Autonomous Replication of the Conjugative Transposon Tn916

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ABSTRACT

Integrative and conjugative elements (ICEs), also known as conjugative transposons, are self-transferable elements that are widely distributed among bacterial phyla and are important drivers of horizontal gene transfer. Many ICEs carry genes that confer antibiotic resistances to their host cells and are involved in the dissemination of these resistance genes. ICEs reside in host chromosomes but under certain conditions can excise to form a plasmid that is typically the substrate for transfer. A few ICEs are known to undergo autonomous replication following activation. However, it is not clear if autonomous replication is a general property of many ICEs. We found that Tn916, the first conjugative transposon identified, replicates autonomously via a rolling-circle mechanism. Replication of Tn916 was dependent on the relaxase encoded by *orf20* of Tn916. The origin of transfer of Tn916, *oriT*(916), also functioned as an origin of replication. Using immunoprecipitation and mass spectrometry, we found that the relaxase (Orf20) and the two putative helicase processivity factors (Orf22 and Orf23) encoded by Tn916 likely interact in a complex and that the Tn916 relaxase contains a previously unidentified conserved helix-turn-helix domain in its N-terminal region that is required for relaxase function and replication. Lastly, we identified a functional single-strand origin of replication (*sso*) in Tn916 that we predict primes second-strand synthesis during rolling-circle replication. Together these results add to the emerging data that show that several ICEs replicate via a conserved, rolling-circle mechanism.

IMPORTANCE

Integrative and conjugative elements (ICEs) drive horizontal gene transfer and the spread of antibiotic resistances in bacteria. ICEs reside integrated in a host genome but can excise to create a plasmid that is the substrate for transfer to other cells. Here we show that Tn916, an ICE with broad host range, undergoes autonomous rolling-circle replication when in the plasmid form. We found that the origin of transfer functions as a double-stranded origin of replication and identified a single-stranded origin of replication. It was long thought that ICEs do not undergo autonomous replication. Our work adds to the evidence that ICEs replicate autonomously as part of their normal life cycle and indicates that diverse ICEs use the same replicative mechanism.

Integrative and conjugative elements (ICEs), also called conjugative transposons, are mobile genetic elements that encode proteins that mediate transfer of the element from the host cell (donor) to a recipient by conjugation. ICEs often contain additional (cargo) genes that can provide a selective advantage to the host cells (reviewed in references 1 and 2). Most ICEs have been identified based on their cargo genes and the phenotypes conferred. For example, many ICEs carry genes encoding antibiotic resistances. The horizontal dissemination of ICEs and their associated cargo genes is a major driver of bacterial genome plasticity and evolution and the spread of antibiotic resistances (for examples, see references 3, 4, 5, and 6).

ICEs are typically found integrated in a host chromosome and are passively inherited by vertical transmission via chromosomal replication and partitioning. When integrated, most ICE genes are repressed. However, under certain conditions, or stochastically, ICE genes required for excision and transfer are expressed and the ICE can excise from the genome. A site-specific recombinase (integrase) catalyzes this excision and the formation of a circular plasmid species that is a substrate for conjugative transfer. All functional ICEs that use a type IV secretion system (i.e., ICEs in organisms other than actinomycetes [7]) contain an origin of transfer *oriT* and encode a cognate relaxase. The relaxase nicks at a site in the *oriT* and becomes covalently attached to the 5' end of the DNA. The nicked double-stranded DNA (dsDNA) is unwound and the relaxase attached to the single-stranded DNA (ssDNA) is transferred via a type IV secretion system out of the donor and into a recipient cell to generate a transconjugant (re-

viewed in references 2 and 8). In the transconjugant, the relaxase catalyzes recircularization of the ssDNA, releasing a ssDNA circle and a free relaxase.

The DNA processing steps accompanying conjugative transfer are similar to the steps underlying rolling-circle replication of some plasmids and phages (reviewed in reference 9). Plasmid rolling-circle replication initiates when a relaxase encoded by the plasmid nicks at the origin of replication in the double-stranded DNA (the double-strand origin) and covalently attaches to the 5' end of the nicked strand. The nicked dsDNA is unwound, and unidirectional replication proceeds around the circle from the free 3' end. The relaxase recircularizes the nicked strand, releasing a ssDNA circle. The ssDNA circle typically contains a single-strand origin of replication (*sso*) that enables priming for DNA synthesis that converts the ssDNA to dsDNA.

ICEs that transfer ssDNA through a type IV secretion system were long thought to lack the ability to replicate autonomously (1,

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FIG 1 Genetic map of Tn916 and schematic for detecting excision products. The ends of Tn916 are indicated by black rectangles. (A) Map of Tn916. Gray and black arrowheads represent the genes in Tn916 and the direction of transcription. *orf23* and *orf22* encode homologues of the helicase processivity factor (HelP from ICEBs1), and *orf20* encodes the relaxase needed for DNA transfer and replication. All other gene names (numbers) are indicated above the corresponding genes. *oriT*(916) (44) and *sso916* are indicated with black lines. (B) Cartoon of the qPCR strategy to measure Tn916 replication. Excision of Tn916 from the chromosome of strain LDW173 produces (i) circular Tn916 containing site *att1*Tn916 and (ii) vacated chromosome site *att1*. The products are detected via qPCR using primers b and c (primers oLW526 and oLW527) or a and d (primers oLW542 and oLW543), which are represented by small arrows.

8, 10). ICEs can be maintained as integrated chromosomal elements, and it appeared that ICEs could rely exclusively on vertical transmission for inheritance. Furthermore, it is difficult to detect ICE replication, because activation and excision of most ICEs occur in a small fraction of donor cells.

However, there is compelling evidence that at least two ICEs undergo autonomous replication. When derepressed, ICEBs1 from *Bacillus subtilis* undergoes autonomous rolling-circle replication (11–13). This replication initiates at the origin of transfer (*oriT*) after nicking by the ICEBs1-encoded relaxase NicK (11). Processive unwinding of the nicked DNA is dependent on the host translocase PcrA and the ICEBs1-encoded helicase processivity factor HelP (12). ICEBs1 contains a single-strand origin of replication (*sso*) that enables second-strand synthesis (13). Recently, the ICE R391, a member of the SXT/R391 family of ICEs from *Vibrio cholerae* and *Providencia rettgeri*, was found to replicate autonomously in *Escherichia coli*, and the relaxase and *oriT* of R391 are important for R391 replication (14). The copy number of circularized SXT is also greater than the number of chromosomal sites from which it excised (15), indicating that SXT undergoes autonomous replication and that replication is a conserved feature of the SXT/R391 family of ICEs. R391 also encodes a conserved, functional plasmid partitioning system (14), also consistent with autonomous replication and segregation. Other ICEs and ICE-like elements may also be capable of autonomous, plasmid-like replication (16–18). However, most ICEs activate and excise in a small fraction of cells (15, 19–21), thereby hindering detection of replicating intermediates in population-based assays.

We were interested in determining if other ICEs found in Gram-positive bacteria are capable of autonomous replication. We focused on Tn916 (Fig. 1A), the first conjugative transposon identified (22, 23) and one of the most widely studied ICEs (8, 24). Tn916 and related elements (e.g., Tn1545) contain a gene conferring resistance to tetracycline, exhibit a broad host range (25), are found in many clinical isolates of *Enterococcus faecalis*, *Clostridium difficile*, and *Streptococcus pneumoniae* (reviewed in reference 26),

and can function in *B. subtilis* (27, 28). Unlike many ICEs (e.g., ICEBs1 and R391) that have a specific integration site, Tn916 can integrate into multiple sites in a host genome, with a preference for AT-rich regions (29, 30).

We found that Tn916 is capable of autonomous replication in *B. subtilis*. Replication was dependent on the relaxase encoded by Tn916 *orf20*. In addition, we found that the conjugative origin of transfer of Tn916, *oriT*(916), could also function as an origin of replication, and we identified a functional *sso* in Tn916. Our results demonstrate that Tn916 replicates autonomously by rolling-circle replication. These findings strengthen the model that many, and perhaps all, functional ICEs undergo autonomous replication as part of their normal life cycle.

MATERIALS AND METHODS

Media and growth conditions. *Bacillus subtilis* cells were grown in LB medium or the MOPS (morpholinepropanesulfonic acid)-buffered S7₅₀ defined minimal medium (31) as indicated. Cultures of cells containing pHP13-derived plasmids were grown for imaging and chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) in medium containing 2.5 µg/ml chloramphenicol to select for the plasmid as described previously (13). Cells containing *myc*-tagged *orf20* alleles were grown in medium containing 50 µg/ml spectinomycin to maintain the single-cross-over integrations. Where indicated, tetracycline (2.5 µg/ml) was added to Tn916-containing cells to increase gene expression and excision (32). Antibiotics were otherwise used at the following concentrations: kanamycin, 5 µg/ml; chloramphenicol, 5 µg/ml; spectinomycin, 100 µg/ml; tetracycline, 10 µg/ml; and a combination of erythromycin at 0.5 µg/ml and lincomycin at 12.5 µg/ml to select for macrolide-lincosamide-streptogramin (*mls*) resistance.

Strains. *E. coli* strains used for plasmid construction were AG1111 (MC1061 F' *lacI^q lacZM15* Tn10) (33) and TP611 (*thi thr leuB6 lacY1 tonA21 supE44 hsdR hsdM recBC lop-11 cya-610 pcnB80 zad::Tn10*) (34).

B. subtilis strains were derived from JH642 (*pheA1 trpC2*) (35, 36) and are listed in Table 1. Most strains were derived from JMA222, a derivative of JH642 that was cured of ICEBs1 (37). The *ssb-mgfpmut2* fusion is expressed from the *rpsF* promoter, and PrpsF-*ssb-mgfpmut2* was inserted by

TABLE 1 *Bacillus subtilis* strains used

Strain	Relevant genotype ^a (reference[s])
BS49	<i>metB5 hisA1 thr-5 att(yufKL)::Tn916^b att(ykyB-ykuC)::Tn916</i> (27, 40, 43)
JMA222	ICEBs1 ⁰ (37)
CMJ129	pHP13, <i>lacA::[(PrpsF-rpsF-ssb-mgfpmut2) tet]</i> (13)
LDW173	<i>att1::Tn916</i> [same as <i>att(yufKL)::Tn916</i>]
LDW631	<i>att1::Tn916 Δorf20-631</i> ; deletes most of <i>orf20</i> , leaves a functional <i>oriT</i>
LDW737	<i>amyE::[(att1Tn916) spc]</i>
LDW815	pLW805 [<i>oriT</i> (916) Pspank- <i>orf23-orf22-orf20 spc lacI</i>]
LDW818	<i>att1::Tn916::pLW805 [oriT(916) Pspank-orf23-orf22-orf20 spc lacI]</i>
LDW853	<i>att1::Tn916 orf20-3UAA</i>
LDW872	pLW862 (pHP13 <i>ssiA cat mls</i>), <i>lacA::(PrpsF-rpsF-ssb-mgfpmut2 tet)</i>
LDW878	pLW868 (pHP13 <i>sso916 cat mls</i>), <i>lacA::(PrpsF-rpsF-ssb-mgfpmut2 tet)</i>
LDW879	pLW859 [<i>oriT</i> (916) Pspank- <i>orf23-orf22-orf20-His₆ spc lacI</i>]
LDW894	pLW890 (pHP13 <i>sso916R cat mls</i>), <i>lacA::(PrpsF-rpsF-ssb-mgfpmut2 tet)</i>
LDW929	<i>att1::Tn916 Δorf20-631 lacA::[mls Pspank(hy)-orf20-myc, pLW920 spc]</i> ; pLW920 is integrated into <i>orf20</i> to generate <i>orf20-myc</i>
LDW930	<i>att1::Tn916 Δorf20-631 lacA::[mls Pspank(hy)-orf20ΔHTH-myc, pLW920 spc]</i> ; pLW920 is integrated into <i>orf20ΔHTH</i> to generate <i>orf20ΔHTH-myc</i>
LDW931	<i>att1::Tn916 orf20-3UAA lacA::[mls Pspank(hy)-orf20-myc, pLW920 spc]</i>
LDW932	<i>att1::Tn916 orf20-3UAA lacA::[mls Pspank(hy)-orf20ΔHTH-myc, pLW920 spc]</i>

^a All strains except BS49 are derived from JH642 and contain the *trpC2 pheA1* alleles (35, 36). Strains do not contain Tn916 unless Tn916 is specifically indicated.

^b *att1* is the same as *att(yufKL)* and is located between *yufK* and *yufL*.

double crossover at *lacA*, as described previously (38). Strains were constructed by natural transformation (39).

B. subtilis strains containing Tn916. Tn916 host strain LDW173 was generated by natural transformation of strain JMA222 with genomic DNA from strain BS49 (40) and selecting for resistance to tetracycline as previously described (41). The Tn916 genomic integration site was mapped by inverse PCR essentially as described previously (42). As in the parental strain (43), Tn916 is integrated between chromosomal genes *yufK* and *yufL* at coordinate 3209748 (36). Tn916 is oriented such that transcription of *orf24* and *int* of Tn916 is codirectional with that of *yufL*. This insertion site is identical to one of the two Tn916 insertion sites found in strain BS49 (43). The second Tn916 insertion in BS49 between *ykyB* and *ykuC* is not present in LDW173 and likely was not transferred during transformation.

orf20. We constructed two mutations in the *orf20* (relaxase) gene. (i) *Δorf20-631* is a markerless deletion that fuses the first 90 codons of *orf20* with the *orf20* stop codon, deleting the intervening 306 codons and preserving *oriT*(916) (44). Two ~1-kb fragments containing DNA flanking the deletion endpoints were PCR amplified and inserted into the BamHI and EcoRI sites of pCAL1422 (a plasmid that contains *E. coli lacZ*) via isothermal assembly (45), as previously described (12, 13). The resulting plasmid, pLW625, was integrated into the chromosome of LDW173 (wild-type [WT] Tn916) via single-crossover recombination. Transformants were screened for loss of *lacZ*, indicating loss of the integrated plasmid, and PCR was used to identify a *Δorf20* clone. (ii) The *orf20-3UAA* nonsense mutation replaces the third codon of *orf20*, GAA, with the stop codon UAA. *orf20-3UAA* was also constructed by allelic replacement by using essentially the same strategy as for the *Δorf20-631* allele. Approximately 1-kb fragments containing DNA flanking the point mu-

tation site were PCR amplified using primers containing the G-to-T mutation or its reverse complement. The two PCR products were inserted into the BamHI and EcoRI sites of pCAL1422 via isothermal assembly, and the isothermal assembly product was transformed directly into LDW173 (contains wild-type Tn916) cells. Transformants were screened for loss of *lacZ*, and mutants containing the G-to-T point mutation were identified by sequencing. One such mutant (strain LDW853) was then used for experiments.

We fused various alleles of *orf20* to the LacI-repressible IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter Pspank(hy) and used these to test *orf20* function. Constructs included Pspank(hy)-*orf20* (wild type), present in strains LDW929 and LDW931, and Pspank(hy)-*orf20ΔHTH-myc* (missing the N-terminal helix-turn-helix domain), present in strains LDW930 and LDW931. The wild-type *orf20* coding sequence begins at the CUG codon, whereas the *orf20ΔHTH* coding sequence begins at the annotated AUG start codon (see below). Both the *orf20* and *orf20ΔHTH* expression constructs include the 24 bases upstream of the presumed CUG start codon, including the putative ribosome binding site ATTGGAGG. Both *orf20* and *orf20ΔHTH* were PCR amplified from LDW173 genomic DNA, and the PCR fragments were inserted into the SphI and SacI sites of pCJ80 by isothermal assembly. pCJ80 contains Pspank(hy) and the repressor *lacI*, an *mls* cassette marker, and flanking homology for insertion by double crossover into the chromosome at *lacA*. The alleles were inserted into the chromosome by double-crossover, producing *lacA::[Pspank(hy)-orf20 mls]* or *lacA::[Pspank(hy)-orf20ΔHTH orf20 mls]*.

orf20 and *orf20ΔHTH* were tagged with three *myc* epitopes at the C terminus, producing *lacA::Pspank(hy)-orf20-myc* alleles, by single-crossover integration of a pCAL812-derived plasmid as previously described (46). Briefly, ~1 kb of *orf20* encoding the C-terminal end of the protein that is common to both the wild type and *orf20ΔHTH* alleles was PCR amplified and inserted into the XhoI and EcoRI sites of pCAL812 by isothermal assembly, resulting in plasmid pLW920. pLW920 was transformed into *lacA::[Pspank(hy)-orf20]* or *lacA::[Pspank(hy)-orf20ΔHTH]* by selecting for the spectinomycin resistance gene on pLW920.

att1Tn916. A copy of *att1Tn916* (the circular junction in Tn916 that is generated after excision from *att1*) was inserted at *amyE::[(att1Tn916) spc]* in LDW737, a control strain for qPCR. *att1Tn916* was PCR amplified from DNA from LDW173 cells, which contain a small amount of excised Tn916 circles. The PCR product was inserted via isothermal assembly into the EcoRI and HindIII sites of pAS24, an *amyE* insertion vector that contains a *spc* cassette.

Plasmids. Plasmids pLW805 and pLW859 are pUS19 based and contain *oriT*(916) and *orf23*, *orf22*, and *orf20* driven by Pspank. In pLW859, *orf20* is tagged with six histidine residues at the 3' end. Pspank, *lacI*, and an intervening multicloning site from pDR110 were inserted into the HindIII and EcoRI sites in pUS19 to make pCAL799 (pDR110 is from D. Rudner, Harvard Medical School) (47). *orf23* and *orf22* were PCR amplified from LDW173 and inserted into the NheI and SphI sites in pCAL799 to make pLW521 (pUS19, Pspank-*orf23-orf22 lacI*). A fragment encompassing *oriT*(916) (44) and *orf20* was PCR amplified from LDW173 and inserted into the SphI site downstream of *orf22* in pLW521 to make pLW805 and pLW859. In pLW859, the His tag was added to *orf20* with the downstream PCR primer.

We constructed pHP13 derivatives to test for *sso* activity as previously described (13). pLW868 (pHP13*sso916*) and pLW890 (pHP13*sso916R*) contain *sso916* in the functional and reverse orientation, respectively, relative to the direction of leading-strand DNA synthesis of pHP13 (48). We PCR amplified a 663-bp fragment from 89 bp upstream of the 3' end of *orf19* through the first 458 bp of *orf18* and inserted the fragment into the BamHI and EcoRI sites in pHP13. *ssiA*, which forms a primosome assembly site, was PCR amplified from pHV1610-1 (49) to make pLW862 (pHP13*ssiA*).

Tn916 excision and replication. We used qPCR to measure Tn916 excision and replication (Fig. 1B). Excision was measured using primers

oLW542 (5'-GCAATGCGATTAATACAACGATAC) and oLW543 (5'-TCGAGCATTCATCATACATTC) (Fig. 1B, primers a and d) to quantitate the vacant insertion site *attI*. *attI* amplification was normalized to a control chromosomal region in *mvpG*, which is 15 kb downstream of *attI*. *mvpG* was amplified with primers oLW544 (5'-CCTGCTTGGGATTCTCTTATC) and oLW545 (5'-GTCACTTGCACACTTCTCTC).

The copy number of the Tn916 circle was measured with primer pair oLW526 (5'-AAACGTGAAGTATCTTCCTACAG) and oLW527 (5'-TCGTCTATCAAAGCTCATTC) (Fig. 1B, primers b and c) to quantitate the unique *attI*Tn916 junction formed via site-specific recombination, and the average copy number of circular Tn916 per cell was calculated by normalizing *attI*Tn916 amplification to *mvpG*. To determine if Tn916 was replicating, we determined the ratio of the number of copies of circular Tn916 to the number of copies of the excision site.

Copy numbers of *attI*Tn916 and *attI* were determined by the standard curve method (50). Standard curves for *attI*Tn916, *attI*, and control chromosome locus *mvpG* were generated from genomic DNA of LDW737, which contains one copy of each amplicon in the chromosome. LDW737 contains an ectopic copy of *attI*Tn916 inserted at *amyE*. LDW737 does not contain Tn916 and therefore contains a copy of the unoccupied chromosome site *attI*. DNA for standard curves was prepared from stationary-phase LDW737 with an *oriC/terC* ratio of 1.3, verifying that *amyE*::*attI*Tn916, *attI*, and *mvpG* were represented in ~1:1:1 ratios.

Determination of copy number of an *oriT*(916) plasmid. The copy number of plasmid pLW805 [*oriT*(916), Pspank-*orf20-orf22-orf23*] was determined essentially as described previously (12). We used primers oLW128 (5'-ATGGAGAAGATTCAGCCACTGC) and oLW129 (5'-GCCATTATGGATTCTGTCAGAGG), which are specific to *spcE* in the plasmid, and normalized the amount of *spcE* to that of the chromosomal locus *mvpG*. Strain LDW818 contains pLW805 inserted into Tn916 by single crossover and was used to represent a plasmid copy number of one and to generate standard curves to calculate plasmid copy number.

Identification of the XRE-like helix-turn-helix domain. We searched for conserved domains within *orf20* using the CUG start codon upstream of the annotated AUG (see Fig. 3A) using the NCBI Conserved Domain Database (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). The helix-turn-helix domain cd00093 was identified (E value = 3.50e-10) along with the expected relaxase domain pfam02486. A search for Orf20 homologues within ICEs using HMMER3::phmmer and the ICEberg database yielded 101 hits, including relaxases containing the XRE-like helix-turn-helix domain (51). A HMMER3::phmmer search of reference proteomes yielded additional relaxase homologues that contained the XRE-like helix-turn-helix domain and are present in a variety of sequenced Gram-positive species (<http://www.ebi.ac.uk/Tools/hmmer/>).

Orf20-His₆ purification and mass spectrometry. Orf20-His₆ was purified from *B. subtilis* cells containing pLW859 [*oriT*(916) Pspank-*orf23-orf22-orf20*-His₆ *spc*]. Cells were grown to mid-exponential phase in LB medium containing 1 mM IPTG and 100 µg/ml spectinomycin. Cells from 250 ml of culture were pelleted, washed with 1× phosphate-buffered saline (PBS), recentrifuged, and stored at -80°C. Pellets were thawed on ice and lysed in 25 ml binding buffer (300 mM NaCl, 50 mM sodium phosphate buffer, pH 7.4) containing 0.1 mg/ml lysozyme and the protease inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) at 1 mM at 37°C for 15 min. Lysates were sonicated for 5 min total (0.3-s pulses, 15% output), followed by centrifugation to remove insoluble material.

Lysates were preincubated with 0.5 ml Talon Superflow cobalt resin (GE Healthcare) on a rotating platform for 1 h at 4°C. Resin and lysate were loaded onto a Poly Prep column (Bio-Rad), and Orf20-His₆ was purified according to GE Healthcare's protocol for batch, gravity-flow purification under native conditions. Elution fractions were exchanged into buffer consisting of 20 mM HEPES and 150 mM KCl, pH 7.5, using PD-10 desalting columns (GE Healthcare).

Purified protein was precipitated using trichloroacetic acid (TCA) and solubilized in 8 M urea, 100 mM Tris, pH 8.5. Cysteines were reduced and

alkylated using Tris 2-carboxyethyl phosphine HCl (TCEP) and iodoacetamide, respectively. The sample was then digested for 4 h at 37°C with endopeptidase Lys-C (Roche). The sample was then diluted to 2 M urea and digested with trypsin (Promega). Peptides were identified using the LTQ XL linear ion trap mass spectrometer (Thermo Fisher) using MudPIT and SEQUEST software as previously described (52). Tandem mass spectra were searched against a database of predicted open reading frames (ORFs) from the genome of *B. subtilis* strain 168 (NCBI accession no. ASM904v1) and from Tn916 (accession no. U09422.1), including the predicted full-length Orf20 sequence.

Measurement of single-strand-origin activity. We used live-cell microscopy and ChIP-qPCR to analyze the association of Ssb-green fluorescent protein (GFP) with pHP13-derived plasmids containing DNA fragments with candidate *sso*'s (13). ChIP-qPCR was carried out as described previously (13). For imaging, cells were placed on a slice of agarose dissolved in 1× Spizizen's salts [2 g/liter (NH₄)₂SO₄, 14 g/liter K₂HPO₄, 6 g/liter KH₂PO₄, 1 g/liter Na₃ citrate-2H₂O, 0.2 g/liter MgSO₄·7H₂O] (39) essentially as described previously (53). Images were acquired on a Nikon Ti-E inverted microscope using a CoolSnap HQ camera (Photometrics). GFP fluorescence was generated using a Nikon Intensilight mercury illuminator through Chroma filter set 49002. Image processing was performed using ImageJ software.

RESULTS

Rationale. Excision of Tn916 from a site in the chromosome yields two products (Fig. 1B): (i) the vacated (empty) chromosomal attachment (integration) site and (ii) the circular plasmid form of Tn916. We reasoned that if Tn916 replicates autonomously, then the copy number of the circular form following excision would be greater than that of the chromosomal location that was vacated upon excision. We used quantitative PCR (qPCR) to measure the amount of the empty chromosomal integration site (called here *attI*) relative to a nearby gene (*mvpG*) and the amount of the Tn916 circle relative to the amount of *attI* (see Materials and Methods). We also reasoned that if Tn916 replicates by rolling-circle replication, then the Tn916 relaxase encoded by *orf20* (Fig. 1A) would be required for replication.

Excision of Tn916 from a site in the *B. subtilis* chromosome. We measured the excision frequency of Tn916 from a chromosomal site in *B. subtilis* during exponential growth and entry into stationary phase. *B. subtilis* strain LDW173 contains a copy of Tn916 inserted between chromosomal genes *yufK* and *yufL* (*malK*) (see Materials and Methods). We used qPCR to monitor excision from the *yufK-yufL* integration site, *attI*, and normalized *attI* amplification to that of a nearby chromosomal locus, *mvpG*, which should be unaffected by excision. We considered a strain without Tn916 to represent 100% excision (all cells had *attI*), and we used this to generate standard curves to calculate excision frequencies (see Materials and Methods).

There was a basal level of excision of Tn916 in cells growing exponentially in rich (LB) medium (Fig. 2A). *B. subtilis* cells (strain LDW173) growing exponentially in LB medium were diluted to a low density (optical density at 600 nm [OD₆₀₀], ~0.05) in the absence or presence of tetracycline (see below). In the absence of tetracycline, we detected approximately 0.003 empty *attI* sites per *mvpG*, and excision increased to approximately 0.01 *attI* sites per *mvpG* during exponential growth (Fig. 2A). After cells entered stationary phase, the amount of *attI* per *mvpG* appeared to decline (Fig. 2A). Based on these results, we infer that Tn916 had excised from *attI* in ~0.3 to 1% of cells during exponential growth.

Excision of Tn916 from a site in the *B. subtilis* chromosome

FIG 2 Products generated following excision of Tn916 from the chromosome. Cells containing Tn916 (strain LDW173; triangles) or Tn916 Δ *orf20* (LDW631; circles) were grown in the presence (filled symbols) or absence (open symbols) of 2.5 μ g/ml tetracycline. Quantitative PCR data (black lines, left axes) and growth phase as determined by OD₆₀₀ (gray lines, right axes) are shown. Strain LDW737 contains one copy of each qPCR amplicon (*att1*Tn916, *att1*, and *mrpG*) and was used to generate standard curves for qPCR (see Materials and Methods). (A) Excision of Tn916. The vacated chromosomal site *att1* was amplified by qPCR, and *att1* signal was normalized to the signal of an unrelated chromosomal locus, *mrpG*. (B) Average copy number of circular Tn916 per host chromosome. The *att1*Tn916 junction present in the circular (excised) form of Tn916 was amplified by qPCR, and the qPCR signal was normalized to that of chromosomal gene *mrpG*. (C) Tn916 circles per excision event. Circular Tn916 (*att1*Tn916) was amplified by qPCR, and the *att1*Tn916 signal was normalized to that of the *att1* site formed from excision. Data are means $\pm$ standard deviation of results of ≥ 4 independent experiments.

is stimulated by tetracycline. Excision of Tn916 requires the expression of *int* and *xis* (20), and these genes are downstream of *tetM* (encoding tetracycline resistance) (Fig. 1A). The presence of tetracycline enhances transcription of the genes needed for excision (32) and enhances excision of Tn916 from the chromosome of *Enterococcus faecalis* (54). Conjugative transfer of Tn916 also increases in the presence of tetracycline (32, 54–56), perhaps as a result of increased excision and/or increased expression of the conjugation genes that become fused to the promoters driving *int* and *xis* after excision and circularization. Interestingly, the amount of excision and conjugation and the amount of stimulation by tetracycline is different for different insertion sites (54).

To determine the effects of tetracycline on excision of Tn916 from *att1* in *B. subtilis*, exponentially growing cells were diluted to a low density into tetracycline (2.5 μ g/ml), as described above. Growth in the presence of tetracycline caused an increase in the amount of *att1* per *mrpG* compared to that of cells grown in the absence of tetracycline (Fig. 2A), indicating that the presence of tetracycline caused an increase in the excision frequency. This increase was most apparent during entry into and during early stationary phase (Fig. 2A). There was a decline in the amount of *att1* later in stationary phase. This decline could be indicative of the death of cells from which Tn916 had excised, thereby causing a decrease in the amount of *att1* (present in ~ 1 to 3% of the cells) with relatively little overall effect on the amount of *mrpG* (present in almost all cells). Alternatively, the decrease in *att1* could indicate that Tn916 reintegrated into *att1*.

Autonomous replication of Tn916. To measure the amount of the circular Tn916 after excision, we used a control that would mimic 100% excision and a copy number of one Tn916 circle per chromosome. To generate this control, we cloned the circular junction generated by excision of Tn916 from *att1* (*att1*Tn916) (Fig. 1B) and integrated a single copy of this DNA into the *B. subtilis* chromosome. We considered a strain with *att1*Tn916 in single copy (and without any copies of Tn916; strain LDW737) to represent 100% excision and a copy number of one. This strain was used in the determination of the copy number of the circular form of Tn916 (see Materials and Methods).

We used qPCR to measure the amount of the circular form of Tn916 relative to the chromosomal locus *mrpG* from the same samples used to determine the amount of *att1* (see above). We found that similar to the amount of *att1*, the amount of the circular form of Tn916 increased during growth and reached a maximum as cells approached and entered stationary phase (Fig. 2B). The increase in copy number was most dramatic early in stationary phase, where there was a peak of ~ 0.2 copies of the circular form of Tn916 per copy of *mrpG* (Fig. 2B). This increase in stationary phase was apparent following growth in the presence or absence of tetracycline, although the copy number appeared to be ~ 2 -fold higher in the presence of tetracycline (Fig. 2B).

We found that the copy number of the circular form of Tn916 was greater than that of the vacated chromosomal site (*att1*) (Fig. 2C), indicating that there might be autonomous replication of the excised form of Tn916. During exponential growth, this ratio was approximately 2 to 3 and increased to ~ 10 circles per *att1* in stationary phase (Fig. 2C). These results indicate that the copy number of the circle was greater than that of the empty chromosomal site and that the excised Tn916 was most likely replicating autonomously. The increased copy number in stationary phase compared to exponential growth is probably due to continued replication and decreased cell growth.

The increased copy number of the Tn916 circle was dependent on the relaxase encoded by Tn916 *orf20*. Plasmids that use rolling-circle replication require a plasmid-encoded relaxase (sometimes called the initiator or replicase) that nicks DNA in the origin of replication (the double-strand origin or *dso*) to initiate unidirectional replication (9). Replicative relaxases are homologous to conjugative relaxases (57–59). Some replicative relaxases can function in conjugation (60–62), and some conjugative relaxases can function in replication (11), thereby blurring the distinction between replicative and conjugative relaxases.

We found that the conjugative relaxase of Tn916 (encoded by

orf20) was needed for autonomous replication of Tn916. The copy number of the Tn916 circle was significantly reduced in the *orf20* mutant (Fig. 2B and C). There appeared to be a similar amount of excision in the relaxase (Δ *orf20*) mutant as judged by the amount of *att1* compared to *mrpG* (Fig. 2A), indicating that loss of the relaxase did not affect excision. However, the average copy number of the circular form of Tn916 Δ *orf20* relative to the excision site was 0.39 ± 0.06 , and the copy number remained relatively constant during the entire time course of the experiment (Fig. 2B and C). This is considerably different from the ratio for the wild-type Tn916 (*orf20*⁺). Together, these results indicate that Tn916 normally undergoes autonomous rolling-circle replication after excision from the chromosome and the Tn916-encoded relaxase is required for this replication.

The Tn916 origin of transfer *oriT* can function as an origin of replication. If Tn916 uses its relaxase for rolling-circle replication from *oriT*, then we expected that *oriT* and *orf20* could function to support replication of a heterologous plasmid that is otherwise missing an origin of replication. By analogy to ICEBs1 (12), we also expected that this replication would also require the homologs of the ICEBs1 helicase processivity factor *HelP* that are encoded by Tn916 *orf22* and *orf23*.

We found that the Tn916 origin of transfer *oriT*(916) could function as an origin of replication. We cloned *oriT*(916) into a plasmid that is otherwise incapable of autonomous replication in *B. subtilis*. The parent plasmid, pUS19 (47), contains a pUC origin that is not functional in *B. subtilis* but is functional in *E. coli*. pUS19 also contains *spcE*, conferring resistance to spectinomycin in *B. subtilis*. In addition to *oriT*(916), we cloned the genes *orf20* (relaxase) and *orf22* and *orf23* (*helP* homologues) from Tn916 to generate plasmid pLW805. In this plasmid, transcription of *orf20*, *orf22*, and *orf23* is controlled by the LacI-repressible, IPTG-inducible promoter Pspank (Pspank-*orf23-orf22-orf20*), making expression of these genes dependent on IPTG.

We transformed cells lacking Tn916 with pLW805 [*oriT*(916) Pspank-*orf23-orf22-orf20*] DNA (which had been isolated from *E. coli*) and selected for spectinomycin-resistant transformants. Transformants were obtained in the presence of IPTG (enabling expression of *orf20*, *orf22*, and *orf23*), but no transformants were obtained in the absence of IPTG. These results indicate that *oriT* was capable of supporting replication and that replication was likely dependent on expression of the relaxase and perhaps the *helP* homologues. The plasmid copy number was approximately 4 ± 1 per cell, as determined by qPCR of *spcE* plasmid DNA relative to the chromosomal locus *mrpG*.

The *oriT*(916) plasmid (pLW805) was unstable, even when cells were grown in IPTG and spectinomycin. The fraction of plasmid-containing cells was determined by counting CFU on LB agar plates containing IPTG and spectinomycin or on LB agar without additives. After 7 or 8 generations of exponential growth in liquid LB medium with IPTG and spectinomycin, approximately $17\% \pm 6\%$ of cells were able to form colonies on LB plates with IPTG and spectinomycin (the total number of cells was determined by counting CFU on LB plates with neither spectinomycin nor IPTG), indicating that the plasmid had been lost from $\sim 83\%$ of the cells growing in culture. This is not surprising for a plasmid that has a relatively low copy number, replicates by rolling-circle replication, lacks a single-strand origin of replication, and lacks partitioning functions.

The *oriT*(916) plasmid (pLW805) was even more unstable

when cells were grown nonselectively and in the absence of IPTG (causing decreased expression of *orf20*, *orf22*, and *orf23*). Plasmid-containing cells were transferred to medium lacking IPTG and spectinomycin, and after 7 or 8 generations of exponential growth without inducer or selection, only 0.2% of cells were resistant to spectinomycin, indicating that the *oriT* plasmid was lost in $>99.5\%$ of cells. These results indicate that expression of the relaxase and perhaps the predicted helicase processivity factors was needed for plasmid propagation. Based on what is known about rolling-circle replication and the functions of the relaxase and helicase processivity factors (e.g., see reference 12) and the finding that the relaxase was needed for replication of Tn916 (Fig. 2C), we conclude that replication from *oriT*(916) was dependent on the relaxase (*orf20*) and probably at least one of the *HelP* homologues (*orf22*, *orf23*). Results below indicate that the relaxase and both *HelP* homologues are associated with the plasmid replicating from *oriT*(916).

Analysis of Orf20 reveals a conserved N-terminal helix-turn-helix domain. Tn916 *orf20* (relaxase) is annotated to start with an AUG codon (Fig. 3A and B) (63). We noticed that *orf20* lacks an obvious ribosome binding site (RBS) upstream of the putative start codon. However, there is a potential ribosome binding site and CUG start codon upstream of the annotated AUG start (Fig. 3B). The predicted protein generated using this CUG start includes a helix-turn-helix (HTH) domain that is found in the xenobiotic response element (XRE)-like family of DNA-binding proteins (e.g., the repressor Xre of the *B. subtilis* defective phage PBSX; λ cI and Cro) (NCBI accession no. cd00093) (64–67).

The XRE-like helix-turn-helix domain is conserved in many homologues of the *orf20*-encoded relaxase (see Materials and Methods), including the relaxases of Tn916-related elements present in multidrug-resistant *C. difficile* strain 630 (CTn7) and in pathogenic strains of *S. pneumoniae* (Tn5253), and in relaxases present in putative mobile elements from several Gram-positive species (6, 26, 68) (Fig. 4). We suspect that some *orf20* homologues were misannotated based on the initial annotation of Tn916 *orf20*. In the reference genomes (e.g., relaxase *orf26* in CTn1) (Fig. 4), there are sequences encoding a potential HTH motif in or immediately upstream of the annotated start codon (6, 69, 70), consistent with the notion that the actual relaxase is larger than that originally annotated. Other *orf20* homologues, including the relaxase NicK from ICEBs1, do not contain an XRE-like helix-turn-helix domain.

We postulated that the Tn916 relaxase was larger than previously predicted and contained a conserved helix-turn-helix domain. To test this, we analyzed peptide fragments from purified relaxase. We fused a hexahistidine tag to the C terminus of the relaxase (Orf20-His₆) in the *oriT*(916) plasmid (generating pLW859). Like the parent plasmid, the plasmid with *orf20*-His was capable of autonomous replication in *B. subtilis* (strain LDW879), indicating that Orf20-His₆ was functional. We purified Orf20-His₆ from *B. subtilis* and analyzed the protein by mass spectrometry. We identified peptides from both the N-terminal helix-turn-helix and C-terminal relaxase regions (Fig. 3A; Table 2). These results indicate that cells produce Orf20 starting with the CUG codon and containing the helix-turn-helix region.

To verify that the helix-turn-helix region was part of the relaxase, we made a nonsense mutation in this region of *orf20*. We mutated the third codon downstream of the presumed CUG start codon to a stop codon (*orf20*-3UAA) (Fig. 3B). Like the *orf20*

FIG 3 The relaxase encoded by *orf20* contains a conserved N-terminal helix-turn-helix region. (A) The revised amino acid sequence of the Tn916 relaxase (Orf20). The methionine previously thought to be the start of the protein is represented by a bold M. Peptides identified by mass spectrometry were mapped to the amino acid sequence of Orf20 and are underlined. The bold underline indicates peptides that overlapped the junction between the N-terminal region and what was previously thought to be the initiating methionine. (B to D) Maps of *orf20* and *orf20* overexpression alleles used here. Gray arrows correspond to the *orf20* coding sequence and the direction of transcription. The dark gray rectangles represent the conserved XRE-like helix-turn-helix (HTH) and relaxase regions in *orf20* as determined using the NCBI Conserved Domain Database (see Materials and Methods). The black rectangles represent the putative ribosome binding site (rbs) that is present in Tn916 and preserved in the myc-tagged alleles. (B) *orf20* in Tn916 and upstream sequence. The jagged arrow represents the C terminus of *orf21*. The CUG codon and previously proposed AUG start codons are indicated. The relative location of the UAA nonsense mutation in *orf20*-3UAA is marked with an asterisk. (C and D) Myc-tagged *orf20* alleles with (C) (WT *orf20*) or without (D) (*orf20* Δ HTH) the N-terminal helix-turn-helix region. The C-terminal myc tags are not shown. *orf20*-myc alleles were driven by promoter Pspank(hy) (black arrow). (C) WT *orf20*-myc contains the entire coding sequence as depicted in panel A and noncoding DNA upstream of the CUG start. (D) *orf20* Δ HTH-myc contains the *orf20* coding sequence starting at the previously proposed AUG start codon as depicted in panel A and noncoding DNA upstream of the CUG start.

deletion, the *orf20*-3UAA nonsense mutation abolished replication of Tn916 (Table 3). Replication was restored to both Tn916 Δ *orf20* and Tn916 *orf20*-3UAA when full-length *orf20* (*orf20*-myc), starting with the CUG codon and containing a C-terminal myc tag, was expressed from Pspank(hy) (Table 3). Abrogation of relaxase function with the *orf20*-3UAA mutation indicates that the annotated AUG start codon in Tn916 (Fig. 3B) does not initiate translation of a functional relaxase and that the start codon is most likely upstream of the position of the nonsense mutation.

We also overexpressed the previously annotated *orf20*, missing the helix-turn-helix domain (*orf20* Δ HTH-myc), from Pspank(hy). The Pspank(hy)-*orf20* Δ HTH-myc allele was unable to complement the replication defects of relaxase-deficient Tn916 (Table 3). The simplest interpretation of these results is that the helix-turn-helix domain of Orf20 is required for replication.

Based on the results above, we conclude that the actual *orf20* open reading frame encodes the helix-turn-helix motif found in many XRE-like proteins. Furthermore, the open reading frame most likely begins at the CUG codon that is preceded by a potential ribosome binding site (Fig. 3). It seems reasonable to retain the name *orf20* for the Tn916 gene encoding the conjugative (and replicative) relaxase, recognizing that in some of the literature, this refers to the shorter open reading frame, but in many cases, the exact coding sequence is not so important for the genetic analyses. It would also be reasonable to change the name of the Tn916 gene for the relaxase, perhaps calling it *nick*, adopting the name used for the ICEBs1-encoded relaxase. Here, we continue to refer to the full-length relaxase gene as *orf20*.

Association of Help homologues Orf23 and Orf22 with re-

laxase Orf20-His₆. The plasmid replicating from *oriT*(916) with *orf20*-His (pLW859) also contained *orf23* and *orf22* from Tn916, the homologues of ICEBs1 *help*. Mass spectrometry of affinity-purified Orf20-His₆ identified peptides from both Orf23 and Orf22 (Table 2). Copurification of the Help homologues with functional relaxase indicates that the Help proteins are part of the relaxosome and are likely important for replication from *oriT*. These data are consistent with the model that Tn916 replicates by a rolling-circle mechanism and uses helicase processivity factors to facilitate unwinding of the DNA strands after relaxase nicking, analogous to autonomous replication of ICEBs1.

Identification of a single-strand origin in Tn916. Because Tn916 replicates by rolling-circle replication, we expected it would have a single-strand origin of replication. Rolling-circle replicating plasmids and phages contain an *sso* or encode a primase that enables conversion of ssDNA to dsDNA (71–74). ICEBs1 has a single-strand origin that enables second-strand synthesis (13).

We tested parts of Tn916 for *sso* activity using a plasmid-based assay. pHP13 is a rolling-circle replicating plasmid that lacks an *sso* and accumulates ssDNA (75). In cells expressing a fusion of the host single-stranded DNA binding protein to GFP (Ssb-GFP), accumulation of ssDNA can be visualized as large fluorescent foci of Ssb-GFP in most pHP13-containing cells (Fig. 5A and B, strains CMJ118 without plasmid and CMJ129 with pHP13). We previously found that when pHP13 contains *sso1* from ICEBs1, there is a reduction in the size and intensity of Ssb-GFP foci (13). Similarly, *ssiA* from pAM β , which is a primosome assembly site (49), reduces pHP13 ssDNA (Fig. 5C, pLW862, strain LDW872), show-

FIG 4 Alignment of several relaxase homologues. The amino acid sequences of the relaxases from Tn916, Tn5253, CTn7, CTn1, and ICEBs1 were aligned with the Clustal Omega algorithm (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) (83). Black-shaded residues are identical in all five relaxases, and gray-shaded residues are similar in all relaxases. The helix-turn-helix region present in four of the five relaxases is boxed. Previously proposed N-terminal methionines encoded in *orf20* of Tn916 and *orf26* of CTn1 are bold and boxed. The output alignment was shaded using BoxShade (http://www.ch.embnet.org/software/BOX_form.html). The order of sequences (Tn916 Orf20 to ICEBs1 NicK) reflects the order of the original input queries; closely related sequences were not computationally grouped in the final alignment.

ing that this fluorescence microscopy-based assay, in conjunction with the pHp13 vector, can be used to rapidly screen single-strand origins of multiple types (RNAP dependent versus primase dependent).

Most *sso*'s in rolling-circle replicating plasmids are found in intergenic regions and are orientation specific (71). Therefore, we cloned several intergenic regions from Tn916 into pHp13 and screened for a reduction in ssDNA accumulation as visualized by a reduction in the size of Ssb-GFP foci. One of the regions we screened, encompassing the intergenic region between *orf19* and *orf18* (Fig. 1A), reduced ssDNA (pLW858, strain LDW878) (Fig.

5D). In addition, we found that the Sso activity of the “*orf19-orf18*” region was orientation specific. That is, the fragment cloned in the opposite orientation into pHp13 (pLW890, strain LDW894) did not reduce the size or intensity of Ssb-GFP foci (Fig. 5E). The predicted secondary structure of the sequence in this region did not appear to resemble any of the three common types

TABLE 2 Mass spectrometry of affinity-purified Orf20-His₆ shows that the HelP homologues are associated with the relaxase

Protein	% sequence coverage ^a	No. of peptides ^b	Molecular size (kDa) ^c
Orf20 relaxase	51.5	84	46.8
Orf22 HelP	67.2	33	14.1
Orf23 HelP	69.6	18	11.8

^a Percentage of the protein sequence detected by mass spectrometry. Amino acid sequences were based on Tn916 genes (GenBank accession no. U09422.1, except that for Orf20, which was based on the reannotated gene starting at CUG and encoding the N-terminal helix-turn-helix region (Fig. 3B).

^b Number of total peptides detected.

^c Predicted molecular size.

TABLE 3 Complementation of the Tn916 replication defects of relaxase Δ *orf20* and *orf20*-3UAA mutants

Line	Tn916 genotype	Pspank(hy) ^a	No. of circles per excision ^b
1	Wild type		6.05 ($\pm$ 0.43)
2	Δ <i>orf20</i>		0.43 ($\pm$ 0.12)
3	<i>orf20</i> -3UAA		0.61 ($\pm$ 0.26)
4	Δ <i>orf20</i>	<i>orf20</i> -myc	8.03 ($\pm$ 2.94)
5	<i>orf20</i> -3UAA	<i>orf20</i> -myc	6.98 ($\pm$ 1.60)
6	Δ <i>orf20</i>	<i>orf20</i> Δ HTH-myc	0.55 ($\pm$ 0.08)
7	<i>orf20</i> -3UAA	<i>orf20</i> Δ HTH-myc	0.46 ($\pm$ 0.17)

^a *orf20* complementation alleles were expressed in *trans* at the *lacA* locus and driven by promoter Pspank(hy).

^b The number of circles per excision was quantified by qPCR by measuring the amount of the circular junction *attI*Tn916 relative to the vacant chromosome site *attI* (Fig. 1A). Data are averages of results from 3 independent experiments ($\pm$ standard deviation). Strains were LDW173, LDW631, LDW853, LDW929, LDW931, LDW930, and LDW932 (lines 1 to 7, respectively).

FIG 5 Ssb-GFP to visualize ssDNA and single-strand-origin activity. All cells contain ssDNA-binding protein Ssb fused to GFP. Phase contrast (top panels) and GFP fluorescence (bottom panels) are shown. (A) No plasmid, strain CMJ118. In cells without a plasmid, Ssb-GFP forms small foci at the replication forks (arrow) (38). (B) pHP13, strain CMJ129. Ssb-GFP forms large foci (arrowhead) in cells containing pHP13, which does not contain a functional *sso* and accumulates ssDNA (13). (C) pHP13ssiA, strain LDW872. The primosome binding site *ssiA* from pAM β , which can function as an *sso* in rolling-circle replicating plasmids and reduce ssDNA (49), was cloned into pHP13 to make pHP13ssiA (pLW862). Cells containing pHP13ssiA had small foci of Ssb-GFP (arrow). (D) pHP13sso916, strain LDW878. Cells containing pHP13 with the *sso* from Tn916 (pLW868) did not accumulate large Ssb-GFP foci (arrow), indicating reduced ssDNA. (E) pHP13sso916R, strain LDW894. Cells containing pHP13 with *sso916* cloned in the reverse orientation (pLW890) had large Ssb-GFP foci (arrowhead), indicating accumulation of ssDNA. Data are representative images from ≥ 3 independent experiments.

of *sso*'s, namely, *sso_A*, *sso_U*, or *sso_T* (71), whereas *sso1* from ICEBs1 resembles that from pTA1060 (13) and belongs to the *sso_T*-family.

We quantified the Sso activity of the “*orf19-orf18*” fragment (referred to as *sso916*) by immunoprecipitating Ssb-GFP and determining the amount of plasmid DNA bound to Ssb (ssDNA) using qPCR (Fig. 6). *sso916* (present in pLW858) reduced the amount of Ssb-bound plasmid DNA ~ 30 -fold, similar to *sso1* from ICEBs1 (13). In contrast, in cells containing pLW890 (pHP13 with *sso916R*, i.e., *sso916* in the opposite orientation), the amount of plasmid DNA bound to Ssb-GFP was similar to that of the parent plasmid (pHP13) without an *sso* (Fig. 6). The differences in the amount of Ssb-GFP bound to each of the plasmids was not due to differences in plasmid copy number. The copy number of pLW858 (pHP13 with *sso916*) was approximately the same as that of pHP13, and that of pLW890 (pHP13 with *sso916R*) was approximately 1.4-fold that of pHP13.

We found that *sso916* is conserved in other Tn916-like ICEs. We searched for sequences similar to the 116-bp intergenic region that contains *sso916* and found that 15 additional ICEs contained a region with 94 to 100% sequence identity (Table 4). The conju-

FIG 6 *sso916* reduces the amount of Ssb-GFP bound to plasmid DNA. Plasmid DNA associated with Ssb-GFP. Ssb-GFP was immunoprecipitated following cross-linking with formaldehyde. The amount of plasmid DNA that was coimmunoprecipitated (with Ssb-GFP) was amplified with qPCR using primers to the *cat* gene in pHP13. The amounts of PCR products were normalized to the amount of plasmid DNA in total lysates, essentially as described previously (13). Data are means $\pm$ standard deviation of biological triplicates.

gation genes from all of these ICEs are highly similar ($\geq 80\%$ identity) to those in Tn916, but each contains accessory (cargo) genes different from those in Tn916. Based on these results, we conclude that *sso916* is a functional single-strand origin of replication in Tn916 that has been conserved during genetic diversification of Tn916-like elements.

DISCUSSION

Tn916 replicates autonomously. We found that the broad-host-range conjugative transposon Tn916 undergoes autonomous rolling-circle replication. The excised circular form of Tn916 is multicopy, and replication is dependent on the relaxase encoded by *orf20*. The Tn916 origin of transfer *oriT*(916) supports replication of a plasmid that is otherwise incapable of replication in *B. subtilis*.

TABLE 4 *sso916* is conserved in other Tn916-like ICEs

ICE ^a	Organism ^b
Tn6009	<i>Klebsiella pneumoniae</i> 41
ICESpnH034800032	<i>Streptococcus pneumoniae</i> H034800032
CTn6009	<i>Streptococcus cristatus</i>
ICESpn9409	<i>S. pneumoniae</i> 9409
ICESpn11928	<i>S. pneumoniae</i> 11928
ICESpnMalM6	<i>S. pneumoniae</i> Mal M6
ICESpn11930-2	<i>S. pneumoniae</i> 11930
ICESpn23771	<i>S. pneumoniae</i> 23771
ICESpn11876	<i>S. pneumoniae</i> 11876
ICESpn11930	<i>S. pneumoniae</i> 11930
ICESsu(BM407)1	<i>Streptococcus suis</i> BM407
ICESsu(BM407)2	<i>S. suis</i> BM407
ICESp23FST81	<i>S. pneumoniae</i> ATCC 700669
ICESsu(SC84)	<i>S. suis</i> SC84
Tn5397	<i>Clostridium difficile</i> 630
Tn1545	<i>S. pneumoniae</i> BM4200

^a ICEs with regions similar to the intergenic sequence containing *sso916* were identified by using WU-BLAST 2.0 and searching the ICEberg v1.0 database of ICE nucleotide sequences (51). The search identified *sso916* in Tn5251, which is essentially identical to Tn916 ($>95\%$ identity at the nucleotide level) and is not included in the table.

ICESpnH034800032 and CTn6002 are listed separately in ICEberg and were identified in different organisms but are essentially identical elements ($>95\%$ identity at the nucleotide level). Conservation of each putative *sso* with *sso916* is 100%, except for Tn5397 and Tn1545, which have 94% and 96% identity, respectively.

^b Each ICE was initially identified in the indicated species and strain.

Replication appears to be dependent on the relaxase and at least one and perhaps both of the helicase processivity factor homologues Orf23 and Orf22. Copurification of the relaxase and both HelP homologues indicates that both of the HelP homologues are likely functioning in DNA unwinding. We do not know if one or the other or both are required or if they are redundant. Lastly, Tn916 contains a functional *sso*, *sso916*. Our results support a model in which relaxase Orf20 initiates rolling-circle replication from *oriT*(916) and the HelP homologues facilitate processive unwinding of the nicked strand, analogous to the role of the relaxase and HelP in ICEBs1 (11, 12). After recircularization of the unwound strand, Sso activity would be used to initiate priming of lagging-strand DNA synthesis.

Tn916 was thought to be incapable of autonomous replication. Previous studies may have failed to detect replicating Tn916 because, like many ICEs, Tn916 excises in a small fraction of host cells (19, 20). The circular form of Tn916 was detected using Southern blotting when *xis* and *int* were overexpressed (20). The ratio of Tn916 circles per excision event was reported to be 1.8 Tn916 circles per excision site. We observed a similar ratio when nutrients were nonlimiting (~1 to 3 circles per excision site during exponential growth) (Fig. 2C).

Tn916 excision and copy number peaked during early stationary phase. Likewise, maximal excision of Tn916 in *E. faecalis* and *Listeria monocytogenes* occurs during late exponential phase (19), consistent with the notion that activation of Tn916 is dependent, in part, on cell growth phase. Other ICEs also have growth-phase-dependent excision due to nutrient limitation, in response to cell-cell signaling, or both (17, 37, 76–78).

Identification of an N-terminal helix-turn-helix domain in the Tn916-encoded relaxase. We identified a conserved helix-turn-helix domain in the N-terminal region of the relaxase Orf20. This domain is conserved in many relaxase homologues, and our results indicate that this region is essential for relaxase function.

A purified form of Orf20 from Tn916 nicks *oriT*(916) nonspecifically *in vitro*, and coinubation with the recombinase Int then generates strand- and sequence-specific nicking (63). However, because *orf20* was misannotated, Orf20 used in these experiments was purified without the N-terminal helix-turn-helix domain. Our results indicate that Orf20 contains an N-terminal helix-turn-helix domain. Because *oriT*(916) functions as an origin of replication in the absence of *int*, we suggest that Int is not involved in nicking *oriT*(916) and that the helix-turn-helix domain in Orf20 likely facilitates recognition of *oriT*(916).

A class of conjugative relaxases from plasmids that contain an N-terminal helix-turn-helix motif has recently been described (79), although the domain is not a member of the XRE-like family present in Orf20. Mutation of a highly conserved glutamate residue in the helix-turn-helix domain of representative relaxase TraX prevented relaxase binding to *oriT* (79), consistent with the model that the N-terminal helix-turn-helix domain in Orf20 is needed for proper recognition and nicking of *oriT*(916).

Replication and maintenance. Replication of ICEBs1 and ICE R391 is required for maintenance of the elements in dividing host cells (11, 14), and studies with other ICEs found that the relaxase is required for stability of the cognate ICE (e.g., reference 17). However, we did not observe a significant loss of a Tn916 Δ orf20 mutant (missing the relaxase). This apparent stability could indicate that the circular form of Tn916 might cause growth arrest or possibly cell death. No genes in Tn916 that cause such a phenotype

have been identified, but there are several genes with unknown function. We also observed a decrease in signal of *attI* during stationary phase. This is consistent with Tn916 reintegration into *attI* or death of cells in which Tn916 has excised. We do not favor the first hypothesis because Tn916 can integrate into multiple sites (29, 80, 81), and we have observed that Tn916 does not have a preference for reintegration into *attI* in transconjugants (L. D. Wright and A. D. Grossman, unpublished results).

Some ICEs are known to cause growth arrest and/or cell death. For example, when activated, ICE_{clc}, an ICE active in *Pseudomonas* species, can inhibit host cell growth (~50% of activated cells stop dividing) and cause cell lysis (77, 82). Despite the damage incurred by host cells, 75% of donors with excised ICE_{clc} that contact a recipient cell successfully transfer the element (82). Single-cell microscopy studies, such as those conducted on ICE_{clc}, are required to assess the effect of Tn916 induction on host cell fate.

Autonomous replication of integrative and conjugative elements is conserved. Growing evidence indicates that several ICEs replicate autonomously by a common mechanism. ICEBs1 and Tn916 both replicate by a rolling-circle mechanism using similar machinery. However, ICEBs1 and Tn916 are very different elements, with different regulatory mechanisms, different modes of integration, and different cargo genes. The ICE R391 also replicates autonomously in Gram-negative *E. coli*, and its relaxase and *oriT* are important for replication, indicating that R391 likely also uses rolling-circle replication. Since all functional ICEs that use a type IV secretion system have an origin of transfer and a cognate relaxase, the accumulating findings support the notion that many, and perhaps all, ICEs are capable of autonomous rolling-circle replication.

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