Control of SXT Integration and Excision

Vincent Burrus and Matthew K. Waldor*

*Department of Microbiology and Medicine, Tufts University School of Medicine,
and Howard Hughes Medical Institute, Boston, Massachusetts 02111*

The *Vibrio cholerae* SXT element is a conjugative self-transmissible chromosomally integrating element that encodes resistance to multiple antibiotics. SXT integrates in a site-specific fashion at *prfC* and excises from the chromosome to form a circular but nonreplicative extrachromosomal form. Both chromosomal integration and excision depend on an SXT-encoded recombinase, Int. Here we found that Int is necessary and sufficient for SXT integration and that *int* expression in recipient cells requires the SXT activators SetC and SetD. Although no *xis*-like gene was annotated in the SXT genome, Int was not sufficient to mediate efficient SXT chromosomal excision. We identified a novel SXT Xis that seems to function as a recombination directionality factor (RDF), facilitating SXT excision and inhibiting SXT integration. Although unrelated to any previously characterized RDF, Xis is similar to five hypothetical proteins that together may constitute a new family of RDFs. Using real-time quantitative PCR assays to study SXT excision from the chromosome, we determined that while SXT excision is required for SXT transfer, the percentage of cells containing an excised circular SXT does not appear to be a major factor limiting SXT transfer; i.e., we found that most cells harboring an excised circular SXT molecule do not act as SXT donors. In the absence of *prfC*, SXT integrated into several secondary attachment sites but preferentially into the 5′ end of *pntB*. SXT excision and transfer from a donor containing *pntB*::SXT were reduced, suggesting that the SXT integration site may also influence the element's transmissibility.

Integrative and conjugative elements (ICEs) are now recognized as a large and diverse class of mobile genetic elements in both gram-negative and gram-positive organisms (9). ICEs excise from the chromosomes of their hosts, transfer to a new host via conjugation, and then integrate into the chromosome again. These elements encode diverse excision, recombination, and conjugation systems, as well as many other properties, such as resistance to antibiotics (25, 30), nitrogen fixation (27), and degradation of aromatic compounds (24). ICE integration can be more or less site specific, and a large variety of sequences are used as targets for integration.

The SXT element is a *Vibrio cholerae*-derived ICE that has also been referred to as a conjugative transposon (29) and a constin (17). SXT was originally isolated in 1993 from MO10, a *V. cholerae* serogroup O139 clinical isolate. The MO10-derived SXT, SXT^{MO10}, encodes resistance to sulfamethoxazole, trimethoprim, chloramphenicol, and streptomycin (29). After the extensive cholera outbreaks on the Indian subcontinent caused by *V. cholerae* O139 in late 1992 and 1993, *V. cholerae* O1 reemerged and was found to harbor an ICE, designated SXT^{ET}, that is very similar to SXT^{MO10} but contains different antibiotic resistance genes (15). Other SXT variants that lack antibiotic resistance genes have also been recognized in recent *V. cholerae* O139 isolates. SXT-related ICEs are currently found in virtually all *V. cholerae* clinical isolates from the Indian subcontinent. Also, SXT-related elements have been detected in recent *V. cholerae* clinical isolates from Mozambique and South Africa (11).

SXT-related elements are not unique to *V. cholerae*. We found SXT in *Providencia alcalifaciens* clinical isolates from patients in Bangladesh (15). R391, which mediates kanamycin and mercury resistance and was originally derived from a 1972 South African *Providencia rettgeri* isolate (10), and other IncJ elements are functionally and genetically related to SXT elements (14). In fact, our recent comparison of the complete DNA sequences of SXT^{MO10} and R391 revealed that both of these elements contain a highly conserved genetic backbone that mediates their regulation, excision-integration, and conjugative transfer (3, 4, 6). In the laboratory, SXT can be transferred by conjugation to a variety of gram-negative organisms (29). In addition, SXT can mediate the transfer of certain mobilizable plasmids and of chromosomal DNA in an Hfr-like fashion (16).

An autonomously replicating extrachromosomal form of SXT^{MO10} has not been isolated. Instead, this 99.5-kb ICE is found integrated into the 5′ end of the chromosomal gene *prfC*, which encodes the peptide chain release factor 3 (RF3) involved in translation regulation (17). SXT integration disrupts the 5′ end of *prfC* and provides a novel 5′ coding sequence for *prfC* and a promoter that leads to expression of a functional RF3. All the SXT-related ICEs that have been studied, including R391, are also integrated into *pfrC* (14). These elements encode a tyrosine recombinase, Int, which is related to the λ family of site-specific recombinases. In a previous study, it was shown that the excision and integration mechanisms of SXT resemble those of lambdoid phages (17). Like λ, the integrated SXT excises from the chromosome to form a circular extrachromosomal molecule. This episomal form of SXT appears to be an intermediate required for transfer. Recombination occurs between short sequences that are nearly identical on the circular form of the element (*attP*) and at the

* Corresponding author. Mailing address: Dept. of Microbiology, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111. Phone: (617) 636-2370. Fax: (617) 636-0337. E-mail: matthew .waldor@tufts.edu.

TABLE 1. *E. coli* K-12 derivative strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype[a]	Reference or source
Strains		
CAG18439	MG1655 *lacZU118 lacI42*::Tn*10*	26
KB1	MG1655 *recA56 gutA52 gutR*::Tn*10*	K. Bettenbrock, unpublished data
HW220	CAG18439 *prfC*::SXT	17
BI533	MG1655 Nal^r	16
BW25113	*lacI^q rrnB$_{t14}$ ΔlacZ$_{WJ16}$ hsdR514 ΔaraBAD$_{AH33}$ ΔrhaBAAD$_{LD78}$*	12
VI11	CAG18439 *prfC*::SXT Δ*s002*	This study
VI38	CAG18439 *prfC*::SXT Δ*s003*	This study
VI61	CAG18439 Δ*lacZ*::*attP-cat*	This study
VI100	CAG18439 *prfC*::SXT Δ*xis*	This study
VI141	CAG18439 Δ*prfC pntB*::SXT	This study
VI152	KB1 Δ*prfC ysgA*::SXT	This study
SM10λ*pir*	F$^-$ *recA*::RP4-2-Tc::Mu Km λ*pir*	20
Plasmids		
pGP704	*oriR6K mobRP4* Ap^r	20
pVI4	pGP704 *s003-int-attP*	This study
pVI5A	pGP704 *s003-s002-int-attP*	This study
pVI6A	pGP704 *s002-int-attP*	This study
pVI6AM1	pVI6A *xis'*	This study
pVI6AM2	pVI6A *s002'*	This study
pVI8A	pCRII-TOPO *attP*	This study
pVI11	pKD3 *attP* Cm^r PCR template for one-step chromosomal gene activation	This study
p*attP*	pGP704 *attP*	This study
pInt33	pBAD33 *int*$_{SXT}$$^+$	B. Hochhut, unpublished data
pSetCD	pBAD-Topo *setCD*	4
pSetCD33	pBAD33 *setCD*	J. Beaber, unpublished data
pXis	pBAD-Topo *xis*	This study
pKD3	Cm^r PCR template for one-step chromosomal gene activation	12
pKD4	Km^r PCR template for one-step chromosomal gene activation	12

[a] Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Km^r, kanamycin resistant; Nal^r, naladixic acid resistant.

chromosomal site (*attB*) during integration and between *attL* and *attR* during excision. Int is necessary both in the donor cells for SXT excision and in the recipient cells for SXT integration. Both SXT excision and integration occur independent of *recA*.

The ~99.5-kb DNA sequence of SXT^{MO10} was recently determined, and SXT genes involved in conjugative transfer and chromosomal excision were identified (4). SXT utilizes a conjugation system related to that encoded by the F plasmid; two SXT loci, *setC* and *setD*, encode transcriptional activators required for SXT excision and transfer. Although the annotation of the SXT genome did not identify any genes encoding a recombination directionality factor (RDF) (19), most of the integrating elements that have been studied, including phages and ICEs, encode an RDF. The λ RDF, Xis, is required during the Int-catalyzed recombination reaction between *attL* and *attR* to facilitate phage excision, and it inhibits recombination between *attP* and *attB*, preventing integration (2). λ Xis binds to and bends DNA in *attP* and *attR* to influence the architecture of the nucleoprotein complexes at these sites to either inhibit or favor recombination. In the present study, we investigated which SXT genes are required for the element's integration and excision by using real-time quantitative PCR to measure the frequency of SXT excision. While Int proved to be necessary and sufficient for integration, excision required a novel SXT-encoded Xis. The SXT Xis acts as an RDF, promoting SXT excision and inhibiting SXT integration. Determination of the percentage of cells containing an excised circular SXT revealed that the majority of such cells do not act as SXT donors.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are described in Table 1. Bacterial strains were routinely grown in Luria-Bertani (LB) broth at 37°C on a roller drum incubator and were maintained at −80°C in LB broth containing 15% (vol/vol) glycerol. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 50 μg/ml; nalidixic acid, 40 μg/ml; streptomycin, 200 μg/ml; sulfamethoxazole, 160 μg/ml; trimethoprim, 32 μg/ml; and tetracycline, 12 μg/ml.

Bacterial conjugation. Conjugation assays were performed by mixing equal volumes of overnight cultures of donor and recipient strains. The cells were harvested by centrifugation and resuspended in 0.05 volume of LB broth. The cell suspensions were poured onto LB agar plates supplemented, when required, with 0.02% arabinose or 0.2% glucose. The conjugation mixtures were incubated at 37°C for 6 h. The cells were then resuspended from the plates in 1 ml of LB medium, and serial dilutions were plated on the appropriate selective media to determine the numbers of donors, recipients, and exconjugants.

Plasmid and strain construction. Plasmids pVI4, pVI5A, and pVI6A (Table 1) were constructed by PCR amplification of the SXT *attP* region that resulted from excision of SXT with primers repKOF and orfX1 (Table 2) and template DNA from a Δ*s002* SXT (VI11), a wild-type SXT (HW220), and a Δ*s003* SXT (VI38), respectively. Then PCR products were purified from a 1% agarose gel by using a QIAquick gel extraction kit (Qiagen) and cloned into the TA cloning vector pCRII-TOPO (Invitrogen) according to the manufacturer's instructions. The subcloned fragments were removed from the pCRII-TOPO vector by digestion with *Kpn*I/*Xba*I (pVI4) or *Eco*RI (pVI5A and pVI6A) and then ligated to *Kpn*I/*Xba*I-digested or *Eco*RI-digested pGP704. pGP704 contains the RP4 mobilization locus, the R6K conditional origin of replication, and an ampicillin resistance gene (20). Frameshift mutations in *s002* and *xis* were introduced by in vitro site-directed mutagenesis of pVI6A by using a QuikChange XL site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The *s002* frameshift mutation in pVI6AM2 was created with primers O2QCF and O2QCR, and the *xis* frameshift mutation in pVI6AM1 was created with primers MVI1F and MVI1R. pVI8A was constructed by PCR amplification of the SXT *attP* region with primers CAttPF and CAttPR. After purification, the PCR product was cloned into the TA cloning vector pCRII-TOPO. pVI11 was

TABLE 2. DNA sequences of the oligonucleotides used in this study

Primer	Nucleotide sequence (5′ to 3′)
repKOF	TTGCTCTGGTAAATCGGCTAGG
orfX1	TACGCAGCCCCTTGCTAAAG
XisTF	TGATTTTCAGGAGAATGCAGA
XisTR2	TCAGTCCTCTTTCTTCTCATAC
CAttPF	TTCGAAGGTTTAGCCACAGTTGTTTATGAGTG
CAttPR	TTCGAATATTCCGCTTTTGTAATGTCGAAA
LacZWF	TTGTGAGCGGATAACAATTTCACACAGGAAACAGCTGTGTAGGCTGGAGCTGCTTCG
LacZWR	GCGAAATACGGGCAGACATGGCCTGCCCGGTTATTACATATGAATATCCTCCTTA
XisWF	GATGATAGCTTTACATTGATTTTCAGGAGAATGCAGGTGTAGGCTGGAGCTGCTTCG
XisWR	CAACTGGCGTTTCGGACAATAAAAGAAAGAGGCCATATGAATATCCTCCTTA
PrfCWF	TAGCCGCAATTTTTCGTTTTCAACAAGCGCGGCGGGTGTAGGCTGGAGCTGCTTCG
PrfCWR	CCGTAAGCGGCTAATAAGGAAGGGAAATTGACAGGGCATATGAATATCCTCCTTA
MVI1F	GATGTTAGAAAAGGGGTACCTGGTGCGCTACTG
MVI1R	CAGTAGCGCACCAGGTACCCCTTTTCTAACATC
O2QCF	GTTGTCGTCAACATTCCCCCTAGGGAGCCACCCTGAACTGC
O2QCR	GCAGTTCAGGGTGGCTCCCTAGGGGGAATGTTGACGACAAC
ARB1	GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT
ARB2	GGCCACGCGTCGACTAGTAC
SXTR1	GCCAATTACGATTAACACGACG
SXTR2	CGGATTTGACAAGCGAAGAACTG
PrfCQF	GCTCAAAGGGCTGGTACAGC
PrfCQR	TTGTTGGAGATTGGACGGAAC
EattBF	GCCGCACTTTTGCCATTATT
EattBR	AGCAGCACCTTCTCGGTGAT
SXTJF	GCGAAGGACCTTTGCTATCATC
SXTJR	TGGTTTTAAGCGTTGAAAGGC

constructed by introducing the 600-bp *Bst*BI *attP*-containing fragment of pVI8A into *Bst*BI-digested pKD3. pKD3 contains a *cat* gene flanked by FLT recognition target (FRT) sites (12). p*attP* was constructed by introducing the *Sac*I/*Xba*I fragment of pVI8A into *Sac*I/*Xba*I-digested pGP704. pXis was constructed by cloning the *xis* gene amplified by PCR with primers XisTF and XisTR2 into the TA cloning expression vector pBAD-TOPO (Invitrogen) according to the manufacturer's instructions. The Δ*xis* mutation was introduced into SXT by using the one-step chromosomal gene inactivation technique (12) with primers XisWF and XisWR and the pKD4 template as previously described (4). Δ*prfC* derivatives of *Escherichia coli* MG1655 were created by using the same technique with primers PrfCWF and PrfCWR and pKD3 as the template. Similarly, *E. coli* strain VI61, used as a calibrator for the real-time quantitative PCR, was constructed by using primers LacZWF and LacZWR and pVI11 as the template. The PCR product was introduced into the chromosome of *E. coli* CAG18439 as described by Datsenko and Wanner (12), generating a substitution of *attP* and *cat* in place of the *lacZ* gene.

Molecular biology methods. Plasmid DNA was prepared with either a Qiaprep Spin miniprep kit or a Qiaprep miniprep kit (Qiagen), and chromosomal DNA was prepared with a G Nome DNA kit (Q-Biogene) as described in the manufacturer's instructions. Southern blotting was performed as described previously (28) with probes conjugated to horseradish peroxidase and detected with a chemiluminescent substrate (Amersham). The secondary integration sites of SXT were determined by performing arbitrary PCR as described by O'Toole and Kolter (22), with the following modifications. The first round of amplification was performed by using the arbitrary primer ARB1 (22) and the primer unique to the right end of SXT, SXTR1 (Table 2), in 50-μl PCR mixtures with a HotStarTaq Master Mix kit (Qiagen) and 30 ng of template DNA. The first-round PCR conditions were (i) 15 min at 95°C, (ii) 6 cycles of 20 s at 95°C, 30 s at 30°C, and 90 s at 72°C, (iii) 30 cycles of 20 s at 95°C, 30 s at 40°C, and 2 min at 72°C, and (iv) 5 min at 72°C. The second round of amplification was performed like the first round with 1 μl of the first-round reaction mixture as the template and primers ARB2 (22) and SXTR2, which was identical to the rightmost end of SXT (Table 2). The second-round amplification conditions were (i) 15 min at 95°C, (ii) 30 cycles of 20 s at 95°C, 30 s at 45°C, and 2 min at 72°C, and (iii) 5 min at 72°C. The purified PCR products were sequenced by using the SXTR2 primer at the Tufts Medical School DNA Sequencing Core Facility as described previously (29) and were compared with the GenBank DNA sequence database by using the BLASTN program (1).

Real-time quantitative PCR assays for relative quantification of *attB* and *attP*. Real-time quantitative PCR assays were developed to measure the percentages of cells in a culture that contained unoccupied SXT *attB* sites and the SXT *attP*

sequence (a measure of the amount of excised circularized SXT). The amounts of both *attB* and *attP* were normalized to the amount of chromosomal DNA in each sample; the latter quantity was determined by amplifying the 3′ end of *prfC* by using real-time quantitative PCR. An *E. coli* strain, VI61, containing single chromosomal copies of *attB* and *attP*, was constructed to calibrate the real-time quantitative PCR assays. Primer Express V1.5a (Applied Biosystems) was used to design primers EattBF and EattBR, primers SXTJ2F and SXTJ2R, and primers PrfCQF and PrfCQR, which were used for amplifying the 67-bp *attB* fragment, the 63-bp *attP* fragment, and the 66-bp *prfC* fragment, respectively (Table 2). The ABI PRISM 7700 sequence detection system (Applied Biosystems) was used to quantify the increase in fluorescence emission during PCR that resulted from binding of the dye SYBR Green I to double-stranded DNA. Sequence Detector software (version 1.7; Applied Biosystems) was used for data acquisition and analysis. Each 20-μl reaction mixture contained 10 μl of 2× SYBR Green PCR Master Mix (Applied Biosystems), each primer at a concentration of 500 nM, and 8 ng of the DNA template. The PCR conditions were (i) 2 min at 50°C, (ii) 10 min at 95°C, and (iii) 45 cycles of 15 s at 95°C and 1 min 60°C. Three reactions were performed for each sample. The mathematical model used for analysis of the data was developed by Pfaffl (23). Briefly, a standard curve was generated for each of the three primer sets by using VI61 DNA as the template by plotting the C_t values as a function of the concentration input of DNA (the C_t value was defined as the PCR cycle number at which the fluorescence rose above the background level). The PCR efficiencies (E) for particular primer sets were calculated by using the equation $E = 10^{(-1/\text{slope})}$ (slopes were calculated from the standard curves). The relative ratio of the amplified *att* sequences was calculated based on E and the C_t deviation (ΔC_t) of an experimental sample compared with the control sample (VI61 chromosomal DNA) and was expressed in comparison to the calibrator sequence (the 3′ end of *prfC*) by using the following equation:

$$R_{att} = \frac{(E_{att})^{\Delta C_{t(att)}}}{(E_{prfC})^{\Delta C_{t(prfC)}}}$$

where E_{att} is the PCR efficiency of the *att* site tested (*attB* or *attP*), E_{prfC} is the PCR efficiency of the calibrator sequence (the 3′ end of *prfC*), $\Delta C_{t(att)}$ is the difference between the C_t value of the reference strain and the C_t value of the strain tested [$C_{t(VI61)} - C_{t(sample)}$] for the *att* site tested, and $\Delta C_{t(prfC)}$ is the difference between the C_t value of the reference strain and the C_t value of the strain tested [$C_{t(VI61)} - C_{t(sample)}$] for the calibrator sequence. For convenience, the ratio R_{att} was expressed as a percentage (i.e., as the copy number of *att* sites per hundred chromosomes). The measured PCR efficiencies for the amplifica-
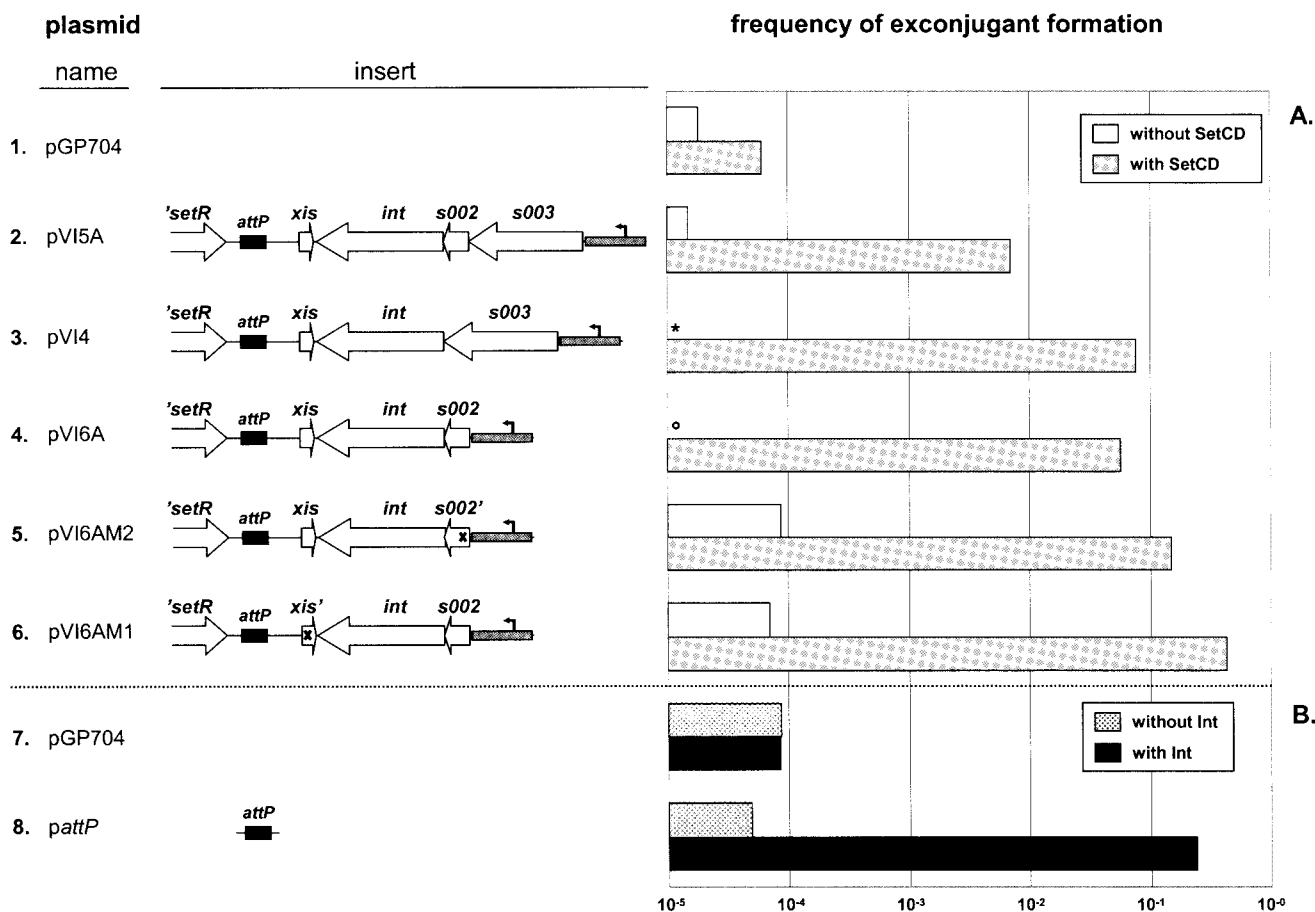
FIG. 1. Genes required for SXT integration. Integration of replication-deficient plasmids containing the SXT *attP* site was assessed by a conjugation assay. The inserts in these plasmids, all of which were derived from the mobilizable suicide vector pGP704, are indicated. In the diagrams of the inserts, open reading frames are indicated by arrows, the SXT *attP* site is indicated by a black box, the putative promoter region upstream of the *int*-containing operon is indicated by a gray box with an arrow, and the frameshift mutations are indicated by x's. The frequency of exconjugant formation was obtained by dividing the number of exconjugants (Tc^r Ap^r CFU) by the number of recipients (Tc^r CFU). In all the cases, the donor strain was *E. coli* SM10λ*pir* and the recipient strains were *E. coli* CAG18439 derivatives. For the plasmids in panel A, the recipient cells harbored pSetCD33, which contained *setCD* under control of P$_{BAD}$. For the plasmids in panel B, the recipient cells harbored pInt33, which contained *int* under control of P$_{BAD}$. To induce expression of *setCD* or *int*, the conjugation assays were carried out by using media supplemented with 0.02% arabinose. The bars indicate the mean values obtained from two independent experiments. The asterisk and the circle in lines 3 and 4 indicate that the frequencies of exconjugant formation were less than 10^{-5} exconjugant/recipient; in line 3, the frequency was 9.7×10^{-6}, and in line 4, the frequency was 7.0×10^{-6}.

tion of *attB*, *attP*, and *prfC* were 1.89, 2.00, and 1.97, respectively (ideally, the values are 2.00).

RESULTS

SetCD is required for *int* expression, and Int is sufficient to mediate SXT integration. In previous studies, it was determined that *int*, which lies near one end of SXT, is required for SXT integration (17). *int* is part of a putative operon downstream of *s002* and *s003*, two hypothetical genes that are apparently not required for SXT transfer (4, 17). We constructed several plasmids based on the mobilizable Ap^r suicide vector pGP704 (20) to investigate whether *int* is sufficient to mediate integration of the SXT attachment site (*attP*) into *prfC*. This vector requires the product of *pir* to replicate, so after its conjugative transfer from a *pir*$^+$ host to a *pir* host, Ap^r exconjugants are isolated only following plasmid integration. The largest of the inserts in the plasmids (pVI5A) contained *attP*

plus 3.6 kb of adjacent sequence, including *int*, *s002*, and *s003* (Fig. 1A). The frequency of exconjugant formation for each of the plasmids tested did not differ from the background frequency of exconjugant formation observed with pGP704 (~10^{-5}) (Fig. 1A). This background frequency likely resulted from random integration of pGP704 into the recipient strain. Thus, the entire putative *int*-containing operon and *attP* were insufficient to promote site-specific integration of the plasmids into the chromosome. A control conjugation assay demonstrated that all the plasmids shown in Fig. 1 could be transferred at the same frequency to a *pir*$^+$ host, indicating that their different inserts did not alter their capacities to be mobilized (data not shown).

Since we previously reported that SetC and SetD stimulate the expression of *s003* and presumably *int* as well (4), we hypothesized that expression of *setCD* in recipient cells might be required to promote integration of the plasmids described

above. To test this idea, we introduced a SetCD expression vector into the recipient strain described above. When this new recipient was used, we observed an increase of nearly 3 orders of magnitude in the frequency of exconjugant formation with pVI5A, the plasmid containing the largest insert (Fig. 1A). Deletion of either or both of the hypothetical genes located 5′ of *int*, *s002*, and *s003* did not reduce the SetCD-dependent formation of exconjugants (Fig. 1A, lines 3 to 5). In fact, pVI5A derivatives that lacked *s002* and/or *s003* appeared to integrate with greater efficiency than pVI5A, suggesting that *s002* and/or *s003* may decrease integration efficiency. Alternatively, expression of *int* from the presumed promoter upstream from *s003* may have been altered in these plasmids. Overall, these results indicate that *int*-mediated integration of the SXT *attP* site requires SetCD in the recipient. This integration process is not dependent on homologous recombination in the recipient as similar results were obtained in a *recA* recipient (data not shown).

To assess whether SetC and SetD have a direct role in integration or function solely as regulators of *int* expression, we tested if the integrase itself, expressed in the recipient strain under control of an arabinose-inducible promoter, was sufficient to catalyze the integration of p*attP*, a pGP704-based plasmid containing a 600-bp fragment encompassing the SXT *attP* site. The frequency of exconjugant formation in a recipient expressing *int* was more than 3 orders of magnitude higher for p*attP* than for pGP704 (Fig. 1B). These results indicate that Int is the only SXT-encoded protein required to mediate SXT integration; the lack of integration initially observed in the experiments described above was most likely due to a lack of *int* expression. These observations also corroborate the idea that there is a SetCD-regulated promoter upstream of *s003* that regulates *int* expression.

Int alone does not promote efficient excision. Neither the annotated SXT (4) nor R391 (6) genome sequence was reported to encode a potential RDF (Xis-like protein). Since genes encoding Xis proteins are often located near *int* genes, we investigated whether pVI4, pVI5A, pVI6A, pVI6AM2, and p*attP*, once integrated into the chromosomes of the exconjugants described above, excised with equal efficiencies. To do this, we developed real-time quantitative PCR assays to measure the amounts of unoccupied *attB* sites and *attP* sequences (i.e., the amounts of excised circularized plasmid). In these assays, the amounts of *attB* and *attP* DNA were normalized to the copy number of chromosome DNA, which was determined by amplifying the 3′ end of *prfC*. The assays were also performed with a control SXT-free *E. coli* strain, strain VI61 (Table 1), which contains single chromosomal copies of *attB* and *attP*.

SetC and SetD were found to be required for excision of four of the five integrated plasmids, pVI4, pVI5A, pVI6A, and pVI6AM2 (Fig. 2). In the absence of these activators, virtually no *attB* was detected in cultures of cells containing the integrated forms of these plasmids. This was expected since *int* is required for SXT excision (17) and, as shown above, SetC and SetD are required for *int* expression. In the presence of SetCD, all four plasmids excised, and more than 90% of the cells contained an unoccupied *attB* (Fig. 2). Only a low percentage of *attP* was detected in these cells (data not shown), probably because the excised plasmids were lost by dilution due to an

inability to replicate in a *pir* background. Thus, all four of these integrated plasmids contained the genes and sequences necessary and sufficient to mediate plasmid excision.

In marked contrast to the SetCD-activated excision of these four plasmids, we detected almost no Int-activated excision of integrated p*attP*. Int was necessary and sufficient for integration of this plasmid, which contained only the SXT *attP* site (Fig. 1B). The pronounced difference between the excision of p*attP* and the excision of the four plasmids discussed above suggests that Int alone is not sufficient to promote excision. Rather, a sequence absent from p*attP* and present in the other plasmids is necessary. Apparently, neither *s002* nor *s003* encodes this other function since pVI6AM2, which lacks both functional *s002* and *s003*, excised at least as well as pVI5A (Fig. 2).

Identification of an SXT Xis. The analyses described above suggested that a gene between the 3′ end of *int* and *attL* that facilitates SXT excision was overlooked in our annotation of the SXT genome. In fact, a small open reading frame of 195 nucleotides directed toward *int* was identified 14 bp downstream from the *int* stop codon by using the gene prediction program GeneMark 2.4 (7). This sequence is preceded by a suitable ribosome binding site (AGGAGA), which is located 6 bp upstream from the ATG start codon. The putative 64-amino-acid protein that we called Xis is predicted to have a basic pI (pI 8.10) and to contain a helix-turn-helix DNA-binding motif (Dodd-Egan score, 3.32 [13]).

We assessed the involvement of *xis* in SXT excision by introducing a frameshift mutation in *xis* in pVI6A, which yielded pVI6AM1. The frameshifted *xis* should have produced only the 11 N-terminal amino acids of the putative native Xis protein. In striking contrast to cells harboring integrated pVI6A, cells harboring integrated pVI6AM1 contained virtually no detectable unoccupied *attB* sites even in the presence of SetCD (Fig. 2). This observation demonstrated that *xis* plays a critical role in facilitating SXT excision. In addition, *xis* appeared to have a negative effect on integration, since pVI6AM1 formed exconjugants more efficiently than pVI6A formed exconjugants (Fig. 1A, lines 4 and 6). Together, these observations suggest that *xis* encodes an RDF.

We further demonstrated that *xis* plays a critical role in excision of the entire SXT element. A Δ*xis* derivative of SXT was constructed by using the one-step chromosomal gene inactivation technique (12). As shown in Fig. 3, the amount of *attB* and *attP* per chromosome was reduced by more than 3 orders of magnitude in the Δ*xis* mutant of SXT compared to the amount in the wild-type SXT. Complementation analyses confirmed that *xis* promotes excision. As shown in Fig. 3, introduction of pXis, a plasmid containing *xis* under control of P_{BAD}, into the Δ*xis* SXT mutant markedly increased the percentage of cells containing the excised SXT element. Apparently, very few Xis molecules are required to enhance SXT excision, since the presence of pXis increased the percentage of cells containing *attP* and *attB* even in the absence of arabinose (Fig. 3). In contrast to the Xis-mediated increases in SXT excision observed in the Δ*xis* SXT strain, overproduction of SetCD in this background led to only small increases in SXT excision. This suggests that activation of *int* expression is not sufficient to complement the excision deficiency in the Δ*xis* background. Confirmation of this hypothesis was obtained
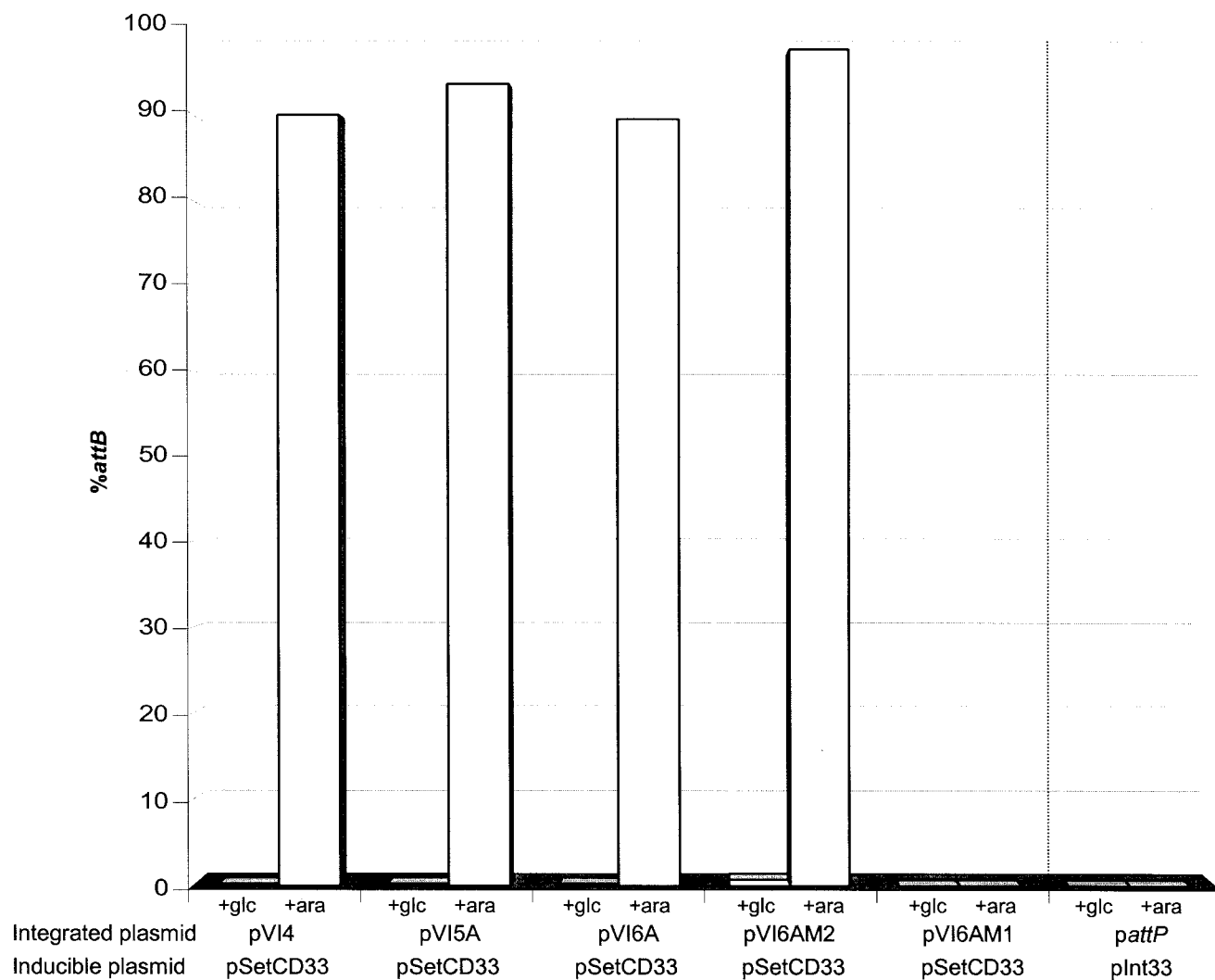
FIG. 2. Xis augments excision of integrated SXT-derived plasmids. The amount of an unoccupied *attB* site was quantified by real-time quantitative PCR. The means of triplicate measurements performed with each sample are shown. *E. coli* CAG18439, which contained one copy of *attB* per chromosome, was used as a control strain. DNA templates were extracted from strains containing the integrated plasmids described in Fig. 1 and pSetCD33 or pInt33 expressing *setCD* or *int* under control of P_{BAD}. Strains were grown overnight in LB medium supplemented either with 0.2% glucose (+glc) to repress expression or with 0.02% arabinose (+ara) to induce expression.

when we found that overexpression of Int under control of P_{BAD} did not enhance the frequency of excision of a wild-type SXT (data not shown).

Deletion of *xis* from SXT or overexpression of *xis* in the recipient reduces SXT transfer. The frequency of transfer of the Δ*xis* SXT mutant was reduced by nearly 3 orders of magnitude compared to the wild-type SXT transfer frequency (Fig. 3), suggesting that circular excised SXT molecules are required for SXT transfer. However, unlike the frequency of transfer of a Δ*int* mutant (17), the frequency of transfer was not zero for the Δ*xis* mutant. This observation is consistent with the finding that there is a detectable, although greatly reduced, amount of *attB* in the Δ*xis* SXT, suggesting that there is a very low level of Xis-independent SXT excision. The transfer frequency of the Δ*xis* SXT could be restored to nearly wild-type levels by provision of Xis in *trans* from pXis (Fig. 3). However, there did not appear to be a direct correlation between the number of copies

of excised circularized SXT per chromosome and the SXT transfer frequency. Overexpression of *xis* from pXis increased the percentage of cells containing detectable *attB* and *attP* to levels that were nearly 10-fold greater than those observed without *xis* expression, yet the SXT transfer frequency was increased only about twofold (Fig. 3C and E). In contrast, overexpression of *setCD* had a minimal effect on the percentage of cells containing *attB* and *attP* but led to a marked increase in SXT transfer (Fig. 3F). These results suggest that the frequency of SXT transfer is controlled both by regulation of SXT excision and by regulation, via SetCD, of the formation of the conjugal machinery.

The results of the plasmid transfer experiments discussed above suggested that Xis may lower the efficiency of exconjugant formation by reducing integration into the recipient chromosome (Fig. 1, lines 4 and 6). Further support for the idea that Xis negatively influences SXT integration was provided by
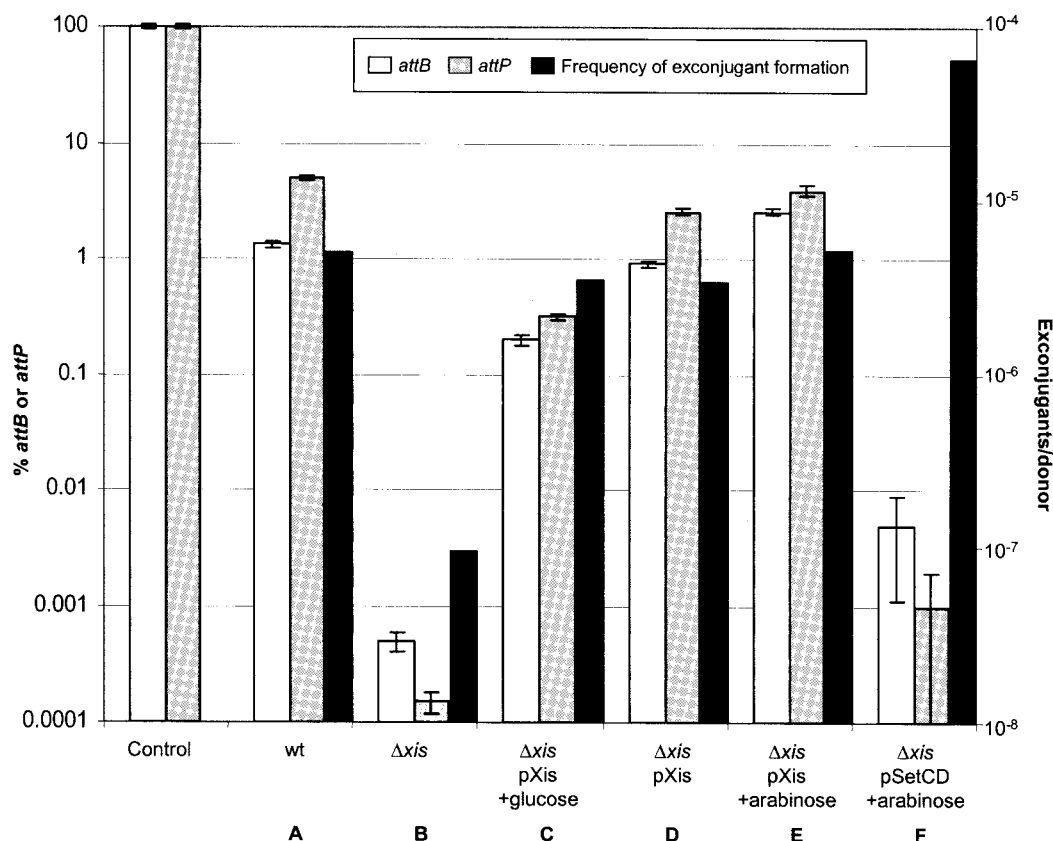
FIG. 3. Xis promotes excision and transfer of SXT. Real-time quantitative PCR was used to determine the percentage of unoccupied *attB* sites and *attP* sequences resulting from circularization of SXT. Triplicate measurements were obtained for each sample, and the mean and standard deviation are shown for each assay. The control strain *E. coli* VI61 (CAG18439 Δ*lacZ::attP-cat*) contained a single chromosomal copy of *attB* and *attP*. *E. coli* HW220 (CAG18439 *prfC*::SXT) was used as the wild-type (wt) strain. The Δ*xis* strain was *E. coli* VI100 (CAG18439 *prfC*::SXT Δ*xis*). DNA templates were prepared from overnight LB broth cultures containing 0.2% glucose or 0.02% arabinose where indicated. The frequencies of exconjugant formation were obtained by dividing the number of exconjugants (Nal[r] SXT[r] CFU) by the number of donor cells (Tc[r] CFU). In all the mating experiments, the recipient strain was *E. coli* BI533 (MG1655 Nal[r]). The solid bars indicate the mean values obtained from two independent mating experiments. The expression of *xis* by pXis and the expression of *setCD* by pSetCD in the donor strains were repressed with 0.2% glucose or were induced with 0.02% arabinose.

experiments in which *xis* was overexpressed from pXis in recipient cells. We found that the presence of Xis in recipients reduced the frequency of exconjugant formation by about 700-fold (data not shown).

SXT integrates into alternate sites in the absence of *prfC*. To examine whether SXT integration is limited to the *prfC* locus and whether integration at this site modulates SXT transfer, we constructed a Δ*prfC* mutant of *E. coli* MG1655. We first tested whether plasmid p*attP* (described in Fig. 1) integrates into this strain (Int was provided in *trans* in the recipient for these experiments). In the absence of *prfC*, p*attP* integration, which was measured by the frequency of exconjugant formation, was reduced by more than 2 orders of magnitude (Table 3). Despite this dramatic reduction in p*attP* integration into the Δ*prfC* background, this plasmid integrated more efficiently than pGP704 integrated (Table 3). The presence of *recA* in the recipient strain did not augment integration of p*attP*, suggesting that Int-mediated recombination of p*attP* with alternative *attB* sites accounts for integration of the plasmid in the Δ*prfC* background.

SXT integration into Δ*prfC* recipients was also reduced by more than 2 orders of magnitude (Table 3). Since SXT is

unable to replicate autonomously, the exconjugants recovered in these experiments likely represented integration into alternative chromosomal attachment sites. The SXT integration sites in two of these exconjugants (VI141 and VI152) were determined by sequencing the junctions between the right end of SXT and the chromosome. The resulting sequences were then compared to the complete sequence of the genome of *E. coli* K-12 (5). In the case of VI152, SXT was integrated into the putative gene *ysgA*, which encodes a protein of unknown function. SXT integration totally disrupted the open reading frame of *ysgA*, suggesting that it was no longer functional in VI152. In the case of VI141, SXT was integrated into the 5′ end of *pntB*, the last gene of the *pnt* operon, which encodes a pyridine nucleotide transhydrogenase. As a result of the integration, 30 amino acids from the N terminus of the predicted protein should have been replaced by 21 amino acids provided by SXT. Interestingly, in the absence of *prfC*, the 5′ end of *pntB* appeared to be a preferred alternate SXT integration site, since Southern analysis revealed that SXT was integrated into this site in 5 of the 12 SXT[r] exconjugants tested; all the other alternate integration sites were unique (data not shown).

The frequency of SXT excision from the secondary integra-

TABLE 3. Effect of the Δ*prfC* mutation on the frequency of
exconjugant formation

Donor strain[a]	Recipient strain[b]		Frequency of exconjugant formation $(10^{-5})^c$
	recA	*prfC*	
pGP704	+	+	8
p*attP*	+	+	25,000
	−	+	43,000
	−	−	205
	+	−	78
SXT	+	+	44
	+	−	0.1

[a] The donor strain was *E. coli* SM10λ*pir* containing either pGP704 or p*attP* or
E. coli BW25113 *prfC*::SXT.
[b] The recipient strains were the MG1655-derived strains CAG18439 (*recA*[+])
and KB1 (*recA*) and their Δ*prfC* derivatives. When pGP704 and p*attP* were used,
the recipient strains expressed Int from pInt33, as shown in Fig. 1.
[c] The frequency of exconjugant formation was calculated by determining the
number of exconjugants per recipient and is the mean from two independent
experiments. The mating experiments were carried out for 6 h at 37°C.

tion site *pntB* in VI141 was about 3.5-fold lower than the
frequency of SXT excision from *prfC* in HW220. Whereas
HW220 contains about 2.19% circular SXT molecules, VI141
was found to contain only 0.62% circular SXT molecules.
Thus, the frequency of SXT excision seems to be modulated by
the sequence within which SXT is integrated. The reduction in
SXT excision from VI141 may at least in part explain the
10-fold reduction in the frequency of SXT exconjugants ob-
served when VI141 was used as a donor (3.3×10^{-5} exconju-
gant/donor) compared to the frequency when HW220 was used
as a donor (3.0×10^{-4} exconjugant/donor). All eight SXT
exconjugants derived from the VI141 donor were found to
harbor SXT integrated into the 5′ end of *prfC*, its primary
integration site (data not shown). Thus, integration of SXT
into *pntB* in the donor did not alter the specificity for the 5′ end
of *prfC* in the recipient strain.

DISCUSSION

SXT integration into and excision from the chromosome of
host cells are critical steps in SXT maintenance and transmis-
sion. Int, the SXT integrase, is closely related to λ Int and like
this phage recombinase was found to be necessary and suffi-
cient for SXT integration. SetC and SetD, the SXT-encoded
transcription activators, were also found to be required for *int*
expression and thus for SXT integration. Int was also required
for SXT excision (17), but it was not sufficient for this process.
Instead, as is the case for numerous prophages and ICEs (19),
another protein, designated Xis, was also needed for efficient
SXT excision. Xis not only promoted SXT excision but also
appeared to inhibit SXT integration, suggesting that this novel
gene product functions as an RDF. Although SXT Xis is not
related to any known RDF, like λ Xis it is predicted to be a
small basic protein. It may therefore function like λ Xis to alter
the architecture of the integrase-DNA complex. Xis-mediated
SXT excision and SetCD-activated production of the SXT con-
jugal machinery appear to be independently controlled steps
that regulate SXT transfer.

Int was necessary and sufficient to promote integration of
mobilizable plasmids carrying the SXT *attP* site. *s003* and *s002*,
which are 5′ of *int* in SXT, were not required to promote

integration of these plasmids, and their functions remain ob-
scure. SetC and SetD were required in recipient cells to acti-
vate *int* expression from plasmids harboring *attP*, *int*, and the
presumed SetCD-activated promoter upstream of *s003*; this
result is consistent with the idea that *s003*, *s002*, and *int* con-
stitute an operon. The requirement for SetCD in recipient cells
to mediate integration of these plasmids suggests that SetCD-
activated de novo expression of *int* from SXT transferred into
recipient cells is required for SXT integration into the chro-
mosome. Since it was previously shown that *int* is required in
both donor and recipient cells for SXT transfer (17), such a
requirement for *int* expression in recipient cells differs from the
requirement for Tn*916*, where Int is thought to be transported
along with Tn*916* DNA through the mating pore into recipient
cells (8). Our findings may suggest that *setC* and *setD* are
transcribed very soon after SXT enters recipient cells. How-
ever, other experiments suggest that in the transfer of the
entire SXT element, *setC* and *setD* are not required in the
recipient cells (J. W. Beaber and M. K Waldor, unpublished
results). This finding indicates that activation of *int* expression
may result either from the transfer of SetCD from the donor to
recipient cells or from the early transcription of another acti-
vator encoded by SXT and absent from the inserts in the
plasmids used in this study.

Since the RDFs that have been characterized are a highly
diverse group of small proteins (19), it is not surprising that we
failed to annotate the SXT *xis* gene in our initial annotation of
the SXT genome (4). The SXT Xis protein is unrelated to any
previously described Xis protein and does not belong to any of
the RDF subgroups outlined by Lewis and Hatfull (19). How-
ever, *orf4* from R391, an ICE closely related to SXT (4, 6, 14),
is virtually identical to *xis* and is located in the same relative
position in the R391 genome as *xis* is in SXT. Our experimental
analyses indicate that *orf4* functions like SXT *xis* and that the
SXT *xis* gene restored wild-type levels of excision and transfer
to an R391 Δ*orf4* mutant (data not shown).

By using BlastP, five small hypothetical proteins from *My-
cobacterium leprae*, *Vibrio cholerae*, *Sinorhizobium meliloti*, *Meso-
rhizobium loti*, and *Brucella melitensis* were found to be similar
to Xis, exhibiting 34, 28, 45, 41, and 45% identity to Xis,
respectively. Like Xis, all but one of these putative proteins
was predicted to contain an N-terminal helix-turn-helix motif
by the Dodd-Egan method (13). In addition, a ClustalW align-
ment of Xis with these proteins indicated that their predicted
helix-turn-helix domains are related (Fig. 4). Since the genes
encoding RDFs are often linked to genes encoding integrases
in mobile elements, we looked for recombinase-encoding
genes near the *xis*-like genes. Whereas no recombinase genes
were close to *ml2429* of *M. leprae* and *vc1785* of *V. cholerae*, the
three genes encoding the other three hypothetical Xis-like
proteins were each closely linked to three different genes en-
coding related CP4-like tyrosine recombinases. This observa-
tion provides further evidence that these three *xis*-like genes
encode RDFs. The *xis*-like gene of *S. meliloti* and the linked
putative *int* gene are part of an unannotated 20,494-bp inte-
grative element. This element is apparently integrated into the
3′ end of a tRNA^Thr gene, since 14 bp of the 16-bp direct
repeat that flanks the element constitutes the 3′ end of this
tRNA. The SXT Xis and the five Xis-like proteins that we

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                                           HTH
                                  ┌────────────────────────┐
xis      1 ----------------MQMTAVAHQVTP---FLTSYEVMARYHISYTTLWRRIKDGSLPQP-----RINRNTRNKLWHIEDLEEYEK-KED-------- 66
VC1785   1 ------------------MGQQDIGEHP-MRFLKLKEVMEKTALSRSAIYRKMNDGEFPQSVSLGERAIAWVESEVDEWMDFCLKQR---------- 68
ML2429   1 MTSTNGPSARDSAGKSVRDTGSFEGQQPRTQFTTVAEVATLMRVSKMTVYRLVHNGELPAV-----RVGRSFRVHAKAVHDMLETSYFDVG-------- 86
SMc02201 1 ----------------------MKITDP---LLTVRFSAEVLQISVPTFWRHVADGTLPRDVKIG-GLSRWPQSEIIAVIERAKASRSNVA-------- 65
msr5461  1 --------------------MNFDHQDP---LLRDIEAAAILGMSKPTFWRRVRDKTAPQDVKIG-GMSRWPRSEILAVIERAKAAR-NAA-------- 66
BMEI1700 1 --------------------MQVTDP---LLIAREGAKYLQVSLPTFYRRIADGTLPKEIKMG-SLSRWSQSEILAAVEYAKAKR-NAGSPSPVKEG 72
```
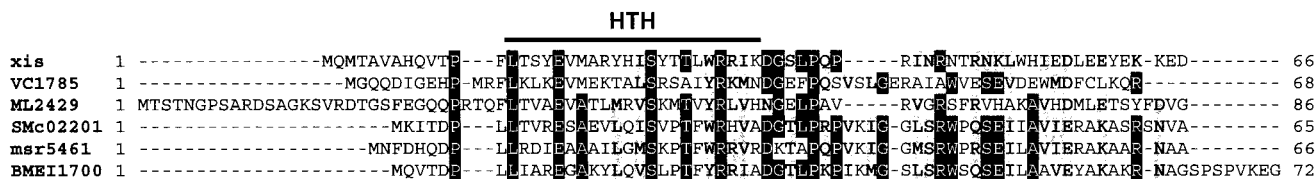
FIG. 4. ClustalW alignment of SXT Xis with five hypothetical proteins from *V.cholerae* (VC1785), *M.leprae* (ML2429), *S.meliloti* (SMc02201), *M.loti* (msr5461), and *B.melitensis* (BMEI1700). Amino acid residues that are identical in at least two-thirds of the sequences are indicated by a black background, and residues that are similar in at least two-thirds of the sequences are indicated by a grey background. The bar indicates the predicted helix-turn-helix (HTH) DNA-binding motif in SXT Xis, VC1785, ML2429, SMc02201, msr5461, and BMEI1700. These peptides have Dodd-Egan scores of 3.32, 4.13, 4.24, 2.57, 4.70, and 2.26, respectively.

identified may constitute a new subgroup of RDFs distinct from the 11 subgroups described by Lewis and Hatfull (19).

In most of the integrating mobile elements that have been studied, *xis* and *int* are part of the same operon and thus are cotranscribed. In SXT, *xis* and *int* are convergent genes that do not appear to be coregulated. While *int* expression requires SetCD, this does not seem to be the case for *xis*. The β-galactosidase activity of a *lacZ* fusion integrated at *xis* in SXT increased less than twofold following expression of *setCD* (data not shown).

As both Xis and Int are required for SXT excision, factors that influence the amounts and/or activities of these proteins are likely to control SXT excision, at least in part. As in SXT integration, *s002* and *s003* do not appear to have a significant effect on SXT excision. However, our data suggest that there may be an SXT-encoded factor that reduces Xis production or activity. We observed a much larger SetCD-mediated increase in the percentage of cells containing an unoccupied *attB* in the experiments with integrated plasmids (Fig. 2) than in the experiments with the entire integrated SXT (Fig. 3 and data not shown). A potential explanation for this difference is that SXT encodes a factor that is absent from the integrated plasmids that negatively regulates the Xis function. In λ, Xis levels are controlled by the Lon and FtsH proteases (18). Interestingly, SXT encodes a Lon orthologue (*s039*), but we do not know yet whether this presumed protease affects Xis levels. Besides the amounts and activities of Xis and Int, the site of SXT integration also appears to influence SXT excision. We observed a small reduction in SXT excision (3.5-fold) when SXT was integrated at *pntB* in a Δ*prfC* host.

While SXT excision is required for SXT transfer, the number of excised circular SXT molecules does not ordinarily appear to be a major factor limiting SXT transfer. We found that about 5% of cells grown in rich medium contained the SXT *attP*, yet the frequency of SXT transfer from these cells was less than 10^{-5} (Fig. 3A). The discrepancy between these frequencies indicates that most cells that harbor an excised circular SXT molecule do not act as SXT donors. Our data suggest that the factors that strongly influence SXT transfer are SetCD regulated since overexpression of these transcription activators greatly enhanced SXT transfer, even in the Δ*xis* SXT background (Fig. 3F), in which the percentage of cells harboring the SXT *attP* was greatly reduced. Presumably, the SetCD-regulated factor is the conjugative machinery, as all of the SXT *tra* genes that have been characterized are regulated by SetCD (4). The apparently distinct control of the formation of circular SXT molecules and the SXT conjugative machinery contrasts

with the control of Tn*916* excision and conjugation. In this well-characterized ICE, transcription of *xis*, *int*, and *tra* genes is usually coupled, since *tra* gene expression requires Tn*916* excision (21).

It was hypothesized previously that SXT integration into *prfC* may influence SXT function since *prfC* encodes RF3, a protein involved in regulation of translation (17). Integration of the *E. coli* pathogenicity island Pai I into *selC* (encoding a selenocysteine-specific tRNA) has been shown to have functional implications for the regulation of Pai I-encoded genes (25a). We found that SXT has a relaxed specificity of integration when *prfC*, the primary integration site, is absent. At least nine different secondary SXT integration sites were detected, and one of them, the 5′ end of the *pntB* gene, is a preferred target. SXT integrated at *pntB* in a Δ*prfC* background was still transmissible, indicating that RF3 is not required for either SXT excision or SXT transfer. However, RF3 may have subtle effects on SXT transfer. The strain harboring SXT integrated into *pntB* exhibited about a 10-fold reduction in the ability to transfer SXT compared to the ability of a strain containing SXT integrated into *prfC*. It is possible that the absence of RF3 from the *pntB*::SXT strain may account for this reduction. The site of integration of other ICEs, such as Tn*916*, has also been shown to influence the transmissibility of these elements (21).

The existence of alternative SXT attachment sites has potential implications for the transmissibility of SXT, as well as chromosomal sequences. Alternative attachment sites may broaden the SXT host range to include organisms that do not have conserved *prfC* orthologues. Finally, since SXT is able to mobilize chromosomal DNA in an Hfr-like manner, the existence of several alternative SXT integration sites may allow mobilization of genes located at virtually all positions in many prokaryotic genomes.

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