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Cloning and Sequencing of the Genes for Shiga Toxin from *Shigella dysenteriae* Type 1

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The structural genes for Shiga toxin, designated *stx* A and *stx* B, were cloned from *Shigella dysenteriae* type 1 3818T, and a nucleotide sequence analysis was performed. Both *stx* A and *stx* B were present on a single transcriptional unit, with *stx* A preceding *stx* B. The molecular weight calculated for the processed A subunit was 32,225, while the molecular weight of the processed B subunit was 7,691. Comparison of the nucleotide sequences for Shiga toxin and Shiga-like toxin I (SLT-I) from *Escherichia coli* revealed that the genes for Shiga toxin and SLT-I were greater than 99% homologous; three nucleotide changes were detected in three separate codons of the A subunits. Only one of these codon differences resulted in a change in the amino acid sequence: a threonine in Shiga toxin at position 45 of the A subunit compared with a serine in the corresponding position in SLT-I. Furthermore, Shiga toxin and SLT-I had identical signal peptides for the A and B subunits, as well as identical ribosome-binding sites, a putative promoter, and iron-regulated operator sequences. These findings indicate that Shiga and SLT-I are essentially the same toxin. Southern hybridization studies with total cellular DNA from several *Shigella* strains and internal toxin probes for SLT-I and its antigenic variant SLT-II showed that a single fragment in *S. dysenteriae* type 1 hybridized strongly with the internal SLT-I probe. Fragments with weaker homology to the SLT-I probe were detected in *S. flexneri* type 2a but no other shigellae. No homology between the Shiga-like toxin II (SLT-II) probe and any of the *Shigella* DNAs was detected. Whereas SLT-I and SLT-II are phage encoded, no phage could be induced from *S. dysenteriae* type 1 or other *Shigella* spp. tested. These results suggest that the Shiga (SLT-I) toxin genes responsible for high toxin production are present in a single copy in *S. dysenteriae* type 1 but not in other shigellae. The findings further suggest that SLT-II genes are absent in shigellae, as are toxin-converting phages.

Shiga toxin is a cell-associated protein toxin composed of one copy of an A subunit (molecular weight estimated as 32,000) and five copies of a B subunit (molecular weight estimated as 7,700) (8, 38). The toxin inhibits protein synthesis in eucaryotic cells by cleaving the N-glycosidic bond at adenine 4324 in 28S rRNA (Y. Endo, K. Tsurugi, T. Yutsudo, Y. Takeda, T. Ogasawara, and K. Igarashi, Eur. J. Biochem., in press). The mode of action of Shiga toxin is therefore identical to that of the plant toxin ricin (10). The biological and biochemical properties of Shiga toxin have recently been reviewed by O'Brien and Holmes (27).

Shigella species other than *Shigella dysenteriae* type 1 and certain strains of *Escherichia coli*, *Salmonella typhimurium*, *Vibrio cholerae*, and *Campylobacter jejuni* produce low levels of a cytotoxin(s) that is neutralizable by antibodies against purified Shiga toxin from *S. dysenteriae* type 1 (30, 32; A. D. O'Brien, M. E. Chen, R. K. Holmes, J. Kaper, and M. Levine, Letter, Lancet, i:77–78, 1984; M. A. Moore, M. J. Blazer, G. I. Perez-Perez, and A. D. O'Brien, submitted for publication). Some strains of *E. coli* produce toxin in amounts equivalent to those produced by strains of *S. dysenteriae* type 1 (23, 30). *E. coli* that produce such elevated levels of toxin have been associated with outbreaks and sporadic cases of diarrhea (5, 37), hemorrhagic colitis (23, 34), and hemolytic uremic syndrome (19, 23).

Toxins produced by organisms other than *S. dysenteriae* type 1 that are neutralizable with antibodies against purified Shiga toxin are referred to as Shiga-like toxins (SLTs). The SLT produced at high levels by *E. coli*, SLT-I, is also referred to as Vero toxin or Vero toxin type 1 due to its cytotoxic activity on Vero cells. The immunological, biological, and biochemical properties of purified Shiga toxin and purified SLT-I from *E. coli* are virtually identical (28, 42; A. D. O'Brien, T. A. Lively, T. W. Chang, and S. L. Gorbach, Letter, Lancet ii:573, 1983).

The *E. coli* SLT-I structural genes (*slt*-I A and *slt*-I B) are carried on temperate bacteriophages (31, 41). The amino acid compositions of the SLT-I A and B subunits predicted from the DNA sequences (3, 7, 18) are nearly identical to the amino acid compositions of the A and B subunits of Shiga toxin experimentally determined by Donohue-Rolfe et al. (8). In addition, the predicted amino acid sequence of the B subunit of SLT-I (3, 7, 18) is identical to the amino acid sequence of the B subunit of Shiga toxin experimentally determined by Seidah et al. (38), and the amino acid sequence of the A subunit shares limited homology with the A subunit of ricin (3, 7).

An antigenic variant of SLT-I, referred to as SLT-II or Vero toxin type 2, has recently been described in enterohemorrhagic strains of *E. coli* (43; S. M. Scotland, H. R. Smith, and B. Rowe, Letter, Lancet ii:885–886, 1985). The structural genes of SLT-II, *slt*-II A and *slt*-II B, share approximately 55% overall nucleotide and amino acid sequence homologies with the structural genes of SLT-I (17). Recent data on the mode of action of SLT-II indicate that, like ricin and Shiga toxin, it is a 28S rRNA glycosidase (Endo et al., in press).

The genes for high-level production of Shiga toxin, desig-

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nated *stx*, have recently been cloned in vivo and mapped to a location near *pyrF* on the chromosome of *S. dysenteriae* type 1 (39). Here we report the in vitro cloning of the structural genes for Shiga toxin, *stx* A and *stx* B, from total cellular DNA of *S. dysenteriae* type 1 3818T. The nucleotide sequences of the Shiga toxin genes were compared with the previously reported sequences of SLT-I. In addition, Southern hybridization studies were conducted to assess the prevalence of SLT and toxin-converting phage sequences in various *Shigella* species.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. dysenteriae* type 1 3818T (Centers for Disease Control enteric reference number 3818-69), *S. dysenteriae* type 1 60R, *S. flexneri* type 2a M4243, *S. boydii* type 1, and *S. sonnei* were obtained from S. B. Formal of the Walter Reed Army Institute of Research (Washington, D.C.). In this report the last two strains are designated *S. boydii* SF-b1 and *S. sonnei* SF-s1. *S. dysenteriae* type 1 3818T is toxigenic and invasive. It was isolated in 1969 from the stool of a patient with dysentery during an epidemic of shigellosis in Central America (24). The *E. coli* minicell-producing strain P678-54 described by Adler et al. (1) was obtained from C. L. Pickett of the Uniformed Services University (Bethesda, Md.).

Cosmid pHC79 (15) was used for the initial cloning; and plasmids pBR322, pBR328, pACYC184, pUC18, and pUC19 were used for subcloning as described by Maniatis et al. (22). Recombinant plasmid pJN25 was described by Newland et al. (26) and carries the intact structural genes for SLT-I. Recombinant plasmids pJN31, containing a 250-base-pair (bp) *Hin*dIII to *Hpa*I internal fragment from the *slt*-I A gene, and pNN76, containing a 5-kilobase-pair (kbp) *Eco*RI fragment, including the *slt*-II A and B genes, were obtained from J. W. Newland of the Walter Reed Army Institute of Research.

Media, enzymes and biochemicals. Bacteria were routinely cultured in LB medium (22). Strains tested for toxin production were grown in Chelex-treated glucose syncase medium (30) or Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). Media were supplemented as indicated in the text with the following antibiotics (Sigma Chemical Co., St. Louis, Mo.) at the indicated concentrations: ampicillin, 100 μg/ml; tetracycline, 15 μg/ml; and chloramphenicol, 30 μg/ml.

Restriction enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, Md.); Boehringer Mannheim Biochemicals (Indianapolis, Ind.); or New England BioLabs, Inc. (Beverly, Mass.). DNA polymerase I (Klenow enzyme), calf intestinal alkaline phosphatase, and T4 DNA ligase were purchased from Boehringer Mannheim. The enzymes were used according to the recommendations of the supplier. Lambda in vitro packaging kits and nick-translation kits were purchased from Bethesda Research Laboratories and were used according to the instructions of the supplier.

[α-^{35}S]dATP (500 Ci/mmol) and [^{35}S]methionine (800 Ci/mmol) were purchased from New England Nuclear Research Products (Boston, Mass.). [α-^{32}P]dCTP (3,000 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, Ill.).

Preparation of plasmid, cellular, and phage DNA. Plasmid and cellular DNAs were prepared by the method described by Maniatis et al. (22). Phage from *E. coli* C600(933J) or *E. coli* C600(933W) was obtained after induction with mitomycin C by previously described methods (12). Phage DNA was extracted with formamide by previously published procedures (6). Lambda DNA was purchased from Bethesda Research Laboratories.

Phage induction. Experiments to induce phage from *S. dysenteriae* type 1 3818T and 60R, *S. flexneri* M4243, *S. sonnei* SF-s1, and *S. boydii* SF-b1 with UV light or mitomycin C were performed as described previously (12, 31). *E. coli* HB101 and *E. coli* C600 were used as hosts to plaque phage.

Cloning and subcloning. Cloning of the Shiga toxin genes from *S. dysenteriae* type 1 3818T and all manipulations of toxin-positive clones were conducted under BL3-EK1 containment (11). Cosmid cloning was performed essentially as described by Maniatis et al. (22). Transductants were screened for toxin production with a colony blot assay which immunochemically detects the B subunit of Shiga toxin (42). Colony blot-positive transductants were tested for biologically active toxin by the HeLa cell cytotoxicity assay (14). Neutralization of cytotoxicity was performed with rabbit antiserum against purified Shiga toxin (29), rabbit antiserum against crude SLT-II (43), a mixture of anti-Shiga and anti-SLT-II, and monoclonal antibody (MAb) 13C4 produced against SLT-I (42). Normal rabbit serum and MAbs against cholera toxin (16) were used as control antibodies.

Cytotoxicity and neutralization assays. Bacteria were assayed for toxin as described previously (30), and neutralization assays were performed by previously published methods (23).

Minicell analysis and immunoprecipitation. Minicell experiments were performed by the method described by Meagher et al. (25). The labeled proteins were extracted with polymyxin B (100 μg/ml) for 45 min at 37°C and then analyzed on sodium dodecyl sulfate–15% polyacrylamide gels (21). Immunoprecipitation of the extracted proteins was performed essentially as described by Kessler (20). Rabbit antisera (100 μl of a 1:100 dilution of anti-Shiga or normal rabbit serum) or MAbs (100 μl of ammonium sulfate-concentrated culture supernatants) of anti-SLT-I (MAb 13C4 or MAb 16E6) or anti-cholera toxin (MAb 32D3) were mixed overnight at 4°C with the labeled proteins (10^6 cpm per sample). The following day a second antibody (1 μl of goat anti-mouse immunoglobulin G [IgG] and IgM heavy and light chains; Boehringer Mannheim) was added to facilitate precipitation. The samples were incubated for an additional hour at 37°C, and then the antibody-antigen complexes were precipitated with Pansorbin (Calbiochem-Behring, La Jolla, Calif.) as described previously (20).

Southern hybridizations. Southern transfer of DNA and DNA-DNA hybridizations were performed as described by Maniatis et al. (22). *Eco*RI-restricted DNA samples from SLT-I phage 933J and from SLT-II phage 933W were nick translated and used as probes to detect homologous phage sequences in *Eco*RI-digested total cellular DNA preparations. Internal DNA probes from the SLT-I and SLT-II A subunit genes were prepared by published procedures (9). The SLT-I probe was a 250-bp *Hin*dIII to *Hpa*I fragment from pJN31, and the SLT-II probe was an 800-bp *Sma*I to *Pst*I fragment from pNN76.

Nucleotide sequence analysis. Subfragments of the recombinant plasmid pNAS4 were cloned into the M13 mp18 and mp19 replicative-form vectors (36) to allow determination of the nucleotide sequence of the Shiga toxin genes. Synthetic oligonucleotides (model 380A DNA synthesizer; Applied Biosystems, Inc., Foster City, Calif.) specific to the SLT-I nucleotide sequence (18) were used as primers in the dideoxy chain-terminator method (2). The sequencing strategy is depicted in Fig. 1. The amino acid sequences and biochem-

ical properties of the Shiga toxin A and B subunits were deduced with a DNA computer program (IBI/Pustell). A computer search of GenBank (MicroGenie; Beckman Instruments, Inc., Palo Alto, Calif.) revealed no significant homologies with bacterial or bacteriophage sequences.

RESULTS

Cloning of toxin genes. The genes for Shiga toxin were cloned from total cellular DNA isolated from *S. dysenteriae* type 1 3818T with the cosmid vector pHC79. Transductants were initially screened for production of the B subunit with a colony blot assay. Of 750 transductants screened, 3 carried recombinant plasmids, designated pNAS1, pNAS2, and pNAS3, which produced the B subunit. Transductants carrying pNAS2 or pNAS3 produced biologically active toxin, as assayed in the HeLa cell cytotoxicity assay. The cytotoxin produced by these two transductants was neutralizable with polyclonal antibodies against purified Shiga toxin and MAbs against SLT-I. The toxin was not neutralizable with polyclonal antibodies against crude SLT-II.

Digestion of plasmid DNAs from pNAS1, pNAS2, and pNAS3 with *Hin*dIII, *Eco*RV, and *Eco*RI revealed common restriction fragments. Digestion of pNAS2 and pNAS3 with *Eco*RI showed a common fragment of approximately 5 kbp that was absent in the nontoxigenic plasmid pNAS1. This 5-kbp *Eco*RI fragment was subcloned from pNAS2 into the vector pACYC184 to produce the hybrid plasmid pNAS4 which carried the intact genes for Shiga toxin production. A restriction map of the 5 kbp *Eco*RI fragment and the strategy for subcloning and sequence analysis are shown in Fig. 1. From the subcloning and sequence analyses, the A- and B-subunit genes for Shiga toxin were located within a 3.2-kbp *Nco*I to *Eco*RI fragment, between the *Bgl*II and *Hin*cII restriction sites.

Minicell analysis. The polypeptides encoded by pNAS4 were analyzed in minicell experiments (Fig. 2A). The results with minicells showed that pNAS4 encodes polypeptides with the same apparent molecular weights as the SLT-I A and B polypeptides encoded by pJN25, a recombinant plasmid carrying the intact structural genes for SLT-I (26). An A polypeptide with a molecular weight of approximately 32,500 and a B polypeptide with a molecular weight of approximately 8,000 were identified from the minicells transformed with pNAS4. Higher-molecular-weight forms (presumed to be unprocessed polypeptides with signal sequences) of the Shiga toxin and SLT-I A and B subunits were also encoded by minicells (Fig. 2A). The processed and unprocessed forms of the A and B polypeptides were absent in the minicells carrying the vector plasmid pACYC184.

The radiolabeled toxins produced by minicells containing pNAS4 or pJN25 were immunoprecipitated with either rabbit antiserum against purified Shiga toxin or MAbs against SLT-I, and were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Polypeptides corresponding in molecular weights to the processed A subunit (M_r, 32,500), an A_1 fragment (M_r, 27,500) which is the product of proteolytic nicking (28), and the processed B subunit (M_r, 8,000) were immunoprecipitated by anti-Shiga or anti-Shiga-like antibodies (Fig. 2B). No polypeptides were precipitated by the control antibodies (normal rabbit serum and anti-cholera toxin MAbs).

Sequence analysis of toxin genes. In Fig. 3 the nucleotide and deduced amino acid sequences of *stx* A and *stx* B are presented and compared with the *slt*-I A and *slt*-I B sequences as reported previously (18). The nucleotide and amino acid sequences for *stx* A and *slt*-A differed by three nucleotides, shown at positions 223, 359, and 850 in Fig. 3. Only one of the three nucleotide differences between *stx* A and *slt*-I A caused a change in the amino acid sequences. The adenine to thymine transversion at nucleotide 359 resulted in a substitution (albeit conservative) of a threonine in the Shiga toxin sequence for a serine in the SLT-I sequence. Nucleotide sequences (thus far determined) upstream and downstream to the *stx* operon were identical to those reported previously for the *slt*-I operon cloned from phage 933J (18). The Shiga toxin A and B subunits possessed signal peptides of 22 and 20 amino acids, respectively (designated by negative values in Fig. 3), which were identical to the SLT-I A- and B-subunit signal peptides. Separation of the signal peptides from the mature polypeptides at putative *E. coli* signal peptidase I cleavage sites (33) resulted in a processed Shiga toxin A subunit of 293 amino acids, with a calculated molecular weight of 32,225, and a processed B subunit of 69 amino acids, with a calculated molecular weight of 7,691. The amino acid sequence of the Shiga toxin B subunit deduced from the nucleotide sequence was iden-

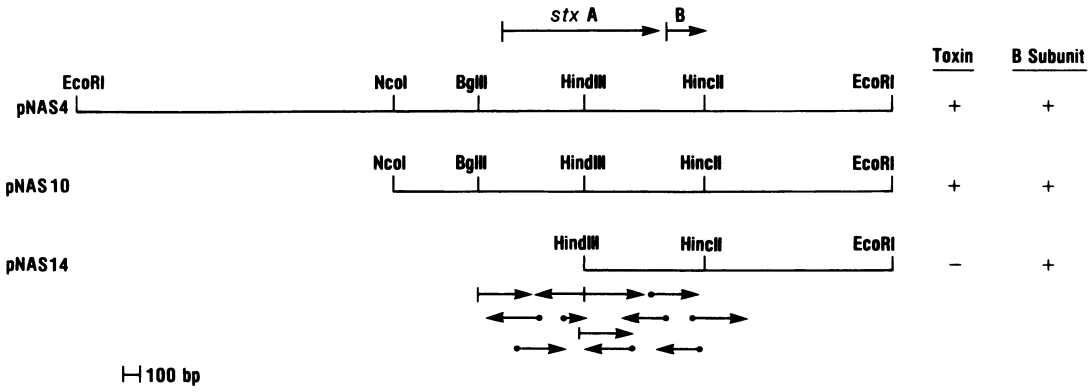

FIG. 1. Restriction map of Shiga toxin genes. Restriction sites are indicated within the 5-kbp *Eco*RI fragment isolated from the recombinant plasmid pNAS4. Subclones carrying regions of the 5-kbp *Eco*RI fragment are shown, and their ability to produce biologically active toxin and antigenically reactive B subunit is indicated on the right of the figure. The Shiga toxin structural genes, *stx* A and *stx* B, are oriented above the restriction map. The strategy used to sequence the coding and noncoding strands of *stx* A and *stx* B is depicted by the arrows below the map. Arrows preceded by a vertical line indicate subfragments sequenced by using the M13 universal primer. Arrows preceded by a dot indicate where synthetic oligonucleotides specific to the SLT-I sequence were used as primers.

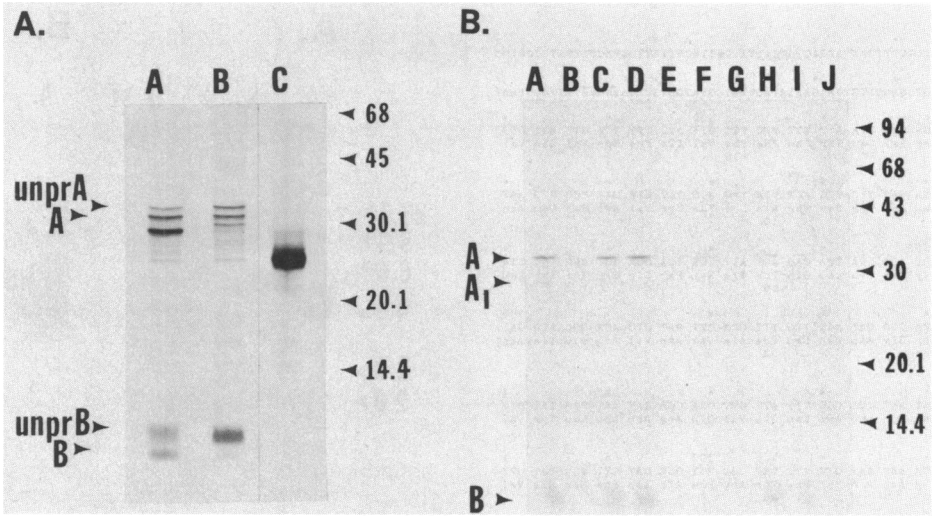

FIG. 2. (A) Analysis of [^{35}S]methionine-labeled proteins produced in minicells transformed with plasmids carrying the genes for SLT-I or Shiga toxin. Proteins were separated with a sodium dodecyl sulfate–15% polyacrylamide gel and visualized following autoradiography. Lane A, Polypeptides encoded by pJN25, which carries the SLT-I genes; lane B, polypeptides encoded by pNAS4, which carries the Shiga toxin genes; lane C, polypeptides encoded by the vector pACYC184 (control for pNAS4). Sizes and positions of molecular size standards (in kilodaltons; low-molecular-weight standards; Bio-Rad Laboratories, Richmond, Calif.) are indicated on the right. The location of the processed and unprocessed A (unprA) and B (unprB) subunits are indicated on the left. (B) *Staphylococcus aureus* protein A immunoprecipitation of the [^{35}S]methionine-labeled polypeptides produced by minicells carrying the SLT-I or Shiga toxin genes. Lanes A through E, Polypeptides encoded by pJN25 that were specifically precipitated by rabbit anti-Shiga toxin (lane A), normal rabbit serum (lane B), anti-SLT-I MAb 16E6 (lane C), anti-SLT-I MAb 13C4 (lane D), and anti-cholera toxin MAb 32D3 (lane E); lanes F through J, polypeptides encoded by pNAS4 that were specifically precipitated by anti-Shiga toxin (lane F), normal rabbit serum (lane G), anti-SLT-I MAb 16E6 (lane H), anti-SLT-I MAb 13C4 (lane I), and anti-cholera toxin MAb 32D3 (lane J). The proteins were separated on a sodium dodecyl sulfate–15% polyacrylamide gel and visualized following autoradiography. The positions of molecular size standards are indicated on the right; and the positions of the A, A_1, and B polypeptides are indicated on the left.

tical to that determined previously by using the purified protein (38).

The structural genes of the Shiga toxin operon were translated in frame and were organized tandemly with the open reading frame of *stx* A 5′ to the open reading frame of *stx* B. An untranslated space of 12 nucleotides separated *stx* A and *stx* B. A putative promoter sequence (35) was identified 5′ to *stx* A (at nucleotides 19 and 42; underlined in Fig. 3). This promoter was the same promoter identified for the *slt*-I operon (3, 7). It has been proposed that the *slt*-I operon is repressed under iron replete conditions by the *fur* gene product (4), and a putative binding site for the Fur protein was identified in a region of dyad symmetry within the *slt*-I promoter (4, 7). Because the promoter sequences of the *stx* and *slt*-I operons were identical, it is likely that the negative effect of iron on the expression of Shiga toxin is also mediated by the *fur* gene product.

Two sequences homologous to the 3′ end of *E. coli* 16S rRNA (40) which could serve as ribosome-binding sites were identified for both *stx* A and *stx* B (at nucleotides 145 and 1107, respectively; overlined in Fig. 3). Both the sequence and position of the *stx* ribosome-binding sites were identical to those reported previously for the *slt*-I operon (18). The proposed mode of expression for the Shiga toxin operon was identical to that proposed previously (18) for the *slt*-I operon; i.e., the individual subunits are synthesized from a polycistronic mRNA that is regulated by the promoter 5′ to *stx* A. Translation to give the individual A and B subunits would be initiated from the independent ribosome-binding sites. The existence of an additional ribosome-binding site for *stx* B may function to give the holotoxin subunit ratio of 5B:1A (8). However, the effect of two different genetic backgrounds, i.e., *S. dysenteriae* type 1 compared with *E. coli*, on the expression of the Shiga toxin (*slt*-I) operon and on the assembly of the holotoxin has yet to be determined.

Toxin production, phage induction, and Southern hybridization experiments. *S. dysenteriae* type 1 3818T and 60R, *S. flexneri* M4243, *S. boydii* SF-b1, and *S. sonnei* SF-s1 were studied for the production of cytotoxin that was antigenically related to SLT-I or SLT-II and for DNA sequences that were related to the genes for SLT-I or SLT-II. The results of these experiments are summarized in Table 1. Various levels of cytotoxin(s) were detected among all four species of *Shigella*. However, high-level toxin production ($>10^5$ 50% cytotoxic dose per ml of sonic lysate) was observed for *S. dysenteriae* type 1 3818T and 60R only. The cytotoxic activity detected for all strains of *Shigella* tested was neutralizable with MAbs to SLT-I and not by polyclonal antibodies to crude SLT-II. Southern hybridization experiments performed under conditions that permitted no more than a 30% base-pair mismatch showed strong homology between an internal probe for SLT-I and single fragments of different sizes from *S. dysenteriae* type 1 3818T and 60R (Fig. 4A). With an extended time (11 days), additional bands of weak homology were also detected among *S. dysenteriae* type 1 3818T and 60R and *S. flexneri* M4243 (Fig. 4B), but not among *S. sonnei* or *S. boydii*. These fragments appeared to be of the same or similar size in *S. dysenteriae* type 1 and *S. flexneri*. Under the hybridization conditions described above, no homology was detected between the internal SLT-II probe and the cellular DNAs from the five strains of *Shigella* (data not shown).

The strains described above were also tested for UV- or mitomycin C-inducible phages and the presence of DNA

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                                                  50
TGACCAGATATGTTAAGGTTGCAGCTCTCTTTGAATATGATTATCATTTTCATTACGTTATTGTTACGTTTATCCGGTGC

          100                                                    150
GCCGTAAAACGCCGTCCTTCAGGGCGTGGAGGATGTCAAGAATATAGTTATCGTATGGTGCTCAAGGAGTATTGTGTAAT

                                                 200
ATG AAA ATA ATT ATT TTT AGA GTG CTA ACT TTT TTC TTT GTT ATC TTT TCA GTT AAT GTG
Met Lys Ile Ile Ile Phe Arg Val Leu Thr Phe Phe Phe Val Ile Phe Ser Val Asn Val
-22                                          -10

  G                       250
GTT GCG AAG GAA TTT ACC TTA GAC TTC TCG ACT GCA AAG ACG TAT GTA GAT TCG CTG AAT
Val Ala Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala Lys Thr Tyr Val Asp Ser Leu Asn
      +1                                 10

          300
GTC ATT CGC TCT GCA ATA GGT ACT CCA TTA CAG ACT ATT TCA TCA GGA GGT ACG TCT TTA
Val Ile Arg Ser Ala Ile Gly Thr Pro Leu Gln Thr Ile Ser Ser Gly Gly Thr Ser Leu
    20                                 30

        350          T                                          400
CTG ATG ATT GAT AGT GGC ACA GGG GAT AAT TTG TTT GCA GTT GAT GTC AGA GGG ATA GAT
Leu Met Ile Asp Ser Gly Thr Gly Asp Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp
    40                 Ser             50

                                                450
CCA GAG GAA GGG CGG TTT AAT AAT CTA CGG CTT ATT GTT GAA CGA AAT AAT TTA TAT GTG
Pro Glu Glu Gly Arg Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn Asn Leu Tyr Val
    60                                 70

                                                500
ACA GGA TTT GTT AAC AGG ACA AAT AAT GTT TTT TAT CGC TTT GCT GAT TTT TCA CAT GTT
Thr Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr Arg Phe Ala Asp Phe Ser His Val
    80                                 90

                    550
ACC TTT CCA GGT ACA ACA GCG GTT ACA TTG TCT GGT GAC AGT AGC TAT ACC ACG TTA CAG
Thr Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp Ser Ser Tyr Thr Thr Leu Gln
    100                                110

          600
CGT GTT GCA GGG ATC AGT CGT ACG GGG ATG CAG ATA AAT CGC CAT TCG TTG ACT ACT TCT
Arg Val Ala Gly Ile Ser Arg Thr Gly Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser
    120                                130

        650                                                    700
TAT CTG GAT TTA ATG TCG CAT AGT GGA ACC TCA CTG ACG CAG TCT GTG GCA AGA GCG ATG
Tyr Leu Asp Leu Met Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met
    140                                150

                                                750
TTA CGG TTT GTT ACT GTG ACA GCT GAA GCT TTA CGT TTT CGG CAA ATA CAG AGG GGA TTT
Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg Gly Phe
    160                                170

                                800
CGT ACA ACA CTG GAT GAT CTC AGT GGG CGT TCT TAT GTA ATG ACT GCT GAA GAT GTT GAT
Arg Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val Met Thr Ala Glu Asp Val Asp
    180                                190

                    C
CTT ACA TTG AAC TGG GGA AGG TTG AGT AGT GTC CTG CCT GAC TAT CAT GGA CAA GAC TCT
Leu Thr Leu Asn Trp Gly Arg Leu Ser Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser
    200                                210

          900
GTT CGT GTA GGA AGA ATT TCT TTT GGA AGC ATT AAT GCA ATT CTG GGA AGC GTG GCA TTA
Val Arg Val Gly Arg Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly Ser Val Ala Leu
    220                                230

        950                                                   1000
ATA CTG AAT TGT CAT CAT CAT GCA TCG CGA GTT GCC AGA ATG GCA TCT GAT GAG TTT CCT
Ile Leu Asn Cys His His His Ala Ser Arg Val Ala Arg Met Ala Ser Asp Glu Phe Pro
    240                                250

                                               1050
TCT ATG TGT CCG GCA GAT GGA AGA GTC CGT GGG ATT ACG CAC AAT AAA ATA TTG TGG GAT
Ser Met Cys Pro Ala Asp Gly Arg Val Arg Gly Ile Thr His Asn Lys Ile Leu Trp Asp
    260                                270

                                    1100
TCA TCC ACT CTG GGG GCA ATT CTG ATG CGC AGA ACT ATT AGC AGT TGA GGG GGT AAA
Ser Ser Thr Leu Gly Ala Ile Leu Met Arg Arg Thr Ile Ser Ser ---
    280                                290

                        1150
ATG AAA AAA ACA TTA TTA ATA GCT GCA TCG CTT TCA TTT TTT TCA GCA AGT GCG CTG GCG
Met Lys Lys Thr Leu Leu Ile Ala Ala Ser Leu Ser Phe Phe Ser Ala Ser Ala Leu Ala
-20                                -10

          1200
ACG CCT GAT TGT GTA ACT GGA AAG GTG GAG TAT ACA AAA TAT AAT GAT GAC GAT ACC TTT
Thr Pro Asp Cys Val Thr Gly Lys Val Glu Tyr Thr Lys Tyr Asn Asp Asp Asp Thr Phe
+1                                 10                                      20

        1250
ACA GTT AAA GTG GGT GAT AAA GAA TTA TTT ACC AAC AGA TGG AAT CTT CAG TCT CTT CTT
Thr Val Lys Val Gly Asp Lys Glu Leu Phe Thr Asn Arg Trp Asn Leu Gln Ser Leu Leu
                                   30                                      40

  1300                                                   1350
CTC AGT GCG CAA ATT ACG GGG ATG ACT GTA ACC ATT AAA ACT AAT GCC TGT CAT AAT GGA
Leu Ser Ala Gln Ile Thr Gly Met Thr Val Thr Ile Lys Thr Asn Ala Cys His Asn Gly
                                   50                                      60

                        1400
GGG GGA TTC AGC GAA GTT ATT TTT CGT TGA CTCAGAATAGCTCAGTGAAAATAGCAGGCGGAGATTCAT
Gly Gly Phe Ser Glu Val Ile Phe Arg ---

          1450                                                   1500
AAATGTTAAATACATCTCAATTCAGTCAGTTGTTGCCGGTTCTGATAATAGATGTGTTAGAAAATTTCTGCATG
```

FIG. 3. Nucleotide sequence of the *stx* A and *stx* B structural genes. The nucleotides are numbered above the sequence. Translation of the sequence gave an open reading frame for the A-subunit gene between nucleotides 161 and 1106 and an open reading frame for the B-subunit gene between nucleotides 1118 and 1385. The amino acids are numbered below the sequence. The amino-terminal residues of the processed A and B subunits are depicted as +1. Three nucleotide differences between *stx* A and *slt*-I A are shown above the Shiga toxin sequence, at positions 223, 359, and 850. The single amino acid difference between Shiga toxin and SLT-I is shown below the sequence, at residue 45. A putative promoter sequence (35) is underlined at nucleotides 19 to 24 (−35) and 42 to 47 (−10). Two potential ribosome-binding sites (40) are overlined beginning at nucleotides 145 (5′ to *stx* A) and 1107 (5′ to *stx* B). The nucleotide sequence of SLT-I reported previously (18) was corrected here. This sequence contained three fewer T residues in the first 150 bp.

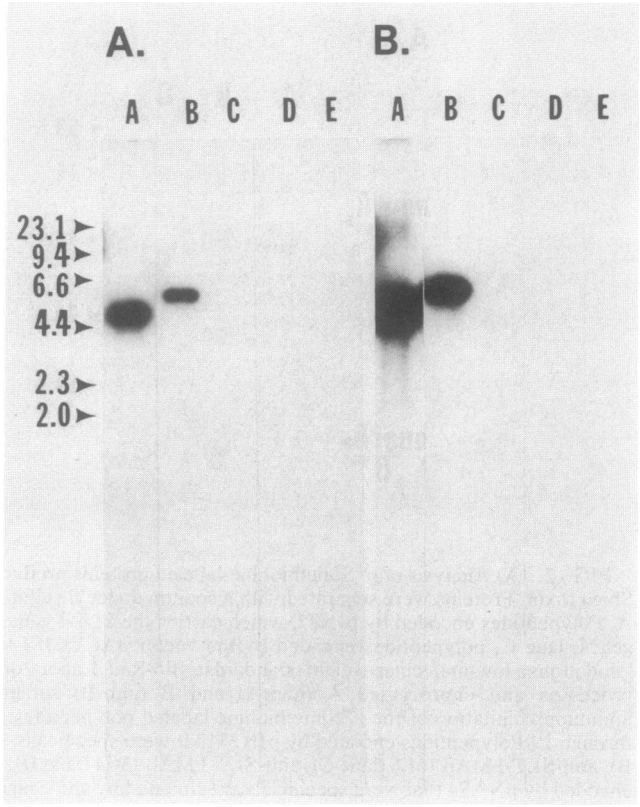

FIG. 4. Southern blot analysis of *Eco*RI-restricted total cellular DNA from *S. dysenteriae* type 1 60R (lanes A), *S. dysenteriae* type 1 3818T (lanes B), *S. flexneri* type 2a M4243 (lanes C), *S. boydii* SF-b1 (lanes D), and *S. sonnei* SF-s1 (lanes E) probed with the ^{32}P-labeled, 250-bp *Hin*dIII-*Hpa*I fragment internal to *slt*-I A. (A) Exposure for 2.8 days. (B) Exposure for 11 days, demonstrating homology between the SLT-I probe and total cellular DNA from *S. flexneri* (lane C). The positions of lambda-*Hin*dIII fragments (size markers) are indicated on the left of the figure.

sequences related to the SLT-I- or SLT-II-converting phages from *E. coli* 933. No phages were detected by using *E. coli* K-12 C600 and HB101 as the host bacteria for the plaquing experiments (Table 1). Total cellular DNA from the five strains of *Shigella* was probed under hybridization conditions that permitted up to 30% base-pair mismatch with genomic DNA from SLT-I-converting phage 933J or SLT-II-converting phage 933W. Sequences related to phage 933J were detected in *S. dysenteriae* type 1 3818T and 60R and *S. flexneri* M4243, but not in *S. sonnei* SF-s1 or *S. boydii* SF-b1. No sequences related to phage 933W were detected in any of the strains tested (data not shown).

DISCUSSION

In the studies reported here, the structural genes for Shiga toxin from *S. dysenteriae* type 1 3818T were cloned and the

TABLE 1. Toxin production and phage induction in *Shigella* spp.

Strain	Toxin production (CD_{50}/ml) of[a]:		Neutralization by:			Phage induction by:	
	Lysate	Supernatant	Rabbit anti-Shiga	MAb anti-SLT-I	Rabbit anti-SLT-II	UV light	Mitomycin C
S. dysenteriae type 1							
3818T	10^6–10^7	10^6	+	+	−	−	−
60R	10^6–10^7	10^6	+	+	−	−	−
S. flexneri type 2a M4243	320	NK[b]	+	+	−	−	−
S. boydii SF-b1	80	NK	+	+	−	−	−
S. sonnei SF-s1	80	NK	+	+	−	−	−

[a] CD_{50}, Cytotoxic dose of 50%.
[b] NK, No kill in the HeLa cell cytotoxicity assay.

nucleotide sequences were determined. The nucleotide sequences of three members of the Shiga toxin gene family, SLT-I (18), SLT-II (17), and Shiga toxin, have now been determined. These sequence analyses define a relationship among the Shiga toxin and SLT genes and suggest models of evolution. We have shown previously (17) that SLT-I and SLT-II share limited regions of high nucleotide sequence homology (greater than 70%) but are only 55% homologous overall. This indicates that the SLT-I and SLT-II genes diverged from each other in the distant past, prior to the evolution of *S. dysenteriae* type 1 and *E. coli* as separate genera. In contrast, the Shiga toxin and SLT-I nucleotide sequences were greater than 99% homologous, and translation of the nucleotide sequences revealed that Shiga toxin and SLT-I were essentially identical toxins. This high degree of genetic relatedness would be expected to exist between an individual gene isolated from two separate bacterial strains of the same species.

At this time it is not possible to ascertain the origin of the Shiga toxin or SLT-I genes. Since SLT-I is encoded by a temperate coliphage, transduction could serve to disseminate the toxin structural genes among related bacteria. Although attempts to induce phage from several strains of *Shigella* were unsuccessful (Table 1), DNA sequences homologous to the SLT-I-converting phage 933J were detected within *S. dysenteriae* type 1 and *S. flexneri* type 2A (data not shown). These sequences may represent (i) a defective or incomplete phage in *Shigella* species which is no longer inducible or (ii) a related phage that is not inducible with either UV light or mitomycin C or does not form plaques on *E. coli* K-12 HB101 or C600.

The results of this study support the nomenclature established previously (30) for the Shiga-related toxins because they reveal the near identity between the sequences of SLT-I and Shiga toxin. *Shigella* toxin was described in 1903, prior to the discovery of any related cytotoxins in *E. coli* or related genera. To avoid confusion, the designation SLT should be used for toxin variants isolated from bacteria other than *S. dysenteriae* type 1 which share antigenic or biologic properties with Shiga toxin. The designations SLT-I, SLT-II, etc., should be assigned on the basis of the sequence relatedness of a new antigenic variant to the prototypic cytotoxin genes described previously.

A definitive role for Shiga toxin in diarrheal disease has not yet been demonstrated. Studies with nonisogenic strains of *Shigella* differing in toxin production or invasiveness (an essential virulence trait) have produced inconclusive results about the role of toxin in the pathogenesis of shigellosis (13). The data reported here should facilitate the construction of isogenic strains of *Shigella* that differ only in the ability to produce toxin. These strains could be used in animal studies to clarify the role of Shiga toxin in the pathogenesis of shigellosis and hemolytic uremic syndrome.

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