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Genomic and Functional Analyses of SXT, an Integrating Antibiotic Resistance Gene Transfer Element Derived from *Vibrio cholerae*

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SXT is representative of a family of conjugative-transposon-like mobile genetic elements that encode multiple antibiotic resistance genes. In recent years, SXT-related conjugative, self-transmissible integrating elements have become widespread in Asian *Vibrio cholerae*. We have determined the ~100-kb DNA sequence of SXT. This element appears to be a chimera composed of transposon-associated antibiotic resistance genes linked to a variety of plasmid- and phage-related genes, as well as to many genes from unknown sources. We constructed a nearly comprehensive set of deletions through the use of the one-step chromosomal gene inactivation technique to identify SXT genes involved in conjugative transfer and chromosomal excision. SXT, unlike other conjugative transposons, utilizes a conjugation system related to that encoded by the F plasmid. More than half of the SXT genome, including the composite transposon-like structure that contains its antibiotic resistance genes, was not required for its mobility. Two SXT loci, designated *setC* and *setD*, whose predicted amino acid sequences were similar to those of the flagellar regulators FlhC and FlhD, were found to encode regulators that activate the transcription of genes required for SXT excision and transfer. Another locus, designated *setR*, whose gene product bears similarity to lambdoid phage CI repressors, also appears to regulate SXT gene expression.

The term conjugative transposon (CTn) encompasses a diverse and growing group of mobile genetic elements. Though transmissible via conjugation, CTns, unlike conjugative plasmids, are not thought to have an autonomously replicating extrachromosomal form; instead, CTns integrate into their hosts' chromosomes. Chromosomal integration by CTns is mediated by recombinases of either the integrase (27, 28) or resolvase (33) family. CTn integration can be more or less site specific, depending on the particular CTn as well as the host background. In some cases, the molecular details of CTn integration have been characterized in some detail (28). CTn transfer is thought to occur via a nonreplicative circular intermediate that forms following the excision of the element from the chromosome. The gene products that mediate conjugative transfer of these elements have not been extensively studied; however, DNA sequence and genetic analyses of the two most studied CTns, the Tn*916*-related CTns derived from gram-positive bacteria and the *Bacteroides*-derived CTnDOT-related CTns, suggest that CTn-encoded conjugative transfer systems are not related to those encoded by known conjugative plasmids (3, 8).

In addition to containing DNA sequences coding for their self-transmissibility, CTns impart a variety of properties to their hosts. CTns provide an important means for dissemination of genes encoding resistance to antibiotics (27) and heavy metals (6) among gram-negative and gram-positive bacteria. Additionally, CTns have been found to encode pathways for the degradation of aromatic compounds (22), sucrose metabolism (12), nitrogen fixation and vitamin biosynthesis (31), and nisin synthesis (25).

In *Vibrio cholerae*, the gram-negative bacterium that causes the severe diarrheal disease cholera, resistance determinants to sulfamethoxazole, trimethoprim, chloramphenicol, and streptomycin are carried on a CTn-like element called SXT (32). This element was initially detected in the newly emerged O139 serogroup of *V. cholerae*, but SXT or very closely related elements are now found in virtually all clinical *V. cholerae* isolates from the Indian subcontinent (13). We also recently detected SXT (or at least a very similar element) in *Providencia alcalifaciens* isolates from patients with diarrhea in Bangladesh (13). SXT appears to be representative of a family of closely related conjugative, self-transmissible, chromosomally integrating elements. One of these, R391, which was initially described in 1972 in a South African *Providencia rettgeri* isolate and carries kanamycin and mercury resistance determinants (6), integrates into the same chromosomal site as SXT, the 5′ end of *prfC* (11).

In the laboratory, SXT is transmissible by conjugation to a variety of gram-negative organisms, and it can mediate the transfer of certain mobilizable plasmids, as well as chromosomal DNA, in an Hfr-like fashion (14). A circular but nonreplicative extrachromosomal form of SXT is thought to be an intermediate in its transfer (15). The formation of this extrachromosomal form of SXT requires the SXT-encoded site-specific recombinase Int, which is closely related to the integrases found in the λ family of bacteriophages. This integrase is required for SXT transfer but not for SXT-dependent transfer of mobilizable plasmids or chromosomal DNA (14). Similar to lambdoid phages, SXT integrates site specifically into the chromosome in an *int*-dependent, *recA*-independent fashion (15).

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype	Reference or source
E. coli K-12		
CAG18439	MG1655 *lacZU118 lac142*::Tn*10*	30
HW220	SXT^+ exconjugant of CAG18439	15
JO216	HW220 Δ*traI*	This study
BI533	MG1655 Nal^r	14
TOP10	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 deoR araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA I nupG*	Invitrogen
BW25113	$lacI^q$ $rrnB_{t14}$ $\Delta lacZ_{WJ16}$ *hsdR514* $\Delta araBAD_{AH33}$ $\Delta rhaBAAD_{LD78}$	7
JO193	SXT^r exconjugant of BW25113	This study
BI957	JO193 Δ*rumB′-rumA*::*cat*	This study
JO296	JO193 Δ*s024-s040*	This study
JO248	JO193 Δ*traD-s043*	This study
JO246	JO193 Δ*traD*	This study
JO247	JO193 Δ*s043*	This study
JO356	JO193 Δ*s044-s045*	This study
JO352	JO193 Δ*traL-traA*	This study
JO207	JO193 Δ*traA*	This study
JO355	JO193 Δ*s052-traN*	This study
JO249	JO193 Δ*s060-s073*	This study
BI918	JO193 Δ*s074*	This study
JO316	JO193 Δ*traG*	This study
JO251	JO193 Δ*s079-s084*	This study
JO313	JO193 Δ*setDC*	This study
JO212	JO193 Δ*floR*	This study
JO359	JO212 Δ(*traL-traA*)::*lacZ*	This study
JO360	JO212 Δ*s003*::*lacZ*	This study
JO386	JO212 Δ*traG*::*lacZ*	This study
JO427	JO212 Δ*traN*::*lacZ*	This study
JO400	JO212 Δ*setCD*::*lacZ*	This study
JO397	JO212 Δ*floR*::*lacZ*	This study
Plasmids		
pSU4628	CloDF13::Tn*A* Δ*Eco*RV	5
pCR2.1	Ap^r Kn^r	Invitrogen
pOriT	pCR2.1 containing putative SXT origin of transfer	This study
pKD46	Ts plasmid expressing lambda Red recombinase from arabinose-inducible promoter	7
pKD3	Cm^r PCR template for one-step chromosomal gene inactivation	7
pJB20	*lacZ* Cm^r PCR template for one-step chromosomal gene inactivation	This study
pKD4	Kn^r PCR template for one-step chromosomal gene inactivation	7
pDTraI	pWM91 (20) derivative for allele exchange of *traI*	This study
pBAD-TOPO	Arabinose-inducible expression vector	Invitrogen
pSetCD	pBAD-TOPO containing *setCD*	This study
pSetC	pBAD-TOPO containing *setC*	This study
pSetD	pBAD-TOPO containing *setD*	This study

In this study, we have determined and annotated the DNA sequence of SXT. A nearly comprehensive set of SXT gene deletions was constructed to characterize SXT sequences and genes involved in conjugative transfer and chromosomal excision. These studies revealed that the SXT conjugative apparatus is related to that encoded by the F plasmid and that over half of the SXT genome is not required for its transmissibility. Two SXT loci, designated *setC* and *setD*, were found to encode regulators that activate transcription of genes required for SXT excision and transfer.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are described in Table 1. Nearly all of the *Escherichia coli* strains harboring deletions in SXT were constructed using the one-step chromosomal gene inactivation technique (7) and are derivatives of BW25113. An exception was JO216, which was constructed using an allele exchange vector, pDTraI, a derivative of the *sacB*-containing vector pWM91 (20). The presence of the appropriate chromosomal deletion in all cases was confirmed using PCR. The *cat* marker was left in BI957 to allow selection of the transfer of this element in conjugation assays. Bacterial strains were routinely grown in Luria-Bertani (LB) broth (2) at 37°C on a roller drum incubator. Bacterial strains were maintained at −70°C in LB broth containing 20% (vol/vol) glycerol. Antibiotics were used at the following concentrations: ampicillin, 100 mg $liter^{-1}$; kanamycin, 50 mg $liter^{-1}$; sulfamethoxazole, 160 mg $liter^{-1}$; trimethoprim, 32 mg $liter^{-1}$; tetracycline, 10 mg $liter^{-1}$.

Molecular biology procedures. Plasmid DNA was prepared by using the Qiaprep Spin miniprep kit and Qiaprep miniprep kit (Qiagen). Recombinant DNA manipulations were carried out by standard procedures (2). The TA cloning kit and pBAD TOPO TA cloning kit (Invitrogen) were used for the cloning of PCR products.

DNA sequencing and analysis. A previously constructed cosmid library of partially *Sau*3AI-digested DNA from the SXT^+ *V. cholerae* O139 strain MO10 (32) was screened to identify overlapping cosmids whose inserts, in aggregate, spanned the entire SXT sequence. Initially, cosmids mediating resistance to sulfamethoxazole and trimethoprim were isolated on selective media, leading to the identification of a cosmid spanning the left SXT-chromosome junction (15, 32). Subsequent rounds of colony hybridization with probes derived from the sequences of the inserts of the initial cosmids were used to find cosmids spanning the remainder of the element. A combination of vector-derived primers and plasmid walking was used to obtain the SXT DNA sequence. AssemblyLIGN

(Oxford Molecular Group, Campbell, Calif.) and ContigExpress (InforMax, North Bethesda, Md.) were used to assemble DNA sequences. Automated DNA sequencing was carried out at the Tufts Medical School DNA Sequencing Core Facility as described previously (32). Open reading frames (ORFs) were determined using Vector NTI (InforMax) and GeneMark (http://opal.biology.gatech.edu/GeneMark/). Protein sequences were analyzed for motifs with the SMART program (http://smart.empl-heidelberg.de). The BLAST programs (1) were used to assess similarity between SXT sequences and the GenBank database.

Bacterial conjugations. Conjugation experiments were conducted as previously described (32). Briefly, overnight cultures of differentially marked donor and recipient cells were mixed on an LB plate and incubated at 37°C for 5 h. The cells were resuspended in LB broth, and dilutions were plated on selective media, allowing enumeration of donors, recipients, and transconjugants. The SXT transfer frequency was calculated as the number of transconjugants observed per donor cell. Mating assays for CloDF13 transfer were performed as described above, but the donor cells contained CloDF13. The CloDF13 transfer frequency was calculated as the number of CloDF13 transconjugants observed per donor cell.

PCR assay for detection of the circular extrachromosomal form of SXT. PCR primers oriented towards the left and right SXT-chromosome junctions were used for the detection of a circularized, extrachromosomal form of the element as described previously (15). The template for the PCR assay was 1 μl of overnight cell cultures.

R391 complementation of SXT mutations. R391 was introduced into the deletion strain via conjugation as described previously (11). This resulted in the formation of tandem arrays of R391 and SXT. Transconjugants containing R391 and SXT were then tested for the capability to mobilize SXT independently of R391 in the mating assay described above. R391 complementation was carried out only for strains that were unable to mobilize SXT.

Construction of chromosomal deletions and *lacZ* fusions. Deletions were introduced into a chromosomally integrated SXT by use of the one-step chromosomal gene inactivation technique (7). In this technique, the λ Red recombinase is utilized to facilitate the recombination of linear PCR products into the *Escherichia coli* chromosome. These PCR products, introduced into the cell by transformation, contain an antibiotic resistance gene with 36-bp tails identical to the sequences flanking the region targeted for deletion. There are FRT (FLP recognition target) recombination sites on either side of the antibiotic resistance gene, allowing for the elimination of the marker, resulting in an unmarked, nonpolar deletion (7). Chromosomal *lacZ* gene replacements were created by the use of a novel template, pJB20, for the creation of the PCR products. This template contains a promoterless *lacZ* gene introduced into the unique *Bst*BI site downstream of the *cat* gene in pKD3. These PCR products were introduced into the chromosomally integrated SXT as described above (7), generating a substitution of *lacZ* and *cat* in place of the gene of interest.

β-Galactosidase assays. Overnight cultures of cells containing a plasmid with *setDC* under the control of an arabinose-inducible promoter (10) (pSetDC) were diluted 1:100 in LB broth containing 100 μg of ampicillin/ml and grown for 2.5 h. Arabinose was then added to half of the culture to a final concentration of 1 mM, and the cells were grown for an additional 1.5 h. β-Galactosidase activity, reported in Miller units, was measured as described by Miller (21). The values presented are the means of at least three independent experiments.

Nucleotide sequence accession number. The SXT sequence has been deposited in GenBank with accession no. AY055428.

RESULTS

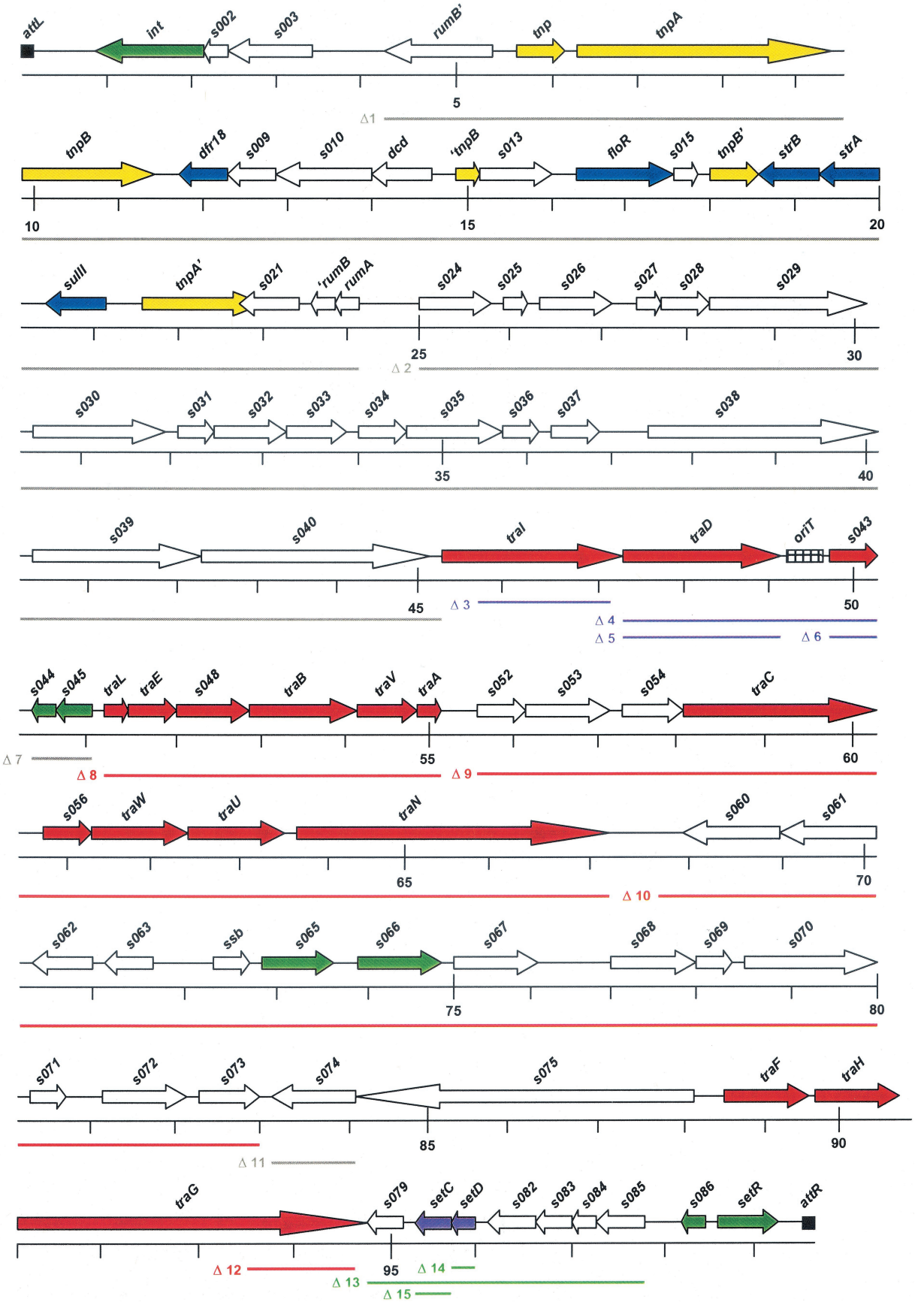
General properties of the SXT sequence. SXT is 99,483 bp in length and contains 87 putative ORFs (Fig. 1 and Table 2). The SXT sequence appears to be a composite of genes derived from plasmids, bacteriophages, and additional diverse and, in many cases, unknown sources (Table 2). While the overall G+C content of SXT (47.1%) is similar to that of the *V. cholerae* genome (47.6%), there are several SXT regions whose G+C contents differ significantly from the rest of the element, again suggesting that genes from numerous sources have become linked within SXT. For example, an insertion within the transfer region (see below) has a G+C content of 42.0% and contains sequences similar to that of DNA from the Ti plasmid from *Agrobacterium tumefaciens* (*s052* and *s053*). SXT's antibiotic resistance genes, which appear to be embedded in a composite transposon-like structure (13) with a G+C content of 51.9%, were also probably acquired via horizontal gene transfer.

SXT is organized in a modular fashion, with clusters of genes with related functions. The antibiotic resistance genes (Fig. 1, depicted in blue) are found near the 5′ end of the integrated form of SXT. The transfer-related genes are clustered in four groups in the 3′ half of SXT (Fig. 1, depicted in red). Proteins required for SXT integration-excision and regulation are encoded at the right and left ends of the element, respectively. As expected from our previous studies, SXT does not contain ORFs similar to genes known to be required for either plasmid replication or partitioning, since SXT is not thought to have an autonomously replicating extrachromosomal form (32). In addition, SXT contains 26 genes with putative products that do not have significant similarity to sequences in the GenBank database and 15 genes whose products are similar to those of genes of undetermined function. Many of these hypothetical genes are also found within clusters (Table 2 and Fig. 1).

Experimental approach. To further analyze the roles of individual genes or groups of genes in the transfer, excision, and regulation of SXT, deletions were constructed using *E. coli* K-12 as the SXT host. Previously, we observed a higher SXT transfer frequency with *E. coli* donors than with *V. cholerae* (32), thus facilitating the analysis. Additionally, we were able to construct many in-frame deletions in SXT in *E. coli* with the recently published one-step chromosomal gene inactivation technique, which utilizes the lambda Red recombinase to integrate linear PCR products into the *E. coli* chromosome (7, 34). Using this technique, we constructed a set of strains containing deletions of nearly every SXT ORF. The resulting mutants were then tested for the ability to mediate SXT excision and self-transfer and the ability to mobilize the plasmid CloDF13. CloDF13 is not self-transmissible and requires the presence of a conjugative element to be transferred into recipient cells (5). We tested SXT deletion mutants defective in conjugation for the ability to be complemented by the presence of R391, since our preliminary data indicated that R391 encodes a conjugative system nearly identical to that of SXT (11).

Integrase region. The first ORF found at the 5′ end of the integrated SXT, *int*, codes for an integrase related to those found in lambdoid bacteriophages. Our previous studies revealed that *int* is required for both the element's integration into and excision from the chromosome (15). In SXT and the related element R391, *int* lies at the 3′ end of a putative operon with two other genes, *s002* and *s003*. Deletion of *s002* (15) and *s003* (Table 2) had no effect on SXT transfer or excision. Sequence analysis and deletion studies did not identify any additional *xis*-like genes involved in SXT excision.

Antibiotic resistance region. The antibiotic resistance genes coding for sulfamethoxazole, trimethoprim, streptomycin, and chloramphenicol resistance are clustered together near the 5′ end of the element (Fig. 1) and were the subject of a recent paper (13). We reported that genes that are likely to encode transposases flank the antibiotic resistance genes; the entire 23-kb sequence from *tnp* to *tnpA′* has a structure similar to that of a composite transposon integrated into SXT in *rumB* (13). Deletion of the entire region, including the truncated *rumB′* and *rumA* (Fig. 1, Δ1), did not alter the frequency of SXT


attL
int
s002
s003
rumB'
tnp
tnpA
5
Δ1
tnpB
dfr18
s009
s010
dcd
'tnpB
s013
floR
s015
tnpB'
strB
strA
10
15
20
sulII
tnpA'
s021
'rumB
rumA
s024
s025
s026
s027
s028
s029
25
30
Δ 2
s030
s031
s032
s033
s034
s035
s036
s037
s038
35
40
s039
s040
traI
traD
oriT
s043
45
50
Δ 3
Δ 4
Δ 5
Δ 6
s044
s045
traL
traE
s048
traB
traV
traA
s052
s053
s054
traC
55
60
Δ 7
Δ 8
Δ 9
s056
traW
traU
traN
s060
s061
65
70
Δ 10
s062
s063
ssb
s065
s066
s067
s068
s069
s070
75
80
s071
s072
s073
s074
s075
traF
traH
85
90
Δ 11
traG
s079
setC
setD
s082
s083
s084
s085
s086
setR
attR
Δ 12
95
Δ 14
Δ 13
Δ 15

transfer or excision (Table 3); thus, the putative transposases have no discernible role in SXT transfer. Consistent with this idea, in the closely related element R391, *rumB* is not interrupted by this composite transposon-like structure (13).

Transfer region. Analysis of the SXT DNA sequence revealed that the SXT conjugative transfer system is most similar in its sequence and organization to R27, a conjugative plasmid derived from *Salmonella enterica* serovar Typhi (29), and is also related to the transfer system of the F plasmid. The conjugation-related genes carried by plasmids derived from gram-negative organisms, such as F, share two basic features: (i) they specify the production of a conjugative apparatus that includes a pilus and enables the formation of mating pairs, and (ii) they encode proteins required for the DNA processing steps that enable DNA transport from the donor to the recipient. SXT contains genes (Fig. 1) that, at least by homology, are likely to be important in both of these processes.

SXT's *tra* gene homologs are clustered together in the 3′ half of the SXT sequence in at least three putative operons. The first operon (*traI* to *s043*) encodes functions for DNA processing, while the other two operons (*traL* to *traN* and *traF* to *traG*) are likely to be involved in pilus assembly and mating pair formation. The first putative pilus assembly operon is interrupted by three genes (*s052*, *s053*, and *s054*) not found in the F plasmid. *s052* and *s053* have similarity to ORFs of unknown function in the *A. tumefaciens* Ti plasmid. The *s054* gene product has similarity to DsbC, a disulfide bond isomerase. Interestingly, orthologs of DsbC are also present in the transfer gene regions of plasmids with conjugation systems most closely related to SXT (26, 29; J. P. Dillard, personal communication; T. Murata, T. Hayashi, M. Ohnishi, K. Nakayama, Y. Terawaki, K. Takashima, T. Ara, H. Mori, J. Kaneko, Y. Kamio, T. Miki, C. G. Han, and E. Ohtsubo, Abstr. 101st Gen. Meet. Am. Soc. Microbiol., abstr. H-66, 2001).

As expected, the SXT genes homologous to those found in the F plasmid or other conjugative plasmids are required for its conjugative transfer. Deletions of transfer region genes (Fig. 1, Δ3 to Δ6, Δ8, Δ9, and Δ12) all rendered SXT immobile. However, only a subset of these mutations impaired transfer of the mobilizable plasmid CloDF13 (see below). These deletions had no discernible influence on SXT excision, as we could detect the extrachromosomal circular form of SXT in each of these strains. Introduction of R391 into these strains complemented the transfer defects in all cases except the Δ4 deletion (see below) (Table 3).

As mentioned above, only a subset of the mutations impaired the mobilization of the plasmid CloDF13. Mutations in genes of both putative pilus assembly operons (i.e., Δ8, Δ9, and Δ12) prevented CloDF13 mobilization; however, mutations in individual genes of the putative DNA-processing operon did not prevent CloDF13 transfer (Table 3, Δ3, Δ5, Δ6). Sequence comparison suggests that this operon encodes a TraG family member (designated TraD according to F nomenclature) and a TraI homolog (designated TraI), as well as one protein with no apparent similarity (the product of *orf43*). TraG family members are "coupling" proteins that link cytoplasmically localized DNA-processing functions and membrane-localized mating pair formation functions during conjugative gene transfer (5). TraI proteins are DNA relaxases that are critical for the initiation of conjugative DNA transfer, catalyzing the strand-specific DNA cleavage at the origin of transfer (*oriT*) (16). Whereas most mobilizable plasmids require the coupling protein of a conjugative system, CloDF13 encodes both a coupling protein and a DNA relaxase specific to its *oriT* (5); thus, it is not surprising that CloDF13 mobilization could occur even in the absence of the SXT-encoded proteins. It seems likely that *orf43* also encodes a protein for which CloDF13 can supply a substitute, since, like mutants lacking *traI* and *traD*, an *orf43* mutant could mobilize CloDF13 but not SXT (Table 3).

Identification of the origin of transfer. Surprisingly, the transfer deficiency of the Δ4 mutation (which removed the region from *traD* to *s043*) could not be complemented by R391, even though the deletions of the individual genes within this region (Δ5 and Δ6) could be complemented. This suggested that the Δ4 mutation includes a *cis*-acting site essential for transfer, such as the SXT-specific *oriT*. Therefore, we tested whether the 550-bp intergenic region between *traD* and *s043* contained the SXT *oriT*. This region was introduced into a plasmid (pCR2.1) that is ordinarily not mobilizable. Unlike pCR2.1, the resulting plasmid, pOriT, was mobilizable if SXT was present in the donor cell (Table 4); in addition, mobilization of pOriT did not require that SXT itself be transferred into the recipient cell. The sequence of this 550-bp region contains several features, including direct and indirect repeats and an area with high A+T content, that are commonly found in the origins of transfer in conjugative elements (16). These observations are consistent with the idea that the SXT *oriT* is located within the intergenic region between *traD* and *s043*.

Regions containing novel genes. There are two large SXT regions that primarily contain genes with no known function. The first of these regions, located between the antibiotic cluster and the first transfer region, consists of 17 putative ORFs (*s024* to *s040*), 14 of which have no significant homology to proteins in the GenBank database. Remarkably, deletion of the entire region from *s024* to *s040* had no effect on SXT transfer or excision (Table 3, Δ2). The second of these regions is 21 kb in length and contains 16 putative ORFs (*s060* to *s073*) likely to comprise at least three different operons based on their orientations. This region, which separates the second and third transfer regions, contains several ORFs with similarity to

FIG. 1. Genetic organization and ORF map of SXT. Putative ORFs are depicted by arrows showing the orientations. Colors are used to indicate similarity or function as follows: transposase (yellow), antibiotic resistance (blue), conjugative transfer (red), and regulation (purple). A hatched box indicates the position of the origin of transfer. ORFs of known or unknown function whose best hits by BLAST are found in phage genomes are colored green. The colored lines beneath the map indicate the locations of deletions discussed in Table 3: gray indicates the deletion had a wild-type phenotype, purple indicates mobilization of CloDF13 but no transfer of SXT, red indicates no or greatly reduced transfer of SXT or CloDF13, and green indicates no transfer of SXT or CloDF13 and no excision.

TABLE 2. Putative ORFs in the SXT sequence

ORF no. (gene name)	Coding region	Probable function	Identity (%/range)[a]	Homology[b]		GI accession no.	Required for SXT transfer[c]
				Gene name	Source		
001 (*int*)	836–2074	Integrase	97/413	*int*	R391 (*P. rettgeri*)	12746566	Y
002	2079–2345	Unknown	93/79	*orf2*	R391 (*P. rettgeri*)	12746565	N
003	2351–3322	Unknown	99/324	*orf1*	R391 (*P. rettgeri*)	12746564	N
004 (*rumB′*)	4244–5413	UV repair DNA polymerase	92/372	*rumB*	R391 (*P. rettgeri*) (truncated)	862633	N
005 (*tnp*)	5697–6287	Transposase	41/178	*tnp*	pPHGI (*Pseudomonas putida*) (truncated)	4754812	N
006 (*tnpA*)	6404–9301	Transposase	29/961	*tnpA*	pPHGI (*P. putida*)	7465523	N
007 (*tnpB*)	9799–11289	Transposase	99/496	*orfA*	*E. coli*	10312101	N
008 (*dfR18*)	11656–12207	Dihydrofolate reductase type VIII	59/157	*dhfRVIII*	pLM0226 (*E. coli*)	507215	N
009	12224–12766	Unknown	45/217	*mlr6191*	*Mesorhizobium loti* (truncated)	14025924	N
010	12769–13932	Unknown					N
011 (*dcd*)	13950–14675	Deoxycytidine triphosphate deaminase	44/208	*dcd*	*Pseudomonas aeruginosa*	9949625	N
012 (*′tnpB*)	14935–15264	Transposase	100/110	*orfA*	*E. coli* (truncated)	10312102	N
013	15298–16179	Unknown					N
014 (*floR*)	16399–17610	Florfenicol exporter	99/404	*floR*	*E. coli*	10312100	N
015	17641–17943	Transcriptional repressor	70/100	*orf1*	*S. enterica* serovar Typhimurium DT104 (truncated)	12719030	N
016 (*tnpB′*)	18058–18594	Transposase	100/135	*orfA*	*E. coli* (truncated)	10312101	N
017 (*strB*)	18572–19405	Streptomycin phosphotransferase	100/278	*strB*	Tn*5393* (*Erwinia amylovora*)	420965	N
018 (*strA*)	19408–20022	Streptomycin phosphotransferase	99/267	*strA*	Tn*5393* (*E. amylovora*)	420964	N
019 (*sulII*)	20272–21084	Dihydropteroate synthase type II	100/271	*sulII*	RSF1010 (*E. coli*)	118596	N
020 (*tnpA′*)	21555–22914	Transposase	24/413	*tnpA*	pPHG1 (*P. putida*) (truncated)	7465523	N
021	22639–23358	Methyl-directed mismatch DNA repair	51/173	*mutL*	*V. cholerae* (truncated)	127554	N
022 (*′rumB*)	23514–23831	DNA polymerase UV repair	98/78	*rumB*	R391 (*P. rettgeri*) (truncated)	862633	N
023 (*rumA*)	23791–24162	UV repair	98/149	*rumA*	R391 (*P. rettgeri*)	862632	N
024	24876–25781	Polymerase epsilon subunit	90/250		R391 (*P. rettgeri*)	13992587	N
025	25858–26169	Unknown					N
026	26241–27164	Unknown	36/261	*nmaI157*	*Neisseria meningitides* (group A strain Z2491)	11281285	N
027	27395–27775	Unknown					N
028	27766–28356	Unknown					N
029	28378–30270	Unknown					N
030	30325–31920	Unknown					N
031	32037–32570	DNA recombination	35/64	*orf1*	pNSF-1 (*Shigella flexneri*)	12034665	N
032	32488–33237	Unknown	25/182	*ss01994*	*Sulfolobus solfataricus* (truncated)	13815272	N
033	33237–33992	Unknown					N
034	34209–34676	Unknown					N
035	34672–35868	Unknown					N
036	35852–36229	Unknown					N
037	36399–37010	Unknown					N
038	37601–40231	Unknown					N
039	40251–42323	Lon protease	28/165	*lon*	*Thermus thermophilus* (truncated)	9719397	N
040	42331–45018	Unknown	25/912	*orf74*	pB171 (*E. coli*)	10955424	N
041 (*traI*)	45176–47323	Relaxase	26/314	*xf1753*	*Xylella fastidiosa*	11361836	Y
042 (*traD*)	47396–49192	Coupling factor	29/536	*traG*	R27 (*S. enterica* serovar Typhi)	10957308	Y
043	49753–50385	Coupling factor	29/96	*r0117*	R27 (*S. enterica* serovar Typhi)	10957306	Y
044	50417–50692	Unknown	37/82	*gp48*	Bacteriophage N15	9630515	N
045	50692–51012	Unknown	40/102	*gp49*	Bacteriophage N15	9630516	N

046 (*traL*)	51187–51471	Sex pilus assembly	23/72	*traL*	pNL1 (*S. aromaticivorans*)	10956948	Y*
047 (*traE*)	51471–52094	Sex pilus assembly	26/171	*trhE*	R27 (*S. enterica* serovar Typhi)	10957221	Y*
048	52078–52974	Unknown	25/254	*r0031*	R27 (*S. enterica* serovar Typhi)	10957220	Y*
049 (*traB*)	52980–54266	Sex pilus assembly	40/209	*trhB*	R27 (*S. enterica* serovar Typhi)	10957218	Y*
050 (*traV*)	54266–54913	Sex pilus assembly	23/126	*trhV*	R27 (*S. enterica* serovar Typhi)	10957215	Y*
051 (*traA*)	54913–55296	Pilin subunit	24/85	*trhA*	R27 (*S. enterica* serovar Typhi)	10957223	Y
052 (*ynd*)	55664–56308	Unknown	46/296	*ynd*	pTi (*A. tumefaciens*)	10955130	N
053 (*ync*)	56292–57239	Unknown	36/108	*ync*	pTi (*A. tumefaciens*)	10955129	N
054 (*dsbC*)	57374–58063	Thiol-disulfide interchange	27/192	*dsbC*	*P. aeruginosa* PAO1	9949905	Y*
055 (*traC*)	58066–60462	Sex pilus assembly	27/869	*trhC*	R27 (*S. enterica* serovar Typhi)	10957214	Y*
056 (*trsF*)	60789–61298	Conjugation signal peptidase	28/127	*trhF*	R27 (*S. enterica* serovar Typhi)	10957198	Y*
057 (*traW*)	61312–62433	Sex pilus assembly	28/303	*trhW*	R27 (*S. enterica* serovar Typhi)	10957197	Y*
058 (*traU*)	62420–63445	Sex pilus assembly	36/316	*trhU*	R27 (*S. enterica* serovar Typhi)	10957196	Y*
059 (*traN*)	63683–67144	Mating pair stabilization	25/481	*trhN*	R27 (*S. enterica* serovar Typhi)	10957195	Y*
060	67927–69006	Unknown					N
061	69009–70070	Unknown					N
062	70186–70899	Nuclease	53/205	*nucM*	*Pectobacterium chrysanthemi*	403002	N
063	71011–71610	Unknown					N
064 (*ssb*)	72318–72734	SSB	38/135	*ssb*	Plasmid F (*E. coli*)	8918883	N
065 (*bet*)	72817–73632	DNA recombination	55/195	*bet*	Bacteriophage 933W	9632480	N
066 (*gp47*)	73921–74934	Unknown	25/255	*gp47*	Bacteriophage A118	5823649	N
067 (*cobS*)	75030–76103	Porphyrin biosynthesis	31/288	*cobS*	pMT1 (*Yersinia pestis*)	3883103	N
068	76975–77925	Unknown	20/205	*r0207*	R27 (*S. enterica* serovar Typhi)	10957394	N
069	77990–78427	Unknown					N
070	78500–80152	Unknown	20/265	*r0206*	R27 (*S. enterica* serovar Typhi)	10957393	N
071 (*radC*)	80237–80731	DNA repair	56/151	*radC*	*V. cholerae*	11355878	N
072	81243–82241	Unknown	36/232	*r0204*	R27 (*S. enterica* serovar Typhi)	10957391	N
073	82335–83039	Unknown					N
074	83307–84233	Response regulator	39/289	*rrp-1*	*Borrelia burgdorferi*	2688314	N
075	84242–88045	Histidine kinase	37/1233	*pa4112*	*P. aeruginosa* PAO1	11351830	N
076 (*traF*)	88747–89721	Sex pilus assembly	27/278	*r0126*	R27 (*S. enterica* serovar Typhi)	10957315	ND
077 (*traH*)	89727–91112	Sex pilus assembly	39/451	*trhH*	R27 (*S. enterica* serovar Typhi)	10957316	ND
078 (*traG*)	91119–94685	Sex pilus assembly	21/828	*traG*	R27 (*S. enterica* serovar Typhi)	10956931	Y
079	94724–95173	Unknown					N
080 (*setC*)	95210–95740	Transcriptional activator	31/172	*s006*	DT104 (*S. enterica* serovar Typhimurium)	12719014	Y
081 (*setD*)	95740–96036	Flagellar transcriptional activator	28/97	*flhD*	*Serratia liquefaciens*	2126176	Y
082	96036–96581	Unknown	29/130	*mlr2934*	*M. loti*	13472590	N
083	96571–96936	Unknown					N
084	96929–97231	Unknown					N
085	97221–97739	Unknown					N
085 (*ydaS*)	98147–98395	Unknown	42/59	*ydaS*	Prophage CP-933N (*E. coli*)	12514682	UNK
086 (*setR*)	98513–99157	Transcriptional repressor	46/213	*cI*	Phage 434	420538	UNK

[a] Identity is presented as percent amino acid identity between the SXT ORF and the best hit. The range is the number of amino acids over which this identity exists.
[b] The number of identical amino acids of the respective gene products was determined by BLAST (1). The best hit and its source are presented.
[c] Strains containing single deletions or deletions of multiple genes were scored for the ability to transfer SXT in a mating assay (Table 3). A strain was scored N if transconjugants were observed, Y if transconjugants were not observed, Y* if the gene was part of a larger deletion in which transconjugants were not observed, ND if the deletion was not constructed, and UNK if a single deletion could not be obtained.

TABLE 3. Phenotypic analysis of SXT deletions

Deletion[a]	Region deleted	SXT transfer (10^{-4})[b]	CloDF13 transfer (10^{-4})[c]	Circle formation[d]	R391 complementation[e]
WT		1.0	3.2	+	NA
Δ1	*'rumB–rumA*	8.0	4.6	+	NA
Δ2	*s024–s040*	1.7	1.0	+	NA
Δ3	*traI*	UD	0.1	+	+
Δ4	*traD–s043*	UD	5.6	+	−
Δ5	*traD*	UD	13	+	+
Δ6	*s043*	UD	22	+	+
Δ7	*s044–s045*	0.6	10	+	NA
Δ8	*traL–traA*	UD	UD	+	+
Δ9	*s052–traN*	UD	UD	+	+
Δ10	*s060–s073*	0.0064	UD	+	NA
Δ11	*s074*	0.52	0.75	+	NA
Δ12	*traG*	UD	UD	+	+
Δ13	*s079–s084*	UD	UD	−	+
Δ14	*setD*	UD	UD	−	+[f]
Δ15	*setC*	UD	UD	−	+[f]

[a] All strains are derivatives of BW25113 (7) containing SXT except Δ3, which is a derivative of MG1655. WT, wild type.
[b] SXT transfer frequency was calculated as the number of transconjugants observed per donor cell. UD (undetected) is defined as below the limits of detection of the assay ($\sim 10^{-8}$).
[c] Mating assays were performed as described in note *b*, but donor cells contained CloDF13. The CloDF13 transfer frequency was calculated as the number of CloDF13 transconjugants observed per donor cell.
[d] PCR for detection of a circularized, extrachromosomal form of the element was performed as described previously (15). +, detected; −, not detected.
[e] R391 complementation was carried out only for strains that were unable to mobilize SXT. NA, not applicable. +, complementation; −, no complementation.
[f] Complementation was performed with plasmids expressing the single gene product.

proteins in the GenBank database. One of these is a putative single-stranded-DNA binding protein (SSB), a common element in several conjugation systems (9). Downstream of the SSB are two ORFs (*s065* and *s066*) that are similar to phage proteins: the recombination protein Bet of phage 933W and a gene product of unknown function. *s074* and *s075* encode a putative two-component system, but a role for these genes in the regulation of SXT transfer seems unlikely, as deletion of the response regulator (Table 3, Δ11) did not have any effect on SXT transfer or excision. In contrast, deletion of the region from *s060* to *s073* (Table 3, Δ10) resulted in a 1,000-fold reduction in the frequency of SXT transfer, although excision of SXT was not abolished. Transfer of CloDF13 was reduced to below detectable levels in this mutant (Table 3). These observations indicate that some of the gene products within this region augment but are not essential for SXT transfer. No single-gene deletion in this region has been found that has an effect comparable to the *s060*-to-*s073* deletion (data not shown).

Regulatory region. The 3′ end of SXT was found to play a critical role in regulation of SXT transfer. This region contains nine ORFs, eight oriented in one direction (*s079* to *s086*) and a final divergently oriented gene (*setR*). Only three of the gene products in this region have significant similarity to proteins of known function in the database. SetC and SetD are similar to FlhC and FlhD, the master activators of flagellar transcription (17), and SetR is homologous to CI^{434} and other related lambdoid phage repressors. Deletion of the region from *s079* to *s085* (Δ13) reduced both SXT and CloDF13 transfer to below detectable limits. Additionally, unlike all previously mentioned mutations except the *int* deletion, this mutation resulted in the absence of a detectable excised circular form of the element (Table 3). The deficiency in SXT excision and transfer in the Δ13 mutant strain could be attributed to the activities of two of the eight deleted genes, designated *setC* and *setD*. Single deletions of either *setC* or *setD* abolished SXT excision and transfer, and these properties could be restored to the mutants by complementation with the individual genes on a plasmid (Table 3). The findings that SetC and SetD were indispensable for both SXT chromosomal excision and formation of the conjugative apparatus, two critical and distinct processes required for SXT mobility, suggested that these gene products may function as regulators.

TABLE 4. Isolation of an SXT *oriT*

Donor strain[a]		Transfer frequency (10^{-5})[b]
Plasmid	SXT	
pCR2.1	−	<0.029
pCR2.1	+	<0.024
pOriT	−	<0.046
pOriT	+	8.2

[a] Donor strains were derivatives of BW25115 either lacking (−) or containing (+) SXT.
[b] Transfer frequency was calculated as the frequency of transconjugants per donor. In all cases, the recipients were BI533.

Since SetC and SetD exhibited similarity to FlhC and FlhD, known transcription activators, we tested whether expression of an arabinose-inducible *setDC* could activate expression of chromosomal *lacZ* transcriptional fusions to genes important for SXT excision and transfer, such as the previously described *int* and *tra* genes. These *lacZ* fusions were created by modification of the template used to generate the PCR products for the one-step chromosomal gene inactivation (see Materials and Methods). These fragments were introduced into the chromosomally integrated SXT, generating a substitution of *lacZ* and *cat* (encoding resistance to chloramphenicol) in place of the gene of interest.

Experiments with these reporter strains demonstrated that

TABLE 5. SetD and SetC stimulate SXT transfer gene expression

Site of fusion[a]	β-Galactosidase activity[b]	
	−	+
Δ(*traL-traA*)::*lacZ*	1.3	53
Δ*s003*::*lacZ*	6.5	320
Δ*traG*::*lacZ*	10	340
Δ*setDC*::*lacZ*	8.4	340
Δ*floR*::*lacZ*	46	38

[a] All strains are derivatives of BW25113 (7) containing SXT.

[b] Cells containing a plasmid with *setDC* under control of an arabinose-inducible promoter (pSetCD) were grown in LB broth containing 100 μg of ampicillin/ml for 2.5 h. Arabinose was then added to half of the culture to a final concentration of 1 mM, and the cells were grown for an additional 1.5 h. β-Galactosidase activity, reported in Miller units, was measured as described by Miller (21). The values presented are the means of at least three independent experiments. +, *setDC* expression; −, no *setDC* expression.

SetC and SetD serve as transcriptional activators of genes important for SXT excision and transfer. An arabinose-inducible *setDC* was introduced into strains containing *lacZ* fusions in place of *s003*, which likely represents the first gene in the *int*-containing operon, and in place of *traL-traA* and *traG* (Table 5), which are in two different putative pilus assembly operons. In the absence of arabinose, the β-galactosidase activities of these three fusions were low (≤10 Miller units [Table 5]). Induction of *setDC* expression from the plasmid resulted in a minimum 34-fold activation of *lacZ* expression in these fusions. SetC and SetD also appear to regulate their own expression, since expression of *setDC* resulted in a 40-fold activation of a *lacZ* fusion in place of *setD* (Table 5). In contrast, a *lacZ* fusion in place of *floR*, a gene that confers resistance to chloramphenicol and whose activity is not important for SXT transfer or excision, was not regulated by *setDC* expression. These results indicate that, in either a direct or indirect fashion, SetC and SetD activate the expression of genes required for SXT excision and transfer, and they suggest that the stimulatory effect of SetC and SetD is specific to SXT genes induced during SXT mobilization.

As mentioned above, SetR, the last ORF of SXT, has homology to CI^{434} and other lambdoid repressors. Interestingly, deletion of *setR* from SXT could be accomplished only in a strain complemented in *trans* with a plasmid expressing *setR*. We did not observe loss of the complementing plasmid in the deletion strain. In contrast, this plasmid was unstable in isogenic cells either lacking SXT or containing a wild-type ($setR^+$) SXT (data not shown). These observations suggest that removal of SetR, presumably a CI-like repressor, is deleterious to cell growth in the presence of SXT.

DISCUSSION

The chimeric nature of SXT is evident from analyses of its DNA sequence and functional properties. SXT contains genes related to the F plasmid genes that encode the DNA-processing, pilus assembly, and mating pair formation functions of conjugative DNA transfer. This is not the case for Tn*916* (8) and CTnDOT, two well-characterized CTns whose conjugative transfer genes are unrelated to those found on plasmids. However, SXT is not simply a plasmid that integrates; in contrast to plasmids, its genome did not contain genes required for either autonomous replication or segregation. SXT chromosomal integration and excision and the recombination events underlying these processes closely resemble lambda phage integration and excision (15). Regulation of SXT transfer may also be similar to control of lambda lysogeny. This hypothesis is suggested by our observation that *setR*, the lambda CI-like repressor found in the SXT genome, could not be deleted in the presence of SXT. *setC* and *setD*, SXT-encoded transcription factors with similarity to the chromosomal regulators of flagellar gene transcription FlhC and FlhD, also play central roles in control of SXT transfer.

The mosaic nature of SXT is underscored by the observation that its antibiotic resistance genes and the putative transposases that surround them were apparently a late addition to the element. The composite transposon-like structure in which the SXT antibiotic resistances are found has a different G+C content than the remainder of the element, and deletion of the entire region did not influence SXT mobility. Also, CTns closely related to SXT, such as R391 and the SXT-like elements found in contemporary Asian *V. cholerae* El Tor O1 clinical isolates, contain different antibiotic resistance genes found in different locations in the SXT "backbone" (13).

The SXT-encoded gene products essential for pilus assembly and DNA processing are similar to and maintain genetic organization with several recently published putative conjugation systems found in several other gram-negative bacteria, including the conjugative plasmids R27 from *S. enterica* serovar Typhi (29), pNL1 from *Sphingomonas aromaticivorans* (26), and Rts1 from *E. coli* (Murata et al., Abstr. 101st Gen. Meet. Am. Soc. Microbiol.), and the gonococcal genetic island from the chromosome of *Neisseria gonorrhoeae* (Dillard, personal communication). Many of the genes flanking the antibiotic resistance genes in *Salmonella enterica* serovar Typhimurium DT104 (4) have significant similarity to those encoded in the SXT conjugation system; however, mobilization of the DT104 element has not been detected. Interestingly, all of these conjugation systems (with the exception of the DT104 island) also contain a *dsbC*-like allele within a putative pilus assembly operon, though its role in conjugative transfer has not been demonstrated. In addition, Rts1 (Murata et al., Abstr. 101st Gen. Meet. Am. Soc. Microbiol.) and *S. enterica* serovar Typhimurium DT104 (4) contain a homolog of SetC but lack *setD*. R27, pNL1 and the gonococcal genetic island do not contain homologs of these genes.

SetC and SetD are the first examples of FlhC- and FlhD-related proteins that are involved in the regulation of conjugative transfer. FlhC and FlhD were initially described as regulators of flagellar gene transcription in *E. coli* (17), but more recent studies have revealed that these genes are involved in regulating diverse processes, including cell division (FlhD only), cell shape (24), virulence factor production (18), and anaerobic respiration (23). In *E. coli*, the products of the *flhD* master operon, FlhD and FlhC, form a D_2C_2 heterotetrameric complex that binds to class II promoters of the flagellar regulon and activates transcription (19). Our work suggests that SetC and SetD may function in a similar fashion. Both *setC* and *setD* are required for SXT conjugative transfer. Further studies are required to determine if SetC and SetD oligomerize and directly activate transcrip-

tion, as is the case with FlhC and FlhD. FlhD and FlhC sit at the top of the regulatory hierarchy governing expression of the flagellar operons. It is not possible to discern from our present study whether there is a similar hierarchy regulating expression of the genes encoding the SXT conjugation system. Given the relatively weak similarity of SetC and SetD to FlhC and FlhD, it seems unlikely that these SXT-encoded regulators interact with the chromosome-encoded regulators, though this possibility remains to be examined.

Since SetD and SetC activate SXT transfer, exploring the factors regulating their production will provide insight into how SXT transfer (and thereby antibiotic resistance gene transfer) is regulated. We hypothesize that SetR, the SXT gene product with similarity to the λ CI repressor, may be involved in the regulation of *setDC* expression. Two observations support this idea. First, *setR* could be deleted only when SetR was provided in *trans*, suggesting that SetR represses expression of SXT-encoded genes whose overproduction is toxic. Second, overexpression of *setDC* was toxic in SXT-containing cells. Ongoing work is focused on the role of SetR in the regulation of conjugative transfer of SXT.

Our study illustrates the considerable value of the one-step chromosomal gene inactivation technique to carry out "functional genomics." Using this technique, we deleted large portions of a 100-kb chromosomal element in a simple and rapid fashion. Phenotypic examination of these large deletions facilitated the identification of regions of interest for creation of in-frame deletions of single ORFs. Introduction of the promoterless *lacZ* allele into the vector used to generate the gene replacement product allowed the examination of regulatory circuits without disrupting the native regulatory elements. Similar vectors could be constructed for the introduction of other sequences on the chromosome to create additional transcriptional reporters, as well as fusion proteins (e.g., green fluorescent protein fusions or epitope tags). Thus, with minor modifications, the one-step chromosomal gene inactivation technique allows studies of gene expression and protein production and localization without relying on plasmid constructs.

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