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Cloning of Genes for Production of *Escherichia coli* Shiga-Like Toxin Type II

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Genes controlling production of Shiga-like toxin type II (SLT-II) in *Escherichia coli* were cloned from the SLT-II-converting bacteriophage 933W and compared with the Shiga-like toxin type I (SLT-I) genes previously isolated and described from phage 933J. Subcloning analysis identified a region within the 4.9-kilobase *Eco*RI fragment of phage 933W that was associated with SLT-II production. Experiments with *E. coli* minicells containing these subclones demonstrated that the 4.9-kilobase *Eco*RI fragment encodes the structural genes for SLT-II. These experiments additionally showed the genetic organization of the SLT-II genes to be the same as that of the SLT-I genes, with the coding sequence for the large A subunit adjacent to that for the smaller B subunit. The mobilities of the SLT-II subunits in sodium dodecyl sulfate-polyacrylamide gels were slightly greater than those determined for the SLT-I subunits. Although apparent processing of the SLT-I subunits was observed with polymyxin B treatment of the labeled minicells, no processing of the SLT-II subunits was detected. Southern blot hybridization studies suggested that the DNA fragment carrying the SLT-II structural genes shares approximately 50 to 60% homology with the DNA of the SLT-I structural genes.

A number of *Escherichia coli* strains isolated from cases of enteropathogenic diarrhea, enterohemorrhagic colitis, and hemolytic uremic syndrome are associated with production of cytotoxins which exhibit biological activities characteristic of *Shigella dysenteriae* type 1 (Shiga) toxin (4). Some of these *E. coli* strains produce high levels (10^5 to 10^8 50% cytotoxic doses per ml of sonic lysate) of a cell-associated cytotoxin that is neutralized by antibodies against Shiga toxin (5, 16, 20, 28; A. D. O'Brien, T. A. Lively, T. W. Chang, and S. W. Gorbach, Lancet ii:573, 1983). This toxin has been designated Shiga-like toxin type I (SLT-I) (35) or Vero cell toxin type 1 (VT1) (16; S. M. Scotland, H. R. Smith, and B. Rowe, Lancet ii:885–886, 1985). SLT-I is structurally similar to Shiga toxin, containing an A subunit and multiple B subunits (26, 30). Some of these *E. coli* strains produce moderate levels (10^3 to 10^4 50% cytotoxic doses per ml of sonic lysate) of a cell-associated cytotoxin that is not neutralized by antibody to Shiga toxin but apparently shares DNA homology and functional activity with SLT-I (5, 20, 28, 35). This cytotoxin has been designated Shiga-like toxin type II (SLT-II) (35) or Vero cell toxin type 2 (VT2) (S. M. Scotland, H. R. Smith, and B. Rowe, Lancet ii:885–886, 1985).

Production of SLT-I and SLT-II in some *E. coli* strains is associated with lysogenic toxin-converting bacteriophages (29, 32, 35; H. R. Smith, N. P. Day, S. M. Scotland, R. J. Gross, and B. Rowe, Lancet i:1242–1243, 1984; S. M. Scotland, H. R. Smith, G. A. Willshaw, and B. Rowe, Lancet ii:216, 1983). In the hemorrhagic colitis-associated *E. coli* O157:H7 strain 933, both toxin types are produced (29). Two bacteriophages, 933J and 933W, control expression of the SLT-I and SLT-II cytotoxins, respectively (35). In contrast, other *E. coli* isolates have been described which produce only SLT-I or SLT-II (20). Previous cloning (23) and sequence analysis (15) of the structural genes for SLT-I identified two subunits: an A subunit and a smaller B subunit with predicted molecular weights for the mature proteins of 32,211 and 7,690. The subunit structural genes, *slt*-I A and *slt*-I B, are on a single transcriptional unit with the A subunit gene preceding the B subunit gene and with each subunit apparently possessing its own putative ribosome-binding site and signal peptides (15, 23). The 933J SLT-I genes appear to be identical to the recently described SLT-I structural genes cloned from the closely related bacteriophage H19B isolated from the enteropathogenic *E. coli* H19 (14).

Although the cytotoxicity of sonic lysates from bacteria encoding only SLT-II is significantly less than that of strains synthesizing only SLT-I, the production of either toxin alone has been associated with pathogenesis. An important question with respect to the pathogenicity of these *E. coli* isolates remains, that is, whether the SLT-I and SLT-II toxins represent the same potential pathogenic mechanism or two distinct mechanisms mediated by two different toxins. The purpose of this investigation was to begin to address this question by isolating and characterizing the genes for SLT-II production from phage 933W and then comparing their genetic organization and gene products with those of the SLT-I genes previously described for phage 933J.

MATERIALS AND METHODS

Bacterial strains, bacteriophage, and plasmids. The toxin-converting phage 933W (29, 35) used in this study was obtained from a mitomycin C-induced phage lysate of the hemorrhagic colitis-associated *E. coli* O157:H7 strain 933 (29). *E. coli* 395-1(933W) was constructed by lysogenizing *E. coli* 395-1 (13) with phage 933W. *E. coli* C600 (1) and *S. dysenteriae* type 1 strain 60R (27) have been described previously. *E. coli* HB101 (3) was used as the recipient in transformation and as the host for the recombinant plasmids. The *E. coli* K-12 minicell-producing strain was DS410 (8). Plasmid vectors pUC18 (24) and pBR328 (33) were used for molecular cloning. Recombinant plasmid pJN25, an SLT-I-positive clone derived from phage 933J, has been described previously (23).

Medium and culture conditions. Luria-Bertani (LB) medium (21) with the NaCl reduced to 5 g/liter, with or without

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1.5% Bacto-Agar (Difco Laboratories, Detroit, Mich.), was used as the nutrient medium. Antibiotics (Sigma Chemical Co., St. Louis, Mo.) were used at the following concentrations: ampicillin, 200 μg/ml; tetracycline, 15 μg/ml; and chloramphenicol, 30 μg/ml.

Cloning and subcloning. A high-titered lysate of phage 933W was prepared by using 1 μg of mitomycin C (Sigma) per ml for induction (10) of the 395-1(933W) lysogen. The phage was purified by centrifugation in a 3 M over 5 M CsCl step gradient, and the DNA was extracted from the phage with formamide (7), followed by precipitation in ethanol. *Eco*RI-digested phage DNA (1 μg) was ligated (T4 ligase; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) to *Eco*RI-cleaved pBR328 plasmid DNA (0.5 μg). During subcloning, sequential digestions with endonucleases requiring different buffers were performed, using the low-salt buffer first (18). Endonucleases were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md; International Biotechnologies, Inc., New Haven, Conn.; and New England BioLabs, Inc., Beverly, Mass. Plasmid DNA was prepared by a minilysate procedure adapted from that of Birnboim and Doly (2). When necessary, plasmid DNA was further purified on CsCl-ethidium bromide equilibrium gradients (7). Isolation of specific DNA fragments was performed with an electrophoretic sample concentrator (model 1750; ISCO, Inc., Lincoln, Nebr.) following the instructions of the manufacturer. Transformation of *E. coli* strains was done by calcium shock (6). Initial cloning experiments were done under BL3+EK1 containment (8a).

Cytotoxicity and neutralization assays. *E. coli* HB101 (pNN76), *E. coli* C600(933W), and *S. dysenteriae* type 1 strain 60R were grown overnight at 37°C with shaking in iron-depleted glucose syncase broth (28). The cultures were then diluted to an A_{600} of 0.03 in 50 ml of medium and further incubated for 24 h. The bacteria were harvested by centrifugation, the culture supernatants were collected, and cell lysates were prepared by sonic disruption by published procedures (25) with the exception that the bacteria were suspended in 3 ml of buffer before sonication. Cytotoxicity for HeLa cells was measured by the method of Gentry and Dalrymple (11). Vero cells were also used in the cytotoxicity assay (35). Sonic lysates were tested for neutralizable cytotoxin with rabbit antisera against crude phage 933W SLT-II (35), purified Shiga toxin (27), or a combination of both antisera (35). The protein content of the sonic lysates was determined by the method of Lowry as modified by Markwell et al. (19). Bovine serum albumin fraction V (Sigma) was used as the protein standard.

Nick translation, hybridization, and autoradiography. The DNA fragments used as probes were labeled by nick translation with α-^{32}P-labeled 5′-dCTP with reagents supplied by New England Nuclear Research Products, Boston, Mass. Preliminary screening for SLT-II-producing clones was done by in situ hybridization on no. 541 filter paper (Whatman, Inc., Clifton, N.J.) following the method of Rubin et al. (31). The *Nco*I-*Eco*RV restriction fragment of pJN25 (23) was used as the DNA probe. Southern hybridization with the SLT-II gene fragment between restriction sites *Pvu*II and *Eco*RV (this study) was performed as previously described (34). In both the colony blot and Southern hybridization procedures, the filters were incubated for 18 to 24 h in a hybridization solution containing labeled probe, 6× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate), 5× Denhardt solution (18), 0.25% sodium dodecyl sulfate, and 100 μg of sheared denatured salmon sperm DNA per ml. The colony blot hybridization was incubated at 60°C, and the Southern hybridization was incubated at 52°C. Filters were then washed twice in 6× SSC at 45°C, rinsed once in 1× SSC at room temperature, allowed to dry, and exposed to X-ray film (Eastman Kodak Co., Rochester, N.Y.) in the presence of intensifying screens. For conditions of high stringency, the hybridization solutions were incubated at 68°C overnight and the filters were washed in 0.1× SSC at 62°C.

Analysis of plasmid-encoded proteins in minicells. Plasmids containing selected subclones of the original 4.9-kilobase (kb) *Eco*RI fragment isolated in pNN76 were analyzed with *E. coli* K-12 minicells. Plasmid-encoded proteins were labeled with [^{35}S]methionine in Tricine buffer (New England Nuclear Research Products) as previously described (22) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (17) and autoradiography (7). Polymyxin B extraction (12) of the labeled proteins present in the periplasm was done immediately after radiolabeling of the minicells. A fivefold concentration of the labeled minicells was suspended in M9-methionine assay medium (9, 21) supplemented with 0.4 mg of polymyxin B per ml. The cells were then further incubated at 37°C for 30 min, and the supernatants were recovered after centrifugation to remove the cells.

RESULTS AND DISCUSSION

To isolate the genes controlling production of SLT-II from phage 933W, *Eco*RI fragments of phage 933W were cloned into the plasmid vector pBR328 and transformed into *E. coli* HB101. Digestion of phage 933W DNA produces approximately 12 *Eco*RI fragments. A set of 90 randomly selected transformants were tested by DNA colony blot hybridization to identify those recombinant clones containing phage 933W DNA fragments homologous to the SLT-I gene of phage 933J. The presence of homology between the SLT-I and SLT-II genes had been suggested in earlier studies (35, 37). The probe for this hybridization screening was the *Nco*I-*Eco*RV fragment of plasmid pJN25 (23). This 3.1-kb DNA fragment contains a complete and functional copy of the SLT-I structural genes and the adjacent phage 933J DNA sequences. Only 2 of the 90 transformants tested hybridized with the *Nco*I-*Eco*RV DNA probe. All 90 transformants were tested for elevated toxin production. Only the two clones reacting with the SLT-I gene-containing *Nco*I-*Eco*RV fragment produced elevated levels of cytotoxic activity. These two clones contained the same 4.9-kb *Eco*RI DNA fragment. One toxin-producing recombinant plasmid, designated pNN76, was chosen for further study.

Total amounts of cytotoxic activity produced by *E. coli* HB101(pNN76) were compared with the levels made by the toxin reference strain *S. dysenteriae* 60R (8a) and by an *E. coli* K-12 lysogen of phage 933W (Table 1). Strain HB101(pNN76) produced a sevenfold-greater level of cytotoxic activity than that of the phage 933W lysogen. A similar sevenfold increase in toxin activity relative to that of the corresponding phage lysogen was previously observed during the cloning of the SLT-I structural genes from phage 933J (23). This increase is consistent with an increase in toxin gene copy number caused by the use of a multicopy cloning vector. Neutralization studies (Table 1) confirmed that the cytotoxic activity associated with recombinant plasmid pNN76 was that of SLT-II.

A restriction map of the 4.9-kb *Eco*RI fragment was determined (Fig. 1). To define more precisely within this fragment the location of the nucleotide sequences associated with SLT-II activity, we constructed a series of subclones

TABLE 1. Production of SLT-II by *E. coli* HB101(pNN76)

Strain	Total cytotoxin produced (CD_{50}/ml)[a]	Neutralization of cytotoxicity for HeLa cells[b]		
		Rabbit anti-Shiga	Rabbit anti-SLT-II	Anti-Shiga + anti-SLT-II
E. coli HB101(pNN76)	1.1×10^4	−	+	+
E. coli C600(933W)	1.6×10^3	−	+	+
S. dysenteriae 1 60R	1.2×10^6	+	−	+

[a] Values are the average of four independent samples per group. Total cytotoxic activity was the sum of the activity present in both the culture supernatants and sonic lysates. Protein concentrations of sonic lysates were comparable, ensuring that differences in levels of cytotoxicity were not due to differences in bacterial growth or efficiency of sonication. CD_{50}, 50% cytotoxic dose.

[b] +, Neutralization; −, lack of neutralization. No increase in neturalization was observed when a mixture of both antisera was used.

using the cloning vector pUC18. These subclones and their cytotoxic activity are depicted in Fig. 1. Only subclones containing the contiguous segment of DNA between the *Kpn*I and *Sph*I restriction sites were positive for elevated levels of toxin activity. Attempts to reduce the *Kpn*I-*Sph*I insert to *Pst*I-*Sph*I (see pNN105, Fig. 1) or to *Kpn*I-*Eco*RV (see pNN106, Fig. 1) resulted in the formation of toxin-negative subclones. These findings suggested that the interval between the *Pst*I and the *Eco*RV sites represents a region internal to the complete DNA sequence controlling SLT-II synthesis.

Next, using *E. coli* minicells, we characterized the products from SLT-II-related DNA sequences and compared these products with those of the SLT-I structural genes. In addition to comparing polypeptide sizes, we wished to determine whether processing of the subunits could be detected. Analysis of the DNA sequence of SLT-I led to a prediction that both A and B subunits contain amino acid leader sequences (15). These should be removed during transport to the periplasm. A comparison between the sizes of the subunits present in polymyxin B-derived periplasmic extracts with those present in whole-cell lysates of SLT-

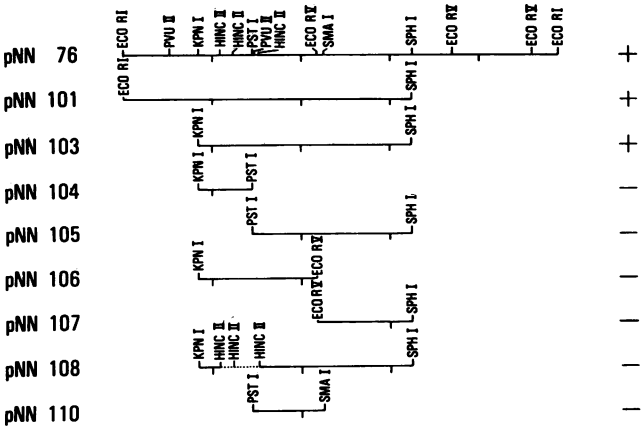

FIG. 1. Restriction endonuclease cleavage maps of the SLT-II-encoding 4.9-kb *Eco*RI DNA fragment of phage 933W and its different subclones. Designations for the recombinant plasmids are indicated in the left column. Symbols for cytotoxic activity are in the right column: +, toxin positive; −, toxin negative. The dotted line present in plasmid pNN108 represents a deletion of the DNA from this portion of the subcloned fragment. One-kilobase increments are indicated below each map.

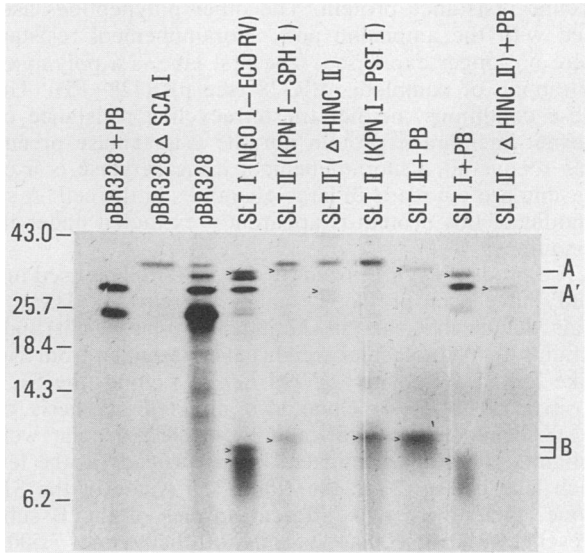

FIG. 2. Autoradiograph of the [^{35}S]methionine-labeled polypeptides synthesized in *E. coli* DS410 minicells containing selected subclones of the 4.9-kb *Eco*RI fragment of pNN76. Each sample loaded onto the gel contained approximately 5×10^5 cpm. Toxin phenotypes of the subcloned fragments are shown in Fig. 1. Vector controls are present in lanes 1 to 3. Cell-free samples obtained by polymyxin B extraction are labeled PB. Numbers on the left indicate molecular masses in kilodaltons of polypeptide standards. Arrowheads at the left of the lanes point to polypeptides encoded by cloned fragments. Toxin subunit designations are labeled on the right of the figure.

producing minicells would enable us to verify this prediction. Any similarities in the properties of the SLT-I and SLT-II products would further help to define the relationship between the two toxins and provide evidence that phage 933W encodes the structural genes for SLT-II.

For these experiments, *E. coli* minicells were prepared containing either SLT-II subclone pNN103, pNN104, or pNN108 or SLT-I clone pJN25 (23). Before conducting the minicell analysis, we wanted to eliminate expression of those vector-encoded gene products with molecular weights similar to that of the A subunit of SLT-I. To achieve this, we recloned the insertions from each of the three SLT-II-related plasmids as *Eco*RI-*Pvu*I fragments from pUC18 into pBR328. By using this combination of restriction sites, both the chloramphenicol and the ampicillin resistance genes of the pBR328 vector were insertionally inactivated. These constructions eliminated the plasmid-encoded gene products associated with these antibiotic resistances from appearing in the minicell analysis and more clearly demonstrated the polypeptides encoded by the cloned DNA fragments. To provide an appropriate vector control in which only tetracycline resistance was expressed, we deleted the *Sca*I fragment of pBR328 (33).

The analysis of plasmid-encoded proteins synthesized by minicells is shown in Fig. 2. Each lane in Fig. 2 is labeled with the corresponding plasmid or cloned fragment which directed protein synthesis in the minicell sample. When done, treatment of a sample with polymyxin B is also indicated (+PB). The complete pBR328 vector produced four labeled bands: tetracycline resistance protein, β-lactamase precursor, mature β-lactamase, and chloramphenicol acetyltransferase. The pBR328 vector containing the *Sca*I deletion (see pBR328Δ*Sca*I) produced only the tetra-

cycline resistance protein. The other polypeptides associated with the ampicillin and chloramphenicol resistances were no longer expressed. The first lane is a polymyxin B extraction of complete pBR328 (see pBR328+PB). Under these conditions, neither the tetracycline resistance cytoplasmic membrane protein nor the β-lactamase precursor was recovered. Chloramphenicol transacetylase is a cytoplasmic protein made in large quantities in the cell. A small amount of this protein is apparently extracted under these conditions.

The products synthesized by the SLT-I clone used in this study have been previously characterized (23). These proteins include an A subunit (32,500 molecular weight) and a B subunit (9,400 molecular weight) as determined from Fig. 2. Like the SLT-I-positive (*Nco*I-*Eco*RV) clone, the SLT-II-positive (*Kpn*I-*Sph*I) clone also directed synthesis of A (33,000 molecular weight) and B (9,800 molecular weight) subunits. These are indicated by arrowheads at the left of each lane in Fig. 2. In the whole-cell lysate of the SLT-I clone, there were two different species of the B subunit present with molecular weights of 9,400 and 7,500. In contrast, only one SLT-II B-subunit polypeptide was seen. Based on these molecular weight estimates, the SLT-II A and B subunits appear slightly larger than the corresponding SLT-I subunits. Thus, the cloned SLT-II sequences direct synthesis of two polypeptides similar but not identical to the A and B subunits of SLT-I, indicating that phage 933W encodes the structural genes for SLT-II.

E. coli(933J) lysogens are approximately 1,000-fold more cytotoxic than *E. coli*(933W) lysogens (35). Because it was possible to visualize the subunits of both SLT-I and SLT-II genes by using identical growth conditions during the minicell analysis, it is unlikely the SLT-II polypeptides were synthesized at an amount 1,000-fold less than those of SLT-I. If such were the case, the SLT-II subunits would not be visible during this analysis, even with the multicopy cloning vehicle contributing to increased toxin expression. This result suggests that quantitative differences between the amounts of SLT-I and SLT-II synthesized do not entirely explain the large differences between levels of cytotoxic activity in whole-cell lysates tested against HeLa cells. Therefore, qualitative differences between the two toxins significantly affect their relative cytotoxicity. One factor distinguishing the toxins may involve processing of the subunits.

In an effort to detect processing of the toxin subunits, we analyzed polymyxin B extractions of the toxin-positive SLT-I and SLT-II plasmids (Fig. 2). Because the experimentally determined molecular weights for the mature and precursor forms of the toxin subunits were in close agreement with those predicted from the DNA sequence of SLT-I (15), we interpret the gel data for the SLT-I samples as follows: (i) compared with the A precursor (32,500 molecular weight), the processed A subunit (31,500 molecular weight) migrates where the β-lactamase precursor of pBR328 normally would be located; (ii) in the periplasmic extract of the SLT-I clone, the missing upper B subunit (9,400 molecular weight) is the unprocessed precursor form of the B subunit (7,500 molecular weight). Unlike the results for SLT-I, the comparison between the sizes of the SLT-II subunits from whole-cell lysates with those present in the polymyxin B extracts failed to detect evidence of processing of the SLT-II subunits. Because the SLT-II polypeptides seen in polymyxin B extracts are apparently the same size as those seen in the whole-cell lysates, it is unclear whether only the mature or only the precursor forms of the subunits are being detected.

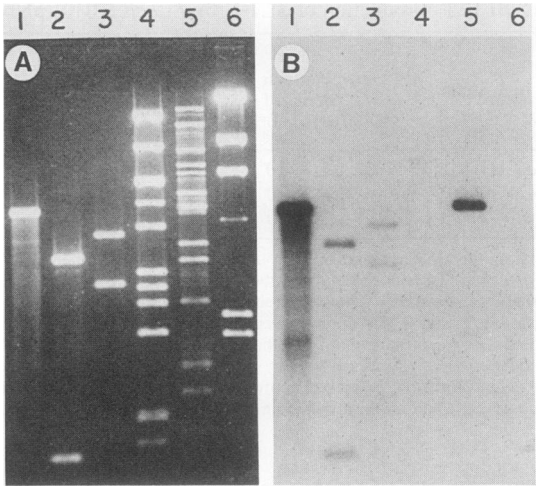

FIG. 3. (A) Agarose gel electrophoresis (0.7%, wt/vol) of restriction endonuclease-digested recombinant plasmids and bacteriophages. Lanes: 1, *Eco*RI-digested pNN76; 2, *Pvu*II- and *Eco*RV-digested pNN112, the clone of the probe; 3, *Nco*I- and *Eco*RV-digested pJN25; 4, *Eco*RI-digested phage 933J; 5, *Eco*RI-digested phage 933W; 6, *Hin*dIII-digested lambda. (B) Autoradiograph of the ^{32}P-labeled *Pvu*II-*Eco*RV DNA fragment from the SLT-II toxin genes hybridized to the Southern transfer of the gel in panel A.

To determine the effect of specific DNA deletions on toxin protein synthesis, we examined the properties of two SLT-II toxin-negative subclones. Plasmid SLT-II (*Hin*cII) has an approximately 400-base-pair *Hin*cII deletion. This deletion has eliminated expression of the B subunit and has reduced the size of the A subunit from a 32,000- to a 28,000-dalton truncated (A′) polypeptide. Plasmid SLT-II (*Kpn*I-*Pst*I) has retained expression of the B subunit while eliminating expression of the A subunit. Based on the polypeptide expression of these subcloned fragments and on their corresponding toxin phenotypes, an approximate location of the SLT-II subunit genes is indicated at the top of Fig. 1. In this arrangement, the A- and B-subunit genes are adjacent. The behavior of the plasmid containing the *Hin*cII deletion suggests that transcription of A proceeds from right to left in Fig. 1. This proposed genetic organization for SLT-II is the same as that previously described for SLT-I (14, 15, 23).

Finally, a series of DNA-DNA hybridization reactions were conducted to further examine the relationship between the structural genes of SLT-II and those of SLT-I. These reactions were also performed to evaluate the efficacy of using internal toxin gene fragments as generic SLT gene probes. For these purposes, the 750-base-pair *Pvu*II-*Eco*RV fragment that is internal to the coding sequence for the A subunit of SLT-II was used to probe Southern blots of DNA samples (Fig. 3A) associated with the production of SLT-I or SLT-II. Under conditions requiring greater than 90% homology (data not shown), the SLT-II fragment reacted only to fragments containing itself (the lower band in lane 2 and the 4.9-kb *Eco*RI fragment of phage 933W in lanes 1 and 5 of Fig. 3A). Because of the similarity in sizes between the pBR328 vector fragment and the SLT-II-positive *Eco*RI fragment of pNN76, only a single band appears in the *Eco*RI digestion in Fig. 3A, lane 1. When conditions of reduced stringency requiring greater than 50% homology were used (Fig. 3B), the probe additionally hybridized to the SLT-I-encoding fragments of pJN25 (*Nco*I-*Eco*RV) and of phage 933J (8.2-kb *Eco*RI fragment) (23). A similar requirement for conditions

of low stringency to detect homology between VT1 sequences and previously uncharacterized VT2-associated sequences present in a 4.7-kb *Eco*RI fragment of phage E32511 had been reported by Willshaw et al. (37). A more recent analysis of these VT2-encoding sequences indicates that they have a restriction map similar to that presented in Fig. 1 (36).

The SLT-II probe was specific because it failed to hybridize with the non-toxin-related restriction fragments of phages 933J, 933W, and lambda (Fig. 3B, lanes 4, 5, and 6, respectively). The additional reactions observed in lanes 1 and 5 are with a 2-kb portion of the toxin-associated fragment which is generated during digestion with excess amounts of *Eco*RI. Under conditions of low stringency, however, the SLT-II probe also reacted unexpectedly with the pBR328 fragments (Fig. 3B, the upper bands in lanes 2 and 3 plus one of the 4.9-kb bands of lane 1). Further investigation showed that both SLT-I- and SLT-II-coding sequences hybridized to sequences flanking the ampicillin resistance gene of pBR328. A comparison of SLT-I (15) and pBR328 (18) sequences revealed no primary sequence relationship between them. Instead, this comparison suggested that multiple copies of poly(dA-dT) regions are responsible for this cross hybridization reaction at reduced stringency. Although the results of our additional investigations indicate that internal DNA fragments from either SLT-I or SLT-II may not be used with confidence as generic SLT probes at low stringency, the fragments do serve as specific probes under conditions of high stringency. Because of the similarities in their genetic organization and subunit sizes, it is doubtful the hybridization observed between SLT-I and SLT-II represents the type of reaction that occurs with pBR328. Our ability to isolate the two SLT-II-producing clones from a set of 90 randomly selected transformants further supports this interpretation. Instead, these hybridizations most likely reflect the true degree of homology between SLT-I and SLT-II, estimated to be 50 to 60% (18). Therefore, the studies presented here further support the conclusion that SLT-I and SLT-II are related toxins that have undergone considerable divergence.

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