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Construction and Characterization of an Isogenic *slt-ii* Deletion Mutant of Enterohemorrhagic *Escherichia coli*

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Enterohemorrhagic *Escherichia coli* (EHEC) produces Shiga-like toxins (SLT), potent protein synthesis inhibitors. To further dissect the role of SLT-II in the course of disease, we have constructed *E. coli* TUV86-2, an isogenic SLT-II-negative mutant of EHEC strain 86-24. The *slt-ii* gene was inactivated by suicide vector mutagenesis. We also isolated derivatives of strain 86-24 that were cured of the phage carrying the toxin genes.

Enteric infections of humans with enterohemorrhagic *Escherichia coli* (EHEC) have a wide spectrum of clinical symptoms, with intestinal as well as extraintestinal manifestations. Within the intestine the infection can vary from an inapparent carrier status to nonbloody or bloody diarrhea and hemorrhagic colitis as the most severe form (1, 21, 38). Systemically, an EHEC infection can lead to neurological symptoms and microangiophathic thrombocytobpenic disorders known as hemolytic uremic syndrome (HUS) (35) or thrombotic thrombocytopenic purpura. HUS affects mostly young children (31) and represents the major cause for acute kidney failure in childhood (35, 36). Thrombocytopenic purpura is more a disease of the elderly (6).

Isolates from outbreak cases usually have the serotype O157:H7 and produce Shiga-like toxins (SLTs) (2, 23, 38, 39). The SLTs are genetically and biochemically related to Shiga toxin produced by *Shigella dysenteriae* type I strains. In general there are two major subclasses of SLTs, SLT-I and SLT-II. These two classes of toxin are differentiated immunologically by their cross-reaction (SLT-I) or lack of cross-reaction (SLT-II) with antisera to Shiga toxin (1). Although at the molecular level the genetics and the mode of action of Shiga-like toxins have been very well dissected, the toxins' role in pathogenesis in both the intestinal and extraintestinal manifestations is still not fully developed. In this study, using suicide vector mutagenesis, we have constructed *E. coli* TUV86-2, an isogenic, toxin-negative mutant of *E. coli* 86-24, an O157:H7 EHEC strain producing SLT-II.

The suicide plasmid pTUV4 was constructed as outlined in Fig. 1. Using the primer pairs UB-3–UB-2 and FG-1–FG-2 (Table 1), two DNA fragments, 610 and 870 bp respectively, were amplified by PCR. They were cloned into pGP704 (25) (Fig. 1) linearized with *Xba*I and *Eco*RI and ligated together at their *Bam*HI restriction site, generating an *slt-ii* operon, with an internal 589-bp deletion. The two PCR-derived DNA fragments covered the entire *slt-ii* toxin operon and flanking upstream and downstream sequences. The internal primers UB-2 and FG-1 were chosen to delete nucleotides 702 to 1290, based on the *slt-ii* gene sequence published by Jackson et al. (17). We deleted the codon for glutamic acid 166 (nucleotides 803 to 805), which is an active-site residue of SLT-II (16), as well as the entire leader sequence of the B-subunit. Furthermore the deletion was designed to result in an out-of-frame reading of the B-subunit. Thus, the product from this gene deletion would result in neither an enzymatic active A-subunit nor a mature B-subunit with the ability to induce apoptosis in certain cells (24). The *sacB* gene from *Bacillus subtilis*, which codes for the enzyme levansucrase, was used as a positive selection system, as described by other investigators (8, 19, 29). Ligation of the *sacB* gene from *B. subtilis* (12) into *Bgl*II (New England Biolabs)-digested pTUV3 yielded pTUV4 (Fig. 1). The plasmid was electroporated into *E. coli* SM10 λ*pir*. *E. coli* SM10 λ*pir*(pTUV4) and EHEC strain 86-24 were grown in Luria-Bertani (LB) media with *E. coli* SM10 λ*pir* and without ampicillin (AMP) (strain 86-24) to an optical density at 600 nm (OD_{600}) of 0.5. The donor strain was washed twice in sterile saline and mixed with the recipient (strain 86-24) in a donor-to-recipient ratio of 2 to 1. An aliquot of 1.5 ml of the bacterial mixture was spun down and resuspended in 100 μl of sterile saline and spread onto a sheep blood agar plate and incubated overnight at 37°C. The entire bacterial lawn from the plate was resuspended in 1 ml of sterile saline and diluted 10-fold to 10^{-5}. A 100-μl aliquot of each dilution was spread onto MacConkey-AMP (200 μg/ml) agar plates. Lactose-positive Amp^r colonies, representing potential *E. coli* 86-24 exconjugants, were picked and streaked on MacConkey-AMP agar plates for single-colony isolation. Bacterial colonies were then replica plated on LB-AMP agar and LB-AMP agar with 10% sucrose and grown overnight at 30°C. Colonies that showed growth on the LB-AMP agar and no growth or inhibited growth on the sucrose-containing plates were further tested in a PCR using the primers UB-3 and FG-2. Exconjugants were lactose positive, Amp^r, and sucrose sensitive and in a PCR with primers UB-3 and FG-2 yielded two bands, 2,069 bp and 1,480 bp in size (*slt-ii* wild-type and mutated genes). An exconjugant was grown in LB medium without antibiotic stress to slight turbidity. Five 10-fold dilutions were spread out on plain LB and LB-sucrose agar plates in parallel. Colonies growing on sucrose plates were further tested for their sensitivity to ampicillin. Ampicillin-sensitive and sucrose-resistant colonies were likely to have undergone a second recombinational event, resulting in the loss of the suicide vector and leaving behind one copy of the toxin genes, either the wild-type or mutated genes. The ratio of sucrose-resistant to sucrose-sensitive colonies was about 10^{-4}, reflecting the frequency of the loss of pTUV4. These numbers

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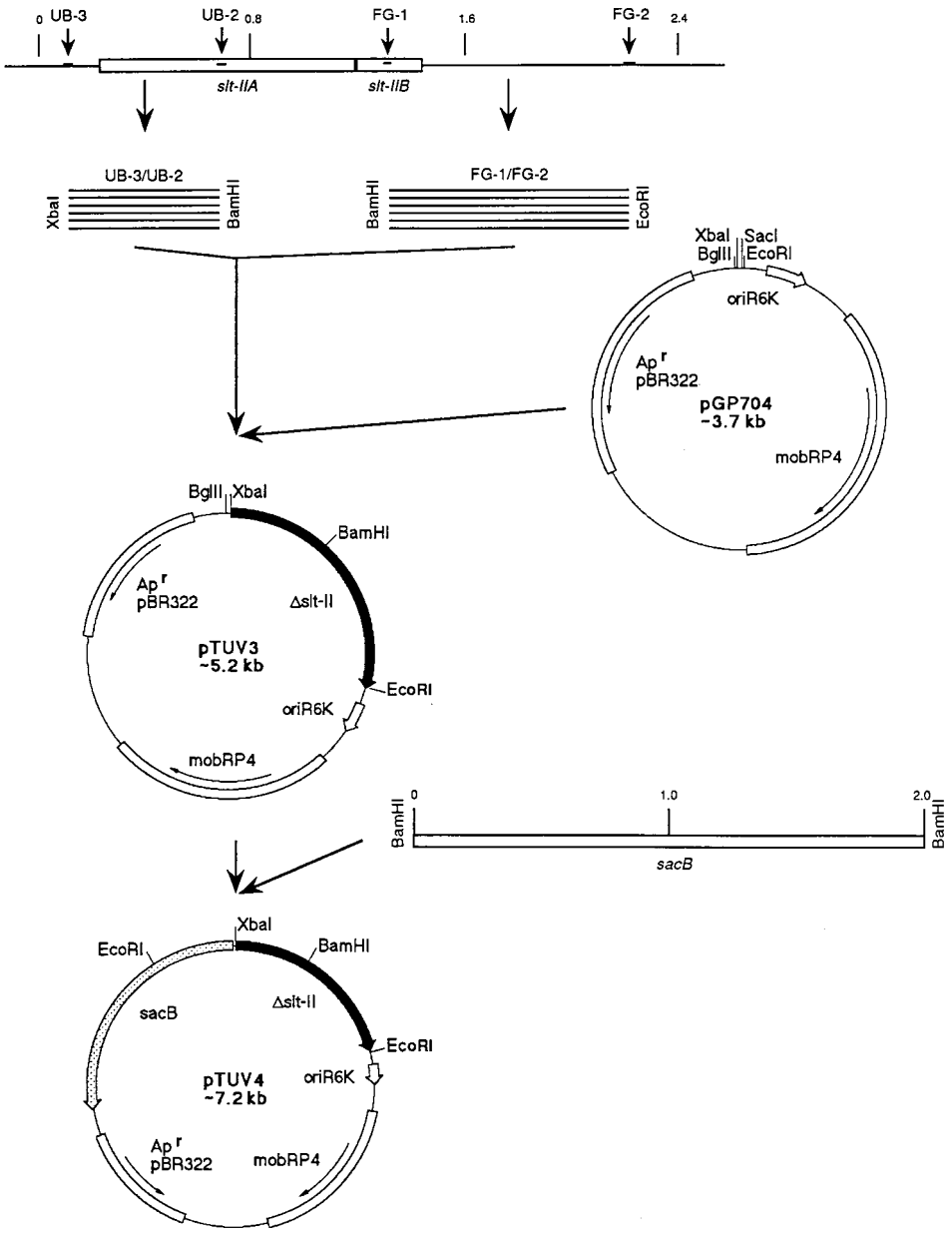

FIG. 1. Construction of pTUV4. PCR fragments generated from *E. coli* 86-24 by utilizing the primer pairs UB-3–UB-2 (610 bp) and FG-1–FG-2 (870 bp) were eluted from agarose gels and digested with *Xba*I and *Bam*HI or *Bam*HI and *Eco*RI, respectively. Cloning of these fragments into pGP704, digested with *Xba*I and *Eco*RI, created a mutagenized *slt-ii* gene copy, harboring a 589-bp internal deletion. The resulting plasmid was named pTUV3. Introduction of the *sacB* selection system into *Bgl*II-digested pTUV3 yielded pTUV4. Δ*slt-ii*, deletion mutation of the *slt-ii* gene; oriR6K, origin of replication from plasmid R6K; mobRP4, oriT from plasmid RP4 (allows mobilization of pTUV4 using the RP4 broad-host-range mobilization system); Ap^r, gene encoding ampicillin resistance from plasmid pBR322; *sacB*, gene from *B. subtilis* encoding the enzyme levansucrase (positive selection system).

were in concordance with published data (3). The sucrose-resistant and ampicillin-sensitive colonies were inoculated into LB supplemented with mitomycin C (400 ng/ml; Sigma Chemical Company, St. Louis, Mo.) and grown overnight to induce phage lysis and toxin release. Of 150 colonies grown, 120 showed low turbidity, with an OD_{600} of ~0.4, and the presence of flocculent debris, indicative of phage induction and cell lysis. Thirty of the isolates, however, showed good growth, with an OD_{600} of >2.5, and no sign of lysis. Similar growth was seen with the nonlysogenic C600 strain. Culture supernatants were tested in triplicate in a toxin capture enzyme-linked immunosorbent assay (ELISA) with the monoclonal antibody 4D1, as described previously (9). Supernatants from the thirty colonies which showed no growth inhibition in mitomycin C-containing LB were all negative by ELISA for SLT-II. Based on the lack of apparent phage induction and the lack of toxin production, we termed this class of mutants phage-cured derivatives of strain 86-24. Only one supernatant from 120 mitomycin C-sensitive cultures was negative in the toxin ELISA. This isolate was termed TUV86-2.

PCR with the primers UB-3 and FG-2 yielded single bands at about 2,100 bp for *E. coli* 86-24 and at about 1,500 bp for *E. coli* TUV86-2 (data not shown). The 600-bp difference reflects the deletion within the *slt-ii* A and B genes. The phage-cured

TABLE 1. List of oligonucleotide primers used in this study

Primer	Sequence (5′ to 3′)[a]	Location (bp)	Reference
UB-3	GC-TCT-AGA-CAG-AGC-AAT-TGC-CTT-CTG-AGC *Xba*I	92–112	17
UB-2	CG-GGA-TCC-GAC-GAC-TGA-TTT-GCA-TTC-CGG *Bam*HI	681–701	17
FG-1	CG-GGA-TCC-GAG-TTT-TCC-AAG-TAT-AAT-GAG *Bam*HI	1,291–1,311	17
FG-2	G-GAA-TTC-TTG-CCT-GGC-TCC-TCT-GGT-GAT *Eco*RI	870 downstream of FG-1	This study
FG-5	ATA-CCA-CTC-TGC-AAC-GTG	645–662	17
SLT-II-1	CTT-CGG-TAT-CCT-ATT-CCC-GG	288–307	27
SLT-II-2	GGA-TGC-ATC-TCT-GGT-CAT-TG	747–766	27

[a] Recognition sites for restriction endonucleases are underlined, and restriction endonucleases are indicated.

derivatives of strain 86-24 yielded no PCR product when the primers UB-3 and FG-2 were used. Nucleotide sequence analysis of the PCR product from TUV86-2, using the primer FG-5, revealed that nucleotides 701 to 1291 were deleted and a *Bam*HI restriction site had been introduced at the gap. Using primers SLT-II-1 and SLT-II-2 (27) and DNA from strain 86-24, a PCR product was obtained and nonradioactively labeled by using the digoxigenin kit Genius 1 from Boehringer Mannheim (Indianapolis, Ind.). The probe was used in Southern hybridizations to detect *slt-ii* gene sequences in chromosomal DNA from strain 86-24 and TUV86-2 digested with *Eco*RI (Fig. 2A) or *Dra*I (Fig. 2B). *Eco*RI recognizes sites outside the toxin operon, and *Dra*I recognizes sites within the toxin operon. With both restriction enzyme digests, the difference between the two strains showed a size shift of 600 bp, reflecting the DNA deletion. The exconjugant strain was also analyzed by Southern blotting. The presence of three probe-positive bands in the *Eco*RI digest suggests that the exconjugant possessed a double insert of the suicide vector. The fragment excised from the suicide vector was 2.1 kb, the size of the smaller band on the Southern blot. TUV86-2 gave a positive signal in a colony blot with an EHEC large plasmid probe (22).

E. coli TUV86-2 was agglutinated with anti-*E. coli* O157 latex particles (Unipath Limited, Ogdensburg, N.Y.), identical to its parental strain. Biochemical profiles, obtained with the BBL CRYSTAL E/NF identification system (Becton Dickinson Microbiological Systems, Cockeysville, Md.), were identical for the two strains. No protein bands comparable in size to either the SLT-II A subunit or an individual SLT-II B subunit could be detected in either sodium dodecyl sulfate protein gel stained with Coomassie blue or Western blots using polyclonal anti-SLT-II serum with supernatants from a mitomycin C-induced bacterial culture of TUV86-2 (Fig. 3). There was no evidence of a new band representing a truncated form of the toxin, suggesting that the truncated A subunit may be rapidly degraded. The supernatants from both TUV86-2 and the phage-cured derivative of strain 86-24 showed no cytotoxicity in a [^{3}H]leucine incorporation assay with HeLa cells (9). The in vivo toxicity of supernatants from the EHEC strain 86-24 wild type and TUV86-2 was examined by mouse lethality assays. Groups of five 2-month-old BALB/c mice received 1-ml intraperitoneal injections of dilutions of sterile filtered supernatant, containing 50 and 5 μg of total protein from EHEC 86-24 or *E. coli* TUV86-2, respectively. All 10 mice receiving injections of supernatants from EHEC strain 86-24 died within 48 h, and all 10 mice receiving injections of supernatants from TUV86-2 survived.

The plaques of *E. coli* TUV86-2 were similar in numbers and shape to those of the *E. coli* 86-24 positive control. A representative from the phage-cured derivatives of strain 86-24 did not release any phage that were capable of forming plaques.

To further investigate the phage-cured nature of this isolate,

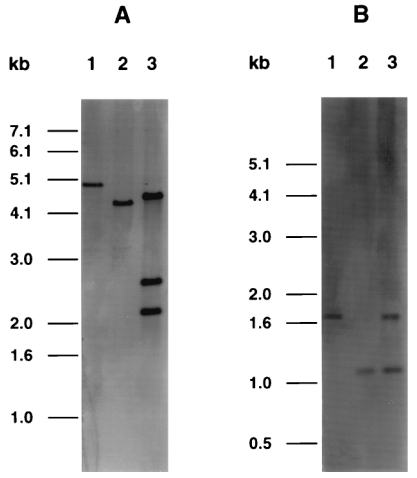

FIG. 2. Southern blot of total chromosomal DNAs from EHEC 86-24 (lanes 1), *E. coli* TUV86-2 (lanes 2), and the *E. coli* 86-24 exconjugant recombined with plasmid pTUV4 (lanes 3). The DNA was digested with either *Eco*RI (A) or *Dra*I (B); a 1-kb DNA ladder served as the molecular size standard.

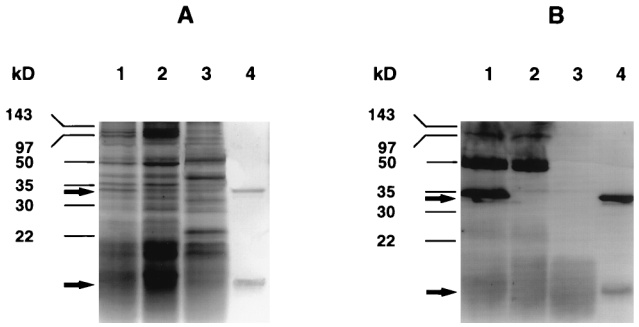

FIG. 3. Proteins from supernatants of mitomycin C cultures and SLT-II holotoxin were separated on a sodium dodecyl sulfate-polyacrylamide gel (A) and exposed to a polyclonal pig anti-SLT-II serum in the corresponding Western blot (B). EHEC strain 86-24 is shown in lanes 1, *E. coli* TUV86-2 is shown in lanes 2, *E. coli* C600 is used as a negative control in lanes 3, and SLT-II holotoxin is used as a positive control in lanes 4. Molecular masses were estimated by utilizing a prestained marker from Bio-Rad. Arrows indicate the position of the SLT-II A- and B-subunits at 32 and 7 kDa, respectively.

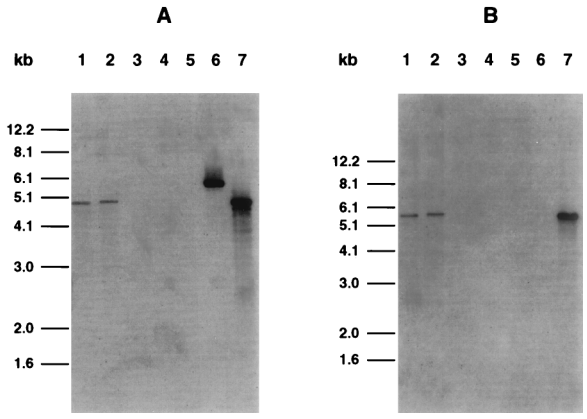

FIG. 4. Southern blot of total chromosomal DNA extracted from various toxigenic and nontoxigenic *E. coli*, digested with *Eco*RI and hybridized with either a 4.9-kb (A) or a 5.5-kb (B) probe from the toxin-converting phage isolated from *E. coli* 86-24. Lanes 1, EHEC strain 86-24; lanes 2, *E. coli* TUV82-2; lanes 3, phage-cured isolate of EHEC strain 86-24; lanes 4, *E. coli* 87-23 (no SLT), lanes 5, *E. coli* C600; lanes 6, phage λ; lanes 7, the SLT-II-converting phage from EHEC strain 86-24. Fragment sizes were estimated by using a 1-kb DNA ladder.

its DNA was subjected to Southern blot analysis using two phage probes derived from the phage within strain 86-24. DNA from *E. coli* bacteriophage containing the *slt-ii* gene was prepared according to standard procedure (32). The DNA was digested with the enzyme *Eco*RI. Two resulting fragments, 4.9 and 5.5 kb, were cloned separately into pUC 18. Neither fragment contained toxin genes. They were labeled with digoxigenin and used as probes in Southern hybridizations with *Eco*RI-digested DNA from *E. coli* 86-24, TUV86-2, the phage-cured isolate, EHEC strain 87-23 (a natural toxin-negative strain), and phage DNA from both λ and the phage from strain 86-24. Both strain 86-24 and *E. coli* TUV86-2 gave positive hybridization bands to both probes (Fig. 4), and the phage-cured isolate showed no hybridization, consistent with the absence of the converting phage. The 4.9-kb but not the 5.5-kb fragment reacted with a fragment from λ DNA.

The production of cytotoxins, termed SLT-I or SLT-II after their relatedness to the Shiga toxin of *S. dysenteriae* type I, was found to be one classical hallmark of EHEC. Molecular biological and biochemical research has produced a lot of information about the genetics of these toxins (4, 26) and their mode of action (10, 11, 13, 15, 18, 33, 34). However, despite all this knowledge, the role of the toxin in the course of the disease or in the development of HUS is still not fully understood.

An isogenic, toxin deletion mutant of an EHEC wild type would be a prerequisite to further dissecting the role of SLTs in suitable in vivo models. An *E. coli* strain, RDEC, that caused diarrhea in rabbits was converted into an SLT-producing strain by making the strain lysogenic with a phage carrying the SLT (37). The resulting mutant was called isogenic and compared in a rabbit model system to its parental strain to investigate the role of the toxin in the course of an infection. The introduction of toxin genes into an *E. coli* strain which causes attaching-and-effacing lesions is not the equivalent of creating an EHEC strain. Besides the site of colonization there may be multiple differences between EHEC and enteropathogenic *E. coli* or other attaching-and-effacing bacteria. Therefore we constructed *E. coli* TUV86-2, a toxin-negative mutant of *E. coli* 86-24, that was fully isogenic to its parental strain.

In the construction of *E. coli* TUV86-2, the majority of our toxin-negative mutants seemed to also be cured entirely of their toxin-converting phages. We tested them for the presence of the phage by PCRs, phage plaque assays, and Southern hybridizations. All tests showed no evidence for the presence of a converting phage. In general, phage curing can be difficult (5) and there are no protocols with guaranteed success available. To date not much is known about the toxin-converting phages except that they are λ-like (7, 14, 28) and about 60 kb in size (30, 41). It has been reported by Karch et al. (20) that SLT-converting phages can be lost upon subcultivation of EHEC strains. However, the rate of phage curing seen in this study with this particular EHEC strain does not explain the rapid loss seen by Karch et al.

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