Cloning of Enterotoxin Gene from *Aeromonas hydrophila* Provides Conclusive Evidence of Production of a Cytotonic Enterotoxin

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Culture filtrates of two *Aeromonas hydrophila* strains which were isolated from patients with diarrhea and assumed to be causative agents of the infections were shown to contain enterotoxic, cytotoxic, and hemolytic activities. Modest heat treatment of the filtrates inactivated the cytotoxic and cytolytic activities, but not the enterotoxic activity. The construction of cosmid gene banks in *Escherichia coli* of DNA from both *A. hydrophila* strains demonstrated that the determinants of the three activities are located on three different segments of the *A. hydrophila* chromosome. Both heated culture filtrates of *A. hydrophila* and nonheated filtrates of an *E. coli* clone containing the *A. hydrophila* enterotoxin gene provoked fluid accumulation in the rabbit ileal loop and suckling mouse models and caused elongation of Chinese hamster ovary cells. Differences in the responses of the models to the *A. hydrophila* enterotoxin and to the heat-labile and heat-stable toxins of *E. coli* indicated that the former is distinct from the latter two types of toxin. These results constitute conclusive evidence for the production by *A. hydrophila* of a cytotonic enterotoxin that is distinct from the *A. hydrophila* cytotoxin and hemolysin and known *E. coli* enterotoxins.

Species of the genus *Aeromonas* are ubiquitous waterborne microorganisms that have been repeatedly implicated as causative agents of clinical infections (6). *Aeromonas hydrophila* has received particular attention because of its association with soft tissue, wound, and blood infections (13, 17, 25), although recent data increasingly implicate it as a cause of acute gastroenteritis (11, 14, 21, 26, 28).

Multiple virulence-associated biological activities, including enterotoxic, cytotoxic, cytolytic, and proteolytic activities, have been detected in culture supernatant fluids of clinical and environmental isolates of *A. hydrophila* (22). Because enterotoxins are central virulence factors of diarrhea-producing bacterial pathogens, it is imperative to determine the prevalence of enterotoxin synthesis in *A. hydrophila* isolates and to examine the role of enterotoxins in the pathogenicity of this species. However, the production of a powerful cytotoxin by *Aeromonas hydrophila* has prevented the use of tissue culture assays (the only rapid assays for *Aeromonas* enterotoxin currently available) in the routine screening of clinical isolates and in basic studies on the enterotoxin. Moreover, despite the increasing body of information on *A. hydrophila*-associated gastroenteritis, the production of an enterotoxin by this organism has still not been unequivocally demonstrated, and even the nature of the putative enterotoxin is controversial: some authors recognize the presence of only cytotoxic activities in culture fluids (8), whereas others have identified both cytotoxic and enterotoxic (as determined by provocation of fluid secretion in rabbit ileal loops) activities but have assigned both properties to a cytotoxic enterotoxin (4). In contrast, it has recently been shown that partially purified enterotoxin preparations provoke fluid accumulation in rabbit ileal loops and the

rounding of Y-1 tissue culture cells, suggesting that the *A. hydrophila* enterotoxin is cytotonic in nature (18).

To resolve some of the controversy surrounding the enterotoxin of *A. hydrophila*, we have exploited the moderate resistance to heat inactivation of the toxin and gene cloning methods to obtain conclusive evidence that the enterotoxin of *A. hydrophila* AH2 and AH1133 is cytotonic and that the enterotoxic, cytotoxic, and hemolytic activities of these strains are distinct and are determined by different genes.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Aeromonas hydrophila* AH2 and AH1133 were isolated from patients in Varanasi, India, presenting diarrhea. *Escherichia coli* K-12 strains used were LE392 (F⁻ *hsd514* [r_k^- m_k^-] *supE44 supF58 lac galK2 galT22 metB1 trpR55* λ⁻) and RB791 W3110 (*lacIq8* λ⁻). *E. coli* H10407 is O78:H111, LT$_h$, ST$_h$, CFAI. Plasmid EWD299 is a hybrid plasmid that contains a heat-labile enterotoxin (LT$_p$) gene of *E. coli* and specifies resistance to ampicillin and tetracycline (5). Plasmid pBR322 encodes resistance to ampicillin and tetracycline (3), whereas cosmids pHC79 (12) and pHSG255 (T. Hashimoto-Gotoh; personal communication) encode resistance to ampicillin and tetracycline and to kanamycin, respectively.

Preparation of culture filtrates. Frozen stock cultures were plated on tryptic soy agar (Difco Laboratories). Single colonies were then used to inoculate 2-ml volumes of tryptic soy broth or L broth, which were then incubated at 37°C for 12 h. From these stationary-phase cultures, 0.1-ml amounts were transferred into 10-ml volumes of fresh broth in 100-ml Erlenmeyer flasks which were then incubated with agitation at 120 rpm on a reciprocal shaker at 37°C for 18 h. The cell densities obtained were ca. 1.5×10^{10} CFU/ml. For cultures prepared for assaying in the rabbit ileal loop and suckling mouse models, polymyxin B (Pfizer Inc.) (final concentration, 50 µg/ml) was added 1 h before the cells were harvest-

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TABLE 1. The effect of heat treatment on the ability of culture filtrates of *A. hydrophila* and *E. coli* LT[+] strains to provoke fluid accumulation in the rabbit ileal loop test

Strain[a]	Fluid accumulation (ml/cm) in cultures[b]:		
	Untreated	Heated at 56°C for 20 min	Heated at 100°C for 10 min
AH2	0.62 ± 0.23	0.49 ± 0.13	<0.1
AH1133	1.07 ± 0.23	0.67 ± 0.18	<0.1
EDW299 (LT$_p$[+])	1.35 ± 0.25	0.21 ± 0.16	<0.1

[a] Cultured for 18 h at 37°C in tryptic soy broth.

[b] Volumes (1 ml) of culture filtrate were inoculated into 7 to 10 cm-long, ligated ileal loops. Results are expressed as mean values ± standard deviations of tests in four rabbits.

ed. Cell cultures were centrifuged at 18,000 × *g* for 20 min at 4°C, and the resulting supernatant fluids were filtered through a membrane filter (Millipore Corp.) (pore size, 0.45 μm). Filtrates prepared in this way were assayed within 2 h. For cultures prepared for tissue culture assays, single colonies carrying independent cosmids were inoculated into 2-ml volumes of L broth containing ampicillin (100 μg/ml) or kanamycin (25 μg/ml) in test tubes (160 by 20 mm), which were incubated with shaking (120 rpm) at 37°C for 18 h.

The addition of polymyxin B to cultures before harvesting them had two advantages: a two- to fivefold increase in released toxin was obtained, and the bactericidal activity of the antibiotic obviated the need to filter large numbers of culture supernatant fluids.

Rabbit ileal loop test. Culture filtrates of strains grown in tryptic soy broth or L broth were tested by the method of Sanyal et al. (28) in ileal loops of adult albino rabbits weighing 1.2 to 1.8 kg. The animals were killed after 8 or 18 h. Each filtrate was tested in four rabbits, unless otherwise specified. Purified cholera toxin (Sigma Chemical Co.) (100 μg/ml) or culture filtrates of *E. coli* H10407 LT$_p$[+] were used as positive controls. Sterile broth containing 50 μg of polymyxin B per ml was employed as a negative control.

Suckling mouse assay. Suckling mouse assays were performed by a modified method of Dean et al. (7): 0.1-ml volumes of each culture filtrate, containing 0.01% (wt/vol) Evans blue dye, was inoculated into the stomach of five 3-day-old mice (Hannover strain; NMRI), which were then deprived of their mothers for 3 h (initial studies on the kinetics of fluid accumulation provoked by culture filtrates from enterotoxigenic *A. hydrophila* isolates showed that fluid accumulation responses were maximal at 3 h under the conditions used [manuscript in preparation]). The mice were killed by cervical dislocation, and their intestines were removed, pooled, and weighed. Fluid accumulation was expressed as the ratio of the weight of the intestines to the remaining body weight. A ratio of ≥0.08 was considered to be a positive reaction. Positive and negative controls were as described above.

CHO tissue culture assay. Culture supernatant fluids of test organisms were examined for cytotoxic and cytotonic responses in Chinese hamster ovary (CHO) cells. Cells were seeded in microtiter plates at a density of 8 × 10³/ml and 150 μl per well in Eagle minimal medium (Serva) supplemented
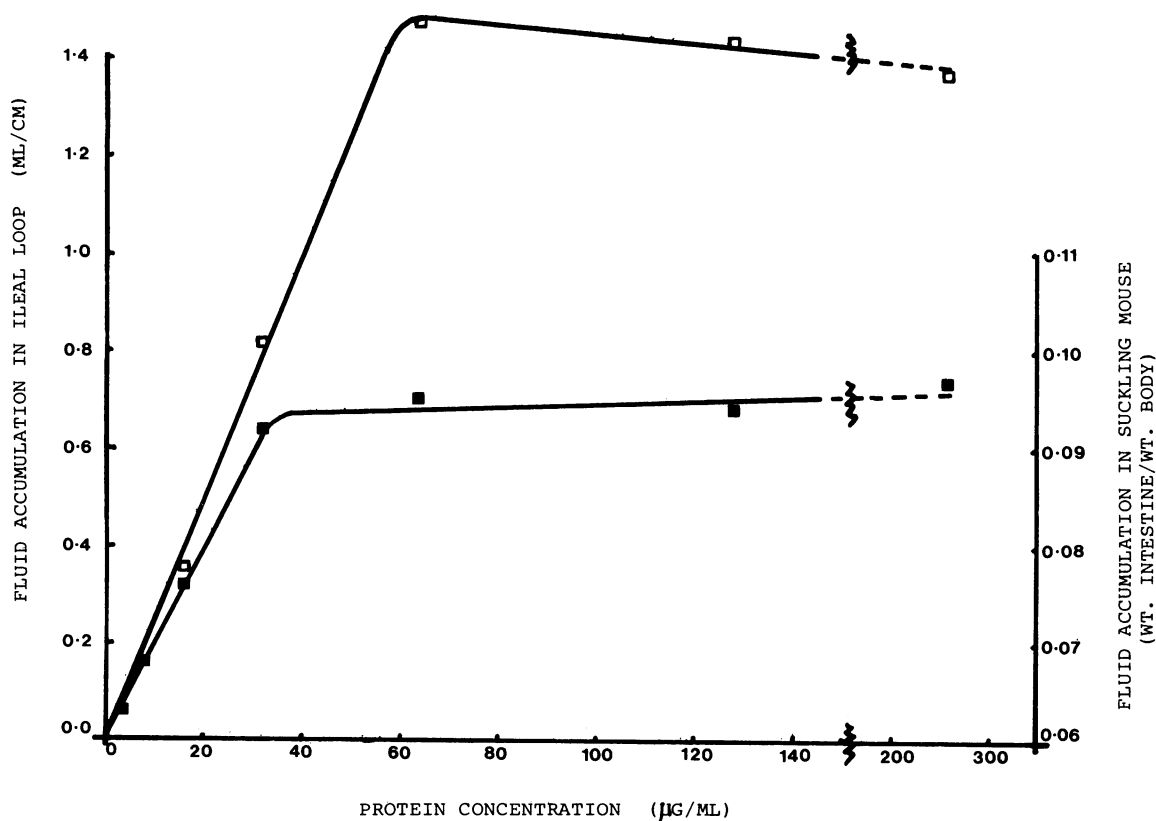

FIG. 1. Dose-response curves for *A. hydrophila* enterotoxin in the rabbit ileal loop (□) and suckling mouse (■) models. Culture supernatant fluids were obtained from strain AH2, concentrated by ultrafiltration, dialyzed against TGED buffer (50 mM Tris-hydrochloride [pH 7.5]–1 mM EDTA–5 mM dithiothreitol–10% [wt/vol] glycerol), held at 56°C for 20 min, and then assayed as described in the text.

with 1% fetal calf serum (Serva) and 50 μg of polymyxin B per ml. Culture filtrates (5, 10 and 20 μl) were added, and the plates were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cultures were examined for morphological changes after 18 h and stained with Giemsa. Positive and negative controls were as described above. CHO cells without any additions served as a further control.

DNA manipulations. Strains were screened for plasmids by the microscale methods of Kado and Liu (15), Portnoy et al. (27), or Birnboim and Doly (2). Plasmid DNA was isolated in quantity by the method of Katz et al. (16).

Restriction endonuclease cleavage reactions were carried out according to recommendations of the manufacturers. The ligation of DNA fragments was performed essentially as reported by Andres et al. (1). Cosmid cloning was carried out as described by Echarti et al. (10). Analysis of plasmids and DNA fragments was carried out by electrophoresis through vertical 0.7% agarose gels in Tris-borate buffer. Phage lambda DNA and phage φX174 DNA replicative forms cleaved with *Hin*dIII and *Hae*III, respectively, were used as molecular size standards.

RESULTS

Characterization of enterotoxigenic *A. hydrophila* strains. The *A. hydrophila* AH2 and AH1133 strains isolated from

TABLE 2. The effect of heat treatment on the ability of culture filtrates of *A. hydrophila* and *E. coli* LT⁺ ST⁺ strains to provoke fluid accumulation in the suckling mouse assay

Strain[a]	Fluid accumulation ratio[b]		
	Untreated	Heated at 56°C × 20 min	Heated at 100°C × 10 min
AH2	0.0865 ± 0.006	0.078 ± 0.004	0.061 ± 0.003
AH1133	0.09 ± 0.01	0.082 ± 0.008	0.065 ± 0.005
H10407 (LT$_h^+$ ST$_h^+$)	0.1035 ± 0.03	0.11 ± 0.005	0.11 ± 0.008
Control (TSB)	0.057 ± 0.006	0.059 ± 0.006	0.061 ± 0.004

[a] Strains were grown in tryptic soy broth for 18 h at 37°C.
[b] The ratio was the weight of intestines to remaining body weight. The fluid accumulation ratio of each of five animals that had received 0.1 ml of culture filtrate was determined. Results are expressed as mean values ± standard deviations for two experiments.

patients with diarrhea were found to be resistant to ampicillin and trimethoprim and to express cytotoxic, hemolytic, and proteolytic activities. Culture filtrates of both strains were consistently positive for enterotoxic activity, which was assayed as fluid accumulation in the rabbit ileal loop assay (Table 1 and Fig. 1). This activity was not significantly reduced by heating the filtrates for 20 min at 56°C but was completely abolished by heating them for 10 min at 100°C. Culture filtrates from a strain which produced the heat-labile
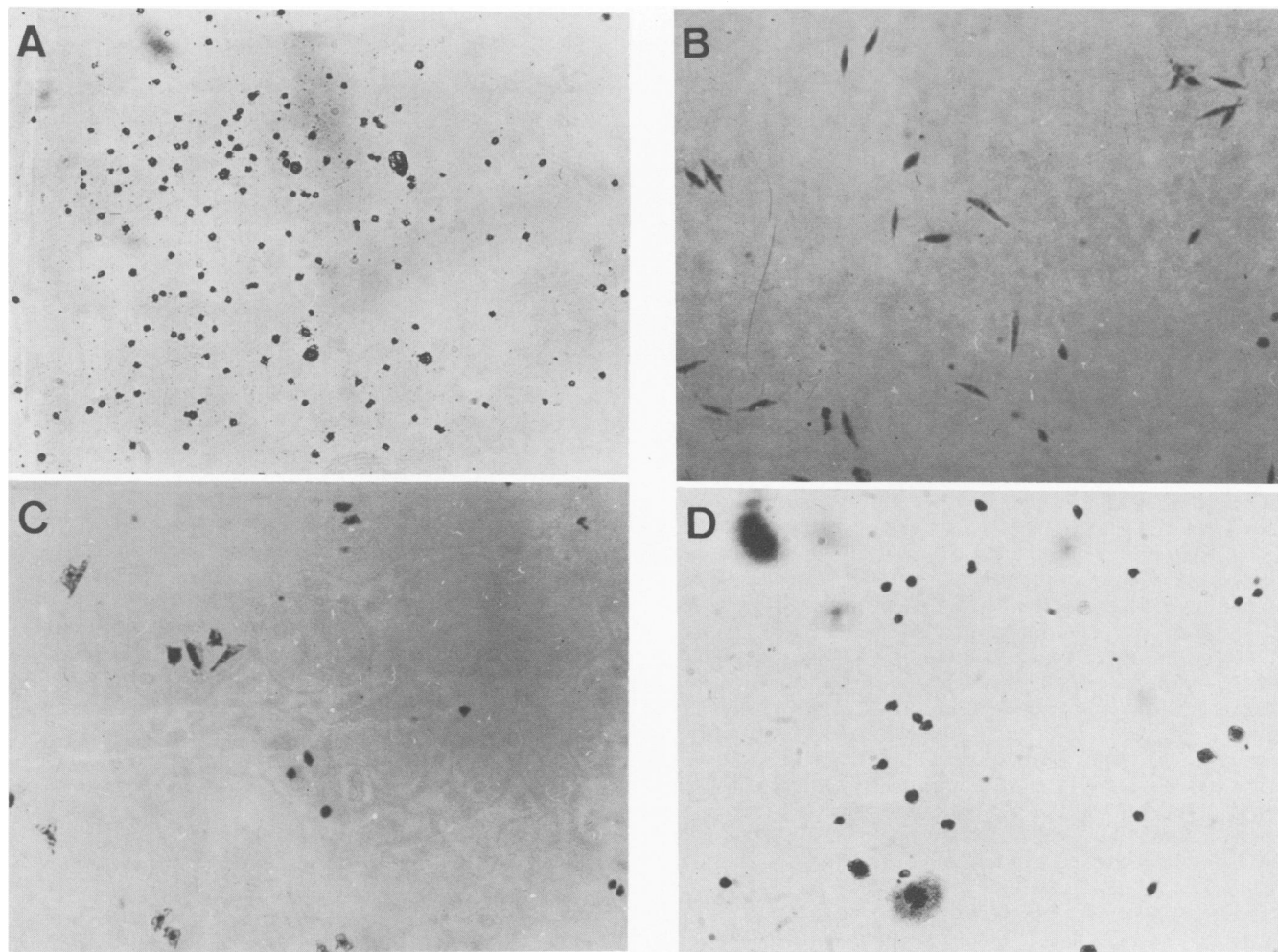

FIG. 2. Morphological changes elicited in CHO cells exposed to unheated and heated culture supernatant fluids of *A. hydrophila* AH2. Results shown are after exposure for 18 h at 37°C. Cells were treated with (A) unheated culture filtrates, (B) culture filtrates heated for 20 min at 56°C, (C) culture filtrates heated for 10 min at 100°C, and (D) tryptic soy broth (control).
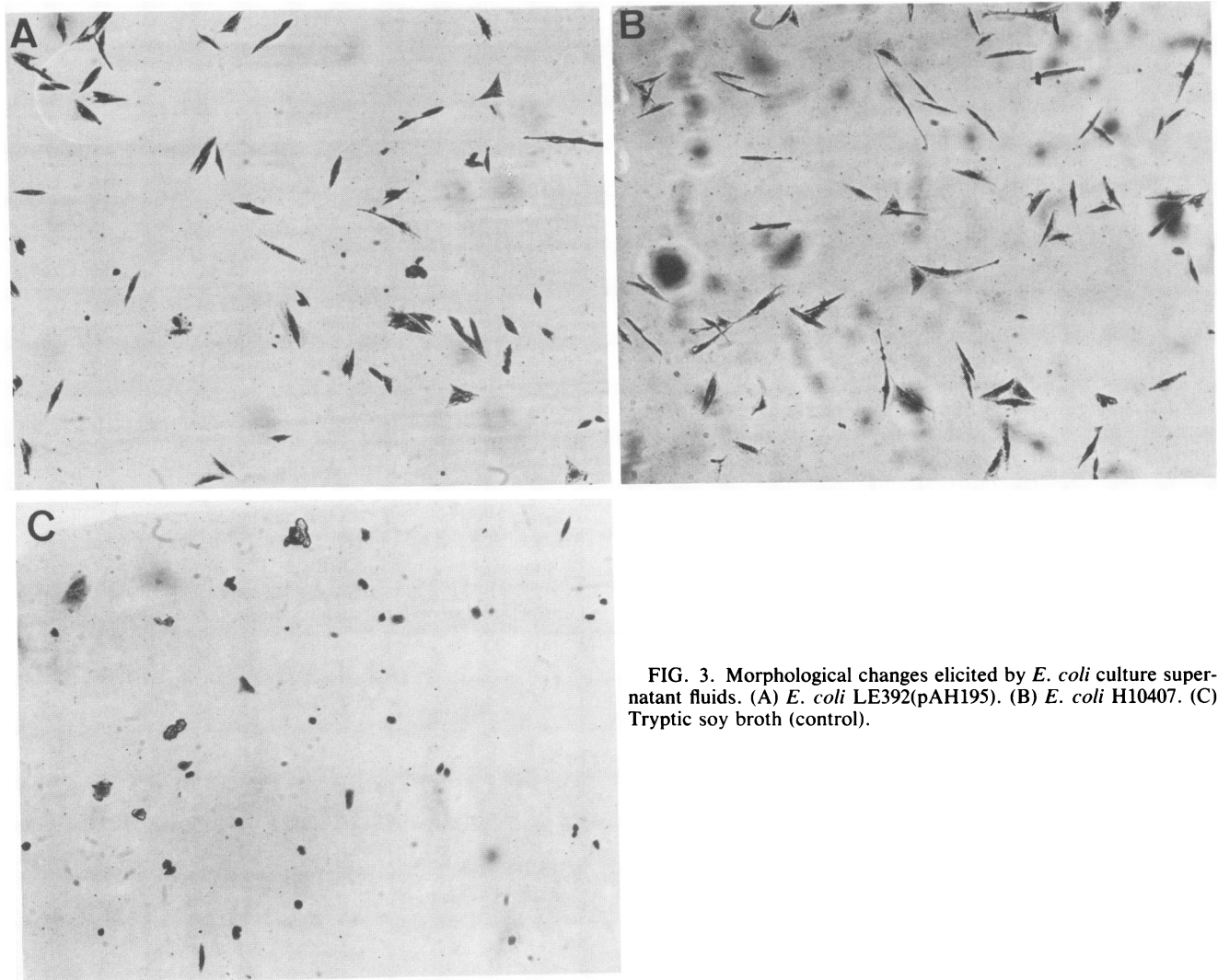
FIG. 3. Morphological changes elicited by *E. coli* culture supernatant fluids. (A) *E. coli* LE392(pAH195). (B) *E. coli* H10407. (C) Tryptic soy broth (control).

toxin of *E. coli* (LT) were sensitive to heat treatments at both temperatures (Table 1).

Unheated filtrates of both *A. hydrophila* strains provoked fluid accumulation in the suckling mouse assay (Table 2 and Fig. 1), although the kinetics of fluid accumulation were found to differ from those of the *E. coli* heat-stable toxin (ST) and LT (T. Chakraborty and U. Bellekes, manuscript in preparation). Heat-treated culture filtrates, held at 56°C for 20 min or 100°C for 10 min, were also positive and negative, respectively, in this assay. Culture filtrates from an ST-producing strain were positive after, and thus insensitive to, heat treatments at both temperatures (Table 2). These results indicate that *A. hydrophila* produces an enterotoxin that is biologically distinct from LT (and, by implication, cholera toxin) and ST of *E. coli*. This conclusion is consistent with colony hybridization studies with cloned LT_p, ST_p, and ST_h gene probes, which failed to demonstrate any homology between these toxin genes and *A. hydrophila* DNA sequences (29). Thus, enterotoxin elaborated by *A. hydrophila* is different from LT and ST.

Tissue culture assay for *A. hydrophila* enterotoxin. To identify the cloned enterotoxin gene in a gene bank of *A. hydrophila* DNA, it was desirable to develop an assay that would facilitate screening of a large number of clones. The

CHO tissue culture assay seemed to be particularly suitable for this purpose because the rounding of cells provoked by proteolytic and cytolytic activities is readily distinguished from the elongation of cells caused by cytotonic enterotoxins.

Unheated culture filtrates of strains AH2 and AH1133 were both cytotoxic in the CHO assay (Fig. 2A): 45 min after addition of filtrates, the cells became rounded and subsequently shrunken; 12 h later they detached from the tissue culture plate surface. Culture filtrates heated to 56°C for 20 min did not provoke the rounding or shrinking of CHO cells but did cause elongation in 50 to 100% of the total cell population (Fig. 2B). Although CHO cell elongation provoked by heated *Aeromonas* culture filtrates was similar to that observed with LT, the magnitude of the elongation was less than that induced by LT (two-thirds as much). No hemolytic activity could be detected in the heated preparations. Culture filtrates heated at 100°C for 10 min provoked no morphological changes in the CHO cells (Fig. 2C). These results indicated that a relatively heat-resistant, cytotonic enterotoxin present in culture filtrates of AH2 and AH1133 is responsible for fluid accumulation in the rabbit ileal loop and suckling mouse models and for the elongation of CHO cells. The results also suggested that the CHO cell assay can be a

valid and rapid assay with which to screen for *Aeromonas* enterotoxin production in a library of cosmid clones generated from the total DNA of strains AH2 and AH1133.

Isolation and identification of an enterotoxin gene of *A. hydrophila*. Cosmid cloning vectors pHSG255 and pHC79 were used to construct gene libraries of DNA isolated from strains AH2 and AH1133, respectively. DNA from each strain was partially digested with restriction endonuclease *Sau*3A, and the fragments thereby generated were ligated into the *Bam*HI sites of the vectors. Hybrid cosmids were subsequently packaged in vitro into phage lambda heads, and the reconstituted lambda particles were used to infect *E. coli* LE392 bacteria. Infectants were selected on plates containing either ampicillin (for pHC79) or kanamycin (for pHSG255). A total of 400 independent pHSG255 cosmid clones carrying AH2 DNA and 1,800 pHC79 cosmid clones carrying AH1133 DNA were obtained.

All clones containing hybrid cosmids were screened for the production of enterotoxin by the CHO tissue culture assay described above. Culture supernatant fluids of two clones, one from each gene bank, caused CHO cell elongation (Fig. 3A). Plasmid DNA was isolated from these clones and analyzed by electrophoresis through an agarose gel. Both clones contained hybrid cosmids with insertions in their *Bam*HI sites. The size of the insert in cosmid pAH195, from the gene bank of AH2, was estimated to be 35 kilobases. Cosmid pAH1287, from the gene bank of AH1133, was unstable and gave rise to deletions on propagation of the clone; it was not studied further. It was noted that strains carrying cosmid pAH195 grew more slowly (30% more slowly) than did plasmid-free LE392 cells (data not shown).

To confirm that the enterotoxin gene of *A. hydrophila* AH2 is carried by cosmid pAH195, the plasmid was transferred by transformation into plasmid-free LE392 bacteria, and culture filtrates of these bacteria were tested in the CHO and rabbit ileal loop and suckling mouse assays (Tables 3 and 4). The results obtained clearly demonstrated that cosmid pAH195 specifies a product that provokes elongation of CHO cells and that it induces fluid accumulation in both animal models. These culture filtrates did not, however, exhibit cytotoxic effects for CHO cells, nor did colonies of pAH195-carrying bacteria produce zones of hemolysis on blood agar plates.

To demonstrate definitively the nonidentity of the cytotoxic and enterotoxic activities in *A. hydrophila*, we identified in the gene bank cosmid clones carrying cytotoxin and

TABLE 3. Ability of culture filtrates of *E. coli* K-12 clones carrying cosmids containing enterotoxin, cytotoxin, or hemolysin genes of *A. hydrophila* to provoke fluid accumulation in rabbit ileal loops

Strain or clone	Fluid accumulation (ml/cm)[a]
H10407 (LT$_h^+$ ST$_h^+$)	1.04 ± 0.37
AH2	0.62 ± 0.07
E. coli LE392(pAH23; Cyt$^+$)[b]	0.08 ± 0.07
E. coli LE392(pAH184; Hly$^+$)	0.11 ± 0.04
E. coli LE392(pAH195; Ent$^+$)	0.54 ± 0.22
E. coli LE392	0.08 ± 0.01
Control (tryptic soy broth)	0.05 ± 0.06
E. coli LE392(pAH23; Cyt$^+$) plus (pAH184; Hly$^+$)[b]	0.09 ± 0.14

[a] Results are expressed as mean values ± the standard deviations of tests in eight rabbits.
[b] Small quantities of sanguinolent fluid were noted in the loops.

TABLE 4. Ability of culture filtrates of *E. coli* K-12 clones carrying cosmids containing enterotoxin, cytotoxin, or hemolysin genes of *A. hydrophila* to provoke fluid accumulation in 3-day-old suckling mice

Strain or clone	Fluid accumulation ratio[a]
A. hydrophila AH2	0.090 ± 0.001
E. coli LE392	0.057 ± 0.006
E. coli LE392(pAH23; Cyt$^+$)[b]	0.065 ± 0.010
E. coli LE392(pAH184; Hly$^+$)[b]	0.061 ± 0.002
E. coli LE392(pAH195; Ent$^+$)[b]	0.080 ± 0.002
H10407(LT$_h^+$ ST$_h^+$)	0.104 ± 0.030
E. coli LE392(pAH23; Cyt) plus (pAH184; Hly$^+$)[b]	0.068 ± 0.030

[a] Fluid accumulation ratio, intestinal weight to remaining body weight; results are expressed as mean values ± standard deviations for four different experiments.
[b] A mortality of 10% of the mice in each experiment was recorded.

hemolysin genes of strain AH2. Culture supernatants of clones that showed cytotoxic effects (crenation and shrinking) on CHO cells or that formed clear halos around colonies on blood agar plates were deemed to identify clones carrying a gene for a cytotoxin. Colonies that produced small but distinct turbid halos on blood agar plates after overnight incubation at 37°C were classified as carrying a gene for a hemolysin. Restriction endonuclease digestion of cosmids isolated from these latter clones demonstrated that their inserts were different (data not shown). A clone carrying a cosmid containing a hemolysin gene (pAH23; *hly$^+$*), and another carrying a cosmid containing a cytotoxin gene (pAH184; *cyt$^+$*) were tested in both animal assays. Neither the hemolysin nor the cytotoxin of strain AH2, alone or in combination, were able to induce fluid accumulation in either model (Tables 3 and 4).

Subcloning of the enterotoxin gene of *A. hydrophila* AH2. Cosmid pAH195 DNA was isolated and cleaved with the restriction endonuclease *Bam*HI. The fragments thereby generated were ligated to *Bam*HI-cleaved pBR322 DNA, and the ligation mixture was used to transform the *E. coli* K-12 strain RB791. Transformants resistant to ampicillin but sensitive to tetracycline were identified and screened for enterotoxin production by means of the CHO tissue culture assay. Plasmid DNA was isolated from two such clones and cleaved with *Bam*HI; the cleavage products were analyzed by electrophoresis through a 0.7% agarose gel. Both plasmids carrying the AH2 enterotoxin gene were found to carry a 3.5-kb *Bam*HI fragment in common. One plasmid, designated pHPC100, was analyzed further, and the locations of the *Eco*RI, *Hin*dIII, *Hin*cII, *Pvu*II, *Xho*I, *Bgl*II and *Pst*I endonuclease cleavage sites were determined (Fig. 4).

DISCUSSION

In this communication, we present evidence which demonstrates that two strains of *A. hydrophila* elaborate a cytotonic enterotoxin which is detectable in the rabbit ileal loop and suckling mouse models (Fig. 1) as well as by the CHO tissue culture assay (Fig. 2). The responses obtained in enterotoxin assays with heat-treated culture filtrates from these organisms suggested that a single toxin type was involved and that this toxin is physiologically different from the ST and LT toxins of *E. coli*. This latter conclusion is supported by three other lines of evidence. (i) Although the morphological changes of CHO cells caused by *A. hydrophila* enterotoxin are typical of a cytotonic enterotoxin, the cell elongation observed is not as pronounced as that pro-
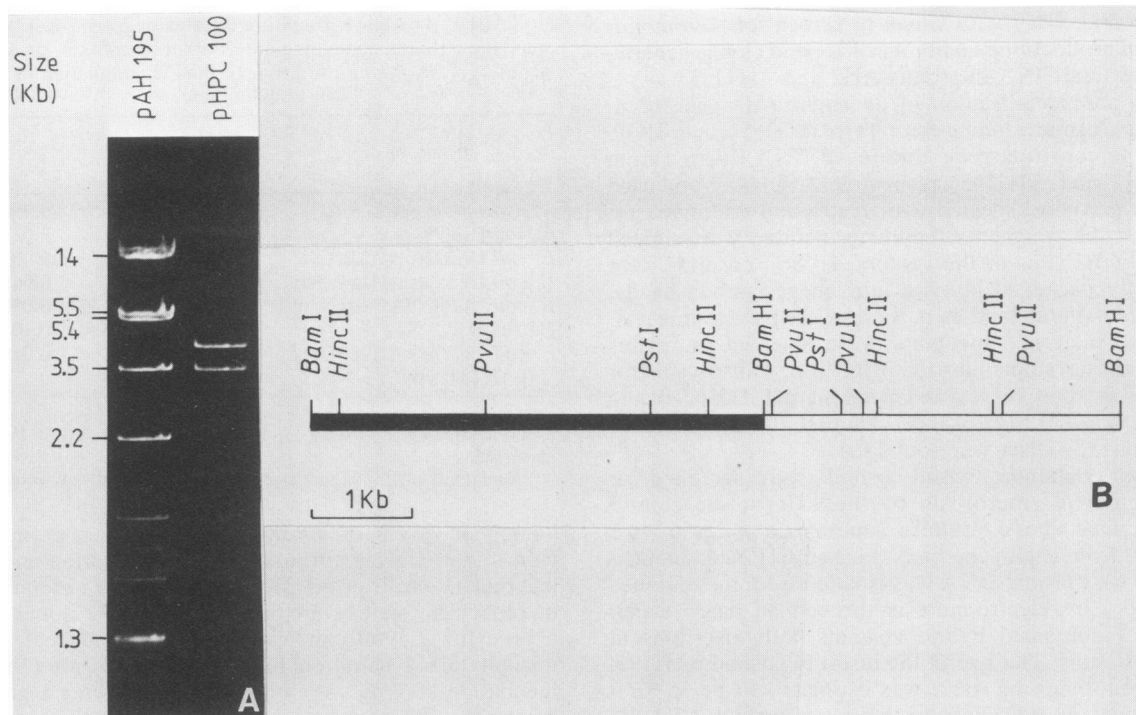
FIG. 4. (A) *Bam*HI endonuclease cleavage patterns of plasmids pAH195 and pHPC100. (B) Restriction endonuclease map of the plasmid pHPC100, a pBR322 hybrid plasmid containing the 3.5-kilobase fragment of pAH195 and specifying the *A. hydrophila* enterotoxin. Solid segment, pBR322 DNA; open segment, *A. hydrophila* DNA.

voked by LT and CT. (ii) The onset of fluid accumulation in the rabbit ileal loop is as rapid as that seen with ST, although its duration is longer and resembles that of cholera toxin (20). (iii) In colony hybridization experiments, no homology was detected between cloned LT and ST gene probes and 70 independent isolates of *A. hydrophila* (29), even under conditions of low stringency that readily permitted detection of cholera toxin genes with the LT gene probe. This finding indicates an absence of relatedness between the *E. coli* and *A. hydrophila* enterotoxins at the genetic level.

With the CHO assay, we have screened for the presence of enterotoxin genes in gene libraries of two enterotoxigenic *A. hydrophila* strains that were established in *E. coli* K-12. One clone that was studied in detail elaborated a product that provoked fluid accumulation in the animal models; however, no hemolytic or cytotoxic activities were detectable in culture filtrates of this clone. Conversely, culture filtrates from clones elaborating either a cytotoxin or a hemolysin failed to show any enterotoxic activity in the animal models. Thus, the cytotoxic, hemolytic, and enterotoxic activities of *A. hydrophila* result from the expression of distinct genes.

A series of enterotoxin-negative insertion mutant derivatives of pHPC100, a pBR322-hybrid plasmid carrying the subcloned *A. hydrophila* enterotoxin gene, were recently generated; these are currently being used for the localization of the enterotoxin gene and in the identification of the enterotoxin protein.

The cloning of the *A. hydrophila* enterotoxin gene in *E. coli* has several important consequences. First, the purification (and hence, the characterization and elucidation of the mode of action) of the enterotoxin has proven to be very difficult, partly because of copurification of the cytotoxin, and partly because of low yields (22). Clearly, the biological

separation of the enterotoxin from the cytotoxin, which was achieved during the cloning, circumvents the former problem, whereas insertion of the cloned enterotoxin gene into a high-expression vector should circumvent the latter. Second, partially purified enterotoxin from *A. hydrophila* has been reported to cause an increase in cyclic AMP levels in the rabbit intestinal loop (9, 18) and to induce steroidogenesis in Y-1 adrenal cells (19). The kinetics and extent of stimulation of cyclic AMP production and steroidogenesis are significantly lower than those obtained with LT or CT. These differences may be due to difficulties in obtaining enterotoxin of high purity or may reflect a mechanism of fluid secretion that is only partially mediated by elevated cyclic AMP levels. The ability to obtain highly purified enterotoxin should facilitate elucidation of its mode of action. Third, the cloned and mapped enterotoxin gene provides a source of a DNA fragment that can serve as a hybridization probe for colony hybridization experiments to detect homologous sequences in various clinical and nonclinical isolates. Such studies will provide important information on the prevalence of enterotoxin production in *A. hydrophila*. Moreover, a number of recent reports describe the detection of a cytotonic enterotoxin-like activity in culture filtrates of *Vibrio fluvialis* (23; our unpublished data) and non-O1 *Vibrio* spp. (24). It will be interesting to use the *A. hydrophila* enterotoxin gene probe to determine whether these other members of the family *Vibrionaceae* produce related enterotoxins. Finally, we have recently developed methods to carry out genetic analyses and manipulation of *A. hydrophila* (unpublished data). The replacement of the intact enterotoxin gene by characterized mutant genes provides a means to obtain isogenic strains differing only in the production of enterotoxin and, thereby, to elucidate the role of the enterotoxin in the virulence of *Aeromonas* bacteria.

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