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# Localization of *stx*, a Determinant Essential for High-Level Production of Shiga Toxin by *Shigella dysenteriae* Serotype 1, near *pyrF* and Generation of *stx* Transposon Mutants

TSUTOMU SEKIZAKI,† SHIGEAKI HARAYAMA, G. M. BRAZIL, AND KENNETH N. TIMMIS*

*Department of Medical Biochemistry, University of Geneva, CH-1211 Geneva 4, Switzerland*

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**Hfr strains of *Shigella dysenteriae* serotype 1 were constructed by transient integration of an RP4 plasmid derivative carrying transposon Tn*501* into the *Shigella* chromosome through Tn*501*-mediated cointegration. The Hfr strains were mated with *Escherichia coli* K-12 recipients carrying various auxotrophic markers, and *E. coli* recombinants which had received prototrophic *Shigella* genes were selected. Some of the *E. coli* transconjugants produced high levels of a cytotoxin which was neutralized by both polyclonal and monoclonal anti-Shiga toxin sera. The determinant for Shiga toxin production, designated *stx*, was first transferred to *E. coli* K-12 and then mapped by Hfr crosses to the *trp-pyrF* region located at 30 min on the *E. coli* chromosome. Bacteriophage P1-mediated transduction analysis of *stx* gave the following gene order: *trp-pyrF-stx*. The level of Shiga toxin production in *E. coli* Stx$^+$ transconjugants and transductants was as high as that of the parental *S. dysenteriae* 1 strain. Stx$^-$ mutants of an Stx$^+$ *E. coli* transductant were generated by random in vivo insertion mutagenesis with a Tn*10* derivative transposon, Tn-mini-*kan*, followed by P1 cotransduction of the kanamycin resistance and PyrF$^+$ markers into a *pyrF Stx*$^+$ *E. coli* K-12 recipient. One *stx*::Tn-mini-*kan* transposon mutation was transferred by P1 transduction from this *E. coli* Stx$^-$ mutant to an *E. coli* K-12 Hfr strain and in turn transferred by conjugation to the original *S. dysenteriae* 1 strain plus two others. All kanamycin-resistant recombinants of *S. dysenteriae* 1 had lost their ability to produce high levels of Shiga toxin. A gene that specifies high-level Shiga toxin production is thus located near *pyrF* on the chromosome of *S. dysenteriae* 1. Stx$^-$ mutants of *S. dysenteriae* 1 exhibited full virulence in the Serény test.**

*Shigella* species and enteroinvasive strains of *Escherichia coli* cause bacillary dysentery, an acute but generally self-limiting invasive disease of humans and subhuman primates. The course of a typical infection involves (i) temporary colonization of the small bowel by the pathogen, which may result in mild abdominal cramps, fever, and a watery diarrhea; (ii) extensive bacterial colonization of the large bowel and invasion of the colonic epithelium; (iii) intracellular multiplication of bacteria, killing of invaded cells, and spread of bacteria to adjacent cells in the mucosa and submucosa; and (iv) initiation of an acute inflammatory response which results initially in further ulceration of the mucosa but which impedes spread of the infection and permits initiation of bowel healing. Acute abdominal pain and the frequent passage of bloody mucoid stools are characteristic of the colonic phase of the disease.

Although cases of bacillary dysentery are generally mild and of infrequent occurrence in developed countries, they can be epidemic and serious in countries where hygiene and nutrition are inadequate and other infections are prevalent. In such conditions, dysentery can be characterized by high mortality rates as a result of its increased severity and because of the development of complications such as hemolytic-uremic syndrome, leukemoid reactions, and sepsis, particularly in infants.

The Shiga bacillus, *Shigella dysenteriae* serotype 1, produces the most severe form of dysentery and is associated with the highest incidence of serious complications. Mortality rates in hospitalized cases of *S. dysenteriae* 1 infections can exceed 10%, despite treatment by recommended methods. At present, a number of developing countries are experiencing a pandemic of *S. dysenteriae* 1 shigellosis which encompasses Central America, much of central Africa, and countries of and adjacent to the Indian subcontinent.

Several bacterial components have been implicated as virulence factors, including outer membrane proteins that may mediate invasion of epithelial cells, O-antigen lipopolysaccharide, an aerobactin iron uptake system, and a cell-bound contact hemolysin. In addition, some *Shigella* and *E. coli* strains produce a powerful cytotoxin, Shiga toxin, that inhibits protein synthesis in susceptible eucaryotic cells, causes fluid secretion in the small bowel of rabbits, i.e., is enterotoxic, and is lethal for various laboratory animals when injected parenterally. Because Shiga toxin has enterotoxic activity, it may be responsible for the diarrheal symptoms of the early phases of some shigellosis infections. Shiga bacillus produces extremely high levels of Shiga toxin, as much as $10^5$-fold more than do other shigellae, and it has been suggested that this may be the reason for the higher virulence of this serotype and the higher incidence of complications such as hemolytic-uremic syndrome associated with infections by this organism (for discussions, see references 2 and 9). However, an essential role for Shiga toxin in *Shigella* virulence has not been established.

The principal difficulty in evaluating the biological role of Shiga toxin is the fact that well-characterized Shiga toxin-negative *Shigella* mutants have not been available. Previous

* Corresponding author.
† Present address: Poultry Disease Laboratory, National Institute of Animal Health, Seki, Gifu 501-32, Japan.

TABLE 1. Bacterial strains

| Strain | Relevant properties | Source or reference |
|---|---|---|
| *S. dysenteriae* 1 | | |
| AA17855 | Isolated from a patient in Bangladesh; $Cm^r$ $Sm^r$ $Tc^r$ | B. Y. Kay |
| TS51 | Temperature-resistant $Km^r$ derivative of AA17855(pMT999) | This study |
| TS52 | Temperature-resistant $Km^r$ derivative of AA17855(pMT999) | This study |
| TS59 | Temperature-resistant $Km^r$ derivative of AA17855(pMT999) | This study |
| 31-81 | $Cm^r$ $Sm^r$ $Tc^r$ | P. J. Sansonetti |
| 11-81 | $Cm^r$ $Tc^r$ $Ap^r$ | P. J. Sansonetti |
| *E. coli* K-12 | | |
| KL208 | Hfr *relA1 lac-42* | 5 |
| KL99 | Hfr *thi-1 relA1 lac-42* | 5 |
| TS50 | $F^-$ *ara leu lac tsx purE gal trp his argG rpsL xyl mtl ilv met* (*metA* or *metB*) *thi gyrA* | Spontaneous $Nal^r$ derivative of CSH57b (6) |
| TS61 | $F^-$ Δ(*lac-pro*) *supF trp pyrF his rpsL thi* | CSH28 cured of F *lac pro* |
| TS67 | As for TS61, but *gyrA* | Spontaneous $Nal^r$ derivative of TS61 (7) |
| *E. coli-S. dysenteriae* 1 hybrids | | |
| TS55 | $Gal^+$ $Stx^+$ $Fla^-$ TS50 transconjugant | Mating between TS52 and TS50 |
| TS60 | $Fla^-$ $Trp^+$ $Stx^+$ TS50 transconjugant | Mating between TS52 and TS50 |
| TS64 | $Trp^+$ $Stx^+$ TS50 transductant | P1 transduction from TS60 to TS50 |
| TS65 | $PyrF^+$ $Stx^+$ TS61 transductant | P1 transduction from TS60 to TS61 |
| TS68 | $Trp^+$ TS61 transductant | P1 transduction from TS60 to TS61 |
| TS70 | $Trp^+$ $Stx^+$ *pyrF* TS65 transductant | P1 transduction from TS68 to TS65 |
| TS73 | $Trp^+$ $PyrF^+$ *stx-1*::Tn-mini-*kan* TS70 transductant | P1 transduction from Tn-mini-*kan* mutagenized TS64 to TS70 |
| TS76 | KL99 Hfr *thi-1 relA1 lac-42 stx-1*::Tn-mini-*kan* | P1 transduction from TS73 to KL99 |
| TS77 | KL208 Hfr *relA1 lac-42 stx-1*::Tn-mini-*kan* | P1 transduction from TS73 to KL208 |

attempts to isolate either spontaneous Shiga toxin-negative mutants (4, 9, 12) or transposon insertion mutants (K. Gamon and K. N. Timmis, unpublished data) failed. A rational strategy for generating such mutants would involve cloning the toxin gene, generation in vitro of deletion mutant derivatives, and substitution, by homologous recombination, of the deletion mutant gene for the normal gene present in a virulent isolate. However, the first step in this strategy was effectively prohibited for several years by successive rulings of a subsection of the U.S. Recombinant DNA Advisory Committee. We therefore took an in vivo approach to generate toxin-defective *Shigella* mutants. In this report, we describe the mapping of a gene that is essential for high-level Shiga toxin production, the generation of transposon Tn*10*-type mutant derivatives of this gene, the introduction of one mutant gene into several *S. dysenteriae* 1 isolates, and the testing of the virulence of the derivatives obtained.

## MATERIALS AND METHODS

**Bacterial strains, bacteriophage, and plasmid.** The bacterial strains used in this study are listed in Table 1. Plasmid pMT999, constructed by M. Tsuda, is a Tn*501*-containing derivative of pTH10 (6), a temperature-sensitive replication mutant of plasmid RP4. It confers resistance to kanamycin, tetracycline, ampicillin, and $HgCl_2$ on host bacteria grown at 30°C but not those grown at 42°C. Bacteriophage P1 *vir* (7) was used for transduction.

**Media.** Luria broth and antibiotic medium no. 3 (Difco Laboratories) were used as complete media, and M9 minimal salts medium containing a carbon source and appropriate supplements was used as minimal medium (3, 7). The concentrations of sugars, amino acids, and vitamins in minimal media were 0.2% (wt/vol), $10^{-3}$ M, and $10^{-5}$ M, respectively (3). The antibiotics added to selective media, at 25 μg/ml, were kanamycin, streptomycin, chloramphenicol, and nalidixic acid. T-broth soft agar (10 g of tryptone (Difco) per liter, 5 g of NaCl per liter, 0.3% agar) was used to test for bacterial motility (5).

**Construction of *Shigella* Hfrs.** The pMT999 plasmid was first introduced from *E. coli* K-12 into the *S. dysenteriae* serotype 1 strain AA17855 by a membrane filter mating (6) at 30°C. *S. dysenteriae* transconjugants exhibited temperature-dependent drug resistance. Temperature-resistant, kanamycin-resistant derivatives were isolated from 100 transconjugants after plating at 42°C; such clones probably carried the pMT999 plasmid integrated into the host chromosome (6, 18). Their ability to conjugally transfer *S. dysenteriae* 1 chromosomal markers to *E. coli* and the approximate locations of the origins of chromosomal transfer were determined as described previously (6). Most temperature-resistant, kanamycin-resistant *S. dysenteriae* transconjugants transferred chromosomal markers and were thus Hfr derivatives.

**Conjugal transfers.** Genetic crosses between *Shigella* Hfr donors and nalidixic acid-resistant *E. coli* recipients were performed by membrane filter mating at 37°C for 4 h. The membrane filters were subsequently suspended in 10 mM $MgSO_4$, and dilutions of bacteria washed from the filters by vortexing were plated on M9 plates containing nalidixic acid and appropriate supplements to select for transconjugants. Genetic crosses of *E. coli* F plasmid-based Hfrs with *E. coli* or *Shigella* recipients were performed in Luria broth at 37°C.

TABLE 2. Transfer properties of *S. dysenteriae* Hfr strains

| Donor | No. of transconjugants obtained/$10^6$ donor cells[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | PurE$^+$ | Gal$^+$ | Trp$^+$ | His$^+$ | ArgG$^+$ | Ilv$^+$ | Met$^+$ | Leu$^+$ |
| TS51 | 1 | 14 | 10 | 3 | 730 | 74,000 | 30 | 1 |
| TS52 | 10 | 290 | 530 | 27 | 210 | 170 | 5 | 4 |
| TS59 | 1,300 | 650 | 8 | 3 | 8 | 3 | 4 | 0.1 |

[a] Filter matings were carried out at 37°C for 4 h with TS50 as the recipient.

The matings were interrupted either 10 or 60 min after the donor and recipient strains were mixed (7). All transconjugants were purified by two single-colony isolations.

**P1 transduction.** P1 transduction was performed with P1 *vir* as described previously at a multiplicity of infection of 0.1 (5).

**Transposon mutagenesis using a Tn*10* derivative.** Transposon mutagenesis of *E. coli* using the Tn*10* derivative Tn-mini-*kan* was carried out as described by Way et al. with the lambda suicide vector NK1105 (20).

**Cytotoxin assay.** Bacterial cells were grown in Luria broth to the stationary growth phase, and culture supernatant fluids were assayed for cytotoxin by a standard procedure with HeLa cells (17).

**Neutralization of cytotoxin by antisera.** The procedure used for neutralization of cytotoxin by antisera was essentially that described by O'Brien et al. (12). Polyclonal anti-Shiga toxin antibodies and monoclonal anti-Shiga-like toxin antibodies (13C4) (16) were generously provided by A. O'Brien. Twofold serial dilutions of serum were mixed with 10 50% cytotoxic doses ($CD_{50}$) of cytotoxin, and the mixtures were incubated at 37°C for 1 h and then at 4°C for 12 h. Samples (40 μl) of the mixtures were then assayed for their cytotoxicity to HeLa cells.

**Virulence assay.** The virulence of *Shigella* strains was assayed by the Serény test (14) and by invasiveness for HeLa cells, as described previously (19).

## RESULTS

**Localization by Hfr crosses of a determinant essential for Shiga toxin production in *E. coli* K-12.** Derivatives of the *S. dysenteriae* serotype 1 strain AA17855 able to transfer chromosomal genes by conjugation were constructed by selection of temperature-resistant clones carrying the Tn*501*-containing RP4 plasmid pMT999, which exhibits temperature-sensitive replication. Most clones could transfer chromosomal markers to *E. coli* recipients and were therefore presumed to have arisen by plasmid integration into the host chromosome. Three *S. dysenteriae* Hfr strains, TS51, TS52, and TS59, were mated with the *E. coli* Rec$^+$ recipient TS50, and *E. coli* transconjugants which had acquired individual *Shigella* chromosomal markers were selected (Table 2). The data are best interpreted as indicating that the points of origin of transfer in *S. dysenteriae* TS51, TS52, and TS59 reside near *ilv*, *trp*, and *purE*, respectively, and that transfer is bidirectional (Fig. 1). For unknown reasons, the numbers of His$^+$ recombinants in these heterologous matings were lower than those expected from the gradient of transmission of other markers.

Previous experiments have suggested that the Shiga toxin gene is located in the *mtl-argE* region of the chromosome of *Shigella flexneri* (13) and near *argE* (17) and *asnA* (and the lysine decarboxylase determinant; K. Gamon and T. Chakraborty, unpublished data) on the chromosome of *S. dysenteriae* 1. Therefore, *E. coli* transconjugants which had acquired *S. dysenteriae* 1 chromosomal markers from this region, i.e., *argG*$^+$, *ilv*$^+$, and *met*$^+$, were selected from a cross of *S. dysenteriae* TS51 with *E. coli* TS50, and 38 Arg$^+$ Ilv$^+$, 96 Ilv$^+$, 18 Met$^+$ Ilv$^+$, 12 Leu$^+$ Ilv$^+$, and 3 Trp$^+$ Ilv$^+$ transconjugants were examined for their production of cytotoxin. Some Ilv$^+$ and Met$^+$ transconjugants expressed cytotoxic activity at low levels (less than 10 $CD_{50}$ per ml of culture supernatant), but the distinction between weak activity and background was small and precluded further analysis of such clones.

Since the ability to synthesize Shiga toxin at levels typical of the donor *Shigella* strain was not cotransferred to *E. coli* K-12 with the *argE* region, its possible cotransfer with other chromosomal markers was examined. Matings were carried out with *Shigella* Hfr strains TS52 and TS59 as the donors, which probably have different origins of chromosome transfer (Fig. 1), and *E. coli* TS50 as the recipient, and PurE$^+$, Gal$^+$, Trp$^+$, or His$^+$ transconjugants were selected. Coinheritance of unselected markers was subsequently examined. *E. coli* cells are motile, and the genes responsible for flagellum synthesis (*fla*) are clustered in three regions, one between *gal* and *trp* and two between *trp* and *his*. *Shigella* strains are nonmotile. Therefore, replacement of *E. coli fla* regions of the chromosome by corresponding regions of the *Shigella* chromosome will probably result in nonmotile *E. coli*. Thus, in addition to cytotoxin production, the Fla phenotype was scored as an unselected marker. In both crosses, transconjugants which produced high levels of cytotoxin (greater than $10^5$ $CD_{50}$ per ml of culture supernatant) were obtained. The ability to produce cytotoxin at a high level was linked with *trp* (Table 3), and the gene responsible was designated *stx* (*S*higa *t*oxin). To establish the linkage of *trp* and *stx*, a Gal$^+$ Stx$^+$ Fla$^-$ transconjugant of *E. coli* TS50, designated TS55, was used as the recipient in a mating with *E. coli* Hfr KL99. This Hfr donor transfers its chromosome in the order *trp-his-purE-gal*. In this cross, the Trp$^+$ and Stx$^-$ traits of *E. coli* KL99 showed 96% coinheritance (Table 3). Interestingly, some Gal$^+$ transconjugants obtained in the crosses TS52 × TS50 and TS59 × TS50, exhibited weak cytotoxin activity but, as before, the scoring of such weak cytotoxin production was difficult.

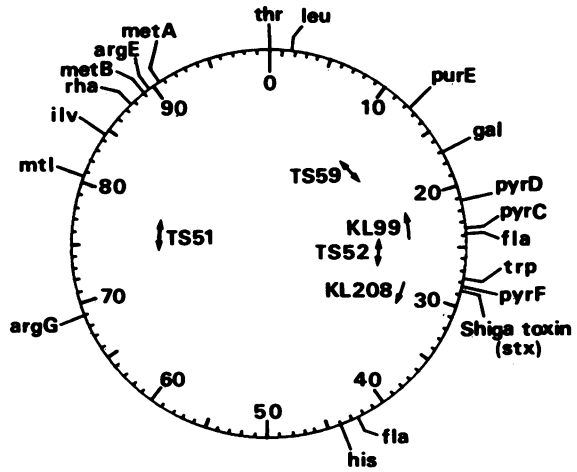


FIG. 1. Linkage map of *E. coli* K-12. The arrows indicate the points of entry and directions of transfer of Hfr strains used in this study. *S. dysenteriae* Hfr strains TS51, TS52, and TS59 probably transfer their chromosomes bidirectionally. Their points of origin were not precisely determined.

TABLE 3. Linkage of *stx* with other chromosomal markers

| 4.2% Donor × recipient[a] | Selected phenotype | Coinheritance of unselected marker phenotype[b] | | | | | | | No. of recombinants examined |
|---|---|---|---|---|---|---|---|---|---|
| | | PurE$^+$ | Gal$^+$ | Fla$^-$[c] | Trp$^+$ | Stx$^+$[d] | Fla$^-$[c] | His$^+$ | |
| TS52 × TS50 | PurE$^+$ | 100 | 1.0 | (4.2) | 10.4 | 0 | (4.2) | 0 | 96 |
| | Gal$^+$ | 0 | 100 | (68.8) | 2.5 | 3.8 | (68.8) | 0 | 80 |
| | Trp$^+$ | 0 | 0 | (7.3) | 100 | 14.6 | (7.3) | 0 | 96 |
| | His$^+$ | 0 | 0 | (22.9) | 1.0 | 1.0 | 22.9 | 100 | 96 |
| TS59 × TS50 | Gal$^+$ | 0 | 100 | (11.3) | 0 | ND | (11.3) | 0 | 80 |
| | Trp$^+$ | 0 | 1.1 | (40.9) | 100 | 2.2 | (40.9) | 0 | 93 |
| | His$^+$ | 0 | 0 | (28.0) | 1.3 | 0 | (28.0) | 100 | 75 |
| | | | | Fla$^+$ | | Stx$^-$ | Fla$^+$ | | |
| KL99 × TS55 | Trp$^+$ | 0 | | (1.3) | 100 | 96.3 | (1.3) | 0 | 80 |
| | His$^+$ | 0 | | (67.5) | 23.8 | 18.8 | (67.5) | 100 | 80 |

[a] In crosses of *Shigella* Hfr strains with the *E. coli* recipient, filter matings were carried out at 37°C for 4 h. In crosses between the *E. coli* Hfr strain and the *E. coli* recipient, matings were carried out in liquid at 37°C for 1 h.

[b] Note that values for percent coinheritance of Fla$^+$ and Stx$^-$ are shown for the KL99 × TS55 mating.

[c] Since *E. coli fla* genes are clustered on both sides of *trp*, values for percent coinheritance with the Fla marker are given in parentheses to indicate that more than one region of the chromosome may be involved in recombination.

[d] Stx$^+$, Ability to express Shiga toxin.

**Establishment of the relative marker order in the *stx* gene region.** Since the results presented above suggested close linkage of *trp* and *stx*, linkage analysis of these loci and the nearby marker *pyrF* was made by P1-mediated transduction. The donor *E. coli* strain used to propagate P1 phage was TS60, which is a Trp$^+$ Stx$^+$ PyrF$^+$ transconjugant obtained by crossing *S. dysenteriae* TS52 with *E. coli* TS50. P1 *vir* Trp$^+$ transductants of TS50 were scored for cytotoxin production: 4 of 120 were found to be positive (Table 4). This means that the *stx* gene is located within 2 min of the *trp* gene. The same phage lysate was also used to transduce the *E. coli* Trp$^-$ PyrF$^-$ strain TS61, and Trp$^+$ or PyrF$^+$ transductants were examined for cytotoxin production. None of 120 Trp$^+$ transductants produced cytotoxin, whereas 55% of the PyrF$^+$ transductants were cytotoxin positive. These results indicated that *stx* is more closely linked to *pyrF* than to *trp* and that the distance between *pyrF* and *stx* is 0.4 min (1). Transduction of Trp$^+$, PyrF$^+$, and Stx$^+$ from TS60 to TS61 constituted a three-point cross; based on the results presented in Table 4, it can be concluded that the gene order on the chromosome of *E. coli* TS60 is *trp-pyrF-stx*.

To confirm that this gene order corresponds to that in the original *S. dysenteriae* 1 strain, a mating was carried out with the *S. dysenteriae* Hfr strain TS52 as the donor and *E. coli* TS67 Trp$^-$ PyrF$^-$ Nal$^r$ as the recipient (since all *S. dysenteriae* isolates that were tested were P1 resistant, analysis by transduction was not possible). Linkage analysis of Trp$^+$ Nal$^r$ and PyrF$^+$ Nal$^r$ transconjugants was consistent with a *trp-pyrF-stx* gene order on the chromosome of *S. dysenteriae* 1 (Table 5).

**Neutralization of the cytotoxin produced by *E. coli* Stx$^+$ with Shiga toxin antibody.** *E. coli* Stx$^+$ transconjugant TS60, *E. coli* Stx$^+$ transductant TS64, and the parental *S. dysenteriae* 1 strain AA17855 were grown overnight in Luria broth, and supernatant fluids of polymyxin B-treated cultures were analyzed for cytotoxin by the HeLa cell assay: all three produced similar levels of cytotoxin, approximately $10^7$ $CD_{50}$ per ml of culture supernatant. The cytotoxic activities of the supernatants of all strains were completely neutralized by polyclonal antibody against Shiga toxin and by monoclonal antibody against Shiga-like toxin. No differences were observed in the serum titers required for complete neutralization of the three toxin preparations (1:3,200 for the polyclonal antibody and 1:10 for the monoclonal antibody).

**Isolation of Stx$^-$ transposon insertion mutants.** The Stx$^+$ PyrF$^+$ *E. coli* derivative TS64 was mutagenized by Tn-mini-*kan*, a transposon derived from Tn*10* (20), by infection with

TABLE 4. Linkage analysis of the *stx* gene region by transduction with P1 *vir*

| Recipient[a] | Selected phenotype | Unselected phenotype | No. (%) of transductants |
|---|---|---|---|
| TS50 | Trp$^+$ | Stx$^+$ | 4 (3) |
| | | Stx$^-$ | 116 (97) |
| TS61 | Trp$^+$ | PyrF$^+$ Stx$^-$ | 4 (3) |
| | | PyrF$^-$ Stx$^-$ | 116 (97) |
| | PyrF$^+$ | Trp$^+$ Stx$^+$ | 24 (30) |
| | | Trp$^-$ Stx$^+$ | 20 (25) |
| | | Trp$^+$ Stx$^-$ | 15 (19) |
| | | Trp$^-$ Stx$^-$ | 21 (26) |

[a] The donor strain was *E. coli* TS60 (Trp$^+$ PyrF$^+$ Stx$^+$).

TABLE 5. Linkage analysis of the *stx* gene region of *S. dysenteriae* 1[a]

| Transconjugants selected | No. (%) of transconjugants |
|---|---|
| PyrF$^+$ | |
| Trp$^+$ PyrF$^+$ Stx$^+$ | 2 (5.0) |
| Trp$^+$ PyrF$^+$ Stx$^-$ | 24 (60.0) |
| Trp$^-$ PyrF$^+$ Stx$^+$ | 1 (2.5) |
| Trp$^-$ PyrF$^+$ Stx$^-$ | 13 (32.5) |
| Trp$^+$ | |
| Trp$^+$ PyrF$^+$ Stx$^+$ | 1 (2.5) |
| Trp$^+$ PyrF$^+$ Stx$^-$ | 2 (5.0) |
| Trp$^+$ PyrF$^-$ Stx$^-$ | 37 (92.5) |

[a] Filter matings were carried out at 37°C for 4 h with TS52 as the donor and TS67 as the recipient.

a lambda suicide vector containing the transposon. Over 10,000 independence kanamycin-resistant transposon mutants of TS64 were pooled, and P1 phage was propagated on a culture of the pooled mutants. The phage lysate was then used to transduce the $Pyr^+$ and kanamycin resistance markers into the *E. coli pyrF* $Stx^+$ strain TS70. The rationale for this strategy was that if there were *stx*::Tn-mini-*kan* mutations present in the pool of kanamycin-resistant mutants of TS64, they should be cotransduced at a high frequency with $PyrF^+$.

Eighty-six $PryF^+$ $Km^r$ transductants thus obtained were examined for cytotoxin production; two were found to be negative, and one was found to produce low amounts of toxin. One cytotoxin-negative transductant, designated TS73, was used as a donor strain for transduction of the kanamycin-resistance marker to the $Stx^+$ *E. coli* strains TS60, TS64, and TS70. All 40 kanamycin-resistant transductants of each of these strains were found to be toxin negative. This means that the Tn-mini-*kan* element in *E. coli* TS73 is inserted into the *stx* gene or into neighboring sequences essential for its expression. The mutant *stx* allele in TS73 was designated *stx-1*.

**Isolation and characterization of $Stx^-$ transposon insertion mutants of *S. dysenteriae* 1.** Because of its positively selectable kanamycin resistance marker, the *stx-1*::Tn-mini-*kan* mutation in *E. coli* TS73 could be readily transferred to other *E. coli* backgrounds by P1-mediated transduction. TS76 and TS77 are *E. coli* Hfr KL99 and Hfr KL208 derivatives, respectively, into which the *stx-1*::Tn-mini-*kan* mutation has been introduced by transduction. Such *E. coli* Hfrs transfer the *stx-1*::Tn-mini-*kan* allele as an early marker (Fig. 1) and were used to introduce it into the parental *S. dysenteriae* 1 strain AA17855. The mating was interrupted 10 min after the donor and recipient cells were mixed to minimize transfer of distal regions of the *E. coli* chromosome. In such matings, kanamycin-resistant (selection), streptomycin-resistant (counterselection) transconjugants appeared at a frequency greater than $10^{-6}$ per donor. Whereas the original *S. dysenteriae* 1 strain produced cytotoxin at a level greater than $10^7$ $CD_{50}$ per ml of culture supernatant, all 40 kanamycin-resistant transconjugant *Shigella* derivatives produced less than $10^2$ $CD_{50}$ per ml of culture supernatant. Blind assays independently carried out by A. O'Brien on two such transconjugants, TS85 and TS86, confirmed these results.

To determine whether the *stx* gene is located in similar chromosomal regions of other *S. dysenteriae* 1 isolates, we introduced the *stx-1*::Tn-mini-*kan* mutation into the *S. dysenteriae* 1 strains 31-81 and 11-81, obtained from P. Sansonetti, Pasteur Institute, Paris, France. Matings were carried out with the *E. coli* KL208 derivative TS77, which was counterselected on the basis of chloramphenicol resistance, as the donor. In both instances, transconjugants were obtained at frequencies greater than $10^{-6}$ per donor. Of 40 transconjugants of each of the two *S. dysenteriae* 1 strains, none produced significant quantities of cytotoxin. Similar results were obtained with the KL99 *stx-1* derivative. Thus, the high frequencies of negative conversion of toxin production in short (10 min) matings using Hfr strains that transfer their chromosomes with opposite polarities strongly support the tentative conclusion discussed above that the position and relative gene order established for *stx* in *E. coli* K-12 is similar to that in different *S. dysenteriae* 1 isolates.

**Virulence of *stx-1* derivatives of *S. dysenteriae* 1.** Two models of bacterial virulence are frequently used to test the pathogenic potential of shigellae and enteroinvasive *E. coli* strains, namely the Serény test (induction of keratoconjunctivitis in guinea pigs; [14]) and invasiveness for tissue culture cells such as HeLa cells. Comparison of the virulence of the parental *S. dysenteriae* 1 strain AA17855 and its *stx-1* derivatives in these two models revealed no qualitative or quantitative differences.

## DISCUSSION

Although Shiga toxin was first described more than 80 years ago, its determinants had not been localized on the *Shigella* linkage map nor had well-characterized toxin-negative mutants been isolated. As a result, the possible functional role of the toxin in dysentery has not been evaluated. Gemski et al. (4) described nontoxigenic mutants of the *S. dysenteriae* 1 strain 725-78 that were isolated on the basis chlorate resistance, but they were subsequently shown to synthesize low levels of toxin (9, 12). Moreover, linkage between the toxin and the chlorate resistance determinants was not demonstrated. The *S. flexneri* 2a strain M4243 produces cytotoxin which is antigenically and biologically similar to Shiga toxin (12), and an *E. coli* K-12 hybrid that had received the *mtl-argE* region of this strain was found to acquire the ability to provoke fluid accumulation in rabbit ileal loops (13). However, proof was not provided that the ability to cause fluid accumulation was due to Shiga toxin production. An *E. coli* strain carrying an R-prime plasmid containing the *argE* region of the chromosome of the *S. dysenteriae* 1 strain 602612 produced Shiga toxin (17). However, it was not possible to generate subclones from this R′ *argE* strain that provoked high-level cytotoxin synthesis, and neutralization of this cytotoxin by anti-shiga toxin serum could not be examined rigorously (T. Sekizaki, T. Chakraborty, W. Goebel, and K. N. Timmis, unpublished observation). Thus, until now, information on the location and importance of determinants specifying the synthesis of normal levels of Shiga toxin was lacking.

In this report, we describe the mapping of *stx*, a gene of *S. dysenteriae* serotype 1 that confers on *E. coli* K-12 the ability to produce Shiga toxin at a high level. This was achieved by the generation of *S. dysenteriae* 1 Hfr derivatives and their use to transfer *stx* by conjugation to appropriately marked *E. coli* K-12 recipients and by P1 transduction analysis of *stx*-bearing *E. coli* transconjugants. Unlike *E. coli* K-12 derivatives bearing R-prime plasmids containing *argE* or *asnA* regions of the *S. dysenteriae* 1 chromosome, K-12 derivatives bearing a chromosomally integrated *stx* gene region stably synthesized high levels of toxin, and this phenotype was readily transferred to other strains of *E. coli* K-12. The *stx* gene is located near *trp* and *pyrF* (about 0.4 min from the latter) at about 30 min on the linkage map of *E. coli* K-12 (1), and the gene order was shown to be *trp-pyrF-stx*. This region of the *Shigella* chromosome had not previously been implicated in virulence.

Having determined the map position of stx in *E. coli* transconjugants, it was possible to select from a culture mutagenized with a kanamycin resistance derivative of Tn*10*, *stx* gene transposon mutants by P1 cotransduction of the $PyrF^+$ character and kanamycin resistance to a $PyrF^-$ $Stx^+$ recipient. Two $Stx^-$ $PyrF^+$ transconjugants were obtained in this way. When one of the *stx*::Tn-mini-*kan* alleles, *stx-1*, was introduced into three different strains of *S. dysenteriae* 1, their levels of cytotoxin production fell by a factor of more than $10^5$. This suggests that there is only one fully functional copy of *stx* per chromosome and that the chromosomal locations of *stx* in these three strains may be similar. DNA fragments carrying the mutant *stx* alleles are being cloned and analyzed.

Certain *E. coli* strains producing Shiga-like toxin were found to be lysogenized by a family of Shiga-like toxin-converting phages (11, 15). The cloning of genes controlling high-level production of Shiga-like toxin from these phages enabled demonstration that the toxin determinants involved are the structural genes for Shiga-like toxin (8). The *stx* gene described here mimics the converting phage: when introduced into *E. coli* K-12, it caused cytotoxin to be produced at a level of $10^7$ $CD_{50}$ per ml of culture supernatant, the same level as that produced by the parental *S. dysenteriae* 1 strain. Thus, by analogy, it was thought that *stx* was probably the structural determinant of the Shiga toxin A and B subunits. However, since *E. coli* K-12 has been reported to synthesize extremely low levels of Shiga toxin (10), the possibility existed that *stx* encodes a positive regulator of expression of the Shiga toxin determinant or that the A and B subunits are encoded by different regions of the chromosome and that Shiga toxin produced in *E. coli* $Stx^+$ transconjugants consists of products of the *stx* gene and an *E. coli* toxin gene. Recent experiments (G. Brazil and K. N. Timmis, manuscript in preparation) have shown that a DNA probe consisting of part of the Shiga-like toxin A subunit coding sequence does not hybridize to *E. coli* K-12 TS50 DNA in colony blots but hybridizes strongly to the *S. dysenteriae* 1 strain AA17855 and to the *E. coli* K-12 $Stx^+$ transductant TS70. Moreover, Southern blotting showed that the DNA fragments of *stx*::Tn-mini-*kan* mutants to which the probe hybridizes are some 2 kilobases larger than the homologous fragments from $Stx^+$ bacteria. Thus, the *stx* gene identified in this report is the structural gene for Shiga toxin.

Although the *stx* determinant that is closely linked to *pyrF* and *trp* was the only locus identified in this study that was able to confer on *E. coli* K-12 recipients the ability to synthesize Shiga toxin at a high level, the *ilv* and/or *met* and *gal* regions of the *S. dysenteriae* 1 chromosome conferred on *E. coli* the ability to produce cytotoxin at a low level. This suggests that *S. dysenteriae* 1 may have more than one determinant for cytotoxin production. Consistent with this possibility was the observation that, in some experiments, *stx-1*::Tn-mini-*kan* mutants of *S. dysenteriae* 1 seemed to synthesize very low amounts of toxin. Unfortunately, the demonstration of cytotoxin production at such low levels was not reproducible, and it was therefore not possible to determine whether these activities are significant and, if so, whether they are neutralized by anti-Shiga toxin antibodies. It has been observed that a toxin-negative mutant of the *S. dysenteriae* serotype 1 strain 725-78 (4) produces a small amount of Shiga-like toxin (12). Whether the cytotoxin produced by this mutant is due to leakiness of the mutation or to the existence of a second copy of the toxin gene has not been determined. The $Stx^-$ mutations described in this paper were induced by transposon insertion and hence should not be leaky. Since they can be readily transferred to many *Shigella* strains because of their kanamycin resistance phenotype, it will be possible to construct $Stx^-$ mutants of strains containing single copies of the Shiga toxin determinant.

Different *Shigella* and *E. coli* strains produce Shiga (-like) toxin at different levels. *E. coli* K-12 has been reported to produce Shiga-like toxin at low levels (less than $10^2$ $CD_{50}$ per mg of bacterial protein [10]), and the *S. flexneri* 2a strain M4243 produces Shiga toxin at a level that is more than a 1,000-fold less than that produced by *S. dysenteriae* 1 (12). It is sometimes assumed that the elevated virulence of *S. dysenteriae* 1 is due to the high levels of cytotoxin it synthesizes. The *stx-1* mutants of *S. dysenteriae* 1 constructed in this work either fail to synthesize Shiga toxin or synthesize it at levels close to the detection limits. Nevertheless, they provoke a normal keratoconjunctivitis in the eyes of guinea pigs (Serény reaction) and invade and multiply normally in HeLa cells. Therefore, Shiga toxin seems not to play a significant role in pathogenesis as measured by these models. Experiments to test its possible role in both the diarrheic and dysenteric phases of the natural disease are in progress. It will be of particular interest to evaluate the possible role of Shiga toxin in the development of severe complications of shigellosis, such as hemolytic-uremic syndrome.

As indicated above, *S. dysenteriae* 1 is presently causing pandemics in some developing countries, and in a number of areas mortality rates are high. One reason for this is an absence of effective antibiotics due to the multiple drug resistances expressed by several of the current epidemic strains of the Shiga bacillus. Thus, there is an urgent need for an efficacious vaccine. One group of live-vaccine candidates under development are aromatic amino acid-dependent ($Aro^-$) *Shigella* mutants that retain their invasive properties but whose metabolic lesions render them nonpathogenic. For *S. dysenteriae* 1, high-level toxin production by such mutants might constitute a hazard in a vaccine strain. In this regard, the Hfr strains of *E. coli* K-12 that donate the *stx-1* transposon insertion mutant allele as an early marker constitute a convenient system with which to mutationally eliminate high-level Shiga toxin production in *S. dysenteriae* 1 strains (and perhaps in other shigellae and Shiga-like toxin-producing isolates of *E. coli*).

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