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Identification and Cloning of a Novel Plasmid-Encoded Enterotoxin of Enteroinvasive *Escherichia coli* and *Shigella* Strains

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We have employed a molecular genetic approach to characterize the nature of enteroinvasive *Escherichia coli* (EIEC) enterotoxic activity, as previously observed in Ussing chambers (A. Fasano, B. A. Kay, R. G. Russell, D. R. Maneval, Jr., and M. M. Levine, Infect. Immun. 58:3717–3723, 1990). The screening of Tn*phoA* mutants of EIEC yielded a single insertion mutant which had significantly reduced levels of enterotoxic activity in the Ussing chamber assay. DNA flanking the insertion was used as a probe to screen for EIEC cosmid clones which conferred secretogenic activity. Such screening resulted in the identification of two overlapping cosmid clones which elicited significant changes in mucosal short-circuit current (Isc). Subcloning and nucleotide sequence analysis of a DNA fragment from one of the cosmid clones led to the identification of a single open reading frame which conferred this enterotoxic activity. By DNA hybridization, this gene (designated *sen* for shigella enterotoxin) was found in 75% of EIEC strains and 83% of *Shigella* strains and was localized to the *inv* plasmid of *Shigella flexneri* 2457T. By PCR, a *sen* gene with 99.7% nucleotide identity was cloned and sequenced from 2457T. A deletion in the EIEC *sen* gene was constructed by allelic exchange, resulting in significantly lower rises in Isc than were elicited by the wild-type parent; however, significant enterotoxic activity remained in the *sen* deletion mutant. To purify the Sen protein, the gene was cloned into the multiple cloning site of the expression vector pKK223-3. Purification of the *sen* gene product yielded a protein with a molecular mass of 63 kDa which elicited rises in Isc in the Ussing chamber. We believe that the *sen* gene product may constitute all or part of a novel enterotoxin in EIEC and *Shigella* spp.

Shigella species and enteroinvasive *Escherichia coli* (EIEC) are recognized diarrheal disease pathogens, causing an estimated 600,000 deaths worldwide each year (8). The most severe manifestation of infection with *Shigella* spp. and EIEC is bacillary dysentery, a syndrome characterized by frequent small-volume stools with blood and mucus (16). Accordingly, research in this field has focused on the mechanism and effects of bacterial invasion. However, most persons infected with *Shigella* spp. or EIEC present with watery diarrhea that may or may not be followed by dysentery (16, 22). In outbreaks of EIEC disease, >90% of patients typically present with watery diarrhea without blood (22). The watery diarrhea is difficult to explain solely on the basis of cell invasion, leading to the hypothesis that *Shigella* spp. and EIEC may produce one or more enterotoxins which elicit secretory diarrhea.

Several investigators have described enterotoxin-like activity in *Shigella* isolates (9, 17, 27). Similarly, Fasano et al. (9) demonstrated that culture supernatants from nine of nine EIEC strains elicited enterotoxic activity in Ussing chambers, which was manifested by significant increases in the transepithelial electrical potential difference and short-circuit current (Isc) without accompanying changes in tissue conductance. Enterotoxic activity was not associated with tissue damage. In these studies, the enterotoxic activity of supernatants was concentrated in the 68- to 80-kDa size range and appeared to be iron regulated, with maximal activity being observed after strains were grown under conditions of iron limitation. These investigators also showed that the level of enterotoxic activity was reduced but not absent in a plasmid-cured *Shigella* strain. More recently, Fasano et al. (10) have described shigella enterotoxin 1 (ShET1), which is encoded by the chromosome and found almost exclusively in *Shigella flexneri*. It has been hypothesized that ShET1 consists of two subunits in 5:1 stoichiometry, with a total molecular mass of 55 kDa.

Herein we describe studies undertaken to characterize the enterotoxic activity described by Fasano et al. in EIEC strains. By molecular techniques, we have identified enterotoxin-related loci and have mutagenized, cloned, and sequenced a gene which, when expressed in *E. coli* K-12, elicits enterotoxic activity in Ussing chambers. By genetic hybridization, we have detected this gene among members of all *Shigella* species and have localized it to the large *inv* plasmid.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used in this study are described in Table 1. EI34 (serotype O136:H−) is a clinical isolate from Brazil which was used as the prototype strain in the studies of Fasano et al. (9). EI37 (O136:H−) is an EIEC isolate from a patient with diarrhea in Japan. 2457T is a well-characterized *S. flexneri* 2a clinical isolate which has been fed to adult volunteers at our institution (18). In such volunteers, several hours of watery diarrhea typically precede the onset of dysentery. All other enteric strains tested were from the strain collection of the Center for Vaccine Development and were isolated from various epidemiologic studies. A nalidixic acid-resistant derivative of EI37 was selected by plating an overnight Luria broth (L broth) culture on L agar containing 100 μg of nalidixic acid medium per ml and by purifying a single resistant colony.

General genetic methods. Basic genetic methods were performed according to

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TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristic(s)[a]	Reference or source
Strains		
EI34	Wild-type EIEC (O136:H−)	8
EI37	Wild-type EIEC (O136:H−)	8
HS4	Nonpathogenic, nontoxigenic *E. coli*	19
HB101	*E. coli* B/K-12 hybrid	6
JM107	*E. coli* K-12, *lacI*^{q}	30
LE392	*E. coli* K-12	25
DH5α	*E. coli* K-12	13
SM10λ*pir*	Tn*phoA* donor *E. coli* strain	28
Plasmids		
pIB307	Temperature-sensitive suicide vector, Ap^r	I. Blomfield
pIB279	Source of *sacB* cassette	I. Blomfield
pBluescript SK II	High-copy-number cloning vector, Ap^r	Stratagene
pKK223-3	*tac* expression vector, Ap^r	Pharmacia
pHC79	Cosmid cloning vector, Ap^r	15
pRT733	Tn*phoA* delivery plasmid, Ap^r, Km^r	28
pJS26	2.8-kb *Hin*dIII fragment of EI34 cloned into *Hin*dIII site of pBluescript	This study
pJS264	2.0-kb *Cla*I-*Hin*dIII fragment of pJS26 cloned into pBluescript	This study
pJS263	0.8-kb *Cla*I-*Hin*dIII fragment of pJS26 cloned into pBluescript	This study
pJS34.1 and pJS34.5	Toxigenic pHC79-derived cosmid clones of EI34 genomic DNA	This study
pJS264K+	2.0-kb insert of pJS264 cloned into *Sma*I site of pKK223-3, in same orientation as *tac* promoter	This study
pJS264K−	2.0-kb insert of pJS264 cloned into *Sma*I site of pKK223-3, in orientation opposite to that of *tac* promoter	This study

[a] Ap^r, ampicillin resistance; Km^r, kanamycin resistance.

standard protocols (3). All chemical reagents were purchased from Sigma Chemical Co., St. Louis, Mo., unless otherwise specified.

Tn*phoA* mutagenesis was conducted by multiple matings of EI37 with strain SM10λ*pir* carrying pRT733, an R6K-derived suicide plasmid harboring Tn*phoA* (28). Matings were plated on L agar containing 100 μg of streptomycin per ml, 100 μg of kanamycin per ml, 40 μg of 5-bromo-4-chloro-3-indolylphosphate (XP) per ml, and 50 μg of ethylenediamine-*N*,*N*′-diacetic acid (EDDA). Iron-regulated fusions were detected by replicating alkaline phosphatase (PhoA)-expressing colonies onto a similar medium without EDDA and selecting those whose levels of activity were visibly decreased.

Electroporation was carried out with a Gene-Pulser electroporation apparatus (Bio-Rad Laboratories, Hercules, Calif.) at settings of 200 Ω and 25.0 μF. Electroporation-competent cells were prepared according to the manufacturer's protocols.

DNA sequencing of EIEC genes was performed by the double-stranded sequencing protocol with Sequenase enzyme according to the manufacturer's instructions (U.S. Biochemicals, Cleveland, Ohio). Sequencing of the *sen* gene from *S. flexneri* was done by direct sequencing of a double-stranded PCR product on an Applied Biosystems model 373A automated sequencer by dye terminator cycle sequencing with *Taq* polymerase (Perkin-Elmer Corp., Norwalk, Conn.) according to the manufacturer's instructions. Automated sequencing was performed in the Biopolymer Laboratory, Department of Microbiology and Immunology, University of Maryland School of Medicine. Sequences were determined on both strands. DNA sequence analysis was performed with Genepro sequence analysis software (version 5.00; Riverside Scientific, Bainbridge Island, Wash.) and the University of Wisconsin Genetics Computer Group sequence analysis package available through the Center of Marine Biotechnology, University of Maryland. The predicted amino acid sequence of each open reading frame (ORF) was compared with proteins listed in EMBL and GenBank with the Genetics Computer Group TFASTA program.

PCR was performed with *Taq* polymerase (Promega Corp., Madison, Wis.) under the following conditions: 94°C for 2 min; 30 cycles of 94°C for 1 min, 45°C for 2 min, and 72°C for 3 min; and an extension at 72°C for 5 min. Primers used for the amplification of *sen* had the following sequences: GGCTACAAAC AATCCAG (5′ end) and GGTACAACAACACTAAG (3′ end).

A gene bank of strain EI34 was constructed in cosmid pHC79 as previously described (15). *Sau*3A partial digests were prepared under conditions which generated fragments of 15 to 25 kb; such fragments were ligated into the *Bam*HI site of the cosmid vector. The ligated DNA was packaged into lambda phage with the Gigapack Gold system according to the manufacturer's protocols (Stratagene Inc., La Jolla, Calif.).

Allelic exchange. A 1.0-kb *Nde*I-*Sph*I deletion that begins 3 bp upstream of the predicted start methionine of *sen* and includes two-thirds of the *sen* ORF was constructed as follows. pJS26 (Table 1) was digested with both enzymes, the protruding termini were digested with mung bean nuclease (Gibco/BRL Laboratories, Gaithersberg, Md.), and the DNA was religated and transformed into *E. coli* DH5α. The now-smaller insert of pJS26 (1.8 kb in size) was isolated as a *Hin*dIII fragment and cloned into the *Hin*dIII site of the temperature-sensitive vector pIB307 (4) (to generate clone pJS26.1). The *sacB* gene (derived from pIB279) was subsequently cloned as a 2.0-kb *Bam*HI fragment into the *Bam*HI site of pJS26.1 (to generate pJS26.2). pJS26.2 was electroporated into EI34, and the transformants were plated at 30°C. After the acquisition of plasmid pJS26.2, the culture was passed at 44°C for 2 days and plated on L agar containing kanamycin; all kanamycin-resistant colonies had undergone homologous recombination with the integration of pJS26.2 into the host genome. Further passage of these colonies on L agar containing 6% sucrose (without NaCl) resulted in a resolution of the merodiploid state. Of the 200 colonies produced by this passage, 4 were found to exhibit the desired deletion.

Biochemical characterization was performed with the API 20E system according to the manufacturer's instructions (Analytab Products, Plainview, N.Y.). The growth rate of mutated EI34 was compared with that of the wild type by measuring the optical density at a 600-nm wavelength with a Spectronic-20 spectrophotometer (Bausch & Lomb, Rochester, N.Y.). Strains were inoculated into L broth in triplicate, and their optical densities were measured at 2-h intervals for 8 h. Optical densities were log transformed and subjected to linear regression analysis; the resulting regressions were compared for their quality of slope with the level of significance at $P < 0.05$. Congo red binding was assessed visually after growth on Congo red agar (Trypticase soy agar with 0.2 g of Congo red per liter).

Ussing chambers. Iron-depleted cultures for testing enterotoxic activity in Ussing chambers were grown overnight at 37°C with agitation in L broth containing 50 μg of EDDA per ml. Experiments were performed as previously described (9). Briefly, New Zealand White rabbits were sacrificed, and segments of ileum were washed and stripped of the serosal and muscular layers and then mounted in Ussing chambers (1.12-cm^2 opening). The chambers were bathed in Ringer's solution at 37°C and gassed with 95% O_2–5% CO_2. After the addition of test samples to the mucosal side, the variations in transepithelial electrical potential difference were directly recorded, conductance was measured for an applied current of 100 μA and the Isc was calculated by Ohm's law ($V = I/C$, where V is potential difference, I is current, and C is conductance). Statistical analysis of Ussing chamber data was performed by the Student t test. The level of significance was $P < 0.05$, with Bonferroni's adjustment for multiple comparisons being made where appropriate. Tissue responses were seen to vary considerably among individual rabbits; therefore, comparisons of experimental and control samples were performed only with the same rabbit.

Protein analysis methods. All chemical reagents for the protein analysis methods were obtained from Sigma Chemical Co. unless otherwise specified. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to standard methods (5). The expression of the *sen* gene product under *tac* control was performed by growing cultures for 3 h in NZCYM (21) medium at 37°C with agitation, adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a 1-mM final concentration, and incubating the mixture for a further 3 h. Bacterial lysates were prepared by boiling them in SDS sample buffer (3) for 20 min. The

protein concentration was measured by the D/C protein assay (Bio-Rad Laboratories). Lysates separated by SDS-PAGE were transferred to Immobilon-P paper (Millipore Corp., Bedford, Mass.) by the method of Towbin et al. (29), and the bands were visualized by staining with Ponceau red (14), excised, and washed thoroughly in distilled water to remove all visible Ponceau red. The Immobilon-P strip was used for N-terminal amino acid sequence analysis by automated Edman degradation at the Protein and Nucleic Acid Facility, Stanford University, Palo Alto, Calif.

Purification of the Sen protein. Purification of the *sen* gene product was performed by isolating inclusion bodies by a modification of previously described methods (20, 23). A 1-liter culture of JM107(pJS264K+) in NZCYM medium was grown for 3 h at 37°C with agitation, and then IPTG was added to a final concentration of 1 mM and the culture was incubated for three more hours. The bacterial growth was harvested by centrifugation, and the pellet was resuspended in 20 mM Tris (pH 7.5)–20% sucrose–1 mM EDTA. This suspension was incubated for 10 min on ice and then centrifuged at 4,000 × *g*. The pellet was resuspended in 50 ml of ice-cold water for 10 min and then centrifuged at 8,000 × *g*. The pellet from this centrifugation was resuspended in 10 ml of cold protease inhibitor solution (phosphate-buffered saline [without Ca^{2+} and Mg^{2+}], 5 mM EDTA, 1 μg of leupeptin per ml, 20 μg of aprotinin per ml, 0.5 mM phenylmethylsulfonyl fluoride [PMSF]). The suspension was then sonicated three times for 30 s each time in a model W-220F sonicator (Heat Systems, Farmingdale, N.Y.) with a microprobe at a setting of 6. RNase T_1 (1.3×10^3 U/10 ml) and DNase I (400 μg/10 ml) were added, and the suspension was incubated for 10 min at room temperature. Forty milliliters of protease inhibitor solution was added, and the suspension was immediately centrifuged at 13,000 × *g* for 30 min. The pellet from this step was resuspended in 40 ml of phosphate-buffered saline (without Ca^{2+} and Mg^{2+})–25% sucrose–5 mM EDTA–6 M urea, incubated on ice for 10 min, and then centrifuged for 10 min at 25,000 × *g*. This washing step was repeated two more times, and the pellet was resuspended in 10 ml of denaturation buffer (50 mM Tris [pH 8.0], 8 M urea, 5 mM EDTA). The suspension was again sonicated for a single 5-s pulse at the settings described above, incubated on ice for 1 h, and then centrifuged at 12,000 × *g* for 30 min. The sedimented inclusion bodies were subjected to renaturation in 100 ml of renaturation buffer (50 mM Tris [pH 8.0], 1 mM dithiothreitol, 20% glycerol, protease inhibitors [leupeptin, aprotinin, PMSF, and EDTA in the quantities described above]). This suspension was stirred gently at 4°C overnight to renature the proteins, which were harvested by centrifugation at 13,500 × *g* for 30 min, and the pellet was subsequently lyophilized by vacuum until ready to use. The preparation was estimated to be ≥90% pure by SDS-PAGE. Further purification was achieved by resolving this preparation by SDS-PAGE, excising the 63-kDa protein, and releasing the protein from the gel by electroelution (14).

Nucleotide sequence accession numbers. Nucleotide sequences have been submitted to GenBank with accession numbers Z54194 (*sen*), Z54195 (ORF 8A), and Z54211 (*sen*-2457T).

RESULTS

Insertional inactivation and localization of EIEC enterotoxin genes. To locate the gene(s) conferring enterotoxin activity in EIEC, we employed a Tn*phoA* mutagenesis strategy. Fasano et al. (9) had previously shown that enterotoxic activity in EIEC was iron regulated; we therefore constructed Tn*phoA* gene fusions in EIEC and screened for those fusions which were optimally expressed in medium with a low level of iron. We found that while prototype EIEC strain EI34 accepted delivery vector pRT733, the acquisition of this plasmid was accompanied by the loss of one of the recipient's native plasmids. So as not to alter the strain in any way other than with a transposon insertion, we looked for other strains which satisfied criteria for Tn*phoA* mutagenesis. We chose EI37, a strain which also produced the EIEC enterotoxin under iron-limited conditions (9).

A nalidixic acid-resistant derivative of EI37 was selected, and the strain was mated with SM10λ*pir*(pRT733). Transconjugants were screened for kanamycin and nalidixic acid resistance. Approximately 1% of the transposon mutants expressed PhoA activity on plates containing 40 μg each of XP and EDDA per ml. All $PhoA^+$ mutants were then screened for the lack of expression of PhoA on L agar plates without EDDA. These experiments yielded 11 Tn*phoA* mutants which demonstrated PhoA activity on EDDA-L agar but exhibited decreased activity levels or no activity on L agar without EDDA. Supernatants of these mutants were prepared by growing 1-liter cultures in L broth with 50 μg of EDDA per ml. One Tn*phoA* mutant (designated 8A) exhibited rises in Isc in the Ussing chamber significantly lower than those of parent EI37 (Fig. 1).

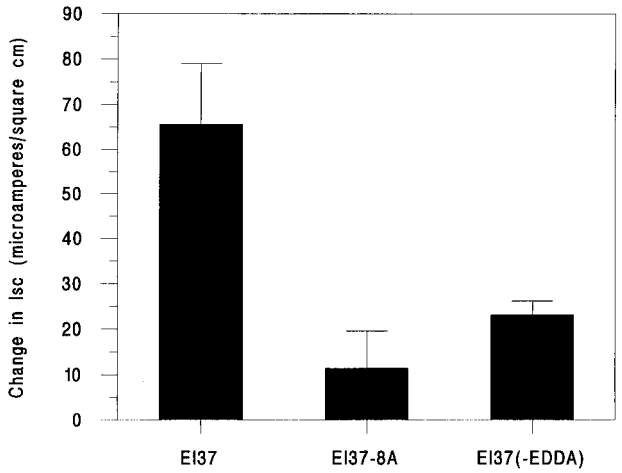

FIG. 1. Insertional inactivation of enterotoxic activity. The changes in Isc by supernatants of parent strain EI37, EI37 mutant 8A, and EI37 grown in L broth without added EDDA as a negative control are shown. ΔIsc values are in microamperes per square centimeter and are the means of three experiments, with the standard errors indicated by T-shaped bars.

Cloning an enterotoxin-related gene from EIEC. DNA flanking the Tn*phoA* insertion was cloned from mutant 8A as a 1.0-kb *Hin*cII fragment, spanning from within the *phoA* gene to 350 bp into the adjacent EI37 DNA. This fragment was used as a probe in colony hybridization to screen an EI34 cosmid library. Seven cosmid clones were found to hybridize with the *Hin*cII fragment; two of the seven (designated pJS34.1 and pJS34.5) elicited rises in Isc in the Ussing chamber (data not shown).

Further subcloning of the enterotoxin gene(s) was accomplished by isolating and cloning restriction fragments of pJS34.5 and pJS34.1 and testing these clones in the Ussing chamber assay. Several such fragments were cloned into the high-copy-number phagemid vector pBluescript SK II, and the chimeras were transformed into *E. coli* DH5α. The smallest cloned fragment which still conferred Ussing chamber activity was a 2.8-kb *Hin*dIII fragment of EI34 DNA; this clone (designated pJS26) elicited reproducible rises in Isc similar to those exhibited by the wild-type parent and by pJS34.5 (Fig. 2). Surprisingly, however, pJS26 did not hybridize with the *Hin*cII probe corresponding to the site of the 8A insertion. A different subclone of pJS34.5 was therefore constructed and did in fact hybridize with the *Hin*cII fragment. With this clone, a complete ORF which corresponded to the gene into which Tn*phoA* had been inserted in mutant 8A was sequenced; this was verified by nucleotide sequence analysis of the *Hin*cII fragment probe. Analysis of this ORF (designated ORF 8A) yielded a predicted peptide 43 kDa in size, featuring a typical *E. coli* signal sequence (7). The predicted protein product of ORF 8A did not show significant homology to any gene product in the GenBank library. When subcloned in its entirety, ORF 8A did not elicit rises in Isc in the Ussing chamber.

To verify this observation, ORF 8A was cloned as a 2.2-kb *Hpa*I-*Sma*I fragment into the *Sma*I site downstream of the *tac* promoter in pKK223-3 and the protein was hyperexpressed in strain JM107. Although a great deal of the 43-kDa protein was secreted into the culture supernatant, the protein did not elicit rises in Isc in Ussing chambers. Suspecting that the 8A inser-

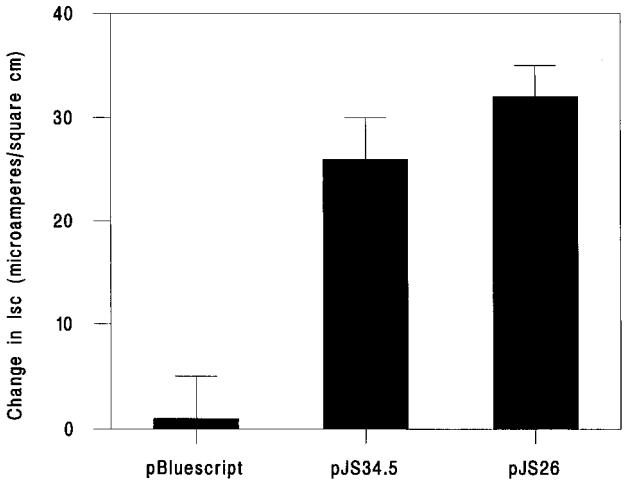

FIG. 2. Cloning of enterotoxin-related gene. The changes in Isc by supernatants of cosmid clone pJS34.5 and subclone pJS26 are shown compared with that of the negative control pBluescript SK II. ΔIsc values are in microamperes per square centimeter and are the means of six experiments, with the standard errors indicated by T-shaped bars.

tion conferred a polar effect on a downstream toxin-encoding gene, we electroporated ORF 8A cloned in pBluescript into the EI34 mutant and tested it in the Ussing chamber. Complementation to wild-type toxigenicity was not observed, consistent with a polar effect (data not shown). To identify the enterotoxin gene, further experiments focused on the toxinogenic clone pJS26.

DNA sequencing of pJS26. The cloned insert of pJS26 was sequenced in its entirety. Analysis of the nucleotide sequence revealed two ORFs encoding putative proteins of 16.1 kDa (ORF1) and 63.1 kDa (ORF2) (Fig. 3). The translated sequences of ORF1 and ORF2 did not reveal significant similarity at the amino acid level to the proteins of any other known genes in the EMBL and GenBank databases.

To further localize enterotoxin activity, the ORFs were subcloned separately for testing in the Ussing chamber. As seen in Fig. 3, a subclone which contained ORF2 produced rises in Isc in the Ussing chamber which were significantly greater than those of the negative control, while ORF1 elicited lower (and not significant) rises in Isc. The activity featured a characteristic lag time of approximately 40 min, followed by a steady rise in the potential difference without a change in the conductance of the tissue; the effect reached a plateau at approximately 120 min. The tissue retained its viability and ability to respond to the addition of glucose.

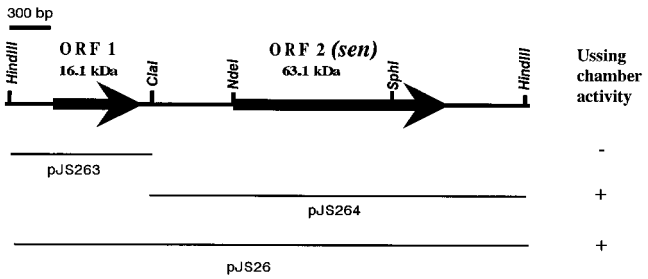

FIG. 3. Potential ORFs of clone pJS26. ORFs were subcloned into pBluescript II SK as indicated. Ussing chamber results are the means of three experiments; a positive result indicates that the change in Isc was greater than that induced by pBluescript ($P < 0.05$).

TABLE 2. DNA probe analysis with the *sen* gene

Category	Probe positive (%)	Probe negative (%)	Total no. tested
EIEC	60 (75)	20 (25)	80
S. dysenteriae	5 (100)	0	5
S. flexneri	11 (73)	4 (27)	15
S. sonnei	11 (100)	0	11
S. boydii	2 (50)	2 (50)	4
Enteropathogenic *E. coli*	0	20 (100)	20
Enterotoxigenic *E. coli*	0	20 (100)	20
Enterohemorrhagic *E. coli*	0	20 (100)	20
Other enteric genera[a]	0	67 (100)	67

[a] This category includes *Campylobacter* spp., *Serratia* spp., *Citrobacter* spp., *Vibrio* spp., *Salmonella* spp., *Enterobacter* spp., and *Pseudomonas* spp.

Neither ORF1 nor ORF2 exhibited a hydrophobic signal sequence at the 5′ end (7); both displayed potential ribosomal binding sites (Fig. 4). The nucleotide sequence of ORF2 predicted a protein of 565 amino acids, including five cysteines. The protein has a pI of 6.36.

Assessment of the prevalence of ORF2 in EIEC and *Shigella* spp. A 2.2-kb ORF2 DNA fragment probe (*Cla*I-*Hin*dIII) was prepared from pJS26 and hybridized against a collection of EIEC and *Shigella* strains under high stringency by the colony blot method. ORF2-homologous sequences were present in 29 (83%) of 35 *Shigella* strains, including members of all four *Shigella* species; homologous sequences were found in 60 (75%) of 80 EIEC strains tested. None of 127 other enteric organisms carried homologous sequences (Table 2). Because of the high level of conservation among *Shigella* spp., the ORF2 gene was designated *sen* (for shigella enterotoxin 2).

To confirm the presence of the *sen* gene in *Shigella* spp., we synthesized oligonucleotide primers upstream and downstream of the *sen* sequence and performed PCR on six wild-type *S. flexneri* isolates. All six yielded identical products of 1.5 kb, similar to the sizes of products obtained from EI34. The nucleotide sequence was determined for the product of *S. flexneri* 2a 2457T. Compared with the sequence from EI34, the 2457T sequence revealed an ORF differing in only five nucleotides out of 1,695 bases in the complete *sen* sequence (i.e., 99.7% identity), also yielding a predicted protein product of 63.1 kDa. The five different nucleotides resulted in only two amino acid changes, indicated in Fig. 4.

To determine the genomic location of the *sen* gene, the *Cla*I-*Hin*dIII probe was used in Southern analysis of plasmid DNA extracted from EI34, *S. flexneri* 2457T, and 2457T lacking its *inv* plasmid. A band corresponding to the large plasmid was seen in the plasmid profiles of the EI34 and 2457T lanes. However, 2457T minus the plasmid did not yield a probe-homologous signal (data not shown). These data suggest that *sen* is encoded by the *inv* plasmid in both EI34 and *S. flexneri* 2457T.

***sen* deletion construction.** In order to gauge the role of *sen* in the expression of enterotoxin activity in EIEC, we constructed a deletion of the N-terminal two-thirds of the gene. Clone pJS26 harboring this deletion mutation (but keeping an intact ORF1) was found to confer significantly lower levels of enterotoxic activity (Fig. 5A). The deletion mutation was then recombined into the EI34 plasmid, replacing the native allele. The growth curve, plasmid profile, Congo red binding, and biochemical profile of the deletion mutant were similar to those of the parent. The results of Ussing chamber experiments on this construct are shown in Fig. 5B. EI34 with *sen* deleted elicited significantly lower rises in Isc than the wild-

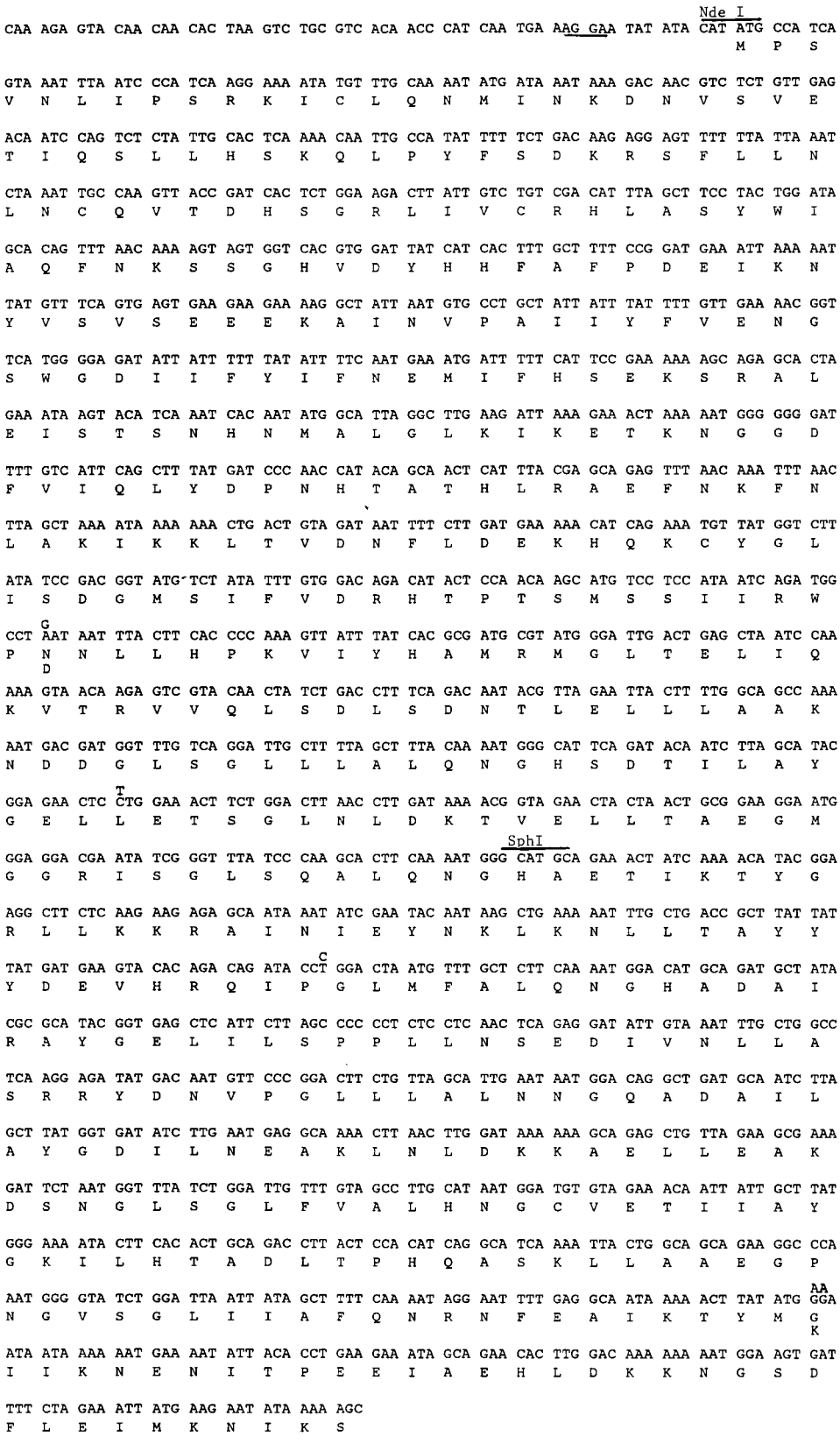

FIG. 4. Nucleotide sequence of the *sen* gene from EI34 and predicted amino acid sequence of its product. The predicted ribosomal binding site is underlined and is located 11 bp upstream from the predicted translational start site. Nucleotides of *S. flexneri* 2457T which differ from this sequence are indicated above the EI34 nucleotide sequence (nucleotides 703, 916, 1130, 1594, and 1595). Amino acid residues of 2457T which differ are indicated below the amino acid sequence. The positions of the *Nde*I and *Sph*I sites used to generate the deletion mutation are indicated.

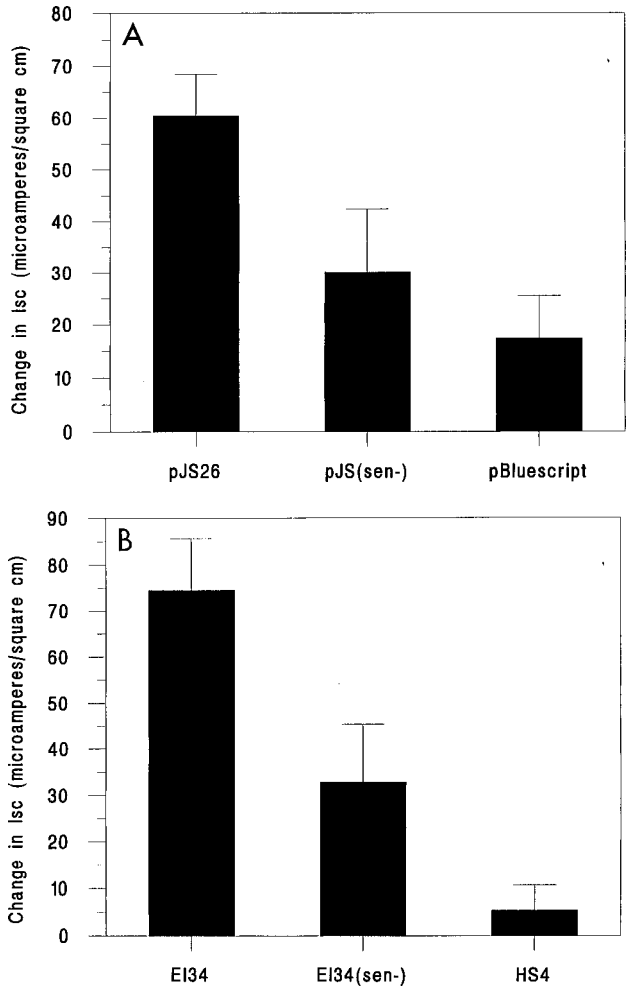

FIG. 5. (A) Ussing chamber activity of the *sen* deletion in pJS26. The supernatants of the *sen* clone pJS26 (positive control), pJS26 carrying a deletion in the *sen* gene [pJS(sen−)], and pBluescript (negative control) were tested in an Ussing chamber. ΔIsc data are the means of three experiments, and the standard errors are indicated by T-shaped bars. (B) Ussing chamber activity of the *sen* deletion in EI34. The supernatants of EI34 (positive control), EI34 carrying a deletion in the *sen* gene [EI34(sen−)], and *E. coli* HS4 (negative control) were tested in the Ussing chamber. ΔIsc data are the means of three experiments, with the standard errors indicated by T-shaped bars.

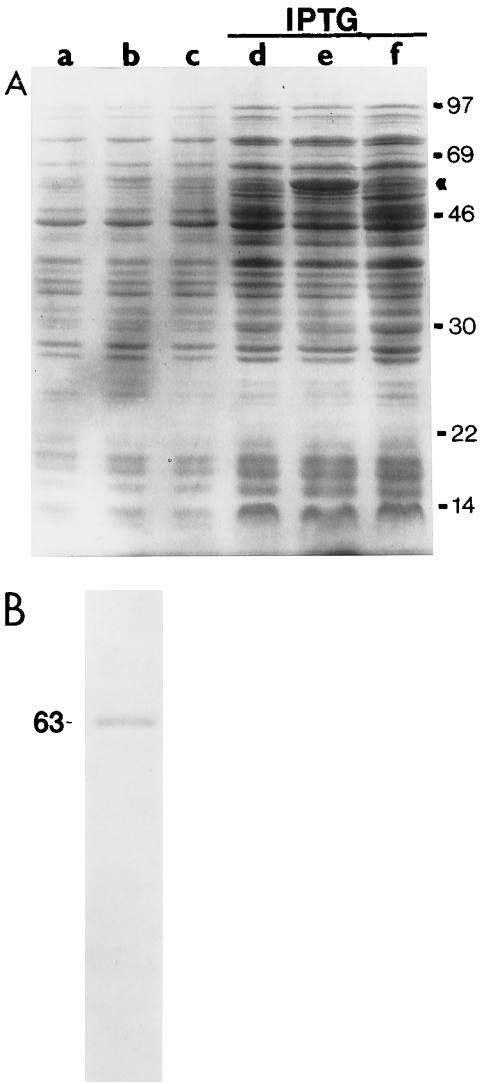

FIG. 6. (A) SDS-PAGE of *sen* gene expression under *tac* control. Cultures were grown in NZCYM for 3 h; for lanes d to f, IPTG was added and incubation was continued for a further 3 h. Cells were harvested and lysed, and total cellular proteins were separated by SDS-PAGE and stained with Coomassie blue. Lanes: a, pKK223-3; b, pJS264K+; c, pJS264K−; d, pKK223-3 after IPTG induction; e, pJS264K+ after IPTG induction; f, pJS264K− after IPTG induction. The arrowhead indicates the position of the 63-kDa (Sen) band present only after the induction of JM107(pJS264K+). See the text for details. (B) Silver-stained SDS-PAGE gel containing purified Sen protein. The 63-kDa protein was purified from pKK264K+ by inclusion body precipitation and electroelution from an SDS-PAGE gel.

type parent. However, the *sen*-negative mutant still elicited significantly higher rises in Isc than the nonpathogenic *E. coli* HS4. Complementation of the *sen* mutation via the reintroduction of clone pJS264 resulted in rises in Isc similar to those of the parent strain (data not shown).

Expression and purification of the *sen* product. The *sen* gene was cloned as a 2.2-kb *Cla*I-*Hin*dIII fragment into the *Sma*I site downstream of the *tac* promoter in the expression vector pKK223-3, to yield clone pJS264K+. Growth of pJS264K+ in host strain JM107 in NZCYM medium containing 1 mM IPTG resulted in the expression of a 63-kDa protein species visualized by SDS-PAGE (Fig. 6A). This protein was less apparent in the absence of IPTG and was not seen in cell lysates of JM107(pKK223-3) or when the insert was cloned in the opposite orientation (pJS264K−). The identity of the protein as the product of the *sen* gene was confirmed by automated N-terminal amino acid sequencing. The Ussing chamber assay of the JM107(pJS264K+) cell lysate did not yield rises in Isc higher than those induced by DH5α(pJS26) (data not shown).

In contrast to results with whole-cell lysates, repeated analysis of the supernatants of JM107(pJS264K+) revealed no protein with a molecular mass corresponding to that predicted for Sen, either by SDS-PAGE or by Ussing chamber experiments. Similarly, cell lysates which were not denatured in SDS did not yield an appropriate protein product. For this reason, we suspected that the expressed protein was hydrophobic and formed inclusion bodies within the bacterial cell. Consequently, a purification procedure was attempted in which inclusion bodies were extracted and the protein constituents were renatured; this method yielded a protein species of 63 kDa by SDS-PAGE. The purified protein was tested in Ussing chambers at serial dilutions over a three-log span of protein

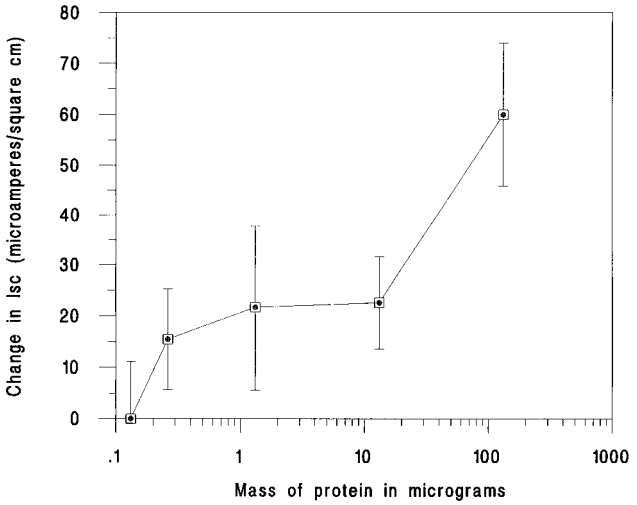

FIG. 7. Dose-response curve of purified Sen protein in the Ussing chamber. Datum points represent the mean ΔIsc of three experiments, with the standard error indicated.

mass (132 ng to 132 μg). As shown in Fig. 7, the change in Isc response in the Ussing chamber correlated with the dose of Sen protein added to the chamber. Further purification by excising and electroeluting the 63-kDa protein from an SDS-polyacrylamide gel also yielded a protein eliciting Ussing chamber activity (Fig. 6B).

DISCUSSION

Substantial work has been done to characterize the pathogenetic mechanisms of shigella and EIEC diarrhea, yet nearly all of this work has focused on the nature and role of mucosal invasion in shigella pathogenesis (11, 12). However, a large proportion of patients with shigella or EIEC disease experience watery diarrhea (16, 22), which is not easily explained on the basis of cellular invasiveness. Several lines of evidence accumulated from the study of both human patients and animal models have suggested that *Shigella* spp. and EIEC may elaborate secretogenic enterotoxins (9, 17, 24). Fasano et al. have used rabbit ileum mounted in Ussing chambers to characterize an enterotoxin-like effect elicited by the supernatants of EIEC and *Shigella* strains (9). Secretogenic activity was found only when cultures were grown under conditions of iron deprivation. In the search for the gene encoding such a toxin, these investigators have recently identified a chromosomal locus of *S. flexneri* which mediates rises in Isc in Ussing chambers, as well as mild fluid accumulation in rabbit ileal loops. This moiety has been termed ShET1 (10).

In this communication, we report further characterization of the toxic activity of EIEC strains and the finding of a second, more prevalent enterotoxin-related locus in *Shigella* and EIEC strains. We constructed iron-dependent Tn*phoA* fusions in an EIEC strain previously shown to elicit iron-dependent rises in Isc in the rabbit mucosal Ussing chamber. Screening such derivatives resulted in the identification of an insertion mutant with significantly reduced levels of enterotoxic activity. When the gene which was interrupted by this insertion mutation was cloned and then hyperexpressed under *tac* control, the gene product did not by itself elicit rises in Isc. However, we also report the identification of a closely linked plasmid locus (*sen*) which encodes a protein that does confer toxigenicity in the Ussing chamber. In addition, significantly less enterotoxic effect was exhibited by the parent EIEC strain when the *sen* gene was subjected to a mutation abolishing the N-terminal two-thirds of the ORF.

sen-homologous sequences were found in 75% of EIEC strains and 83% of *Shigella* isolates, including members of all four *Shigella* species. A 99.7% identical genetic homolog was cloned and sequenced from *S. flexneri*; however, the *sen* gene bears no nucleotide or amino acid homology to ShET1. Unlike ShET1, the *sen* gene is apparently encoded by the large plasmid of *Shigella* spp. and EIEC strains. This plasmid has been shown to encode functions required for the invasion and spread of *Shigella* spp. (11, 12, 26); however, only a relatively small portion of the plasmid has been characterized and it is likely that it may encode other virulence determinants.

Previously, Fasano et al. had shown that a plasmid-cured *Shigella* strain still possessed enterotoxic activity in an Ussing chamber, although the level of such activity was significantly decreased from that of the wild-type parent (9). In this study, we found decreased, but not absent, levels of enterotoxic activity after introducing a deletion in the *sen* gene in EIEC. Since ShET1 has not been found in EIEC, these data suggest that EI34 may possess yet another enterotoxic moiety distinct from both ShET1 and ShET2. In studies of the electrogenic effects induced by the cloned ORFs of pJS26, we found low-level (and not significant) activity associated with ORF1, upstream from *sen*. However, we have found that antibodies raised against the purified Sen protein abolish all toxigenic activity of pJS26 (not shown); thus, we believe that ORF1 is unlikely to confer the residual toxic activity of EI34Δ*sen*. Previous studies suggested that the maximum enterotoxic activity of EI34 was contained in the 68- to 80-kDa supernatant fraction. Our data, suggesting a 63-kDa gene product, may be compatible with the hypothesis that another enterotoxin exists in this range or that the *sen* gene product is part of a larger holotoxin which migrates in the appropriate size range. It is possible that physical characteristics of the 63-kDa protein resulted in its fractionation with larger protein species.

The predicted Sen protein does not exhibit a signal peptide by standard criteria (7). This phenomenon is common for the secreted proteins of the *inv* plasmid, which have been shown to be secreted by the Mxi system and not to require a standard signal peptide (1, 2). The possibility that Mxi is involved in the secretion of Sen is under investigation.

The finding of this putative enterotoxin gene of EIEC and *Shigella* spp. potentially offers insight into the secretory phase of *Shigella* pathogenesis. The deletion of the enterotoxin may be an important adjunct to the deletion of invasion-related genes in the construction of shigella vaccine candidates. Further studies to determine the nature and role of these enterotoxins are under way.

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