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 microangiopathic thrombocytopenic 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 O157:H7 and produce Shiga-like toxins (SLTs) (2, 23, 38, 39). The SLTs 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 at 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 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.

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.
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

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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 XbaI and BamHI or BamHI and EcoRI, respectively. Cloning of these fragments into pGP704, digested with XbaI and EcoRI, 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. _Δslt-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).
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 BamHI 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 EcoRI (Fig. 2A) or DraI (Fig. 2B). EcoRI recognizes sites outside the toxin operon, and DraI 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 EcoRI 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 [3H]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 mg 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,
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-I gene was prepared according to standard procedure (32). The DNA was digested with the enzyme EcoRI. Two resulting fragments, 4.9 and 5.5 kb, were cloned separately into pUC18. Neither fragment contained toxin genes. They were labeled with digoxigenin and used as probes in Southern hybridizations with EcoRI-digested DNA from E. coli 86-24, TUV86-2, the phage-cured isolate, EHEC strain 86-24, 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 that 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, 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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