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Interaction of *Vibrio cholerae* Cells with β-Lactam Antibiotics: Emergence of Resistant Cells at a High Frequency

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Unlike other gram-negative enteric bacteria, *Vibrio cholerae* cells were equally susceptible to penicillin and ampicillin and in general more susceptible than *Escherichia coli* to most of the beta-lactam antibiotics. The turbidity of penicillin-treated cultures continued to increase exponentially for about 3 h, although the cell viability declined rapidly within 30 min of penicillin addition. Prolonged treatment with beta-lactam antibiotics produced cells resistant to these antibiotics. A fluctuation test indicated that this resistance might be due to adaptive mutation. Cells resistant to a beta-lactam exhibited broad cross-resistance to other beta-lactam antibiotics. A new 12,000-Da outer membrane protein was detected both in beta-lactam-resistant cells and in wild-type cells growing in medium containing beta-lactam antibiotics. While the penicillin-resistant cells had all of the penicillin-binding proteins (PBPs) present in the parental cells, significant differences in the relative proportion of low-molecular-weight PBPs were seen. The low-molecular-weight PBPs from resistant cells seemed to form more stable complexes with penicillin than those from the parental strain.

Vibrio cholerae, a noninvasive enteropathogen and the etiological agent of cholera, adheres to epithelial cells of the proximal small intestine and excretes a potent enterotoxin which causes severe diarrhea. Several models simulating the important features of the interaction between the bacterium and the epithelial surface have been proposed (8, 11, 15, 26). The outer membrane of enteric gram-negative bacteria plays an important role in conferring resistance to bile salts and to host defense factors such as lysozyme and leukocyte proteins (19). Studies of the cell surface architecture of *V. cholerae* were thus considered necessary for a better understanding of the cell-cell interaction. It has recently been reported that the cell surface of a hypertoxinogenic strain of *V. cholerae*, 569B, has exposed phospholipids in the outer leaflet of the outer membrane and that these cells are highly sensitive to a wide range of chemicals in general and to hydrophobic compounds and neutral and anionic detergents in particular. The lipopolysaccharide (LPS) moiety has relatively less negative charge (21).

Previous studies have indicated the relatively fragile nature of the murein network of *V. cholerae* (12, 13). These cells lyse rapidly in hypotonic media and in the presence of chelating agents such as Tris and EDTA (13). Purified outer membranes can be directly isolated from whole cells by treatment with protein denaturants such as urea at room temperature (12).

These observations suggest that the murein network of *V. cholerae* is relatively weak and prompted us to examine the effect of beta-lactam antibiotics which inhibit the final stages of murein synthesis in *V. cholerae*. Eleven penicillin-binding proteins (PBPs) were detected in *V. cholerae*, and when they were compared with the PBPs of *Escherichia coli*, several interesting differences were observed (23). The present report is an extension of the previous report (23) and describes several novel features in the interaction of beta-lactam antibiotics with *V. cholerae*.

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MATERIALS AND METHODS

Bacterial strains and growth and viability studies. The parental strain used was *V. cholerae* 569B (serotype Inaba), which carries a streptomycin resistance marker (50 μg/ml). Cells were grown in gyratory shakers at 37°C in nutrient broth (NB) containing 0.18 M NaCl at pH 8.0 as described previously (13). The *E. coli* strain used was C-600, which was grown in NB at pH 7.4. Cell viability was assayed as CFU on nutrient agar plates, and growth was assayed by measuring the A_{585}. A bacterial concentration of 8×10^8 CFU/ml corresponded to an A_{585} of 1.0.

Determination of MICs. The MICs of various antibiotics were determined by both broth dilution and agar dilution assays. For the broth dilution assay, about 5×10^4 cells in NB were treated with different concentrations of antibiotics and shaken for 16 h at 37°C. The minimum concentration at which there was no visible turbidity was taken as the MIC of that antibiotic. In the agar dilution assay, about 10^3 cells were spread on agar plates containing different concentrations of antibiotics, and the minimum concentration which prevented appearance of a single CFU after incubation at 37°C for 18 h was regarded as the MIC. The results of the two assays were similar.

Penicillinase assay. Penicillinase was assayed by the iodometric method (6) with penicillin G (PenG) as the substrate. Under the conditions employed, the limit of detection was 0.1 U of penicillinase (Sigma Chemical Co., St. Louis, Mo.) per ml. Broth cultures (10 ml) of PenG-susceptible and -resistant cells that had been incubated for 16 h were centrifuged, and the amounts of penicillinase in the culture supernatant and in the cell pellet after resuspension in 1.0 ml of 100 mM phosphate buffer (pH 7.0) and mild sonication were measured. Resistant cells were grown both with (5 μg/ml) and without PenG. Colonies of both susceptible and resistant cells (on PenG-containing plates) were also directly assayed for penicillinase by the method of Martine et al. (17).

A penicillinase-producing *E. coli* strain (obtained from AFMC, Pune, India) was used as a control in these experiments.

Lysis of whole cells. Cell lysis was measured by recording the optical density at 585 nm. For monitoring autolysis, PenG-treated and untreated cells were suspended in 10 mM phosphate buffer (pH 7.4) containing 0.18 M NaCl and incubated at 37°C, and lysis was measured as described above.

Isolation of outer and inner membranes. The outer and inner membranes of both PenG-susceptible and -resistant cells were isolated from the crude cell envelope after treatment with 1% Sarkosyl NL97 (12).

Assay of protein. Protein was assayed by the method of Markwell et al. (16) with bovine serum albumin as the standard.

SDS-PAGE. The outer and inner membrane proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12.5% polyacrylamide) by the method of Laemmli (10), except that a slab gel apparatus was used.

Electron microscopy. For thin-section electron microscopy, cells were fixed first with 6% glutaraldehyde (BDH, Poole, England) in 0.125 M phosphate buffer (pH 7.2) 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 Kellenburger buffer, dehydrated with ascending concentrations of ethanol, and embedded in Spurr medium (Polysciences, Inc., Warrington, Pa.) at 70°C for 48 h as described previously (13). Sections were cut with glass knives on a JEOL ultramicrotome, stained with uranyl acetate and lead citrate, and examined under a JEOL 100 C transmission electron microscope at 60 kV.

Analysis of PBPs. PBPs were analyzed with ^{125}I-labeled *p*-hydroxybenzylpenicillin (PenX) as described earlier (23).

Chemicals. The following antibiotics were obtained as gifts: amdinocillin from Leo Pharmaceuticals, imipenem and cefoxitin from F. Kahan, Merck Sharp and Dohme Research Laboratories, and aztreonam from Squibb Institute Medical Research. Other antibiotics and biochemicals were obtained from Sigma Chemical Co. Ingredients of media were from Difco, Detroit, Mich. PenX was a gift from J. T. Park, Tufts University, Boston, Mass.

TABLE 1. Comparison of MICs of beta-lactams for *V. cholerae* and *E. coli*

Beta-lactam	MIC (μg/ml) for:			Ratio[a]
	V. cholerae		*E. coli*	
	Wild type	Pen^{rb}		
Penicillin G	2	50	20	10.0
Ampicillin	2	50	5	2.5
Cephalothin	5	20	20	4.0
Cefoxitin	5	20	20	4.0
Cephalexin	5	20	10	2.0
Cephaloridine	10	20	20	2.0
Aztreonam	5	10	20	4.0
Amdinocillin	5	20	2	0.4
Imipenem	2	2	2	1.0

[a] MIC for *E. coli*/MIC for wild-type *V. cholerae*.
[b] The resistant mutants (Pen^r) were selected with the beta-lactam PenG.

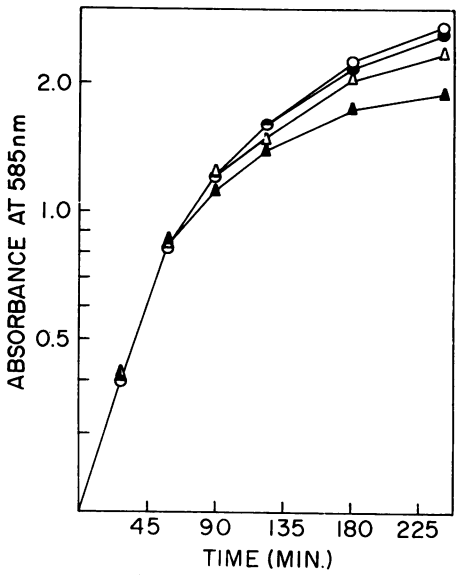

FIG. 1. Change in absorbance in *V. cholerae* cell culture after treatment with different concentrations of PenG. PenG was added during the logarithmic phase of growth. Symbols: ○, untreated cells; ●, 5 μg of PenG per ml; △, 10 μg of PenG per ml; ▲, 20 μg of PenG per ml.

RESULTS

Effect of beta-lactams on *V. cholerae* cells. The susceptibilities of *V. cholerae* cells to various beta-lactam antibiotics were estimated by determining the MICs of these antibiotics. These cells were equally susceptible to PenG and ampicillin, in contrast to other gram-negative organisms, which are more resistant to PenG. In general, *V. cholerae* cells were more susceptible than *E. coli* cells to all beta-lactam antibiotics examined except amdinocillin and imipenem (Table 1).

By using PenG as a representative beta-lactam, the effect

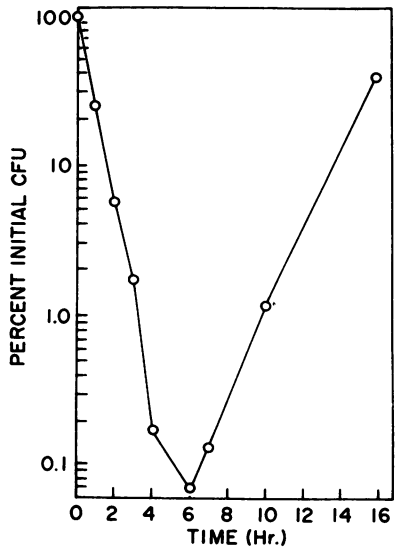

FIG. 2. Survival of PenG-treated *V. cholerae* cells. Exponentially growing cells were treated with 5 μg of PenG per ml, and cell viability was assayed as CFU at different times during incubation at 37°C. The initial titer was 1.6×10^8 CFU/ml.

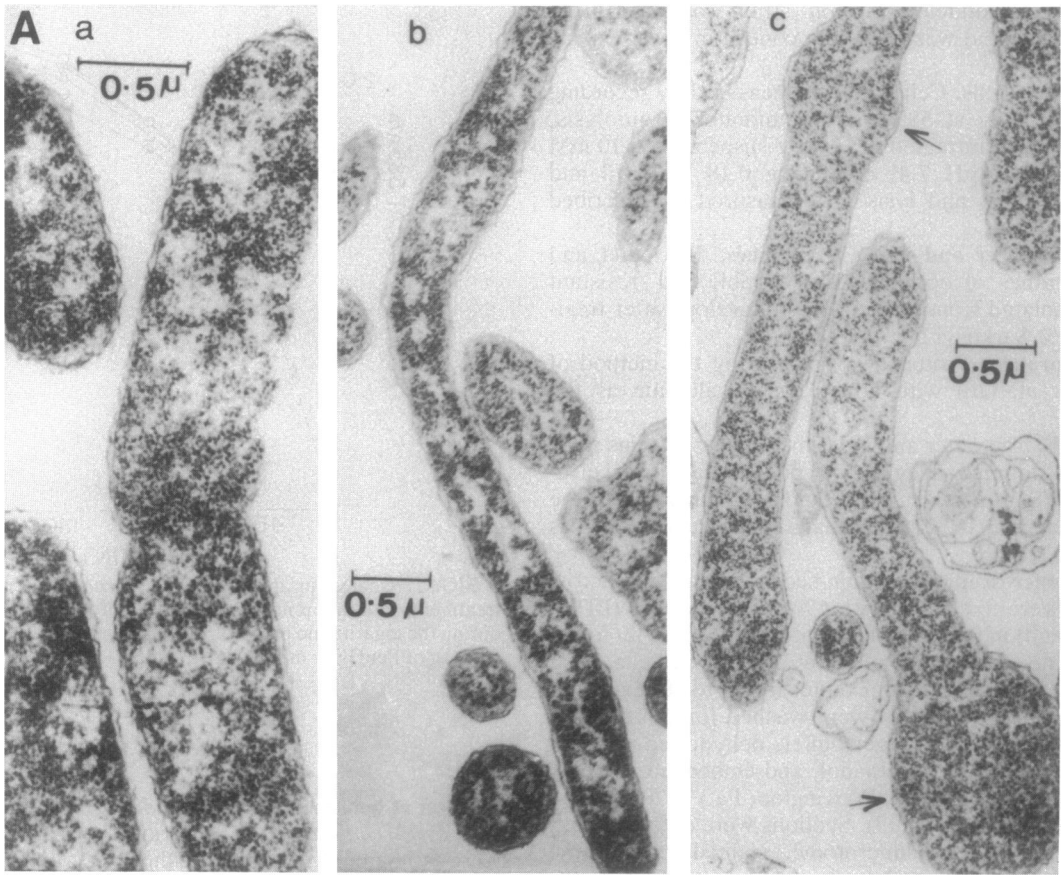

FIG. 3. Thin-section electron micrographs of PenG-susceptible and -resistant *V. cholerae* cells after PenG treatment. (A) Cells were treated with 5 μg of PenG per ml for 15 min (b) or 30 min (c) or were not treated (a). Arrows indicate cylindrical and polar bulges. (B) (a) Susceptible cells after 3 h of treatment with 5 μg of PenG per ml; (b) resistant cells after 30 of min treatment with 10 μg of PenG per ml.

of this group of antibiotics on *V. cholerae* cells was examined. When cells in the logarithmic phase of growth were treated with increasing concentrations of PenG (2.5 to 10 times the MIC), the culture turbidity (Fig. 1) and cellular protein content (measured at 2.5 times the MIC) increased for about 180 min of treatment, as was the case for untreated cells. Furthermore, cells grown in the presence of PenG for 180 min and then suspended in phosphate buffer did not lyse even after 60 min of incubation at 37°C, suggesting that autolysin-induced lysis does not occur in this organism. Cephaloridine, which is known to lyse *E. coli* cells instantaneously (24), also did not lyse *V. cholerae* cells at concentrations of up to five times the MIC. Cell viability, on the other hand, as measured by CFU, declined rapidly in the presence of PenG (2.5 times the MIC) for about 6 to 7 h of treatment (Fig. 2). When PenG treatment was continued for a longer period, there was an increase in CFU (Fig. 2). Similar results were obtained when cells were grown in the presence of other beta-lactams, such as ampicillin, amdinocillin, and various cephalosporins, each at 2.5 times the MIC.

Electron microscopy of cells treated with PenG at different times showed that treatment with 5 μg of PenG per ml for 15 min induced filamentation of cells (Fig. 3A, part b). After about 30 min of penicillin treatment, the filaments became shorter and bulges in both polar and cylindrical regions could be seen (Fig. 3A, part c). After 3 h of treatment, large spherical bodies occurring in clusters were observed (Fig. 3B, part a). These cells had intact outer and inner membranes, and adherence was mediated via the outer membrane. No evidence of disrupted outer membranes or lysed cells could be seen during this period of PenG treatment. These observations support the observed increase in culture turbidity and continued protein synthesis during the early phase of PenG treatment.

PenG-resistant cells. Cells collected from cultures after more than 12 h of PenG treatment were resistant to PenG (up to 50 μg/ml) even after repeated subculturing in antibiotic-free medium. No penicillinase activity could be detected in the PenG-resistant cells when either PenG or imipenem, both of which are potent inducers of penicillinase (22), was used. These cells fully retained their resistance when challenged with PenG in fresh medium. These observations ruled out the possibility of inactivation or depletion of the antibiotic as causative factors in the observed resistance. Instead of treating cells with PenG in liquid culture, in some experiments about 10^8 penicillin-susceptible parental cells were seeded on nutrient agar plates containing 5 μg of PenG per ml. The frequency of cells resistant to PenG was on the order of 50×10^{-8} to 75×10^{-8}. The phenotypes of PenG-resistant cells obtained from plates were identical to those obtained from continued treatment in liquid culture. This experiment was repeated with each of the beta-lactams listed in Table 1, and in every case resistant colonies were readily isolated.

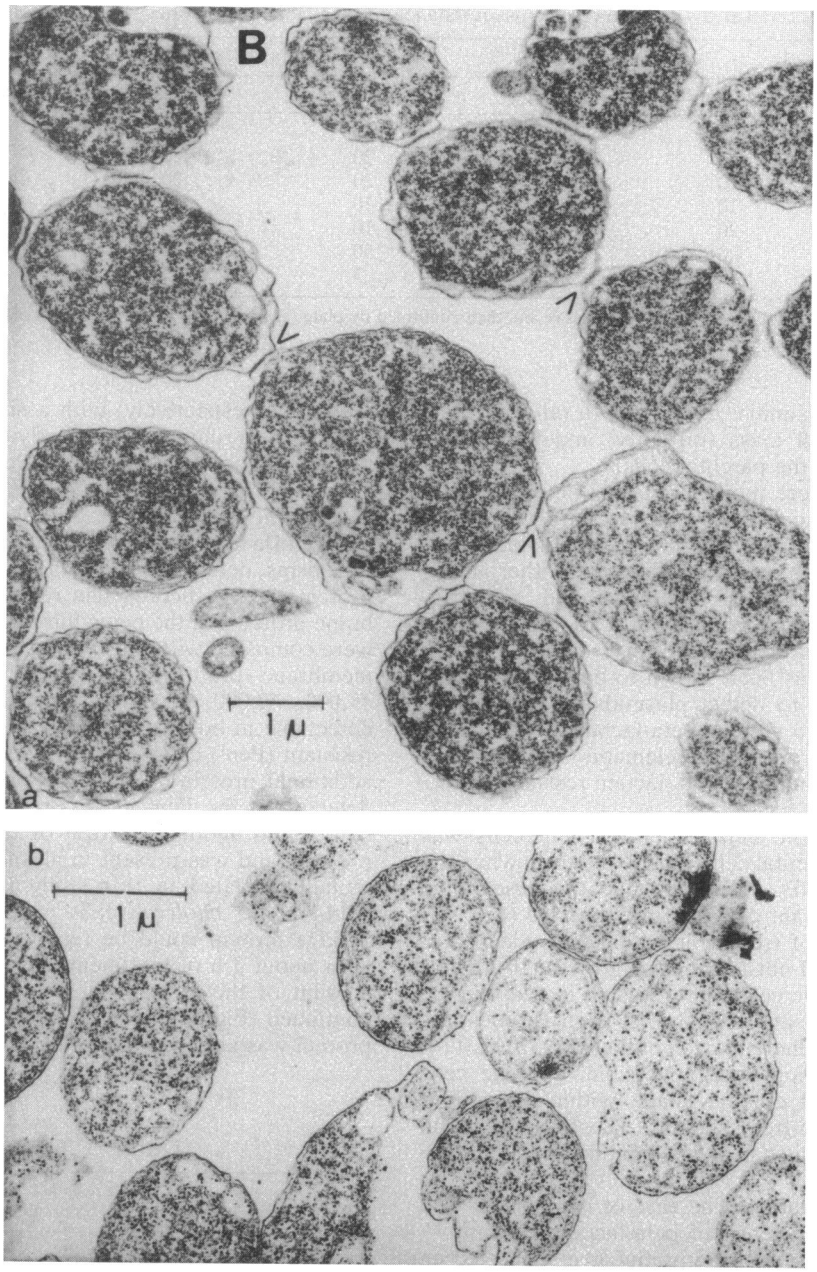

FIG. 3—*Continued.*

The frequency of occurrence of such resistant clones was 20 to $80/10^8$ CFU, depending on the beta-lactam used for selection. The frequency of resistance to other unrelated antibiotics was determined in order to find out whether the high frequency of emergence of resistant cells was unique to the beta-lactam group of antibiotics. When *V. cholerae* cells were treated with neomycin, tetracycline, rifampin, or chloramphenicol at concentrations of two to three times their MICs, the numbers of cells resistant to these antibiotics were on the order of 10^{-9}. Thus, the high rate of emergence of resistant *V. cholerae* cells seems to be unique to the beta-lactam group of antibiotics. To rule out any possible artifact in our method, about 5×10^9 CFU of *E. coli* C-600 was seeded on plates containing ampicillin (10 μg/ml). No colonies were seen after appropriate incubation at 37°C.

The PenG-resistant cells showed cross-resistance to a diverse group of beta-lactams (Table 2). The MICs of the cross-resistant cells varied from 2 to 25 times the MIC of susceptible cells, depending on the beta-lactam tested. Similar results were obtained when other beta-lactams were used for initial screening of susceptible cells. The cross-resistance to beta-lactams thus seemed to be of a general nature and not to be dependent on the antibiotic used in the primary selection. The only exception was imipenem, resistance to which did not yield cross-resistance to other beta-lactams. Interestingly, *V. cholerae* cells are as susceptible to imipenem as *E. coli* cells, and unlike those treated with other beta-lactams, imipenem-treated *V. cholerae* cells do not assume a spherical shape. Phenotypic properties of resistant cells isolated after growth in the presence of different beta-

TABLE 2. Prevalence of cross-resistance to beta-lactam antibiotics in *V. cholerae*

Drug to which resistance was selected	MIC (μg/ml) for[a]:					
	Penicillin G	Ampicillin	Cephalexin	Amdinocillin	Cephaloridine	Imipenem
Penicillin G	50	50	20	20	20	2
Ampicillin	50	50	20	20	20	2
Cephalothin	20	20	20	20	20	2
Amdinocillin	10	10	10	20	20	2
Cephaloridine	20	20	10	20	20	2
Cephalexin	20	20	50	20	20	2
Imipenem	2	2	5	5		10

[a] MICs were first determined by tube (broth dilution) assay and then confirmed by plate (agar dilution) assay (see Materials and Methods).

lactam antibiotics were similar. Their growth rates in terms of both increase in cell mass (turbidity) and CFU were comparable to those of the parental cells.

The resistant cells were more fragile (as determined by lysis) than the parental cells when exposed to 0.01% SDS, 0.005% Triton X-100, 0.01% sodium deoxycholate, or 0.1 mM EDTA. However, their susceptibilities to other antibiotics such as chloramphenicol, nalidixic acid, rifampin, tetracycline, and neomycin were similar to those of the parental cells. Multiple-drug resistance in *V. cholerae* strains isolated from patients has been shown to be plasmid mediated (20). All attempts to detect plasmids in *V. cholerae* 569B isolates resistant to various beta-lactams were unsuccessful. Thus, extrachromosomal elements were not involved in the observed multiple beta-lactam resistance in *V. cholerae* 569B.

The morphology of the beta-lactam-resistant cells was similar to that of the parental cells (Fig. 3A, part a) when the cells were grown in NB. However, like the susceptible parental cells, the resistant cells became rounded (Fig. 3B, part b) within 30 min of treatment with PenG added at a concentration which did not affect their growth (10 μg/ml). In contrast to susceptible cells, there was no increase in the volume of resistant cells after PenG treatment. Besides, the spherical cells did not adhere to each other (Fig. 3B, part b). The change in morphology was reversible, and the cells recovered their normal comma shape within 30 min of suspension in antibiotic-free medium. The transformation from spherical to comma shaped was inhibitable with chloramphenicol.

Mutation versus adaptation. The ease of isolation as well as the high rate of occurrence of beta-lactam-resistant *V. cholerae* cells in the absence of any mutagen suggested an adaptive response. However, the fact that resistance was maintained indefinitely in the absence of beta-lactams and our inability to isolate any sensitive revertant even after screening more than 10^4 isolated colonies suggested a change at the genetic level. To determine which of these possibilities was correct, the fluctuation test (14) was carried out, and the variance in the emergence of resistant colonies from 10 parallel cultures and from a single culture was calculated. According to the logic of this experiment, in the case of a mutation, there should be a large variation in the number of resistant cells from parallel cultures, while such variation should be absent in the case of an adaptive response. Plating aliquots from the single culture is essentially an internal control indicating variation inherent in such an experiment. The data (Table 3) show that the degree of variance with parallel cultures for rifampin-resistant colonies was very high (2,813) compared to that for PenG-resistant colonies (145), while the corresponding figures were comparable (67 versus 89, respectively) with a single culture. Thus, while resistance to rifampin was clearly due to spontaneous mutation, resistance to PenG seemed to be due to some kind of adaptation. It is possible that the beta-lactam resistance of *V. cholerae* represents an example of adaptive mutation (2).

A 12-kDa inducible outer membrane protein. Since in some organisms, defects in the synthesis of major outer membrane proteins lead to beta-lactam resistance (1), the outer membrane proteins of the penicillin-resistant cells of *V. cholerae* were compared with those of the parental cells. Seven outer membrane proteins with molecular weights of 47,000, 45,000, 40,000, 38,000, 35,000, 27,000 and 23,000 were detectable in both susceptible parental cells and penicillin-resistant (Pen^r) cells (Fig. 4A, lanes a and c). However, an additional protein with a molecular weight of 12,000 was detected in the Pen^r cells (Fig. 4A, lane c). This protein constituted about 8 to 10% of the total outer membrane proteins and was present in all of the beta-lactam-resistant mutants isolated in this study (Fig. 4B, lane g). When wild-type *V. cholerae* cells were treated with PenG, the 12-kDa protein could be resolved in the outer membrane after about 3 h of treatment (Fig. 4B, lane c). The relative amount of this protein increased as PenG treatment was continued (Fig. 4B, lanes c to e), and after about 8 h, this protein was abundant (Fig. 4B, lane f). To test whether the

TABLE 3. Fluctuation test

Sample	No. of resistant colonies/10^8 CFU on plates containing indicated drug[a]			
	Parallel culture		Single culture	
	Penicillin G	Rifampin	Penicillin G	Rifampin
1	99	0.65	120	1.33
2	100	0.99	120	1.4
3	115	0.67	127	1.4
4	90	0.48	96	1.18
5	94	0.81	109	1.29
6	102	0.46	111	1.37
7	97	2.65	114	1.1
8	124	1.25	117	1.25
9	82	0.39	122	1.2
10	106	0.8	103	1.37
Variance[b]	145.5	2,813.1	89.44	67.1

[a] See text for description of parallel and single cultures. On plates containing rifampin, 8×10^9 CFU were plated instead of the 1×10^8 CFU used for PenG-containing plates. The concentration of each antibiotic was 5 μg/ml.

[b] Variance was calculated by using the formula $\Sigma(x - \bar{x})^2/(n - 1)$, where x is the observed frequency of resistant cells, $\bar{x}$ is the mean of the observed frequencies of resistant cells and equals x/n, and n is the number of observations.

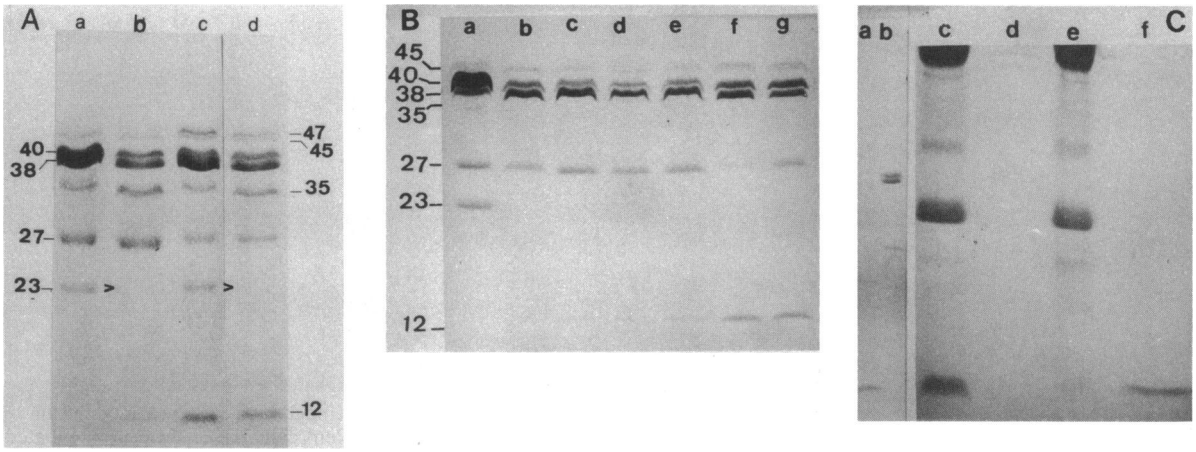

FIG. 4. SDS-PAGE of outer membrane proteins of *V. cholerae* wild-type and beta-lactam-resistant cells grown in NB containing 0.18 M NaCl. (A) Outer membrane proteins of untreated wild-type cells (lane a), wild-type cells treated with 5 μg of PenG per ml for 3 h (lane b), untreated Pen^r cells (lane c), and Pen^r cells treated with 10 μg of PenG per ml for 3 h (lane d). The arrowheads indicate the position of the 23,000-Da protein. (B) Kinetics of appearance of the 12,000-Da protein after PenG treatment of wild-type cells. Shown are outer membrane proteins of untreated wild-type cells (lane a), wild-type cells after 1, 3, 5, 6, or 8 h of treatment with PenG (lanes b, c, d, e, and f, respectively), and cephalexin-resistant cells (lane g). Numbers in panels A and B indicate molecular masses of proteins in kilodaltons. (C) Outer membrane proteins of Pen^r cells treated with 0.01 M Tris-HCl (pH 7.7) buffer containing 2% SDS, 2.5 M NaCl, or 2.5 M LiCl and centrifuged and analyzed as described in Materials and Methods. Lanes a and b, supernatant and pellet, respectively, after treatment of outer membrane with buffer containing 2% SDS; lanes c and d, pellet and supernatant, respectively, of outer membrane treated with 2.5 M NaCl; lanes e and f, pellet and supernatant, respectively, of outer membrane treated with 2.5 M LiCl.

12-kDa protein was synthesized de novo, chloramphenicol and rifampin were added separately to *V. cholerae* cultures just before the addition of PenG. Under conditions where transcription or translation was inhibited, the 12-kDa protein could not be detected, suggesting that this protein was synthesized de novo after the addition of PenG. The 12-kDa outer membrane protein was found to be induced by all beta-lactam antibiotics to which resistance yielded cross-resistance to the other beta-lactams.

The 12-kDa protein was easily extractable with Nikaido's buffer (18) at room temperature (Fig. 4C, lanes a and b) and was the only outer membrane protein which could be solubilized by the chaotropic agent lithium chloride (2.5 M) (Fig. 4C, lane f). This protein is located in the outer membrane, and no trace of it was detected in other cell fractions. Radioactive penicillin did not bind to this protein, showing that this protein is not one of the PBPs of *V. cholerae*.

The 23-kDa outer membrane protein. The 23-kDa outer membrane protein was detectable only when the cells maintained the comma shape. Any perturbation which led to a change from comma-shaped to spherical, such as growth in the presence of beta-lactam antibiotics, resulted in the loss of this protein (Fig. 4A). When the antibiotic-treated cells were washed and incubated in an antibiotic-free medium, the comma shape was restored and the 23-kDa protein was detectable. Preliminary experiments indicate that this 23-kDa protein resembles the OmpA protein of *E. coli*, since it is both heat modifiable and sensitive to exogenous trypsin (data not shown).

PBPs of PenG-resistant cells. The PBPs of *V. cholerae* cells have been described elsewhere (23). To investigate whether the resistance to beta-lactam antibiotics was due to alteration in the penicillin-sensitive enzymes, the PBPs of the resistant cells were compared with those of the parental cells. Crude cell envelope fractions of both the parental and resistant cells were labeled with ^{125}I-PenX and analyzed by SDS-PAGE and autoradiography (23). All 11 PBPs reported for the wild-type cells (23) could also be detected in the PenG-resistant cells and had molecular masses ranging from 22 to 97 kDa (Fig. 5A). The PBPs of the resistant cells were located in the inner membrane.

Although all of the PBPs of the wild-type cells were present in the resistant cells, their relative amounts were different. While PBP 7 accounted for about 40% of the total radioactivity among the PBPs of wild-type cells (23), in resistant cells this PBP was present in a much smaller amount. Moreover, PBPs 2 and 3 were also present in smaller amounts in resistant cells than in wild-type cells. On the other hand, PBPs 9, 10, and 11 were present in resistant cells in much larger quantities than in parental cells, with PBP 11 being the most abundant. The variation in the intensities of bands between wild-type and resistant cells might reflect differences in either the copy number of the proteins, their relative affinity for PenX, or turnover rates. To determine which of these possible factors was responsible for the differences in band intensity, the kinetics of binding of PenX to different PBPs, the rate of release of PenX from bound PBPs, and the effect of preincubation with beta-lactams having high affinities for known PBPs were examined.

There was no significant difference between the kinetics of binding of PenX to PBPs of the resistant cells and that of the parental cells, indicating that the affinities of the PBPs of the resistant cells for PenX were similar to those of the parental cells (23). However, there was a significant difference between the resistant and wild-type cells in release of bound PenX from the low-molecular-weight PBPs (Fig. 5B). ^{125}I-PenX-labeled inner membranes from both the wild-type and resistant cells were incubated at 37°C in the presence of cold PenG (1 mg/ml); samples were removed at different times and analyzed by SDS-PAGE and autoradiography. The pattern of release of bound PenX from high-molecular-weight PBPs (up to PBP 6) was similar to that reported for wild-type cells (23). However, the amount of PenX released

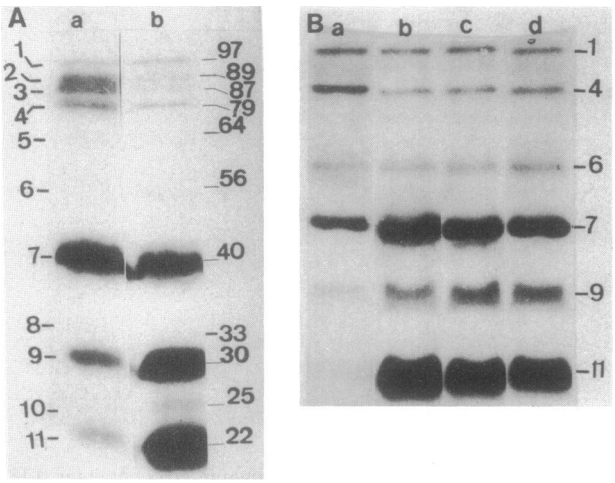

FIG. 5. (A) PBP profile of *V. cholerae* wild-type and Pen^r cells. Cell envelopes of PenG-susceptible and -resistant cells of *V. cholerae* were labeled with ^{125}I-PenX; inner membranes were isolated and analyzed by SDS-PAGE and autoradiography. Lane a, PBP profile of *V. cholerae* 569B wild-type cells. Numbers on the left indicate the different PBPs in order of decreasing molecular mass from top to bottom. Lane b, PBPs of Pen^r cells. Numbers to the right of lane b represent molecular masses in kilodaltons. (B) Release of ^{125}I-PenX from PBPs of *V. cholerae* wild-type and Pen^r cells. Labeled PenX was released from the PBPs of the *V. cholerae* wild-type cell envelope after 30 min of incubation in nonradioactive PenG (1 mg/ml) (lane a) and the Pen^r cell envelope after 0, 15, and 30 min (lanes b, c, and d, respectively) of incubation in nonradioactive PenG (1 mg/ml). Numbers to the right of lane d indicate PBPs affected.

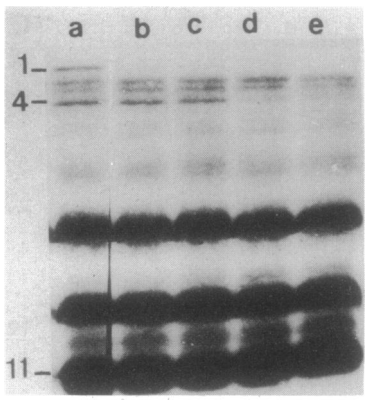

FIG. 6. Effect of preincubation with amdinocillin on binding of ^{125}I-PenX to PBPs of Pen^r cells. Cells were preincubated without amdinocillin (lane a) or with 5 μg (lane b), 10 μg (lane c), 50 μg (lane d), or 100 μg (lane e) of amdinocillin per ml at 37°C. Numbers on the left indicate PBPs of interest.

from the low-molecular-weight PBPs 7, 9, and 11 of the resistant cells (Fig. 5B, lanes b to d) was much smaller than that released from the corresponding PBPs of wild-type cells (Fig. 5B, lane a) (23). Thus, the complex of PenX and these low-molecular-weight PBPs appears to be more stable in the resistant cells.

Preincubation of a membrane suspension with cold PenG (5 μg/ml) saturated the available binding sites for labeled PenX in both wild-type and resistant cells. Preincubation of *V. cholerae* wild-type membranes with amdinocillin affects the binding of PenX to PBPs 1, 4, and 11 (23). However, in the case of Pen^r mutants, preincubation with amdinocillin affected only the binding of PenX to PBPs 1 and 4 (Fig. 6). No effect on binding to PBP 11 was seen even when the amdinocillin concentration was raised to 100 μg/ml.

DISCUSSION

Normally, the smooth-type LPS present in the outer membrane of gram-negative bacteria constitutes an effective permeability barrier for antibiotics such as PenG and novobiocin (19). The outer membrane of parental *V. cholerae* 569B has been reported to have a smooth-type LPS (4), and we have confirmed this. The high susceptibility of *V. cholerae* 569B to many of the beta-lactam antibiotics, including PenG (Table 1), suggests that the outer membrane of *V. cholerae*, with its complement of smooth-type LPS, fails to restrict permeation by these antibiotics. Whether a reduced negative charge on the cell surface of *V. cholerae* (21) plays any role in permeation by exogenous antibiotics, as reported for *abs* mutants of *E. coli* (7), has yet to be examined.

A remarkable feature in the interaction of beta-lactam antibiotics with *V. cholerae* cells was the absence of growth inhibition and cellular lysis. The turbidity of PenG-treated cultures increased at the same rate as that of a control for a period of about 3 h, even though the viable-cell titer declined rapidly over the same period (Fig. 2). No activation of autolysis could be detected in cells grown in the presence of various penicillins and cephalosporins, including cephaloridine, which has been reported to be highly potent in triggering cellular autolysins (9, 23). Also, no evidence of cellular lysis was seen when cells from PenG-treated cultures were examined by electron microscopy (Fig. 3B, part a). Bactericidal action of penicillin without cellular lysis in Lyt$^-$ bacterial strains has been reported (5), but in such cases an inhibition of growth was always observed after addition of the antibiotic.

The loss of the 23-kDa outer membrane protein whenever vibrio cells (both wild type and Pen^r) became spherical and its reappearance when the cells regained their normal shape suggest that this protein might be involved in maintaining the comma shape of *V. cholerae*. This protein has been tentatively identified as being similar to the OmpA protein of *E. coli*, and interestingly the OmpA protein, in conjunction with lipoproteins, has been shown to be necessary for maintaining the rod shape of *E. coli* (3). We are currently investigating the status of outer membrane lipoproteins in control and PenG-treated cells of *V. cholerae*.

The synthesis of a 12-kDa outer membrane protein in *V. cholerae* was invariably seen when *V. cholerae* cells were treated with beta-lactam antibiotics. Synthesis of this protein was sensitive to rifampin and chloramphenicol, indicating that the beta-lactams were effective inducers of its synthesis. This protein was clearly detectable in the various beta-lactam-resistant mutants of *V. cholerae* which were isolated and analyzed. We do not know whether this protein has any direct role in conferring resistance to beta-lactam antibiotics in *V. cholerae*. It might be possible that in the presence of PenG, killing of vibrio cells continues with the concomitant synthesis of this protein until a copy number of this protein sufficient for resistance is synthesized by a small fraction of the cells which survive and emerge as Pen^r cells.

Analysis of the PBP profile of Pen^r cells does not offer any definitive clue to the mechanism of resistance. All of the major PBPs present in susceptible parental cells were present in Pen^r cells, and no significant differences in rates of

binding of PenX to susceptible and resistant cells were noticed (Fig. 5A). The major difference was the much more intense labeling of the lower-molecular-weight PBPs of resistant cells by PenX (Fig. 5A). Low-molecular-weight PBPs are known to have relatively high turnover rates with respect to bound PenG and thus have weak β-lactamase activity (9, 25). However, this possibility was ruled out by the low rate of release of bound PenX from the low-molecular-weight PBPs of resistant cells compared with that from sensitive cells (Fig. 5B) (9). This was also confirmed by the lack of detectable β-lactamase activity in resistant cells. The fact that mutants exhibiting a wide spectrum of cross-resistance against beta-lactams specific for various high-molecular-weight PBPs (PenG, cephaloridine, amdinocillin, and aztreonam, etc.) could be readily isolated suggests that resistance is not due to a modification of the PBPs. We do not think that resistance is related to a permeability problem, as resistance was strictly restricted to antibiotics of the beta-lactam group. Furthermore, the change in morphology of resistant cells grown in the presence of beta-lactams suggests that the antibiotics were not excluded in the resistant cells. The actual mechanism of resistance and the role of the induced 12-kDa outer membrane protein in this process have yet to be established. Finally, the results of the fluctuation test (Table 3) indicate that the beta-lactams may have a directive role in the emergence of resistant mutants.

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