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Cloning, Expression, and Mapping of the *Aeromonas hydrophila* Aerolysin Gene Determinant in *Escherichia coli* K-12

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DNA sequences corresponding to the aerolysin gene (*aer*) of *Aeromonas hydrophila* AH2 DNA were identified by screening a cosmid gene library for hemolytic and cytotoxic activities. A plasmid containing a 5.8-kilobase *Eco*RI fragment of *A. hydrophila* DNA was required for full expression of the hemolytic and cytotoxic phenotype in *Escherichia coli* K-12. Deletion analysis and transposon mutagenesis allowed us to localize the gene product to 1.4 kilobases of *Aeromonas* DNA and define flanking DNA regions affecting aerolysin production. The reduced hemolytic activity with plasmids lacking these flanking regions is associated with a temporal delay in the appearance of hemolytic activity and is not a result of a loss of transport functions. The aerolysin gene product was detected as a 54,000-dalton protein in *E. coli* maxicells harboring *aer* plasmids and by immunoblotting *E. coli* whole cells carrying *aer* plasmids. We suggest that the gene coding aerolysin be designated *aerA* and that regions downstream and upstream of *aerA* which modulate its expression and activity be designated *aerB* and *aerC*, respectively.

The gram-negative organism *Aeromonas hydrophila* is receiving increasing attention because of its association with human disease and foodborne infections (11, 18, 26, 27). Many strains of this species have been shown to elaborate various exotoxins, including two hemolysins, one of which is a cytotoxic hemolysin (aerolysin), enterotoxin, and proteases (20). Although a number of these toxins have been isolated and biochemically characterized, their roles in the pathogenesis of *Aeromonas* infection remain largely unknown.

A large body of literature implicates the cytotoxic hemolysin aerolysin as an important virulence factor (4, 7, 10, 16, 18). Wild-type *Aeromonas* isolates vary widely in their ability to produce the toxin, and toxicity assays performed with mice show a correlation between the amount of toxin produced and mortality in mice (9). More recently, a study of 686 *Aeromonas* strains from various geographical locations has shown a strong correlation between hemolysis and enterotoxicity (5). Nevertheless, discrepancies in the literature exist when equating the cytotoxic phenotype to the hemolytic phenotype (17).

Aerolysin has been isolated and purified to homogeneity (2, 3). The isolated protein has a molecular weight of 52,000 and is toxic for blood erythrocytes and tissue culture cell lines (13). A putative receptor for the toxin on mouse erythrocytes has recently been identified by Howard and Buckley (13, 32). Among the biological activities associated with purified toxin are: elicitation of vascular permeability at the site of injury, edema, and necrotic and lethal activities. These properties are consistent with some of the pathological features associated with *Aeromonas* infections, i.e., local edema, tissue necrosis, septicemia, and mortality (20).

To understand the role of the toxin in *A. hydrophila*-associated infections, we used recombinant DNA techniques to clone the corresponding gene. Our studies are directed at (i) understanding the basis of variation of toxin production in wild-type *Aeromonas* isolates, (ii) dissecting elements involved in transport of the toxin in *A. hydrophila*, and (iii) correlating the presence of the toxin gene and its expression to the virulence of the bacterium. In this paper we report on the isolation, mapping, and expression of the aerolysin determinant in *Escherichia coli*.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* K-12 strains used were LE392 (F⁻ *hsdR514* [*hsdR hsdM*] *supE44 supF58 lacY1 galK galT22 metB1 trpR55* λ⁻), RB308 (F::Tn*1000 thyA deoC rpsL*), CSH26ΔF6 [*ara thi* Δ(*lac-pro*) Δ(*recA-srl*)*F6 rpsL*], MC4100 [*araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25*], JC3272 (*gal his lac lamB lpoB* or *mrc lys trp tsx rpsL* λ⁻), MBM7060 [*araC araD* Δ(*argF-lac*)*U169 trp malB rpsL relA thi supF*], and W3110 (F⁻ *hsdR hsdM*).

A. hydrophila AH2, which produces both hemolytic and cytotoxic activities, has been previously described (6). For the isolation of protein fusions, the plasmid pMLB1034 in *E. coli* MBM7060 (kindly provided by M. L. Berman) was used (35). All other cloning experiments were carried out with the multicopy-number plasmids pBR322, pBR328 (28), and p*tac*11 (1) and cosmid pHSS255 (6).

Media and culture conditions. The medium for maintenance and growth of strains was L broth (1% tryptone, 0.5% yeast extract, 1% NaCl) or the same broth supplemented with 1% agar. Maxicells were grown in M9 medium (21) supplemented with 0.5% glucose and 1 μg of thiamine per ml. When necessary, media were complemented with the following antibiotics at the specified concentrations (micrograms per milliliter): ampicillin, 100; kanamycin, 50; tetracycline, 12.5; streptomycin, 200. Blood agar plates (1% peptone, 1% meat extract, 0.5% NaCl, 1% agar, 5% defibrinated human erythrocytes) were used to identify hemolytic-nonhemolytic colonies. The β-galactosidase chromogenic substrate 5-bromo-4-chloro-3-indoyl-β-D-galactoside was used at a concentration of 40 μg/ml with LB agar medium.

Tissue culture assay. A tissue culture assay was performed as described in reference 6. HeLa cells were grown in Eagle minimal medium (Serva, Heidelberg, Federal Republic of

Germany) with 10% fetal calf serum (Serva) and 50 μg of polymyxin B per ml (Sigma Chemical Co., St. Louis, Mo.).

DNA manipulations. Restriction enzyme cleavage, in vitro ligation, and transformation techniques were carried out as described previously (6). Cosmid cloning was carried out as described by Maniatis et al. (21). Phage lambda DNA cleaved with *Eco*RI and *Hin*dIII served as a molecular size standard.

Cell fractionation and hemolysin assays. Osmotically shocked cells were prepared as described by Neu and Heppel (24). Hemolysin assays were performed as described in reference 34 with 2% washed human erythrocytes. For screening large numbers of samples for hemolysin production, extracts were prepared by the freeze-thaw method of Pearson and Mekalanos (25), except that the extracts obtained were not subjected to centrifugation.

Electrophoretic analysis of plasmid-coded polypeptides. Analysis of plasmid-coded translational products in a maxicell system has been described previously (29). Labeling of polypeptides was carried out in methionine assay medium (Difco Laboratories, Detroit, Mich.) containing 50 μCi of [^{35}S]methionine (970 Ci/mmol; Amersham Büchler, Braunschweig, Federal Republic of Germany).

Samples in gel electrophoresis buffer were placed at 100°C for 5 min to dissociate proteins into individual polypeptides. Samples were analyzed in a 12.5% acrylamide gel dried and used to expose Fuji RX X-ray film.

Immunoblots were performed as described by Towbin et al. (33) with minor modifications. Polypeptides were transferred from sodium dodecyl sulfate-polyacrylamide gels to nitrocellulose filters in 20 mM Tris–150 mM glycine–10% methanol–0.02% sodium dodecyl sulfate. All incubations and washes were performed at room temperature in phosphate-buffered saline containing 0.05% Tween 20. Neutralizing rabbit antisera to aerolysin was the kind gift of S. Notermans (Public Health Laboratory, Bilthoven, The Netherlands) and was used at a 1:1,000 dilution. Horseradish peroxidase-conjugated anti-rabbit immunoglobulin (Nordic Laboratories, Tilburg, The Netherlands) was used at a 1:1,000 dilution. Blots were developed with 3-amino-9-ethylcarbazole (0.4 mg/ml) and 0.015% hydrogen peroxide.

RESULTS

A gene library of strain AH2 was constructed by cloning *Sau*3AI-generated fragments of total cell DNA into the *Bam*HI site of the cosmid vector pHSS255. Hybrid cosmids were packaged in vitro into phage lambda heads, and reconstituted lambda particles were used to infect *E. coli* LE392 bacteria. A total of 816 independent cosmid clones were obtained upon selection for kanamycin resistance.

Cosmid clones were screened for the aerolysin determinant by using blood agar plates and tissue culture cell lines. Recombinant cosmid clones were deemed to carry the gene coding for aerolysin when they produced zones of hemolysis on blood agar plates and caused cytotoxicity in the tissue culture assay. Two cosmid clones were detected that fulfilled both criteria. Plasmid DNA isolated from both recombinant clones showed many common restriction fragments upon digestion with the endonucleases *Hin*dIII, *Eco*RI, *Bam*HI, and *Hin*cII. One cosmid clone, designated pAH507, which makes up 38 kilobases (kb) of AH2 total cell DNA was chosen for further study.

To facilitate mapping of the aerolysin determinant, *Hin*dIII fragments of the cosmid pAH507 were cloned into the *Hin*dIII site of the plasmid vector pBR328 (28). One

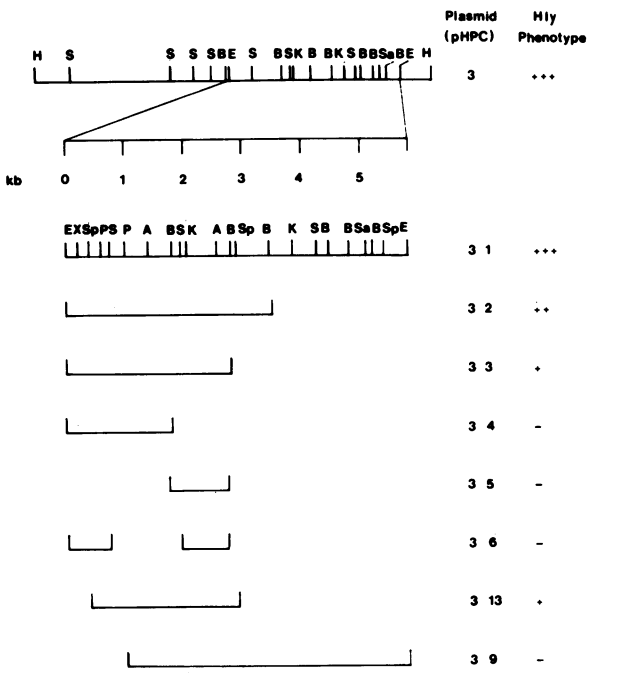

FIG. 1. Physical map of pHPC3 and its derivatives. ⌊⌋, Sequence of *A. hydrophila* DNA subcloned into vector plasmids to form the corresponding recombinant plasmid. Hly phenotype (−, +, ++, +++) is the degree of hemolysis observed with the different deletion mutants. A, *Ava*I; B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; P, *Pst*I; S, *Sma*I; Sa, *Sal*I; Sp, *Sph*I; and X, *Xho*I. Size of inserted *Aeromonas* DNA fragment in plasmid pHPC: 3 = 13.5 kb; 3-1 = 5.8 kb; 3-2 = 3.5 kb; 3-3 = 2.8 kb; 3-4 = 1.8 kb; 3-5 = 1 kb; 3-6 = 1.6 kb; 3-13 = 2.5 kb; 3-9 = 4.8 kb.

recombinant, pHPC3, resistant to ampicillin and exhibiting both hemolytic and cytotoxic activities, was found to harbor a 13.5-kb *Hin*dIII fragment. A partial restriction map of this plasmid is shown in Fig. 1. Subcloning of the 5.8-kb *Eco*RI fragment from pHPC3 into the pBR322-based vector p*tac*11 (1) gave rise to ampicillin-resistant colonies showing clear zones of lysis on blood agar plates. Hemolytic zones were usually obtained after 18 h of incubation at 37°C; further incubation of such colonies resulted in large zones of hemolysis, often 1 to 2 cm in diameter. One recombinant clone, pHPC3-1, whose phenotype was indistinguishable from that of the parental cosmid pAH507 was chosen for further study.

Deletion mapping of the aerolysin gene on pHPC3-1. To delineate the gene on the 5.8-kb *Eco*RI insert of pHPC3-1, we constructed a restriction endonuclease map with several restriction endonucleases (Fig. 1). The map thus obtained was used to construct a set of in vitro deletion mutants of pHPC3-1. These deletion mutants were used to localize the aerolysin gene within the insert.

The deletion plasmids pHPC3-2, -3, and -4 were constructed by digesting plasmid pHPC3-1 with the restriction endonuclease *Bam*HI to completion and religating the DNA fragments at a low DNA concentration. The *tac* promoter was deleted during the construction of plasmids pHPC3-2, -3, and -4. Deletion of the *Bam*HI fragments to the right of the *Bam*HI site located at coordinate 1.8 kb led to a loss of hemolytic activity (Fig. 1, pHPC3-4). Nonhemolytic colonies were also obtained when the *Bam*HI fragment located between coordinates 1.8 and 2.8 kb was cloned into the *Bam*HI site of the vector pBR322. However, all recombi-

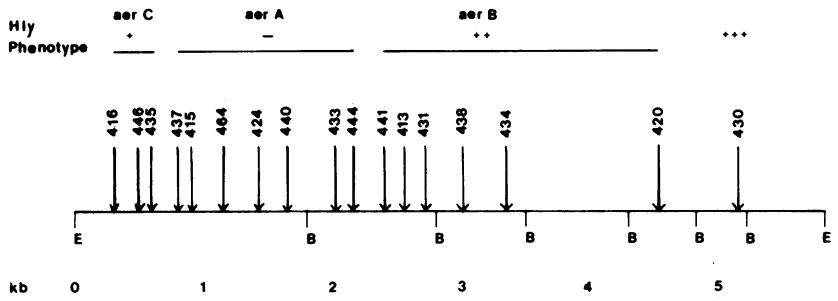

FIG. 2. Linear map of *A. hydrophila* DNA in plasmid pHPC3-1, showing sites of insertion of a number of Tn*1000* derivatives. The probable physical locations of the genes *aerA*, *aerB*, and *aerC*, based on all available data, are shown above the map. E, *Eco*RI; B, *Bam*HI.

nants carrying the *Eco*RI-*Bam*HI-*Bam*HI fragments (pHPC3-3; coordinates 0 to 2.8 kb) were hemolytic. Hemolytic recombinants were obtained by cloning the *Sph*I fragment from pHPC3-3 into the *Sph*I site of pBR322. Plasmid deletions obtained with the restriction endonucleases *Pst*I and *Sma*I produced nonhemolytic recombinants, which suggests that these sites are internal to the aerolysin gene. These results enable the aerolysin gene to be located between coordinates 0.4 and 2.8 kb, transversing the *Bam*HI site located at coordinate 1.8 kb. All recombinants possessing the hemolytic phenotype showed cytotoxicity in the HeLa and CHO tissue culture cell assays. In the course of these experiments, we noted that strains carrying the deletion-derived plasmids pHPC3-2 and pHPC3-3 showed turbid zones of hemolysis after a 24-h incubation at 37°C. This contrasted with the clear zones of lysis obtained with strains harboring the parental plasmid pHPC3-1 incubated over the same period. A closer inspection showed that hemolytic zones were visible earlier for strains harboring pHPC3-2 than for pHPC3-3. However, further incubation of both these strains, up to 72 h, resulted in zones of lysis that were comparable to those of strains harboring pHPC3-1.

Mapping of the aerolysin gene with Tn*1000*. A complementary approach to mapping the aerolysin determinant on plasmid pHPC3-1 was attempted by using insertional inactivation of the gene with transposon Tn*1000*. Mobilization of the pBR322-based recombinant plasmid pHPC3-1 by the F factor occurs by Tn*1000*-mediated cointegration of the two plasmids. Resolution of the cointegrate in recipient cells results in pHPC3-1::Tn*1000* insertion derivatives (12).

Plasmid pHPC3-1 was transformed into *E. coli* RB308 (which contains F::Tn*1000*) to give SBC60. Equal volumes of exponential-phase cultures of SBC60 and JC3272 (Str^r) were mixed and left to stand for 4 h, after which they were plated out onto selective media. Ampicillin-resistant, streptomycin-resistant transconjugants were purified by two cycles of streaking onto fresh selective plates. The sites of insertion of Tn*1000* were mapped by restriction enzyme analysis. Preliminary mapping was done by digestion of plasmids with *Hin*dIII, which cuts both the transposon and the recombinant plasmid at a single site. Fine mapping was performed by digestion with other enzymes (e.g., *Sma*I, *Pst*I) either singly or in combination. Figure 2 shows the location of some of the randomly selected hemolytic and nonhemolytic transconjugants.

Three different phenotypes were detected upon plating these mutants on blood agar plates. No loss of the hemolytic phenotype was detected when insertions were located in the vector backbone or distal to the *Bam*HI site at coordinate 5.3 kb (Fig. 2). Insertions between coordinates 2.4 and 4.6 kb resulted in a reduced hemolytic phenotype similar to that obtained with the in vitro-generated deletion plasmids pHPC3-2 and pHPC3-3. All insertions between coordinates 0.8 and 2.2 kb resulted in the complete loss of the hemolytic phenotype. Surprisingly, colonies with turbid zones of hemolysis were also obtained with transposon insertions between coordinates 0.3 and 0.6 kb.

From the data obtained with transposon insertions, the aerolysin gene can be located between coordinates 0.8 and 2.2 kb of the 5.8-kb *Eco*RI insert of pHPC3-1. These results confirm and extend the observations made with in vitro-generated plasmid derivatives, that is, that adjacent regions both upstream and downstream of the aerolysin structural gene are involved in controlling the expression of its gene product.

To determine the direction of transcription of the aerolysin gene within the cloned insert, aerolysin–β-galactosidase fusions were constructed. For this purpose, we used the fusion vector pMLB1034 (35) to generate hybrid proteins consisting of the N-terminal portion of the aerolysin gene and the C-terminal end of enzymatically active β-galactosidase. The plasmid pHPC3-3 cleaved with the restriction endonucleases *Bam*HI or *Sma*I, singly or in combination with the restriction endonuclease *Eco*RI, was cloned into the vector plasmid pMLB1034 which had been similarly cleaved. All recombinants carrying the 1.8-kb *Eco*RI-*Bam*HI fragment resulted in β-galactosidase-positive fusions, as detected by the appearance of red colonies on MacConkey plates or a blue color upon plating on 5-bromo-4-chloro-3-indoyl-β-D-galactoside plates. No positive β-galactosidase fusions were detected with recombinants harboring either the 0.8-kb *Eco*RI-*Sma*I fragment or the 1.2-kb *Sma*I-*Sma*I fragment of pHPC3-3.

These results enable us to conclude that the aerolysin gene is transcribed in the clockwise direction within the 5.8-kb *Eco*RI fragment from *A. hydrophila*. Furthermore, the promoter region for the aerolysin gene would appear to be at or around the *Sma*I site at coordinate 0.8 kb. Since no known transcription units from the vector pMLB1034 enter the insert (30, 35), the gene is probably transcribed from its own promoter. In this context we note that the *tac* promoter transcribes in the opposite orientation to the promoter of the aerolysin gene in plasmid pHPC3-1.

The β-galactosidase-positive fusion obtained was devoid of both hemolysin and cytotoxic activities. This observation suggests that sequences at the C-terminal end of aerolysin are necessary for expression of these activities.

Analysis of reduced hemolytic phenotype. The reduced hemolytic phenotype obtained by insertions or deletions within regions upstream and downstream of the aerolysin gene is reminiscent of the reduced zones of hemolysis obtained when transport functions of the *E. coli* hemolysin

TABLE 1. Expression and localization of the cloned aerolysin in *E. coli*

Strain[a] or clone	Hemolytic activity[b] (% total activity)		β-Galactosidase activity[c] (% total activity)	
	Osmotic shock fluid[d]	Sonicated cells[e]	Osmotic shock fluid	Sonicated cells
W3110			1.8	98.2
W3110(pHPC3-1)	53	47	1.5	98.5
W3110(pHPC3-2)	61	39	1.2	98.8
W3110(pHPC3-3)	55	45	1.8	98.2

[a] Strains were cultured in 10 ml of broth for 18 h at 37°C.
[b] Hemolysin assays were performed as described in reference 34. Total activity is the sum of activity in osmotic shock fluids and the cell pellet.
[c] β-Galactosidase activity was assayed as described by Miller (23).
[d] Supernatant obtained after suspension in 0.8 ml of cold water and gently shaken for 10 min at 0°C.
[e] Cell pellet from hemolytic activity measurement was suspended in phosphate-buffered saline to a cell density of 10^8 cells per ml. Cells were lysed by ultrasonication (34).

hemolysin have been deleted (34). To test whether this was the case with the aerolysin gene product, we looked for the presence of hemolytic activity in various fractions of osmotically shocked cells. Overnight cultures of strains harboring the recombinant plasmids pHPC3-1, 3-2, and 3-3 were subjected to osmotic shock treatment (24). The osmotically shocked spheroplasts were then sonicated and assayed together with the shocked fluids and hemolytic activity (34). The presence of β-galactosidase, a cytoplasmic protein, was used to monitor the efficacy of the osmotic shock. The results (Table 1) show that, regardless of the plasmid used, hemolytic activity was equipartitioned between the cell extracts and the periplasmic space. Culture supernatants of all three strains were devoid of hemolytic activity.

We next monitored the appearance of hemolytic activity in growing cultures of *E. coli* harboring the abovementioned recombinants. *A. hydrophila* was used as a control, and all cultures were tested for both internal and external levels of hemolysin at various stages of growth. The results obtained are depicted in Fig. 3. Growing cultures of *A. hydrophila* showed strong hemolytic activity in supernatant fluids; no activity was detectable in supernatants of growing cultures of *E. coli* carrying the various recombinants (Fig. 3A). Hemolytic activity decreased in stationary-phase cultures of *A. hydrophila*, and very low activity was detected in the *E. coli* recombinant strains during the same phase of growth. The toxin is therefore not actively secreted in *E. coli* but is released upon lysis of bacterial cells in the late stationary phase of growth.

The levels of hemolytic activity detected internally in the bacterial cell, however, provided a different picture (Fig. 3B). Strains harboring plasmid pHPC3-1 produced detectable levels during the exponential phase of growth; hemo-

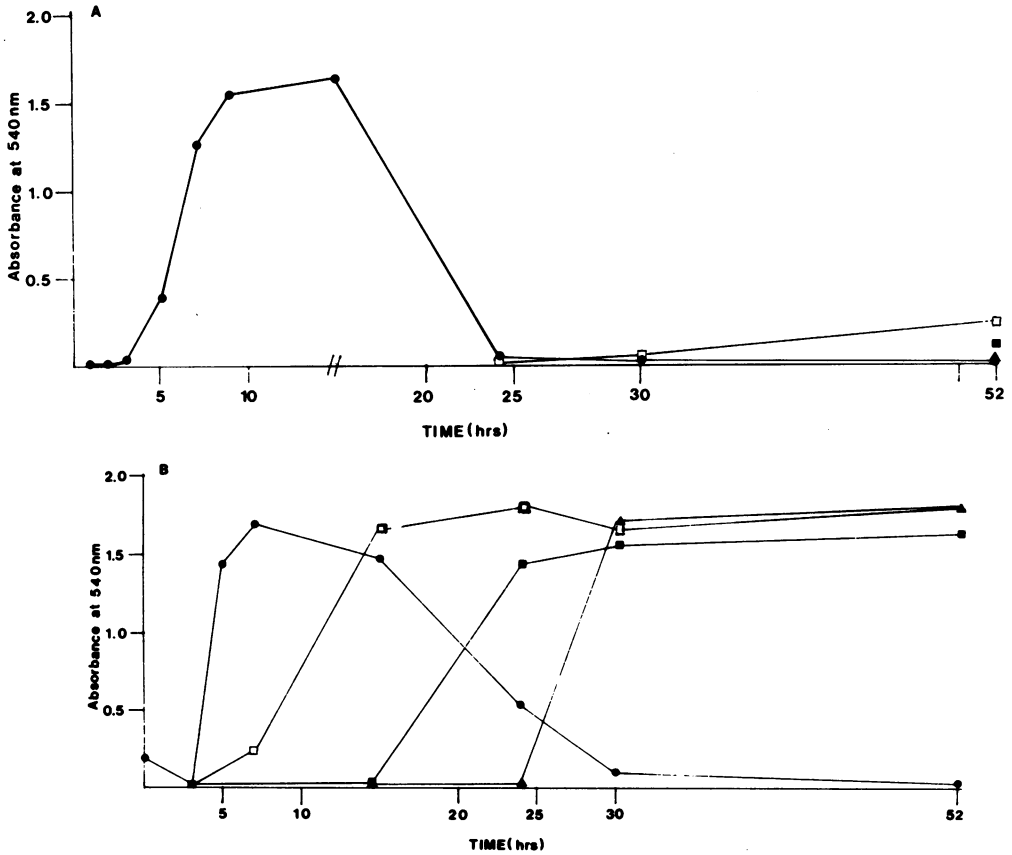

FIG. 3. Accumulation of hemolytic activity in supernatant fluids (A) and sonicated lysates (B) of *A. hydrophila* AH2 and *E. coli* LE392 strains carrying the cloned aerolysin gene during growth. Overnight cultures of all strains were washed and resuspended in phosphate-buffered saline to remove all traces of hemolytic activity. Cells were then diluted in fresh L broth to a density of 10^7 cells per ml. All strains entered the stationary phase of growth after 9 h of growth at 37°C. Growth curves were identical for all strains and are not depicted. Symbols: ●, AH2; □, LE392(pHPC3-1); ■, LE392(pHPC3-2); ▲, LE392(pHPC3-3).

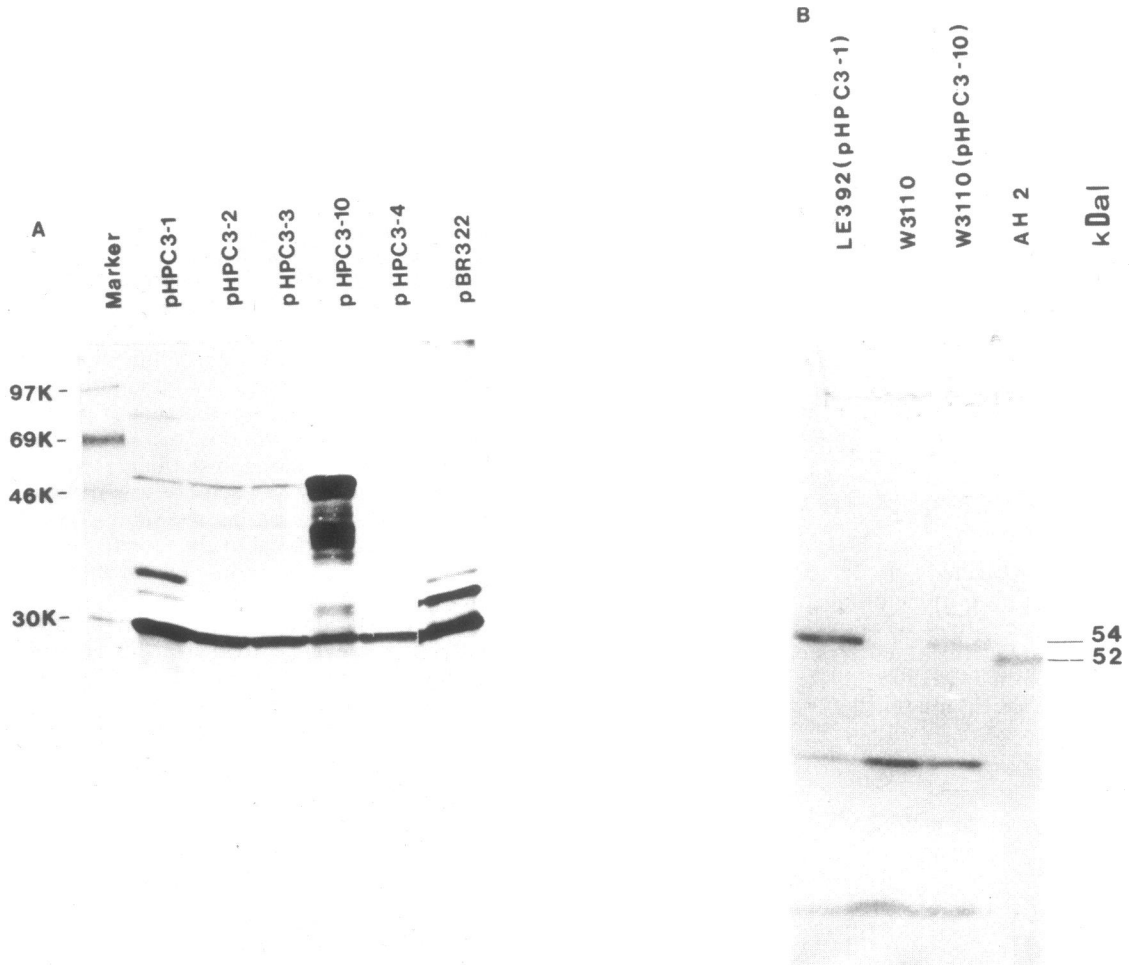

FIG. 4. (A) Autoradiographs of ^{35}S-labeled polypeptides encoded by pHPC3-1 and its derivatives in maxicells. The 54,000-dalton protein is the product of the aerolysin gene. K, Kilodaltons. (B) Immunoblots of whole-cell lysates from *E. coli* K-12 strains harboring pHPC3-1 and pHPC3-10 (in which the 5.8-kb *Eco*RI fragment is in the reverse orientation) and concentrated supernatant fluid of *A. hydrophila* AH2 with rabbit antisera to aerolysin. Cross-reactivity to the *E. coli* peptides in W3110 is noted despite exhaustive cross-adsorption of the antisera with lysed W3110 cells. The same result was obtained with antisera obtained from two other sources (A. Ljungh, Stockholm, Sweden; B. Huhle and T. Chakraborty, unpublished data). The molecular weights ($\times 10^3$) of two forms of the toxin are indicated.

lytic activity was maximal after 15 h of growth and remained high over the period of the experiment. Strains harboring plasmids pHPC3-2 and -3-3 showed considerable lag periods, 15 and 24 h, respectively, before hemolysis was detectable. Maximum activity was observed 8 to 10 h later and remained at high levels thereafter. Apart from the different lag periods observed, the kinetics of toxin production were identical in all three plasmids. Therefore, the total hemolytic activity present in strains harboring the various recombinants decreases in the order pHPC3, pHPC3-2, pHPC3-3. Similar experiments performed with a plasmid with a transposon insertion at coordinate 0.4 kb (pHPC3-435) showed detectable levels of hemolytic activity only during the stationary phase of growth (data not shown). Again, the kinetics of toxin production were identical to those observed above, and the total hemolytic activity was only a fraction of that produced by the parental plasmid pHPC3-1. Our results enable us to propose a genetic nomenclature for the aerolysin determinant in *A. hydrophila*: *aerA* is the structural gene, while *aerB* and *aerC* are regions downstream and upstream of the gene which modulate expression and activity of the gene product, respectively.

Identification of product of the cloned gene as aerolysin. To identify the gene product encoded for by the cloned aerolysin determinant, plasmids pHPC3-1, -3-2, and -3-3 and the vector pBR322 were transformed into the maxicell strain CSH26ΔF6. Polypeptides encoded by the various plasmids were detected by radioactive labeling with [^{35}S]methionine (29). A protein of 54 kilodaltons (kDa) was unique to all plasmids carrying the aerolysin gene (Fig. 4A). In the maxicell strain harboring the plasmid pHPC3-10, which carries the 5.8-kb *Eco*RI fragment in the orientation opposite to that in pHPC3-1, strong expression of the 54-kDa protein was obtained. The gene is therefore probably being transcribed from both the *tac* promoter and its own promoter. Antibody obtained against the cytotoxic hemolysin, purified as described by Asao et al. (2), was shown to specifically react with the 54-kDa protein produced by strains harboring recombinant plasmids carrying the aerolysin gene (Fig. 4B). In W3110 cells, strains harboring the plasmid pHPC3-10 made detectably less aerolysin than strains harboring pHPC3-1. We have, however, observed that similar amounts of aerolysin are detected when the same pair of plasmids are transformed into the *E. coli* K-12 strains LE392 or C600 (H.

Bergbauer and T. Chakraborty, unpublished data). We also found that the toxin present in whole-cell lysates of *E. coli* is larger than that present in supernatant fluids of *A. hydrophila*. Little or no aerolysin could be detected in supernatant fluids of *E. coli* strains carrying the cloned aerolysin gene. The results lead us to conclude that the toxin is both poorly processed and transported in *E. coli*.

DISCUSSION

We report here on the cloning, isolation, and identification of the aerolysin gene and its product in *E. coli*. Using in vitro-generated deletions and Tn*1000*-mediated insertional mutagenesis, we located the gene within a 1.4-kb fragment of *A. hydrophila* whole-cell DNA. The direction of transcription of the gene was established by constructing aerolysin–β-galactosidase gene fusions. Furthermore, regions involved in the expression and modulation of activity of the aerolysin gene product were mapped.

The aerolysin gene product is readily expressed in *E. coli* under the control of its own promoter. Our results obtained from deletion mapping and insertional mutagenesis show that regions both upstream and downstream of the aerolysin structural gene (*aerA*) are involved in the expression of hemolytic activity. We show that the reduced hemolytic activity observed with plasmids lacking *aerB* is associated with a temporal delay in the appearance of hemolytic activity. We emphasize that the difference in hemolytic activity between the plasmid pHPC3-1 and its deletion derivates is not merely a consequence of the loss of the *tac* promoter. First, in pHPC3-1 the *tac* promoter transcribes in the anticlockwise direction into vector DNA sequences, while the putative aerolysin promoter transcribes in the clockwise direction within the insert. Second, reduced hemolytic phenotypes were obtained regardless of whether *aerB* had been deleted (as in pHPC3-2, -3) or mutated by transposon insertions into plasmid pHPC3-1 (which carries the *tac* promoter). Third, even when the *tac* promoter transcribes the aerolysin gene, as in the case in pHPC3-10, no detectable increase in hemolytic activity is observed (unpublished data). We suspect that in these strains increased expression of *aerA* is offset by increased degradation of its gene product (Fig. 4A).

The reduction in hemolytic activity obtained with plasmids lacking *aerB* is not a result of a loss of transport functions. Irrespective of the recombinant used, the distribution of hemolytic activity within the various cell compartments was almost identical. Clearly, transport functions are not contiguous with the *aer* determinant as is the case with the Hly determinant of *E. coli* (35). Recent results obtained with immunoblotting techniques (B. Hule and T. Chakraborty, manuscript in preparation) do not support the notion that plasmids lacking *aerB* produce lower levels of *aerA*. We note that the aerolysin detected in AH2 supernatant fluids is some 2 kDa smaller than the polypeptide produced in *E. coli*. Since the smaller polypeptide produced in AH2 supernatant is obviously a mature toxin form, the larger polypeptide detected in *E. coli* may represent an inactive (or less active) precursor form. In this respect, Howard and Buckley (14, 15) have recently provided evidence for three forms of aerolysin. A precursor form, which when cleaved of it signal sequence (leader peptide), gives rise to a protoxin. The excreted protoxin is apparently proteolytically cleaved to give the active zymogen. Our current studies aim at establishing a precursor-product relationship between the various forms of the toxin in *E. coli*.

Transposon insertions within the region *aerC* also lead to a reduction of the hemolytic phenotype. Since the promoter for the aerolysin gene is probably located close to the points of the insertions, *aerC* may represent either a regulatory region or a gene involved in modulating the expression of the *aerA* gene product.

To date, every exotoxin cloned and introduced into *E. coli* K-12 is neither processed nor excreted efficiently. They include phospholipase C and exotoxin from *Pseudomonas aeruginosa* (8), the hemolysins of *Vibrio cholerae* and *Vibrio parahaemolyticus* (22, 31), cholera toxin from *V. cholerae* (25), cereolysin from *Bacillus cereus* (M. Gilmore, personal communication), and the α-toxin of *Staphylococcus aureus* (19). A similar situation was observed with the aerolysin gene of *A. hydrophila*. This implies that there may be fundamental differences in the secretory and excretory apparatus (for a definition, see reference 22) between *E. coli* and *A. hydrophila*. The cloning of the aerolysin gene will enable us to identify signals for processing and secretion of the protein in *A. hydrophila*.

The availability of cloned gene segments should simplify the detection of the aerolysin gene within members of the family *Vibrionaceae* and other related gram-negative organisms. Many *Aeromonas* strains produce a hemolysin which is distinct in its physiological and genetic properties from aerolysin (D. Schmitt, W. Goebel, and T. Chakraborty, manuscript in preparation). The presence of two hemolytic activities has made a rigorous correlation between the hemolytic phenotype and disease state difficult. Such relationships can now be clearly defined with DNA probes and specific antisera to aerolysin. The role of aerolysin in the pathogenesis of *Aeromonas* infections can now be evaluated by the construction of isogenic strains with mutations and deletions within the *aer* determinant.

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