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Isolation and Characterization of Mutants of a Marine *Vibrio* Strain That Are Defective in Production of Extracellular Proteins

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A marine *Vibrio* strain, *Vibrio* sp. strain 60, produces several extracellular proteins, including protease, amylase, DNase, and hemagglutinin. Mutants of *Vibrio* sp. strain 60 (*epr* mutants) pleiotropically defective in production of these extracellular proteins were isolated. They fell into two classes, A and B. In class A, no protease activity was detected in the cells either, whereas in class B, considerable protease activity was detected in the cells. Gel electrophoretic analysis revealed that the protease detected in class B mutant cells was similar to the protease excreted by the parent strain. In addition, the protease in class B mutant cells was found to be localized in the periplasmic space. These results suggest that the passage of the protease through the outer membrane is blocked in class B mutants. Comparison of membrane protein profiles by polyacrylamide gel electrophoresis revealed that all the *epr* mutants contained an increased amount of a 94,000-M_r protein that may be an outer membrane protein. Four *epr* mutations were mapped in two different regions of the *Vibrio* chromosome by transduction; two class A mutations and one class B mutation were located close to each other, whereas another class B mutation was located in a different region of the chromosome.

Unlike gram-positive bacteria, which often excrete a large amount of proteins into the growth medium, gram-negative bacteria usually do not excrete such large amounts of proteins, most likely because the outer membrane acts as a barrier. Some gram-negative bacteria, however, excrete a variety of proteins into the growth medium (29). The precise mechanism underlying the protein excretion by these bacteria remains to be elucidated, however.

Members of the family *Vibrionaceae* are known to produce many extracellular proteins (9–11, 13, 14, 18, 30, 31, 37). When the *Vibrio* genes encoding these extracellular proteins were cloned into *Escherichia coli*, most of the proteins were not excreted into the medium but accumulated in the *E. coli* cells (3, 11, 16, 21, 24, 27, 33). These observations suggest that the *Vibrionaceae* have some specific mechanism for protein excretion which does not exist in *E. coli*.

We previously isolated a marine *Vibrio* strain, *Vibrio* sp. strain 60, which was found to produce several extracellular proteins, including protease, amylase, DNase, and hemagglutinin (26). With this bacterium, we have recently established gene transfer systems, i.e., a transduction system and a conjugation system, and constructed a genetic map of the chromosome (A. Ichige, S. Matsutani, K. Oishi, and S. Mizushima, unpublished data). To gain insight into the mechanism underlying protein excretion into the medium by gram-negative bacteria, we isolated mutants of *Vibrio* sp. strain 60 which are pleiotropically defective in the production of extracellular proteins. We here report the biochemical and genetic characterization of these mutants.

MATERIALS AND METHODS

Bacteria, plasmids, and phage. *Vibrio* sp. strain 60 was isolated from coastal seawater in Japan and found to produce extracellular hemagglutinin (26). A host range examination for a bacteriophage suggests that *Vibrio* sp. strain 60 is related to *Vibrio anguillarum* (Ichige et al., unpublished data). The taxonomical properties of the former strain have not been examined extensively, however. A spontaneous streptomycin-resistant mutant, MVT606, was then isolated and used as the parent for all the *Vibrio* strains constructed in this study. *Escherichia coli* ME8437 (*man-6*::Tn*9*) was obtained from the Genetic Stock Research Center, National Institute of Genetics, Mishima, Japan, and HB101 (*recA13*) (6) was obtained from H. Kiyohara. All cultures were maintained at −80°C in P broth containing 15% (vol/vol) glycerol. Plasmid RP4 (IncP Tra^+ Cb^r Km^r Tc^r) (4) was provided by K. Yano. Two derivatives of RP4, pIO1 (a Tc^s mutant of RP4; Cb^r Km^r) and pIO6 (pIO1 with Tn*10* inserted into the kanamycin resistance gene; Cb^r Tc^r), were constructed in this laboratory (A. Ichige et al., unpublished data). A generalized transducing phage for *Vibrio* sp. strain 60, As3, was isolated from seawater in this laboratory (A. Ichige et al., unpublished data). Plasmid pIO67 was constructed as follows. RP4 was transferred by conjugation into ME8437, which contains transposon Tn*9* (Cm^r) on its chromosome. Tn*9*-containing derivatives were then obtained by mating ME8437(RP4) with HB101 and selecting for Km^r Cm^r transconjugants. pIO67 (Tra^+ Cb^r Km^r Tc^r Cm^r) was one of the plasmids thus obtained.

Media. Bacterial strains were routinely grown at 30°C in P broth containing 1% polypeptone (Daigo Eiyokagaku Co. Ltd., Osaka, Japan), 0.5% yeast extract (Difco Laboratories), and 1% NaCl, pH 7.0. In transductional experiments, *Vibrio* strains were grown in PMC broth containing 1% polypeptone, 0.5% yeast extract, 3% NaCl, 10 mM $MgSO_4$, and 10 mM $CaCl_2$, pH 7.0. For solid medium, 1.6% agar (Nakarai Chemicals Ltd., Kyoto, Japan) was added. The minimal medium used for genetic mapping was S agar, which contained 0.5% Na_2HPO_4, 0.1% KH_2PO_4, 0.05% $(NH_4)_2HPO_4$, 3% NaCl, 0.001% $FeSO_4 \cdot 7H_2O$, 0.2% glucose, 10 mM $MgSO_4$, and 1.6% agar. When required, antibiotics were added at the following final concentrations (in micrograms per milliliter): tetracycline (Tc), 2.5 for *Vibrio* and 20 for *E. coli*; carbenicillin (Cb), 250; ampicillin (Ap), 50; kanamycin (Km), 100 for *Vibrio* and 50 for *E. coli*; chloramphenicol (Cm), 10 for *Vibrio* and 25 for *E. coli*; and streptomycin (Str), 500 for *Vibrio* and 100 for *E. coli*.

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Isolation of mutants defective in protease excretion. Mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was performed essentially as described by Miller (22). Cells were treated with NTG (25 μg/ml) at 30°C for 30 min. After the NTG treatment, the cells were washed, grown overnight in P broth, and then plated on skim milk agar (P agar supplemented with 1% skim milk [Difco]) to detect mutants defective in extracellular protease production.

Mating. Equal volumes of log-phase donor and recipient cultures were mixed for the transfer of plasmid RP4 or its derivatives, and a 10-μl portion was spotted on a P agar plate. After overnight incubation at 30°C, the cells were suspended in P broth and then plated on an appropriate selective plate.

Generalized transduction with As3. The details of the procedure used will be published elsewhere (Ichige et al., manuscript in preparation). Briefly, a log-phase culture of a recipient strain in PMC broth was mixed with an As3 lysate prepared on a donor strain at a multiplicity of infection of less than 1.0. The mixture was allowed to stand at 30°C for 30 min, diluted, and then plated directly on P agar containing tetracycline for the selection of Tc^r transductants or washed once with 50 mM Na_2HPO_4–3% NaCl (pH 7.0) and then plated on S agar for the selection of prototrophic transductants. When *epr* mutants were used as recipients, the adsorption time was increased to 50 min to improve the transduction frequency. As3 phage lysates were prepared by the liquid culture method as described for *Salmonella* phage P22 (6) except that the *Vibrio* strains were grown in PMC broth at 30°C.

Tn*10* insertion near *epr* mutations. Two derivatives of RP4, pIO1 (Cb^r Km^r) and pIO6 (Cb^r Tc^r), were transferred into MVT606 by conjugation, and then Km^r Tc^r transconjugants were subjected to replica plating six successive times on P agar containing kanamycin and tetracycline to enrich for clones in which Tn*10* was transposed from pIO6 onto the chromosome. Colonies were then pooled, and an As3 lysate was prepared. Individual *epr* mutants were transduced with this As3 lysate, followed by selection for Tc^r Epr^+ to obtain transductants in which Tn*10* was inserted near the *epr* mutations. The insertion of Tn*10* near the *epr* mutations was confirmed by As3 transduction of the Tc^r Epr^+ phenotype from these strains to the original *epr* mutants.

Construction of isogenic paired strains. Two pairs of isogenic strains, MVT1264 (*epr*$^+$)/MVT1265 (*epr-1181*) and MVT1256 (*epr*$^+$)/MVT1257 (*epr-1192*), were constructed as follows. Strains carrying Tn*10* near the *epr-1181* and *epr-1192* mutations were isolated by the method described above, and then each *epr* mutation was transferred to MVT606 by As3 transduction, using tetracycline resistance as a marker. Pairs of Tc^r transductants that were *epr*$^+$ and *epr* were selected.

Osmotic shock procedure. Since the sucrose-EDTA method of Neu and Heppel (23) gave unsatisfactory results with *Vibrio* sp. strain 60 due to the instability of the cells in the absence of NaCl, the modified method described below was used to release periplasmic proteins. A 1-ml portion of an overnight culture was diluted 100-fold with P broth, and then cultivation was continued for 8 h. Cells were harvested by centrifugation, washed once with 10 mM Tris hydrochloride (pH 8.0)–1% NaCl, and then suspended in the original volume of 10 mM Tris hydrochloride (pH 8.0)–7.5% NaCl. EDTA was then added to a final concentration of 1 mM. After standing on ice for 10 min, the cells were recovered by centrifugation and then rapidly suspended in the same volume of cold 10 mM Tris hydrochloride (pH 8.0) containing 0.4% NaCl and 5 mM $MgCl_2$. After standing on ice for 10 min, the shock fluid and shocked cells were separated by centrifugation.

Preparation and fractionation of envelope proteins. Cells harvested by centrifugation were washed twice with 10 mM Tris hydrochloride (pH 8.0)–1% NaCl and then lysed by suspension in 10 mM Tris hydrochloride (pH 8.0). Unbroken cells were removed by centrifugation (8,000 × *g*, 5 min), and the cell envelopes were recovered by centrifugation at 50,000 × *g* for 30 min at 4°C. The cell envelopes were then treated with 1.5% Sarkosyl as described by Filip et al. (8). Sarkosyl-insoluble proteins were recovered by centrifugation (50,000 × *g*, 30 min at 4°C) and then suspended in 10 mM Tris hydrochloride (pH 8.0). Sarkosyl-soluble proteins were precipitated with 4 volumes of cold acetone and then dissolved in 10 mM Tris hydrochloride (pH 8.0).

SDS-PAGE. The method of Laemmli (17) was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), with 10% acrylamide and 0.33% bisacrylamide. Gels were stained with Coomassie brilliant blue by the method of Fairbanks et al. (7).

Enzyme assays. Protease activity was assayed by using azocasein as a substrate by the method of Braun and Schmitz (2). One unit of protease activity was defined as an increase in absorbance at 436 nm of 1.0 per 30 min under the conditions used. β-Lactamase activity was assayed by using the synthetic substrate 7-(thienyl-2-acetamido)-3-[2-(4-*N*,*N*-dimethyl-aminophenylazo)-pyridinium methyl]-3-cephem-4-carboxylic acid (PADAC) (Calbiochem-Behring) (15). One unit of activity was defined as a decrease in absorbance at 572 nm of 1.0 per min under the conditions used. Chloramphenicol acetyltransferase activity was assayed by the method of Lupski et al. (19). One unit of activity was defined as an increase in absorbance at 412 nm of 1.0 per min under the conditions used.

Assay for extracellular products. Colonies were formed on agar plates, and then the extracellular production of proteins around the colonies was detected as follows. Protease was detected on a skim milk agar plate (25). Amylase was detected on a P agar plate supplemented with 0.2% soluble starch. After incubation at 30°C for 24 h, the plate was flooded with an I_2-KI solution (32). DNase was detected on a P agar plate supplemented with 0.2% DNA from herring testes (type XIV; Sigma Chemical Co.). After incubation at 30°C for 24 h, the plate was flooded with 1 N HCl (11). Hemagglutinin was detected as described previously (26) except that the bacteria were grown in HA broth (pH 7.0) containing (per liter) 30 g of polypeptone, 5 g of yeast extract, and 500 ml of artificial seawater (20).

Activity staining for protease. PAGE was performed in the absence of SDS with the buffer system of Davis (5). The gel contained 0.1% gelatin (Difco), 10% acrylamide, and 0.33% bisacrylamide. After electrophoresis, the gel was incubated in 50 mM Tris-glycine buffer (pH 8.3) for 1 h at 37°C, and then proteolytic activity was detected on the gel by staining with 0.2% amido black.

RESULTS

Specific excretion of protease into the culture medium by *Vibrio* sp. strain 60. In addition to hemagglutinin (26), *Vibrio* sp. strain 60 was found to excrete several extracellular proteins, including protease, amylase, and DNase. In order to determine whether these proteins were excreted into the medium in a specific manner, the distribution of protease in a culture of MVT606 was examined. Plasmid pIO67, which

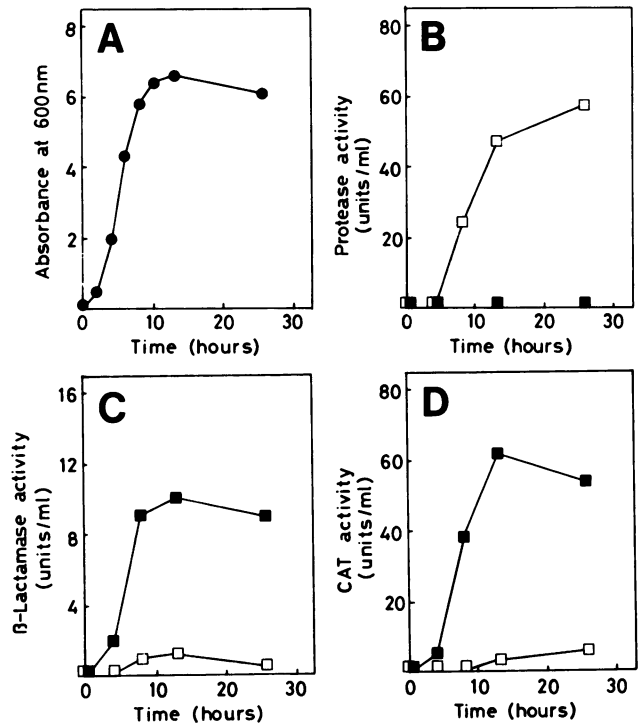

FIG. 1. Distribution of protease, β-lactamase, and chloramphenicol acetyltransferase during the growth of *Vibrio* sp. strain 60. MVT606(pIO67) was grown in P broth. A sample (1 ml) was withdrawn at the indicated times, and then the culture fluid and cells were separated by centrifugation. Cells were then lysed, and the enzyme activities in culture fluids (□) and cell lysates (■) were measured. (A) Growth; (B) protease; (C) β-lactamase; (D) chloramphenicol acetyltransferase (CAT).

carries the genes encoding chloramphenicol acetyltransferase (a cytoplasmic enzyme) and β-lactamase (a periplasmic enzyme), was transferred into MVT606 by conjugation, and the localization of these enzymes as well as that of protease was examined. Almost all the protease activity was located extracellularly, whereas large fractions of both the chloramphenicol acetyltransferase and β-lactamase activities remained in the cellular fraction (Fig. 1), suggesting that *Vibrio* sp. strain 60 excretes the protease into the medium in a specific manner.

Isolation of *epr* mutants. We attempted to isolate mutants that were pleiotropically defective in extracellular protein production (*epr* mutants) as candidates for mutants defective in the process of protein excretion. We first isolated extracellular protease-negative mutants after NTG mutagenesis. Six extracellular protease-negative mutants thus obtained were examined for the production of other extracellular proteins (Table 1). All of the mutants except MVT1188 showed a marked deficiency in the production of all extracellular proteins examined. These five pleiotropic mutants were designated *epr* mutants and studied further.

Biochemical characterization of the *epr* mutants. The distribution of protease activity between the culture fluid and the cell was examined first (Table 2). Although all the *epr* mutants exhibited a highly decreased level of protease activity in the culture fluid, they fell into two classes with regard to the activity detected in the cells; cell-associated protease activity was not detected in class A mutants, whereas the cell lysates of class B mutants exhibited considerable protease activity.

TABLE 1. Production by extracellular proteins by *epr* mutants

Strain	*epr* genotype	Production[a]			
		Protease	Amylase	DNase	Hemagglutinin[b] (HA units)
MVT606	*epr*$^+$	+++	+++	+++	128
MVT1064	*epr-1064*	−	−	−	2
MVT1181	*epr-1181*	+	−	−	16
MVT1188	—	+	+++	+++	128
MVT1192	*epr-1192*	+	−	−	2
MVT1200	*epr-1200*	+	−	−	2
MVT1203	*epr-1203*	−	−	−	2

[a] The production of protease, amylase, and DNase was assayed by agar plate methods and that of hemagglutinin was assayed by the liquid culture method as described under Materials and Methods.
[b] Expressed in hemagglutinating (HA) units.

To clarify the relationship between the protease detected in the class B mutant cells and that excreted by the parent strain, we compared them by SDS-free PAGE followed by activity staining (Fig. 2). At least four to five protease bands were detected for both preparations, and both preparations exhibited similar mobility profiles. In addition, the protease activity in both preparations was sensitive to *o*-phenanthroline, a metal protease inhibitor, and resistant to phenylmethylsulfonyl fluoride, a serine protease inhibitor. Together, these observations suggest that the intracellular protease(s) of the class B mutants and the extracellular protease(s) of the parent strain are closely related, possibly being products of the same gene(s). Thus, it seemed reasonable to assume that the class B mutants had a defect in the process of protein excretion.

The localization of the protease that accumulated in the mutant cells was then examined. Cells of two class B

TABLE 2. Distribution of protease in the parent strain and *epr* mutants

Strain	Phenotype	Protease activity[a] (U/ml)		Class
		Extracellular	Cell-associated	
MVT606	Epr$^+$	38.1	<0.5	Wild type
MVT1064	Epr$^-$	1.6	<0.5	A
MVT1181	Epr$^-$	6.2	5.4	B
MVT1192	Epr$^-$	4.3	5.8	B
MVT1200	Epr$^-$	0.7	5.9	B
MVT1203	Epr$^-$	1.1	<0.5	A

[a] The bacteria were grown in P broth for 16 h.

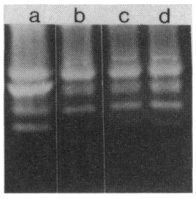

FIG. 2. Activity staining for protease after PAGE. Samples were subjected to PAGE in the absence of SDS, and then protease activity was detected on the gel as described under Materials and Methods. Lanes: a, culture fluid of the parent strain (MVT606); b, c, and d, cell lysates of MVT1181, MVT1192, and MVT1200, respectively. The samples applied to the gel were equivalent to 2.5 μl of the original cultures.

TABLE 3. Localization of protease in class B mutant cells

Strain[a] (mutation)	Fraction	Activity in U/ml (% of total)		
		Protease	β-Lactamase	CAT[b]
MVT1326 (*epr-1192*)	Culture medium	0.6 (13)	1.6 (23)	2.4 (6)
	Shock fluid	1.6 (33)	3.0 (43)	5.0 (13)
	Shocked cells	2.6 (54)	2.4 (34)	31.2 (81)
MVT1328 (*epr-1181*)	Culture medium	1.3 (37)	2.6 (43)	2.1 (8)
	Shock fluid	1.2 (34)	2.4 (40)	4.2 (16)
	Shocked cells	1.0 (29)	1.0 (17)	19.8 (76)

[a] MVT1326 and MVT1328 carry plasmid pIO67.
[b] CAT, Chloramphenicol acetyltransferase.

mutants, MVT1326 and MVT1328, were lysed by suspending them in 10 mM Tris hydrochloride (pH 8.0), and then their membranes were pelleted by ultracentrifugation. For both strains, about 80% of the intracellular protease activity was found in the soluble fraction, i.e., the cytoplasm or periplasm. Cells of these mutants were then subjected to osmotic shock. As shown in Table 3, protease activity was released from the mutant cells by cold osmotic shock with an efficiency approximately the same as that of β-lactamase activity, indicating that most of the intracellular protease was in the periplasmic space.

The periplasmic localization of the protease suggests a possible alteration of the outer membrane composition in the class B mutants. We therefore analyzed the envelope proteins of the mutants and those of the parent strain by SDS-PAGE (Fig. 3). All the mutants examined contained a considerably larger amount of a 94,000-M_r (94K) protein than the parent strain did. It should be noted that an increase in the amount of the 94K protein was also observed for all the class A mutants examined. We then treated the total envelope fraction with Sarkosyl. The 94K protein and several major proteins, which were most likely major outer membrane proteins, were recovered exclusively in the Sarkosyl-insoluble fraction (Fig. 4). It has been shown that the proteins of the cytoplasmic membrane of *E. coli* are, in most cases, solubilized by Sarkosyl, whereas those of the outer membrane are resistant to such solubilization (8). Although it is not clear at present whether the outer membrane proteins of *Vibrio* sp. strain 60 show the same tendency or not, our observations suggest that the 94K protein is an outer membrane protein in both the parent strain and the *epr* mutants.

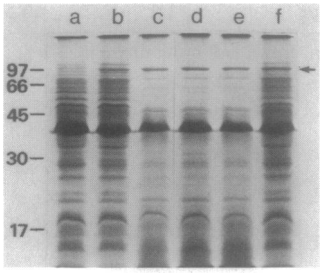

FIG. 3. SDS-PAGE of envelope proteins. The bacteria were grown in P broth for 16 h, and then the proteins in the envelope fraction were analyzed by PAGE. Each lane was loaded with 25 μg of protein. Lanes: a, MVT606, parent strain; b, MVT1064; c, MVT1181; d, MVT1192; e, MVT1200; and f, MVT1203. The arrow indicates position of the 94K protein. The molecular weight standards used were (top to bottom, in thousands) phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, and myoglobin.

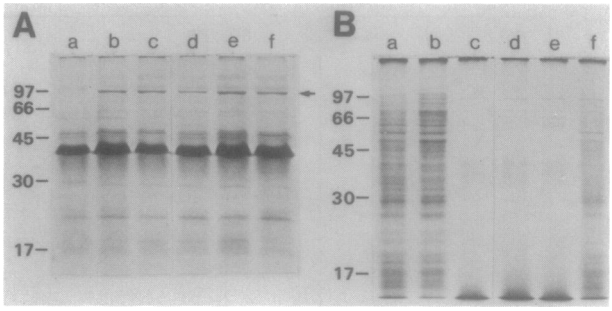

FIG. 4. SDS-PAGE of Sarkosyl-insoluble and -soluble proteins. The envelope fraction was treated with Sarkosyl, and then the proteins in the insoluble (A) and soluble (B) fractions were analyzed by PAGE. Lanes: a, MVT606, parent strain; b, MVT1064; c, MVT1181; d, MVT1192; e, MVT1200; and f, MVT1203. Each lane was loaded with 15 μg of protein. The molecular weight standards were the same as those in Fig. 3. The 94K protein is indicated by an arrow.

Genetic characterization of the *epr* mutants. We recently established a generalized transduction system with phage As3 and a conjugation system in *Vibrio* sp. strain 60. In addition, by means of these gene transfer systems, we have constructed a genetic map of the chromosome of this bacterium, part of which is shown in Fig. 5. To facilitate genetic mapping of *epr* mutations, we first attempted to insert Tn*10* (Tc^r) near the individual *epr* mutations to use Tc^r as a selection marker for As3 transduction. Although all the *epr* mutants exhibited decreased susceptibility to the As3 phage, two such Tn*10* insertions, *chr-1102*::Tn*10* (72% cotransducible by As3 with *epr-1064*) and *chr-1262*::Tn*10* (52% cotransducible with *epr-1181*), were isolated. When *epr* mutants were transduced to Tc^r with As3 grown on MVT1102 (MVT1064 *epr*$^+$ *chr-1102*::Tn*10*), *epr-1192* and *epr-1203* were found to be cotransducible with *chr-1102*::Tn*10* (cotransduction frequency, 68 and 75%, respectively), suggesting that *epr-1064*, *epr-1192*, and *epr-1203* are close to each other. Since *chr-1102*::Tn*10* was mapped between *lys-791* and *cys-676* by As3 transduction (Fig. 5), As3 lysates were prepared on MVT1064, MVT1192, and MVT1203 and used to transduce MVT791 (MVT606 *lys-791*) and MVT676 (MVT606 *cys-676*) to Lys^+ and Cys^+, respectively. Mutations *epr-1064*, *epr-1192*, and *epr-1203* were cotransducible with *lys-791*, with cotransduction frequencies of 5, 7, and 11%, respectively, and with *cys-676*, with cotransduction frequencies of 11, 10, and 11%, respectively, indicating that the three *epr* mutations were also located between *lys-791* and *cys-676* (Fig. 5). The reason for the relatively low efficiency in transduction with *epr* mutations is unclear. On the other hand, *chr-1262*::Tn*10* was mapped in a different region of the chromosome, close to *cys-1149*, with a cotransduction frequency of 85%. When MVT1167, a *cys-1149* strain derived from *Vibrio* sp. strain 60, was transduced to Cys^+ with As3 grown on MVT1181, 58% of the transductants were Epr^-, confirming the proximity of *epr-1181* to *cys-1149*. At present, it is not clear whether the three mutations *epr-1064*, *epr-1192*, and *epr-1203*, located between *lys-791* and *cys-676*, reside in the same gene or not. The fact that two *epr* mutations of the class B mutants were located in different regions of the chromosome suggests that at least two components are involved in the excretion of proteins, possibly through the outer membrane.

To determine whether each of the *epr* mutations was truly responsible for the pleiotropic changes in the phenotype of

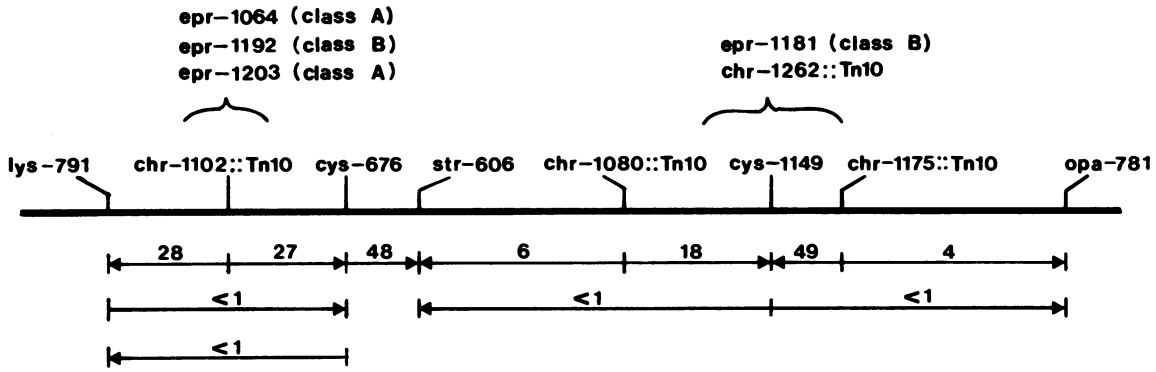

FIG. 5. Part of the genetic map of the *Vibrio* sp. strain 60 chromosome and mapping of *epr* mutations by As3 transduction. The details of the genetic map will be published elsewhere (Ichige et al., in preparation). Only the region around the *epr* mutations is presented. The arrows point from selected to unselected markers, and the numbers representing percent cotransduction frequencies. *chr-1080*::Tn*10* and *chr-1175*::Tn*10* represent Tn*10* insertions that were used for construction of the genetic map.

the class B mutants, two pairs of epr^+ and *epr* strains which were otherwise isogenic were compared. They were MVT1264 (epr^+)/MVT1265 (*epr-1181*) and MVT1256 (epr^+)/MVT1257 (*epr-1192*). The comparison confirmed that each of the *epr* mutations caused the following pleiotropic changes: inability to excrete protease, amylase, DNase, and hemagglutinin into the medium; accumulation of protease in the periplasm; and an increase in the amount of the 94K protein in the envelope.

DISCUSSION

We have isolated mutants that were pleiotropically defective in the production of several extracellular proteins, including protease (*epr* mutants). They fell into two classes, A and B, in terms of their biochemical properties. In class A mutants, the defect did not result in the accumulation of protease in the cells. Class B mutations were found to result in periplasmic accumulation of the protease, the gel electrophoretic profile of which was similar to that of the extracellular protease excreted by the parent strain. These results strongly suggest that class B mutants have a defect in the process of protein excretion across the outer membrane. Mutants similar to the class B mutants have been isolated from *Aeromonas hydrophila* (14), *Erwinia chrysanthemi* (1, 34), and *Pseudomonas aeruginosa* (35). The results also suggest that protease excretion into the growth medium takes place via the periplasmic space. Consistent with this view, the results of a kinetic study suggested that the enterotoxin of *Vibrio cholerae* was excreted from the cells through the periplasmic space (12). We cannot exclude the possibility, however, that the periplasmic accumulation of protease is an artificial event due to blocking of the normal excretion process.

The reason for the lack of accumulation of the protease in class A mutants, even in the cell-associated form, remains to be elucidated. The fact that the class A mutations mapped very close to one of the class B mutations suggests that they are mutations in the same gene. It is possible, therefore, that the difference in phenotype of these class A and class B mutants is a reflection of leakiness of the mutations in the same gene.

It has been shown that the extracellular proteases of *Serratia marcescens* (36) and *Neisseria gonorrhoeae* (28) are first synthesized as a larger precursor containing a considerably large extra domain at the C-terminus. This extra domain was proposed to play an essential role in the translocation of the enzyme across the outer membrane and to be cleaved off as the protein passes through this membrane (28, 36). Provided that the *Vibrio* protease is also synthesized as a larger precursor with an extra peptide, it is probable that the *epr* mutations result in premature processing of this extra peptide, blocking translocation of the enzyme across the outer membrane. At the moment, however, we have no evidence that a larger precursor for the *Vibrio* protease exists.

What is the nature of the alteration in the outer membrane which results in the defect in excretion? Some changes in the composition of outer membrane proteins were reported for one class of *A. hydrophila* mutants defective in protein excretion (14). The only change we observed in the present study was an increase in the amount of a 94K protein possibly located in the outer membrane. The increase was observed for all the mutant strains, irrespective of whether they belonged to class A or B, suggesting that the overproduction of this protein has something to do with the lack of protein excretion. Although the nature of this protein is entirely unknown, it might be a protease, the overproduction of which results in premature processing of extracellular proteins in the periplasmic space. Of course, it is also possible that the increase in the amount of the 94K protein is another consequence of blockage of protein excretion in these mutant cells.

By means of gene transfer systems that were recently established for *Vibrio* sp. strain 60, four *epr* mutations were mapped on the *Vibrio* chromosome. Genetic analysis also revealed that the *epr* mutations caused the observed pleiotropic changes in the phenotype of these mutants. The *epr* mutations were mapped in two distinctly different regions of the chromosome. These mutations may define genes which are involved in protein excretion. In particular, the fact that two class B mutations resided in two different genes indicates that at least two genes are involved in protein excretion into the medium after translocation across the cytoplasmic membrane. The results of biochemical analysis of *A. hydrophila* mutants defective in protein excretion also suggested that at least two gene products are needed for export after protein translocation across the cytoplasmic membrane (14). In *P. aeruginosa*, six genetic loci (*xcp*) involved in the synthesis or release of extracellular proteins have been identified by genetic mapping (35), although the nature of the

gene products remains unknown. Characterization of the *epr* genes and identification of the gene products should provide valuable information on the mechanism of protein excretion by gram-negative bacteria.

Finally, it should be noted that for all the *epr* mutations, some difficulty was encountered in transductional mapping with phage As3, suggesting a change in the cell surface structure. It is possible, therefore, that the *epr* genes are involved in the proper localization of some cell surface components in addition to that of extracellular proteins.

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