numbers represent the average of three independent sets of experiments.
[b] Expressed as the percent dry weight of total LPS.
[c] Expressed as molar ratios of sugars with respect to fructose.
[d] Expressed as percentages of total lipid phosphate.
[e] ND, Not detected.
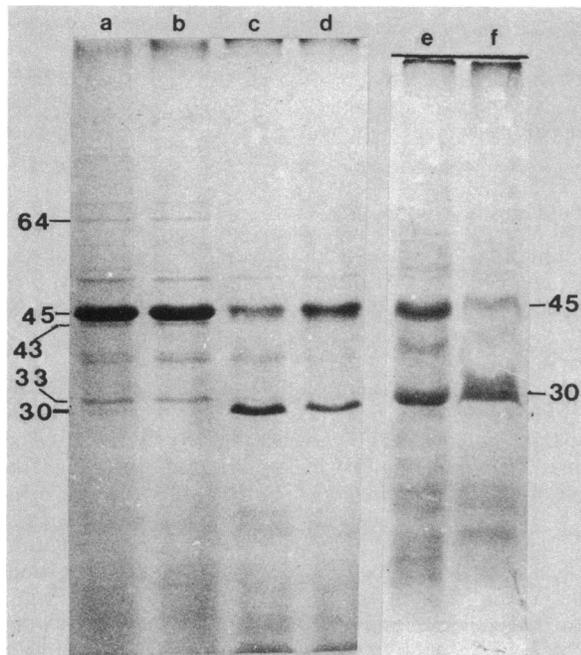[f] Expressed as the percent dry weight of whole cells.

FIG. 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of outer membrane proteins of *V. cholerae* cells. Outer membrane proteins of *V. cholerae* 569B cells grown in LP minus medium (lane a), LP plus medium (lane b), complete minus medium (lane c), and complete plus medium (lane d). Outer membrane proteins of *V. cholerae* strain DC15 cells grown in plus medium (lane f) and minus medium (lane e). The numbers represent molecular weights of the proteins in thousands.

negative bacteria whose peptidoglycan has been damaged by either lysozyme or antibiotics. This is clearly demonstrated by the extensive lysis of *V. cholerae* cells when suspended in distilled water. We have found that 0.09 M NaCl or 0.2 M sucrose was necessary to prevent lysis of these cells. This contrasts with the much higher concentrations of such nonpenetrating solutes, e.g., 0.5 M sucrose, needed to protect *E. coli* cells whose peptidoglycan has been damaged (8). This would indicate that the internal osmotic pressure of *V. cholerae* cells is significantly lower than that of gram-negative bacteria such as *E. coli*. On the other hand, it could also be possible that the internal osmotic pressure of *V. cholerae* cells is similar to other gram-negative bacteria, but the observed lysis could be due to less stable or weaker outer membranes of these cells.

The rate of lysis of *V. cholerae* cells in hypotonic medium is far too rapid for it to be mediated entirely by autolysins. Under nongrowing conditions, the irreversible effect of Mg^{2+} ions in preventing cellular lysis suggests that weak interactions between outer membrane components may be important for stabilizing the cellular structure in these cells. In marine pseudomonads (27), it has been suggested that Mg^{2+} ions form bidentate chelate complexes between two adjacent tetrapeptide subunits in the peptidoglycan, thus stabilizing this structure. In contrast, the protective effect of Mg^{2+} ions on the lysis of *V. cholerae* cells was much less when the growth media were supplemented with 5 mM $MgCl_2$. *E. coli* mutants (*lpo mlpA*) with altered outer membranes have been found to undergo blebbing when grown in low-Mg^{2+} growth medium (39) and require Mg^{2+} ions in growth media to maintain the integrity of the outer membrane. An earlier report (19) has shown that noncovalent interactions are important in stabilizing the cell envelope of *V. cholerae* cells. Although we observed a minimal decrease
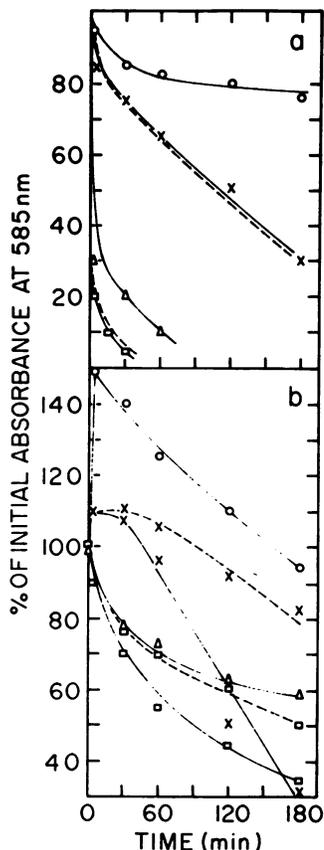
FIG. 7. Lysis of *V. cholerae* DC15. *V. cholerae* DC15 cells were grown in plus medium (a) and minus medium (b), harvested at the exponential phase, and suspended in 0.18 M NaCl (O), 50 mM sodium phosphate buffer (pH 7.20) (×), 50 mM Tris-hydrochloride buffer (pH 8.0) (△), and distilled water (□). Cells were incubated at 37°C (solid lines) or at 10°C (broken lines). Lysis was monitored as described in the legend to Fig. 1.

in the extent of lysis of cells grown in Mg^{2+} ion-supplemented plus and minus media compared with the same media not supplemented with Mg^{2+}, the differential lytic pattern of these cells remained unaltered. No bleb formation was observed in *V. cholerae* cells grown in plus medium (Fig. 2a). This suggests that although the presence of Mg^{2+} ions under growing conditions could stabilize *V. cholerae* cell envelopes to a certain extent, osmotic sensitization induced by growth in plus medium plays a major role in imparting susceptibility to lysis to *V. cholerae* cells.

Susceptibility of *V. cholerae* cells to lysis in hypotonic media under nongrowing conditions prompted us to examine whether these cells could grow in a medium of low osmolarity. Unlike halophilic bacteria, these cells could not only grow in minus medium but also became relatively resistant to lysis under nongrowing conditions (Fig. 1b). This result, along with the fact (Table 1) that there is no major difference in the quantitative amounts of the cell wall constituents of cells grown in plus and minus media, suggests that the internal osmotic pressure of *V. cholerae* cells possibly plays a key role in determining its inherent fragility. However, the electron microscope studies indicate an increase in the rigidity of the outer membrane of cells grown in low-osmolarity medium. Interestingly, no plasmolysis of cells was noticed when minus medium-grown cells were shifted to plus medium after 15 min; however, extensive plasmolysis

was noted if minus medium-grown cells were resuspended in 0.18 M NaCl under nongrowing conditions.

It has been postulated that one of the reasons for the inhibition of autolysis in CAM-treated gram-positive bacteria is the thickening of the cell wall (33). It has also been suggested that *E. coli* cells have a mechanism that can rapidly modify the structure of newly made murein in bacteria that have stopped making protein. Thereafter, the attachment of murein hydrolases to such a murein is blocked, leading to a decreased susceptibility to autolytic degradation (10). However, yet another group has shown that pretreatment with CAM does not affect the rate of shock-induced autolysis in *E. coli* (18). In light of such information, it is intriguing that *V. cholerae* cells show potentiation of lysis upon pretreatment with CAM which acts as a bactericidal agent on cells growing in plus medium. CAM has also been shown to act as a bactericidal agent in *Haemophilus influenzae* cells (7), although formation of sphaeroplast-like bodies has not been reported. It is difficult to explain the formation of such sphaeroplast-like bodies upon treatment with CAM in plus medium. It is also difficult to explain the differential action of CAM on *V. cholerae* cells grown under various osmolarities.

The reciprocal expression of the 45,000- and 30,000-molecular-weight proteins in plus and minus medium is similar to that observed for OmpF and OmpC proteins in *E. coli* (13, 36). The outer membrane proteins from the mutant, however, show a reversal in the expression of the two proteins in plus and minus media compared with the wild type. It is intriguing to note the complete disappearance of the 30,000-molecular-weight protein in cells grown in LP concomitant with the increase of the 45,000-molecular-weight protein. Interestingly, in *E. coli* K-12 mutants (20), it has been observed that protein e functions as the major porin in the absence of OmpC and OmpF proteins. This protein e was later identified as the PhoE protein (25), which was expressed under phosphate starvation in wild-type cells. It is possible that under phosphate-limiting growth conditions in *V. cholerae*, a new protein of molecular weight 45,000 is expressed which replaces the major 45,000- and 30,000-molecular-weight proteins observed in complete medium. This is further supported by the fact that the major outer membrane protein synthesized under phosphate-limiting conditions is not affected by the osmolarity of the growth medium.

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