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Analysis of the Mobilization Functions of the Vancomycin Resistance Transposon Tn*1549*, a Member of a New Family of Conjugative Elements[▽]

Krassimira Tsvetkova, Jean-Christophe Marvaud, and Thierry Lambert*

EA4043 Centre d'Etudes Pharmaceutiques, Université Paris XI, 92296 Châtenay-Malabry, Cedex, France

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Conjugative transfer from *Clostridium symbiosum* to enterococci of Tn*1549*, which confers VanB-type vancomycin resistance, has been reported. This indicates the presence of a transfer origin (*oriT*) in the element. Transcription analysis of Tn*1549* indicated that *orf29*, *orf28*, *orfz*, and *orf27* were cotranscribed. A pACYC184 derivative containing 250 bp intergenic to *orf29-orf30* of Tn*1549* was mobilized in *Escherichia coli recA*::RP4::Δ*nic* provided that *orf28* and *orf29* were delivered simultaneously. These open reading frame (ORF) genes were able to promote mobilization in *trans*, but a *cis*-acting preference was observed. On the basis of a mobilization assay, a minimal 28-bp *oriT* was delimited, although the frequency of transfer was significantly reduced compared to that of a 130-bp *oriT* fragment. The minimal *oriT* contained an inverted repeat and a core, which was homologous to the cleavage sequence found in certain Gram-positive rolling-circle replicating (RCR) plasmids. While Orf29 was a mobilization accessory component similar to MobC proteins, Orf28 was identified as a relaxase belonging to a new phyletic cluster of the MOB_p superfamily. The nick site was identified within *oriT* by an oligonucleotide cleavage assay. Closely related *oriT*s linked to mobilization genes were detected in data banks; they were found in various integrative and conjugative elements (ICEs) originating mainly from anaerobes. These results support the notion that Tn*1549* is a member of a MOB_p clade. Interestingly, the Tn*1549*-derived constructs were mobilized by RP4 in *E. coli*, suggesting that a relaxosome resulting from DNA cleavage by Orf28 interacted with the coupling protein TraG. This demonstrates the capacity of Tn*1549* to be mobilized by a heterologous transfer system.

Tn*1549* is a 34-kb transposon which confers vancomycin resistance in *Enterococcus* spp. (22). This entirely sequenced element, which harbors the *vanB* operon, is transferred passively among enterococci by conjugative plasmids or by the exchange of large chromosomal fragments (9, 17, 22). Tn*1549* has also been reported in Gram-positive anaerobes (18, 46), and transfer from *Clostridium symbiosum* to *Enterococcus* has been demonstrated (28). This element is likely to be a conjugative transposon consisting of three functional modules involved in transposition, transfer, and antibiotic resistance. The transposition module encodes an excisionase and an integrase which cooperatively direct excision of the element. Excision is followed by circularization, resulting in a heteroduplex at the joined ends according to the tyrosine recombinase model of the Tn*916* prototype. Analysis of *Enterococcus* transconjugants indicated that acquisition of VanB-type resistance was due to transposition of Tn*1549* (28). However, it remains to be established if Tn*1549* is a conjugative or a mobilizable transposon, since a single retransfer event has been obtained but in the presence of Tn*916* (28). In both instances, whether active or passive transfer is being considered, presence of a transfer origin (*oriT*) is required. Identification of *oriT* is thus a prerequisite to the elucidation of the transfer process.

oriT is a short specific sequence necessary for conjugative transfer of any mobile genetic element to a recipient cell provided that a conjugation machinery is present. This machinery involves (i) mating-pair formation (Mpf) between the donor and the recipient strains by an exocellular pilus system in Gram-negative bacteria, whereas, in the majority of Gram-positive bacteria, the means to achieve intimate donor-recipient cell contact remains to be identified, and (ii) formation of a channel structure for secretion (14, 30, 35, 36, 41). DNA relaxases are key enzymes in the initiation of the conjugative transfer by catalyzing single-strand cleavage of a phosphodiester bond at the nick site (*nic*) in a specific and reversible manner (2, 3, 5, 34). Many relaxases need an accessory protein, such as MobC, which stimulates strand opening and strand separation, to enhance their activity (52). Conjugative plasmids possess the genes required to promote their self-transfer. Mobilizable elements possess an *oriT* and a cognate relaxase to initiate the relaxosome but need a helper conjugative system which supplies Mpf consisting of the DNA transport machinery for intercellular transfer. Direct interaction between the relaxosome and the transfer machinery occurs by TraG coupling proteins. This family of essential proteins includes TraG of RP4, TrwB of R388, TraD of F, and TraG and VirD4 of Ti in Gram-negative bacteria and TrsK of pGO1 in Gram-positive bacteria (14, 23, 25, 32). Determination of the nucleotide sequence of *tra* regions of conjugative plasmids and of entire genomes from Gram-positive bacteria has revealed homology to genes encoding TraG/TrwB/VirD4 and to the conjugative transfer ATPase VirB4 involved in the transfer DNA translocation process. Homology has also been detected

* Corresponding author. Mailing address: EA4043, Microbiologie, 5 rue Jean-Baptiste Clément, 92296, Châtenay-Malabry, Cedex, France. Phone: (33) 1 46 83 55 49. Fax: (33) 1 46 83 58 83. E-mail: thierry.lambert@cep.u-psud.fr.

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TABLE 1. Bacterial strains and plasmids[b]

Strain or plasmid	Relevant properties	Source or reference
E. coli		
SM10	F^- *tonA21 lacY1 supE44* Mu λ^- [RP4-2 (Tc::Mu)]Km Tra^+	43
Top10	F^- *mcrA* Δ(*mrr-hsd*RMS-*mcr* BC) ϕ80*lacZ*ΔM15 Δ*lacX74 deoR rec araD139* Δ(*ara-leu*)*7697 galU galK rpsL endA1 nupG*	Invitrogen
recA::RP4::Δ*nic*	*chrRP4*::Δ*nic* Km DAP^-	1
C1a	rep^+ ilv^+ Nal	40
BL21λ DE3	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm* (DE3)	Invitrogen
E. faecalis		
JH2-2::Tn*1549*	Fus Rif Vm	28
Plasmids		
$pACYC_{Gm}$	Derivative of pACYC184 in which the *cat-tet* fragment was replaced by *aac(3)-IV*; Gm Apra	Laboratory
pCM105	$pACYC_{Gm}$ Ω250-bp EcoRI-HindIII PCR intergenic fragment *orf29-orf30* from Tn*1549*	This study
pCM105-1	pCM105 (C_{141}→T)[a]	This study
pCM105-2	pCM105 ($G_{152}G_{154}$→ $T_{152}C_{154}$)	This study
pCM105-3	pCM105 ($G_{159}C_{160}$→ $T_{159}G_{160}$)	This study
pCM105-4	pCM105 ($G_{194}C_{195}$→ $T_{194}G_{195}$)	This study
pCM105-5	pCM105 ($C_{216}C_{218}$→ $G_{216}G_{218}$)	This study
pCM106	$pACYC_{Gm}$ Ω28-bp $oriT_{Tn1549}$	This study
pCM107	$pACYC_{Gm}$ Ω130-bp EcoRI-HindIII PCR part of the PCR intergenic fragment *orf29-orf30* from Tn*1549*	This study
pBAD/HisA	Expression vector, *araBAD* promoter; Amp	Invitrogen
pCM108	pBAD/HisA Ω1,328-bp NcoI-XhoI PCR fragment (rlx_{Tn1549})	This study
$pUC18_{Gm}$	Derivative of pUC18 in which *blaT* is replaced by *aac(3)-IV*; Gm Apra	This study
pCM109	$pUC18_{Gm}$ Ω1,879-bp EcoRI-XbaI PCR fragment (intergenic *orf30-orf29* region, *orf29*, and *orf28*) of Tn*1549*	This study
pCM110	$pUC18_{Gm}$ Ω2,664-bp EcoRI-XbaI PCR fragment (intergenic region *orf30-orf29*, *orf29*, *orf28*, *orfz*, and *orf27*) of Tn*1549*	This study
pGB2	Spc Str	15
pCM111	pGB2 Ω372-bp EcoRI-HindIII PCR fragment (*mobC*) of Tn*1549*	This study
pET28a(+)	His tag expression vector; Km	Invitrogen
pCM112	pET28a+ Ω354-bp EcoRI-NdeI PCR fragment (*mobC*) of Tn*1549*	This study
pCM113	pET28a+ Ω1,325-bp NcoI-XhoI PCR fragment (*rlx*) of Tn*1549*	This study
pCM114	pET28a+ Ω741-bp NcoI-XhoI PCR fragment (N_{247}*rlx*) of Tn*1549*	This study
pCR2.1	PCR cloning vector; Amp Km	Invitrogen
pCM115	pCR2.1 Ω2,374 bp PCR fragment (intergenic region *orf30-orf29 mobC rlx orfz*) of Tn*1549*	This study

[a] Sequence was from GenBank accession number AF192329.1, but numbering refers to the *orf30* termination codon.
[b] Amp, ampicillin; Apra, apramycin; Fus, fusidic acid; Gm, gentamicin; Km, kanamycin; Nal, nalidixic acid; Rif, rifampin; Str, streptomycin; Vm, vancomycin.

with integrative and conjugative elements (ICEs) that include conjugative transposons (33). Conjugative transfer of ICEs occurs by a mechanism similar to that of conjugative plasmids (25). In contrast to plasmid *oriT*s, little is known about the initiation of conjugation in ICEs. Recently, the *oriT*s from three ICEs, Tn*916*, ICE*Bs1* of *Bacillus subtilis*, and an element of the SXT/R391 family, have been characterized (12, 29, 39). Similarly to conjugative plasmids, ICEs encode their own relaxase to cleave the *nic* site. Examination of Tn*1549* has shown that Orf28 displays similarity with the MobA relaxase of pC221 (31% identity) and that Orf29 is similar to MobC of pC221 (21% identity). In this study we show that the product of *orf28* acts as a relaxase and we have identified its cognate *oriT*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The origins and properties of bacterial strains and plasmids are listed in Table 1. *E. coli recA*::RP4::Δ*nic* was used as a donor in the mating experiments and was grown on Luria-Bertani (LB) agar supplemented with 0.02% arabinose and 1 mM diaminopimelic acid (DAP). *E. coli* BL21λ DE3 was used as a host for protein expression and purification.

Plasmid methodology, enzymes, and oligonucleotides. Plasmid DNA purification (Qiagen), digestion with restriction endonucleases (New England Biolabs, Invitrogen), amplification of DNA by PCR with *Pfu* (Stratagene) or *Taq* (Invitrogen) DNA polymerases, ligation of DNA fragments with T4 DNA ligase (Invitrogen), and cloning with a TOPO-TA cloning kit (Invitrogen) were performed according to the manufacturers' instructions. *Enterococcus faecalis* JH2-2::Tn*1549* total DNA was prepared with an Illustra kit (GE Healthcare). Recombinant plasmid DNA was introduced into *E. coli* Top10 or *E. coli recA*::RP4::Δ*nic* by electrotransformation. The oligonucleotides used (purchased from Invitrogen) are listed in Table 2.

Reverse transcription. JH2-2::Tn*1549* total RNA was extracted using the RiboPure bacteria kit (Ambion) according to the manufacturer's instructions. To rule out contamination by genomic DNA, total RNA was treated with RNase-free DNase (DNase I) (Invitrogen). The quality and quantity of RNA were measured using the microchip analysis system of an Agilent 2100 bioanalyzer apparatus (Agilent Technologies). Reverse transcription was performed using 2.5 μg total heat-denatured DNA-free RNA, 0.8 μl random primer (3 μg/μl), 1 μl 10 mM deoxynucleoside triphosphates, 4 μl 5× first-strand buffer, 1 μl 0.1 M dithiothrietol, 1 μl RNaseOUT (40 U/μl), 1 μl of SuperScript III reverse transcriptase (200 U/μl) (Invitrogen), and H_2O to a total volume of 20 μl. An aliquot of the cDNA (1 μl of the reverse transcription [RT] reaction mixture) was amplified by PCR with 20 pmol each of primer pairs 30r-29f2, 29r2-28f2, and 28r1-27f in order to determine a putative cotranscription of *orf30*, *orf29*, *orf28*, *orfz*, and *orf27*. Negative controls were performed with 1 μl DNA-free RNA.

Identification of the transcriptional start site for *orf30* and for the relaxase operon. Reverse transcription and rapid amplification of cDNA ends (RACE) were performed using the 5′RACE system kit (Invitrogen) on RNA extracted from *E. faecalis* JH2-2::Tn*1549*. Briefly, for the determination of the transcriptional start site of *orf30*, after a reverse transcription step using an *orf30*-specific primer, Race1, dC-tailed DNA was amplified by PCR with another *orf30*-specific

TABLE 2. Oligonucleotides

Oligonucleotide[a]	Sequence (5′–3′)	Position (start, end)[b]
RlxNco (−)	AGC CAT GGCAGTTACAAAGATACATCC[c]	−21857, −21835
RlxXho (+)	TACTCGAGTAGCTCTTGCGTCCATTCCTTTCCC	20532, 20556
RlxNXho (+)	TACTCGAGCGCTCTGGTGATAACGCGGCC	21116, 21137
RlxpBADXho (+)	TACTCGAGTTCATAGCTCTTGCGTCCATTCCTTTCCC	20529, 20556
C1 (+)	**A**AGGCAGGGACGGGGCAGGCCGTT[c]	22212, 22233
C2 (−)	**G**CCGCGTCCGTTTTGCTTGTTGG	−22209, −22187
FmutNic (+)	**T**AAGCATAGGGAGTGTAGCC	22263, 22245
RmutNic (−)	**C**TACGTTACTGTTTCTCCAC	−22265, −22284
MutAF (+)	**C**ACTCC CGGAAAAACGGCCTG	22245, 22225
MutA1R (−)	**G**CTACAGTACCTATGCTTGCTACGTTACTG	−22246, −22275
MutA2R (−)	**C**ATACACTCCCTATGCTTGCTACGTTACTG	−22248, −22275
MutBF (+)	**A**TCGCGAAACCCCTGAATGGAAAGG	22158, 22176
MutBR (−)	**T**TCCCCAACAAGCAAAACGGAC	−22204, −22183
MobCNdeI (−)	GACATATGGCAAACAGAAAGC G	−22147, −22131
MobCFHindIII (−)	ATAAGCTTGGAAAGGACGGTGAAGCA	−22165, −22147
MobCREcoRI (+)	ATGAATTCTCGATGTAGTCAA GGG C	21793, 21812
30R (−)	ATAAGCTTACTTTTAGCGGGAGCTGC	−22389, −22370
29F (+)	ATGAATTCACCGTCCTTTCCATTCAG	22150, 22170
O30DF (−)	CTAAGCTTGAAACAGTAACGTAGCAAGC	−22279, −22260
27FEcoRI (+)	ATGAATTCATGC CGGAAAAGCCTTGAA	20129, 20148
28RHindIII (−)	ATAAGCTTATGAGTATTCCACGAATGA	−20533, −20514
184OriF/EcoRI (+)	CTGAATTCAGCAAGCATAGGGAGTGTAGCCACTCCCTGCAGAAGCTTTGACA CAGTGCCG	22266, 22239
184R/EcoRI (−)	TAGAATTCCGGATGAGCATTCATCAGG	
HTH-EcoRI (−)	TGAATTCTGGCATCCTCTGTTTTACT	−22404, −22385
RlxXbaI (+)	AATCTAGATACTCATAGCTCTTGCGTCC	20525, 20545
YXbaI (+)	ATCTAGACTATCTCTGTTCCAGCGGGAAG	19742, 19763
Ori34F (+)[d]	TAACGTAGCAAGCATAGGGAGTGTAGCCACTCCC	22272, 22239
Ori34R (−)[d]	GGGAGTGGCTACACTCCCTATGCTTGCTACGTTA	−22239, −22272
28RpACY (−)	ACTCCACCGCTGATGACATCAG	
28FpACY (+)	CTTTATTCACATTCTTGCCCG	
27f (+)[e]	GCTCTAAGTGCGTAGGTTTC	20166, 20185
28f2 (+)[e]	AAACTTTCCCGGCTTGATCT	21231, 21250
28f1 (+)[e]	CCGCAGGAAATCATCAAAAT	21273, 21292
28r1 (−)[e]	GTGCAAGGAAAACGGATTGT	−21435, −21416
29f2 (+)[e]	CAGGGACGCGCAGCACGATT	22108, 22127
29r2 (−)[e]	GAGATACAGAAGATCGGCGT	−21967, −21948
30f (+)[e]	CGATGATCGGCAGGTATTCT	22470, 22489
30r (−)[e]	ATTGACTGGCGCTATCTTGC	−22668, −22649
Race 1(+)[e]	GATGATCGGCAGGTATTCTT	22471, 22490
Race 2(+)[e]	ATAAGCTGTATGACAACCGG	22595, 22614

[a] +, sense primer; −, antisense primer.
[b] Nucleotide numbering according to GenBank accession number AF192329.1.
[c] Restriction sites used in cloning are underlined. 5′-end phosphorylated nucleotides are in bold.
[d] Oligonucleotides used in nicking assay.
[e] Oligonucleotides used in RT-PCR and 5′ RACE assays.

primer, Race2, and a dG-anchor oligonucleotide primer. PCR products were then cloned into pCR2.1 (Invitrogen) and further sequenced. The same protocol was applied to identify the transcriptional start site of the *orf29-orf28-orfz-orf27* operon by the use of (i) *orf28*-specific primer 28f2 for the reverse transcription step, (ii) *orf28*-specific primer 28f1 plus dG-anchor oligonucleotide primer in a first PCR, and (iii) *orf29*-specific primer 29f2 and dG-anchor oligonucleotide primer in the nested PCR step. The PCR products were cloned in pCR2.1 and sequenced.

Plasmid constructions. To localize *oriT*, a 250-bp EcoRI-HindIII PCR fragment containing the intergenic *orf29-orf30* region was cloned in $pACYC_{Gm}$, resulting in pCM105. pCM106 was obtained by PCR amplification with primers 184OriF/EcoRI and 184R/EcoRI and pCM105 DNA as a template. An EcoRI-HindIII PCR fragment obtained with primers 29F and O30DF from *E. faecalis* JH2-2::Tn*1549* was cloned in $pACYC_{Gm}$, resulting in plasmid pCM107.

The *mobC*-like and relaxase genes amplified with primers MobcFHindIII plus MobcREcoRI and RlxNco plus RlxpBADXho were cloned in pGB2 and pBAD/His vectors to generate pCM111 and pCM108, respectively.

A 1,879-pb EcoRI-XbaI-PCR fragment containing the intergenic region *orf29-orf30*, the $mobC_{Tn1549}$ gene, and the *rlx* gene was obtained with primers HTH-EcoRI and RlxXbaI and *E. faecalis* JH2-2::Tn*1549* DNA and cloned in $pUC18_{Gm}$ to generate plasmid PCM109. A 2,662-bp EcoRI-XbaI PCR fragment, containing the intergenic region *orf29-orf30*, the $mobC_{Tn1549}$ gene, the *rlx* gene, *orfz*, and *orf27* was obtained with primers HTH-EcoRI and YXbaI and *E. faecalis* JH2-2::Tn*1549* DNA as a template and was cloned in $pUC18_{Gm}$ to generate plasmid pCM110. To purify $MobC_{Tn1549}$, the relaxase (442 amino acids) and its active N-terminal domain (247 amino acids) proteins were expressed as 6×His-tagged fusions. The EcoRI-NdeI PCR fragment obtained with primers MobcREcoRI and MobCNdeI, the Xho-NcoI PCR fragment obtained with primers RlxNco and RlxXho, and the Xho-NcoI PCR fragment obtained with primers RlxNco and RlxNXho were cloned into pET28a(+). The sequences of the recombinant plasmids were verified to rule out error during DNA polymerization.

Construction of *orf29-orf30* intergenic region (ID) mutants. Site-directed mutagenesis with pCM105 DNA as a template was performed within the intergenic *orf29-orf30* portion to identify nucleotides essential in the mobilization process. Plasmid mutants called pCM105-1 to -5 were obtained with the primer pairs MutAF/MutA1R, MutAF/MutA2R, MutBF/MutBR, C1/C2, and FmutNic/RmutNic, respectively. Each PCR fragment was ligated by T4 ligase and transformed into *E. coli* Top10 (Invitrogen). The presence of the expected mutations was confirmed by DNA sequencing. The various plasmid mutants were then introduced into *E. coli recA*::RP4::Δ*nic*(pCM108, pCM111) by electrotransformation.

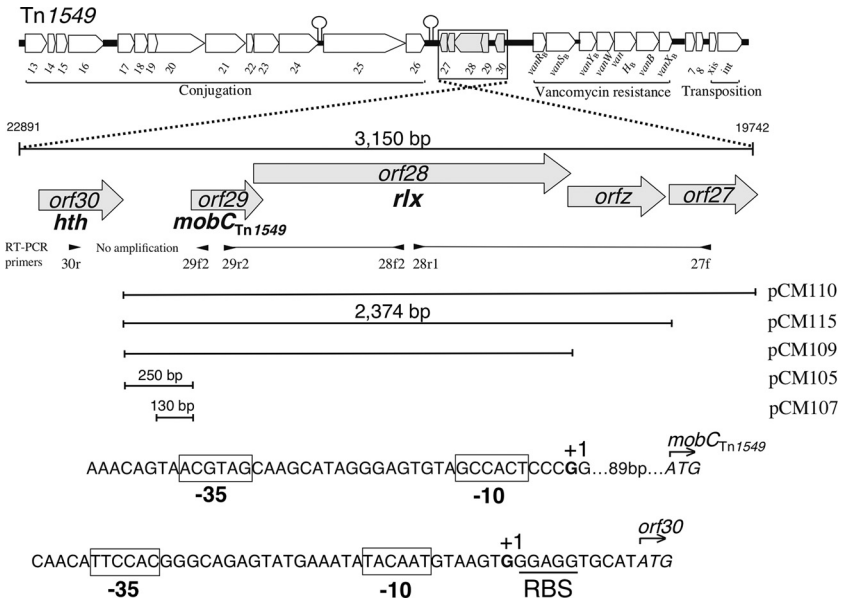

FIG. 1. The schematic representation of Tn*1549* and of its functional modules. Coordinates of the 3,150-bp fragment including the mobilization module are indicated according to GenBank accession no. AF192329.1. Open arrows represent ORFs and indicate the sense of transcription. Large stem-loops are indicated. The relaxase gene cluster is enlarged and inverted to facilitate analysis. The primers used for amplification of transgene fragments from the cDNA are indicated by arrowheads. The resulting PCR products are shown by a thin dark line, which corresponds to a cotranscription. The inserts of plasmids indicated on the right are represented by horizontal bars. Initiation codons of *orf29* and *orf30* are italicized. The beginning of transcription of *orf30* and of the mobilization operon detected by 5′ RACE are indicated in bold, and the −35 and −10 motifs are boxed.

Conjugation experiments. *E. coli recA*::RP4::Δ*nic* was successively transformed with plasmids pCM108 and pCM111. The resulting strain was then transformed independently with the following plasmids: pCM105 and its mutant derivatives, pCM106, and pCM107. Each transformant was used as a donor in conjugation experiments with *E. coli* C1a as a recipient. Mating experiments were carried out on LB agar plates supplemented with 0.02% arabinose and 1 mM DAP incubated at 37°C for 24 h. Transconjugants were selected on LB agar plates containing gentamicin (8 μg/ml) and nalidixic acid (40 μg/ml). Quantifi-

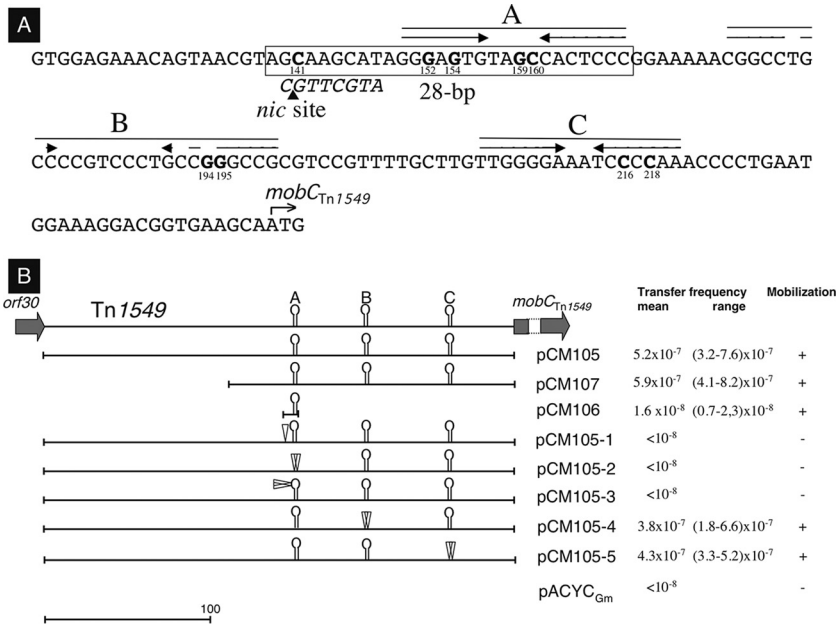

FIG. 2. (A) Portion of the *orf29-orf30* intergenic region upstream from the *mobC* gene. The inverted repeats of hairpins A, B, and C are indicated by arrows. The 28-bp *oriT* is boxed, and the *nic* core site on the antisense strand is italicized. The *nic* site is indicated by an arrowhead. The initiation codon of the *mobC* gene is shown by a bent arrow. Nucleotides which have been substituted in pCM105 derivatives are in bold, and their positions are indicated. Numbering is from GenBank accession no. AF192329.1, but for the sake of simplicity in reading refers to the *orf30* termination codon. (B) Mobilization of pACYC$_{Gm}$ derivatives in *E. coli recA*:RP4::Δ*nic*. Expression of the *rlx* gene was induced by 0.02% arabinose. The intergenic 250-bp *orf29-orf30* fragment is shown with hairpins A, B, and C. Mutations are shown by arrowheads; when they were predicted to decrease the stability of the hairpins, the latter were deleted. Frequencies of transfer were the means of results of three independent mating experiments.

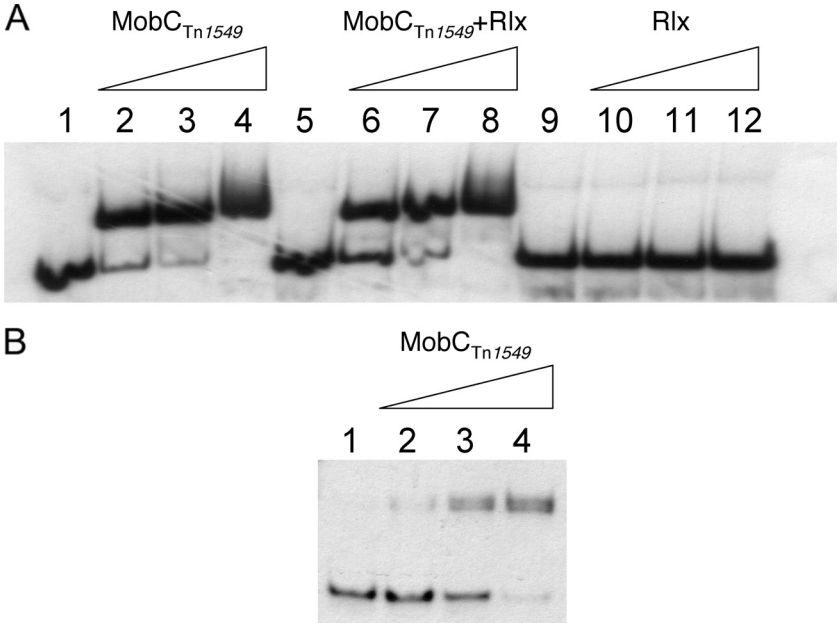

FIG. 3. Electrophoresis mobility shift assay. (A) The labeled 250-bp *orf29-orf30* intergenic fragment was incubated with increasing micromolar amounts of purified proteins $MobC_{Tn1549}$, Rlx, and a mix of MobC $_{Tn1549}$ and Rlx. (B) The 28-bp minimal *oriT* fragment was cloned in $pACYC_{Gm}$, and the PCR product was amplified as part of a 96-bp fragment including flanking sequences of the cloning site. The PCR product was labeled and incubated with increasing micromolar amounts of purified $MobC_{Tn1549}$ protein.

cation of donors was obtained by selection on LB agar plates with 1 mM DAP supplemented with ampicillin (100 μg/ml), streptomycin (20 μg/ml), and gentamicin (8 μg/ml). To evaluate the efficiency of *cis*-acting mobilization proteins, pCM109 and pCM110 were introduced separately into *E. coli recA*::RP4::Δ*nic*. Both transformants were used as donors in conjugation experiments to *E. coli* C1a. Mating experiments were carried out on LB agar plates supplemented with DAP (1 mM). Transconjugants were selected on gentamicin (8 μg/ml) and nalidixic acid (40 μg/ml), and donors were selected on gentamicin (8 μg/ml) and 1 mM DAP. Mating frequencies were calculated by dividing the number of transconjugants by the number of donor cells and expressed as the means of results of three independent experiments.

Purification of relaxase, N-terminal relaxase, and MobC proteins. Plasmids pCM112, pCM113, and pCM114 were introduced into *E. coli* BL21αDE3. The transformants were grown in LB broth containing kanamycin (50 μg/ml) at 37°C. Protein expression was induced by isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM), and each recombinant protein was then purified by immobilized metal affinity chromatography (TALON) resin (Clontech Laboratories, Inc) and was dialyzed after elution, as recommended by the manufacturer.

Electrophoretic mobility shift assays (EMSAs). A 250-bp fragment encompassing the putative *oriT* sequence was amplified by PCR with primers 29F and 30R from JH2-2::Tn*1549* total DNA used as a template. A 96-bp PCR fragment comprising the 28-bp *oriT* sequence (minimal *oriT*) was obtained with primers 28RpACY and 28FpACY and pCM106 DNA as a template; the remaining 68 bp corresponded to the plasmid flanking sequences. Purified fragments were end labeled with [γ-^{32}P]ATP (3,000 Ci mmol^{-1}) and T4 polynucleotide kinase (New England Biolabs). Binding of relaxase or mobilization protein to DNA was carried out in a 20-μl buffer (20 mM Tris-HCl [pH 7.4], 5 mM $MgCl_2$, 50 mM KCl, 25% glycerol, and 0.1 mg/ml sonicated salmon sperm DNA) and 5 nM labeled DNA. The reaction mixtures were incubated at 25°C for 30 min. Samples were loaded onto an 8% polyacrylamide gel (29:1 acrylamide-bisacrylamide, 0.5× TBE) and electrophoresed for 2 h at 120 V. The gels were then dried and visualized by autoradiography.

Oligonucleotide cleavage. Ori34F and Ori34R oligonucleotides were labeled at the 5′ end using [γ-^{32}P]ATP (3,000 Ci mmol^{-1}) and polynucleotide kinase (New England Biolabs). Unbound [γ-^{32}P]ATP was eliminated from the mixture by purification with the mini Quick Spin DNA column (Roche). Nick site cleavage was carried out as previously described (38).

RESULTS

Organization of the mobilization region of Tn*1549*. The sequence of Tn*1549* is available (GenBank accession number AF192329), and a putative relaxase gene (*rlx*) was attributed to *orf28*, which is part of a four-gene cluster transcribed in the same orientation and located upstream from the *vanB* operon (Fig. 1). *orf28* overlaps *orf29* and *orfz* by 39 and 19 bp, respectively. The *orf30* product displays homology with helix-turn-helix XRE-family-like proteins, which include regulatory proteins. Orf29 is a member of the MobC superfamily of bacterial mobilization proteins and shares 20% and 30% identity with MobC of pC221 and MbeC of ColE1, respectively (45). Orf28 belongs to the ColE1 superfamily of relaxases and displays 31% identity with MobA of pC221, which belongs to the MOB_{P7} clade (11). The motif [LF]xxx[GS]xNxNQxAxxxN, described as conserved in mobilization-associated proteins from Gram-positive and Gram-negative bacteria (50), is present in $MobC_{Tn1549}$ except for the [L/F] residue replaced by an isoleucin residue, which is likely an isofunctional amino acid. In contrast, the *orfz* and *orf27* products do not match to any recognized protein family.

Transcriptional analysis of the relaxase-associated genes. The cDNA corresponding to total RNA from *E. faecalis* JH2-2::Tn*1549* was used for PCR to determine if the *orf*s of the mobilization region were cotranscribed (Fig. 1). Amplification of transgene fragments was obtained from cDNA with primer pairs 29r2-28f2 and 28r1-27f, leading to ca. 730-bp and 1,270-bp fragments, respectively. In addition, no amplification was detected with primers 29f2 and 30r located, respectively, in *orf29* and *orf30*. These results summarized in Fig. 1 indicated

that *orf29*, *orf28*, *orfz*, and *orf27* constitute an operon and that there is no cotranscription of *orf30*.

Mapping of the transcriptional start site. Amplification and sequencing of the 5′ RACE products indicated that the end of the mRNA transcribed from *orf30* was found 12 bp upstream from the translational initiation site, suggesting −35 (TTC CAC) and −10 (TACAAT) as promoter sequences separated by 17 bp (Fig. 1). The mRNA for *orf29* was found to begin 91 bp upstream from the start codon, consistent with a promoter composed of −35 (ACGTAG) and −10 (GCCACT) motifs separated by a 17-bp sequence. The −10 (GCCACT) motif was also proposed by computer analysis, since the underlined bases are in agreement with binding sequences of the σ^{70} factor of the *E. coli* RNA polymerase; in contrast, the −35 motif was uncommon. The −10 promoter sequence was within the hairpin A, which in addition to being part of the *oriT* could play a role in the regulation of the mobilization operon. However, an artifact due to RT stopping at the secondary structure should not be completely excluded.

Mobilization experiments with *E. coli*. In a preliminary assay, a 2,374-bp PCR fragment of Tn*1549* beginning from the intergenic region *orf29-30* and ending at the end of *orfz* was cloned in pCR2.1. The recombinant plasmid, pCM115, was mobilizable in *E. coli* SM10 by the transfer apparatus of RP4 (data not shown). This result demonstrated that the cloned fragment contained an *oriT*, and the fact that no homology was detected with the *oriT* region of RP4 suggests that this fragment also encoded mobilization functions. In order to localize the *oriT* and to establish if the $MobC_{Tn1549}$ accessory protein was necessary for transfer, a mobilization assay was carried out with *E. coli recA*::RP4::Δ*nic*(pCM111, pCM108). It consisted of the mobilization of $pACYC_{Gm}$ derivatives in which several portions of the 2,374-bp PCR fragment of Tn*1549* were cloned, while the relaxase was delivered in *trans*. The experiments were also carried out in the absence or in the presence of *orf29* (*mobC*$_{Tn1549}$) under the control of a constitutive promoter provided by pGB2. Mobilization was obtained with a $pACYC_{Gm}$ derivative carrying a 250-bp *orf30-orf29* intergenic fragment, provided that both *rlx*$_{Tn1549}$ and *mobC*$_{Tn1549}$ genes were present (Fig. 2B). Induction by arabinose increased transfer frequency up to 100-fold (data not shown). In contrast, no transfer was observed in the absence of $MobC_{Tn1549}$.

Directed mutagenesis in the *oriT* region. Detailed analysis of the 250-bp *oriT* fragment showed the presence of three hairpin structures upstream from the *mobC*$_{Tn1549}$ gene which were designated A, B, and C. Computer-generated models of the secondary structure formed by the inverted repeats A, B, and C indicated the free enthalpy of the thermodynamically most stable forms to be −6.25, −4.98, and −5.42 kcal mol^{-1}, respectively. Interestingly, inverted repeat A was preceded on the complementary strand by a 8-base-pair stretch with a RYGCTTG′C cleavage motif conserved in rolling-circle replicating (RCR) plasmids such as pNZ4000 of *Lactococcus lactis* (50) and pC221 and pC223 of *Staphylococcus aureus* (10, 11). The impact of the hairpin structures on the activity of *oriT* was evaluated by directed mutagenesis of pCM105 to generate in every hairpin two base substitutions ($G_{152}G_{154}\rightarrow T_{152}C_{154}$, $G_{194}C_{195}\rightarrow T_{194}G_{195}$, and $C_{216}C_{218}\rightarrow G_{216}G_{218}$) that disrupt the secondary structure (Fig. 2A). In addition, the substitution $G_{159}C_{160}\rightarrow T_{159}G_{160}$ was achieved to alter the head of hairpin A. The consequence on transfer was tested by mobilization in *E. coli* as described above. Disruption of inverted repeat A or alteration at the head of the stem-loop abolished transfer. In contrast, disruption of the B and C hairpins did not significantly affect mobilization (Fig. 2B). When the predicted *nic* cleavage site was altered by a substitution ($C_{141}\rightarrow T$), transfer was abolished.

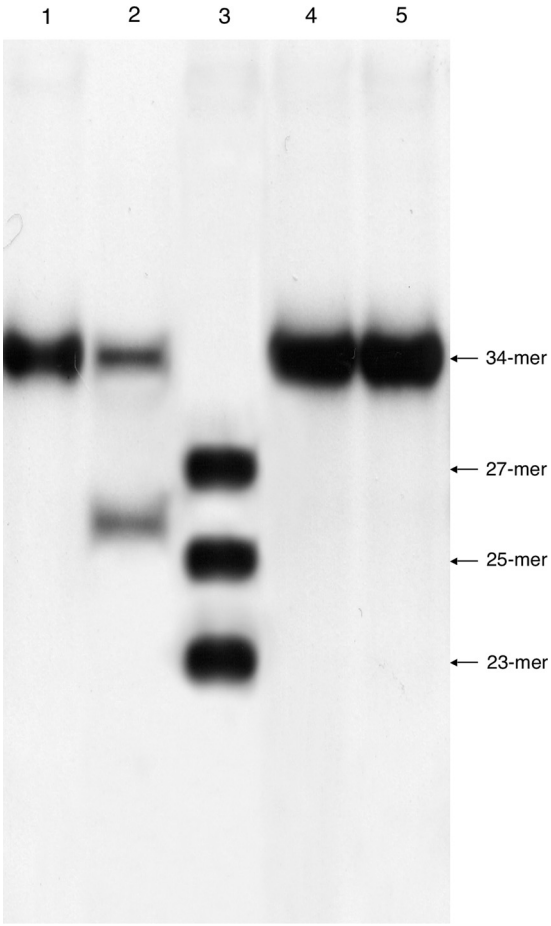

FIG. 4. *In vitro* cleavage of *oriT* by Rlx_{Tn1549}. Reaction mixtures contained 5′-labeled oligonucleotides (10 nM) incubated with or without $RlxN_{247}$ (12.5 μM). Reactions were carried out at 37°C for 3 h as described in Materials and Methods. Lane 1, Ori34R-34-mer (5′-^{32}P-GGGAGTGGCTACACTCCCTATGCTTGCTACGTTA); lane 2, Ori34R-34-mer plus $RlxN_{247}$; lane 3, mix of oligonucleotides 27-mer, 25-mer, and 23-mer used as size standards; lane 4, Ori34F-34-mer (5′-^{32}P-TAACGTAGCAAGCATAGGGAGTGTAGCCACTCCC); lane 5, Ori34F-34-mer plus $RlxN_{247}$. Oligonucleotide control sizes are indicated by arrows.

Identification of a minimal *oriT*. A 130-bp fragment just upstream from *mobC*$_{Tn1549}$ was cloned in the $pACYC_{Gm}$ vector, and the resulting plasmid, pCM107, was found to be as efficient as pCM105 in the mobilization assay (Fig. 2B). Taken together, these results suggest that hairpin A and the adjacent conserved cleavage site were part of the *oriT*. The corresponding 28-bp sequence was introduced following PCR in $pACYC_{Gm}$ to delimit the minimal *oriT*. This fragment was sufficient for mobilization of $pACYC_{Gm}$ in *E. coli recA*::RP4::Δ*nic*[pCM108(*rlx*), pCM111(*mobC*$_{Tn1549}$)], although at a di-

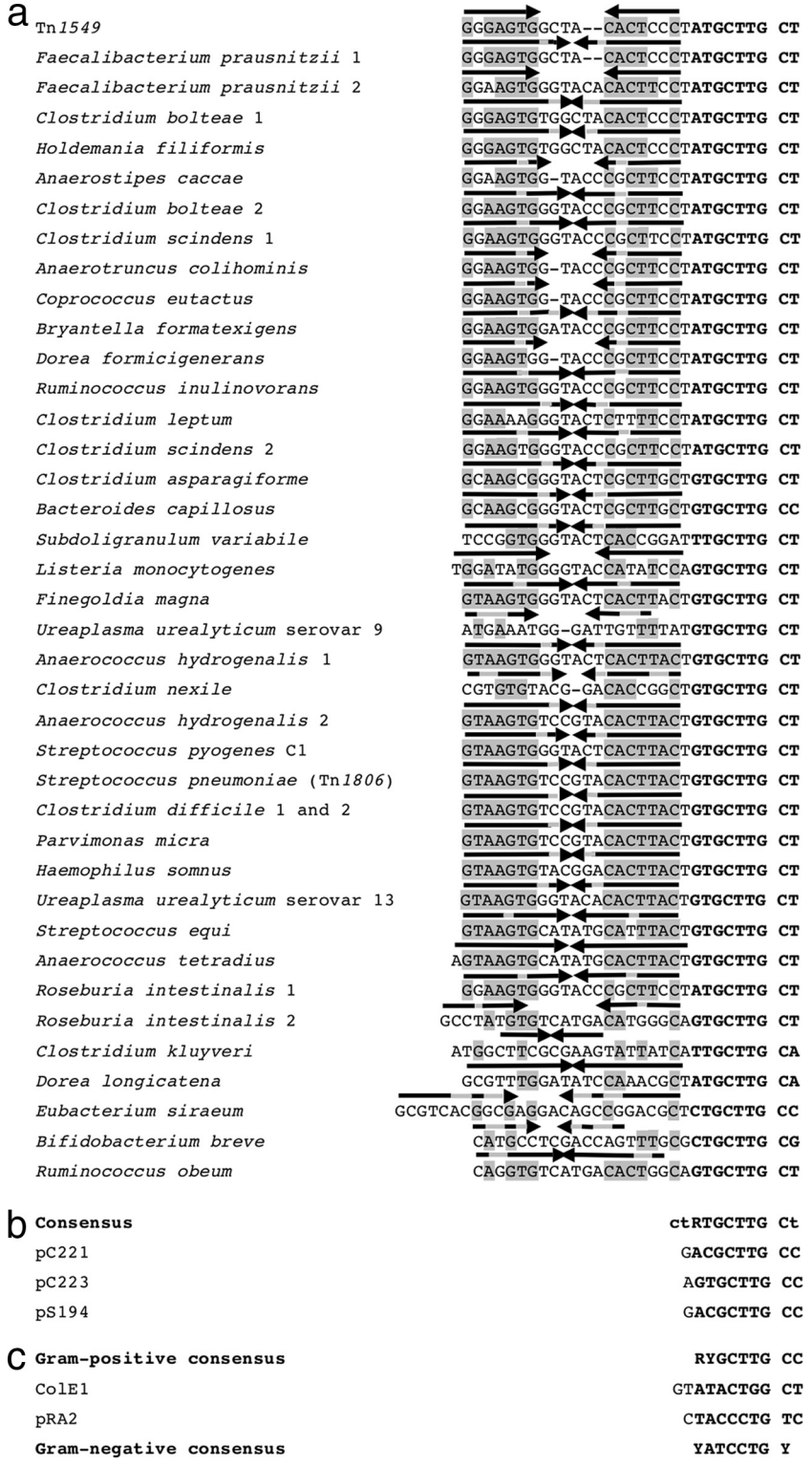

FIG. 5. Alignment of *oriT* regions of the MOB_p superfamily. (a) Putative *oriT* related to $oriT_{Tn1549}$ detected by BLAST searches. Only motifs found in the vicinity of the mobilization genes were examined. Inverted repeats are indicated by arrows: dark, complementary bases; gray, no complementary bases. Conserved nucleotides in the inverted repeats are in gray boxes. Bacterial sources are derived from GenBank accession numbers and are cited in phylogenetic order according to the similarity of their cognate relaxases with Rlx_{Tn1549} (Fig. 6): ABXA01000002.1 and ABXA01000003.1 (*A. hydrogenalis* DSM 7454), ACGC01000033.1 (*A. tetradius* ATCC 35098), ABAX03000014.1. (*A. caccae* DSM 14662), ABGD02000024.1 (*A. colihominis* DSM 17241), AAXG02000015.1 (*B. capillosus* ATCC 29799), ACCG01000027.1 (*B. breve* DSM 20213), ABXX02000003.1 (*B. pseudocatenulatum* DSM 20438), NZ_ACCL01000033.1 (*B. formatexigens* DSM 14469), NZ_ACCJ01000017.1 (*C. asparagiforme* DSM 15981), ABCC02000025.1 (*C. bolteae* ATCC BAA-613), NC_009089.1 (*C. difficile* 630), NC_011837.1 (*C. kluyveri* NBRC 12016), ABCB02000021.1 (*C. leptum* DSM 753), ABWO01000059.1 (*C. nexile* DSM 1787), ABFY02000002.1 (*C. scindens* ATCC 35704), ABEY02000028.1

minished frequency (ca. 30-fold) compared to the 250-bp *orf29-30* intergenic fragment (Fig. 2B); $pACYC_{Gm}$ used as a control could not be transferred.

Mobilization of $pUC18_{Gm}$ by the *cis*-acting *mobC* and *rlx* genes. The ability for certain proteins to act in *trans* can be strongly reduced. Although conjugation proteins are not known to be *cis* acting, TraA *cis*-acting preference has been recently demonstrated for the relaxase of *Rhizobium etli* (38). In order to examine the possibility that *rlx* exhibits a higher efficiency in *cis*, a 1,879-bp fragment which includes the entire *orf30-29* intergenic region, $mobC_{Tn1549}$, and *rlx* was cloned in $pUC18_{Gm}$. The recombinant plasmid, pCM109, introduced by electrotransformation in *E. coli recA*::RP4::Δ*nic*, transferred to *E. coli* C1a at a frequency of 7.2×10^{-4}. Such an efficiency was never obtained when the $mobC_{Tn1549}$ and *rlx* genes were provided in *trans* despite the induction of *rlx* by arabinose. The frequency of transfer was found to be almost identical when the experiments were performed with pCM110, which contains a 2,664-bp fragment delimited by the terminal codons of *orf29* and *orf27*. These data indicate that the presence of *orfz* and *orf27* did not influence mobilization.

Electrophoresis mobility shift assay. The *mobC* and *rlx* genes of Tn*1549* were amplified by PCR and cloned in pET28a(+) expression vectors in order to express 6×His-tagged fusion proteins. In addition, the N-terminal domain (247 amino acids) of the relaxase (Rlx) known to support the transesterase activity was also expressed as a 6×His-tagged fusion (27). The proteins were purified by cobalt ion chelating chromatography, and RlxH6 and $RlxN_{247}H6$ soluble forms were used to test activity. The relaxosome accessory protein $MobC_{Tn1549}$ (M_w, 12,486) is basic (calculated pI = 10.30) like most DNA-binding proteins and was found to bind to the 250-bp *orf29-orf30* intergenic fragment and to the 28-bp minimal *oriT* (Fig. 3). In contrast, RlxH6 alone was unable to bind to these fragments (Fig. 3). It has been proposed that accessory proteins, such as MobC of R1162, denature the *oriT* strands, providing a single-stranded substrate for the relaxase (52) whereas certain relaxases are unable to nick covalently closed circular (CCC) DNA (5).

Site-specific cleavage of oligodeoxyribonucleotides by Rlx. To identify precisely the *nic* site, cleavage assays were performed with single-stranded oligodeoxyribonucleotides spanning the transfer origin's *nic* region incubated with $RlxN_{247}$. The reaction mixtures were separated by electrophoresis in a polyacrylamide gel (Fig. 4). $RlxN_{247}$ cleaved the Ori34R-34-mer oligonucleotide, yielding the 26-mer 5′-^{32}P-GGGAGTG GCTACACTCCCTATGCTTG plus CTACGTTA-3′, but not the complementary oligonucleotide Ori34F-34-mer, indicating that single-strand DNA nicking occurred on the antisense strand upstream from $mobC_{Tn1549}$ and *rlx* genes and that, as expected, *nic* was located at the end of the core site. To study the role of $MobC_{Tn1549}$, the cleavage assay of the Ori34R oligonucleotide was carried out using several quantities of relaxase (0.5 to 12.5 μM) in the absence or in the presence of $MobC_{Tn1549}$ protein (10 μM). Cleavage was more efficient when the concentration of relaxase was increased; in contrast, the reaction was not influenced by the presence of $MobC_{Tn1549}$ (data not shown).

Identification of *oriT* closely related to that of Tn*1549*. $oriT_{Tn1549}$ did not display homology with other *oriT*s from plasmids and transposons (16, 20, 29) except as mentioned at the 8-bp core site found to be conserved with the cleavage motif of RCR staphylococcal plasmids. To test the possibility that $oriT_{Tn1549}$ could be a member of a large family, putative *oriT*s were screened by BLAST in GenBank on the basis of DNA sequence homology with $oriT_{Tn1549}$ in a mobilization gene environment and an identical organization with the mobilization module of Tn*1549*. A total of 39 potential *oriT*s consisting of a consensus motif, C(a)T(a)RTGCTTG′CT, adjacent to an inverted repeat were found to be associated with both putative *mobC* and *rlx* genes as part of ICEs. The genetic elements were distributed in various bacterial species belonging to the *Firmicutes*, and they were also present in *Ureaplasma* species and Gram-negative bacteria such as *Bacteroides* and *Eubacterium* species and *Haemophilus somnus.* In all instances, these putative *oriT*s included a consensus core with the nicking motif composed of 11 conserved nucleotides linked to a variable inverted repeat and were between 28 and 32 bp in size, except in *Eubacterium siraeum* (36 bp). Conserved nucleotides were also found in the inverted repeats but to a lesser extent than in the core site (Fig. 5). Several putative *oriT*s were common to different genera; e.g., the $oriT_{Tn1549}$ was detected in *Faecalibacterium prausnitzii* M21/2 (Fig. 5). *Anaerococcus hydrogenalis* DSM 7454, *Clostridium bolteae* ATCC BAA-613, *Clostridium difficile* 630, *Clostridium scindens* ATCC35704, *F. prausnitzii* M21/2, *Finegoldia magna* ATCC29328, and *Roseburia intestinalis* L1-82 contained two distinct *oriT* mobilization modules. In *H. somnus* 129PT, a putative *oriT* was detected just upstream from a *mobC*-like gene in the absence of a relaxase gene. In contrast, *H. somnus* 2336 harbors a relaxase gene which most probably originated from clostridia (Fig. 6). Since relaxases are key enzymes in the transfer process, a phylogenetic tree was drawn to establish the relationship between the relaxases associated with the putative *oriT*s. The relaxases of *Anaerostipes caccae* DSM1462, *Anaerotruncus colihominis* DSM 17241, *Coprococcus eutactus* ATTC 27759, and *Dorea formicigenerans* ATTC 27755, which are part of a conserved ICE, were found to be identical (Fig. 6). These bacteria also

(*C. eutactus* ATCC 27759), AAXA02000014.1 (*D. formicigenerans* ATCC 27755), AAXB02000001.1 (*D. longicatena* DSM 13814), ABCA03000042.1 (*E. siraeum* DSM 15702), ABED02000029.1 (*F. prausnitzii* M212-2), NC_010376.1 (*F. magna* ATCC 29328), NC_008309.1 (*H. somnus* 129PT), AADR01000004.1 (*L. monocytogenes* 4b H7858), ABEE02000016.1 (*P. micra* ATCC 33270), ABYJ01000019.1 (*R. intestinalis* L1-82), AAVO02000008.1 (*R. obeum* ATCC 29174), NZ_ACFY01000051.1 (*R. inulinivorans* DSM 16841), AM909652 (*S. equi* ICESe2), EF469826 (*S. pneumoniae* AP200 Tn*1806*), NC_008024.1 and FM162351.1 (*S. pyogenes* MAGS10750 and C1), NZ_ACBY01000108.1 (*S. variabile*), AAYQ2000002.1 (*U. urealyticum* serovar 9 ATCC 33175), ABEV01000002.1 (*U. urealyticum* serovar 13 ATCC 33698). The putative *oriT* of *B. pseudocatenulatum* is identical to that of *B. breve*. Core cleavage sequences are indicated in bold. Consensus nick sequences are indicated; Uppercase letters, conserved bases; lowercase letters, bases present in the majority of sequences. (b) Core cleavage sequences of RCR *Staphylococcus* plasmids and consensus sequence. (c) Core cleavage of ColE1 and pRA2 and consensus sequence.

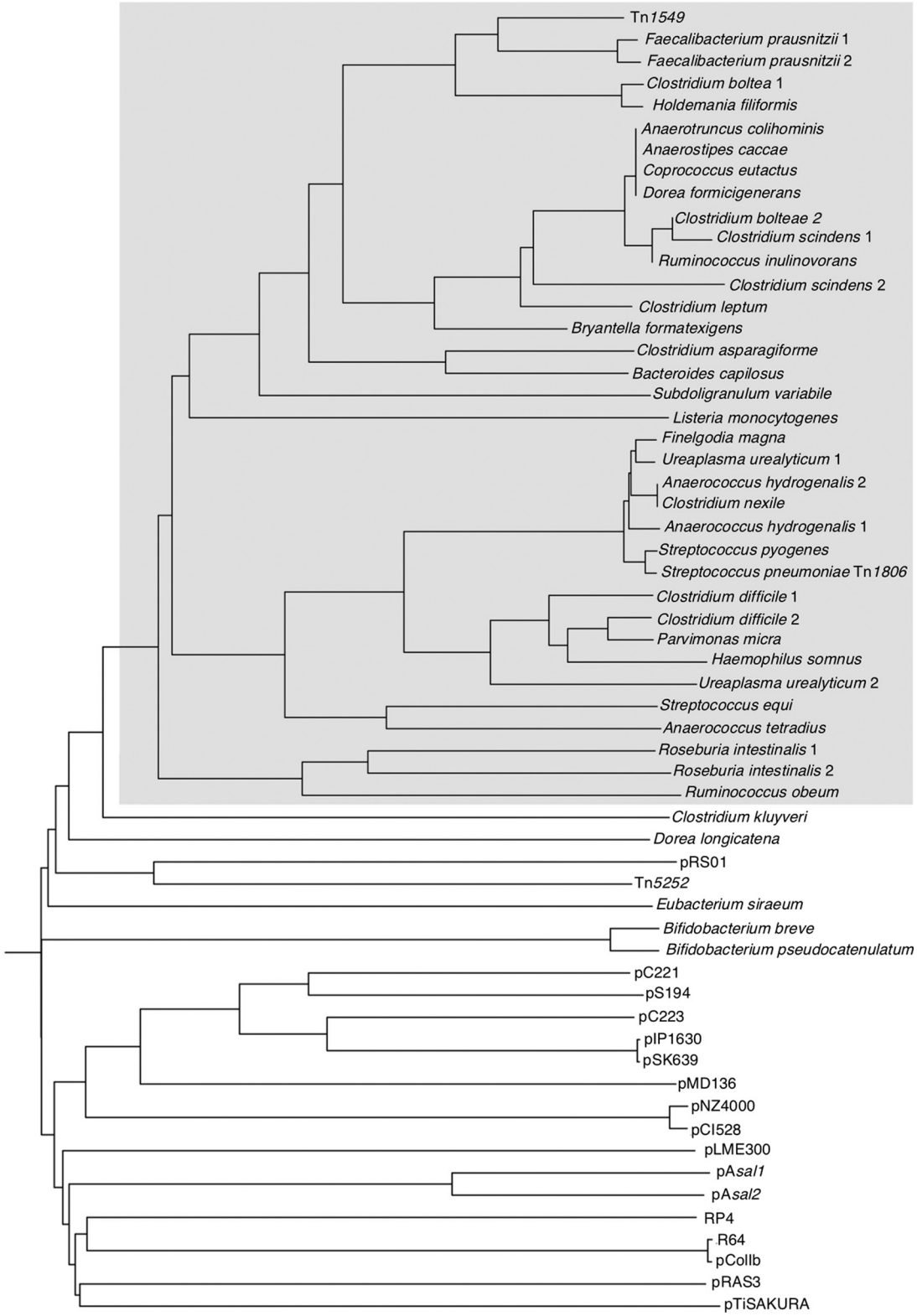

FIG. 6. Phylogenetic relationship among proteins belonging to the 3H family of MOB_p-superfamily relaxases. The tree was constructed from sequences available in data banks by use of the neighbor-joining method. The origin of relaxase-containing plasmids, transposons, or the bacterial host is indicated at each branch. The phyletic group including Rlx_{Tn1549} is gray shadowed. Accession numbers of relaxases: Tn*1549*, AAF72355; *A. hydrogenalis* 1, ZP_03303665.1; *A. hydrogenalis* 2, ZP_03303763.1; *A. tetradius*, NC_ACGC01000033.1; *A. caccae*, ZP_02419708.1; *A. colihominis*, NZ_ABGD02000024.1; *B. capillosus*, ZP_02036891; *B. breve*, ZP_03619377; *B. pseudocatenulatum*, NZ_ABXX02000003; *B. formatexigens*, ZP_03686097; *C. asparagiforme* DSM 15981, NZ_ACCJ01000017.1; *C. bolteae*, ZP_02085866.1 and ZP_02085146.1; *C. difficile*, YP_001088377.1 and YP_001086893.1; *C. kluyveri*, YP_0013933676.1; *C. leptum*, ZP_02081957.1; *C. nexile*, ZP_03288837.1; *C. scindens*, ZP_02429884.1 and

shared a putative minimal *oriT* (Fig. 5). *C. bolteae* ATCC BAA-613 harbors two relaxases, one of which displays 98% identity with this relaxase common to these bacteria. An almost identical relaxase was also found as the product of one of the two intron relaxase genes of *C. scindens* ATCC 35704, whereas the other product was distinct (79% identity). Analysis of the environment of the relaxase genes revealed a sequence highly conserved on ca. 29 kb which was part of an ICE. The presence of this element in these various bacteria supports the notion of recent horizontal transfer. The relaxases of *F. magna* ATCC 29328, *Ureaplasma urealyticum* ATCC 33175, *A. hydrogenalis* DSM 7454, *Clostridium nexile* DSM 1787, Tn*1806* from *Streptococcus pneumoniae* AP200, and *S. pyogenes* MGAS10750 were very similar (≥96% identity) and thus indicate origination from a common ancestor.

DISCUSSION

Emergence of VanA-type resistance in *S. aureus* has required plasmid acquisition, combined with transposition of a Tn*1546*-like element, or acquisition of broad-host-range conjugative plasmids such as Inc18 plasmids bearing the transposon (53). In contrast, VanB-type Tn*1549* is able to disseminate vancomycin resistance among various bacterial genera. Although the transfer origins of conjugative plasmids have been extensively analyzed, only a few *oriT* genes of ICEs have been characterized, i.e., Tn*916* (39), ICE*Bs1* of *B. subtilis* (29), and ICE*St1* and ICE*St3* of *Streptococcus thermophilus* (12). In these elements, the four *oriT* regions contain a highly conserved sequence comprised of a GC-rich inverted repeat separated by ca. 10 bp. In Tn*1549*, a 2,374-bp fragment which includes the *orf29-30* intergenic domain, *orf29* ($mobC_{Tn1549}$), *orf28* (*rlx*), and *orfz* was shown to contain both an *oriT* and the genes for mobilization proteins. In certain plasmids a single protein is required for mobilization, such as MobM of pMV158 in *Streptococcus agalactiae* (19, 24), but in most instances the relaxase needs for DNA cleavage an accessory protein, MobC, which cooperates for strand separation by binding at the *oriT*, generating local distortion of the helix which extends to the *nic* site (52). In the mobilization assay of $pACYC_{Gm}$ derivatives performed with *E. coli recA*::RP4::Δ*nic* by providing rlx_{Tn1549} in *trans*, transfer did not occur in the absence of $mobC_{Tn1549}$. Furthermore, mobilization was enhanced when mobilization genes were delivered in *cis*. Such a *cis* preference has been reported with TraA, the relaxase of symbiotic plasmid pRetCF2d from *Rhizobium etli* (38).

A functional *oriT* was identified on a 130-bp fragment of Tn*1549*. As often reported, minimal changes in base composition in the *oriT* have dramatic consequences for mobilization (3). In Tn*1549*, the alteration of the putative secondary structure A by directed mutagenesis affected mobilization. Interestingly, a minimal *oriT* limited to a 28-bp sequence was found to be sufficient for mobilization of $pACYC_{Gm}$. This minimal *oriT* was a particularly short sequence, consistent with the capacity of $MobC_{Tn1549}$ to bind to the minimal *oriT* as demonstrated by EMSA together with the ability of Rlx_{Tn1549} to cleave short oligodeoxynucleotides. The fact that the transfer frequency was significantly diminished compared to that of the *orf29-30* intergenic region suggests the presence of specific features in the neighboring environment of *oriT* that would enhance the binding of $MobC_{Tn1549}$ and Rlx. Likewise, the 130-bp fragment just upstream from $mobC_{Tn1549}$ contained the binding motifs, since this fragment was as efficient as the entire intergenic *orf29-30* fragment for mobilization of $pACYC_{Gm}$. Rlx_{Tn1549} did not bind to *oriT* alone, but in contrast the minimal *oriT* was found to be sufficient for binding of $MobC_{Tn1549}$. Most of the *oriT*s consist of a short cleavage sequence (*nic* core) associated to an immediately adjacent variable hairpin (37). The *nic* site of Tn*1549* was identified at the 3′ end of a 10-bp arm (core) adjacent to a 7-bp perfect inverted repeat sequence (hairpin A). This sequence shared eight conserved bp with the consensus motif reported in RCR plasmid, whereas *nic* was found in the antisense strand upstream from the relaxase.

Relaxases cleave specifically a single-strand DNA at *oriT* and play a central role in initiation of the relaxosome and DNA transfer of conjugative and mobilizable plasmids. It has been suggested that most mobilizable plasmid relaxases are evolutionarily related (37). The relaxase from Tn*1549* is a member of the MOB_{P7} clade of MOB_p superfamily relaxases (21). MOB_p represents the largest relaxase family and has been divided into seven clades (21). MOB_{P7} is a large clade with profound branches. In this study, we propose to distinguish from the phylogenetic tree a new phyletic cluster in MOB_{P7} with relaxase of Tn*1549* as a prototype (Fig. 6). In this cluster, the putative relaxases from ICEs originate from various anaerobes, *Listeria monocytogenes*, Tn*1806* from *S. pneumoniae*, *S. pyogenes* MGAS10750, ICESe2 from *Streptococcus equi*, and *Ureaplasma* species, and share more than 40% identity. A second cluster includes RCR plasmids from *Staphylococcus*, *Streptococcus*, and *Lactococcus* species, Tn*5252* from *S. pneumoniae*, and ICEs from *Bifidobacterium pseudocatenulatum* and *Ruminococcus obeum*. The third cluster corresponds to plasmids from Gram-negative bacteria. Interestingly, these relaxases were associated with a MobC protein and an *oriT* closely related to that of Tn*1549*. $oriT_{Tn1549}$ is likely a member of a family mainly spread in ICEs from anaerobes which shared a consensus 11-nucleotide sequence *nic* core consisting of a MWRTGCTTG′CT motif. A large portion of this motif is common to the consensus *oriT* RYGCTTG′C characterized in

ZP_02432986.1; *C. eutactus*, ZP_02207623.1; *D. formicigenerans*, ZP_02235231.1; *D. longicatena*, ZP_01994204.1; *E. siraeum*, ZP_02422210.1; *F. prausnitzii*, ZP_02093051.1 and ZP_02090960.1; *F. magna*, YP_001692251.1; *H. somnus*, YP_001784516.1; *H. filiformis*, ZP_03636025.1; YP_0011784516.1; *L. monocytogenes*, ZP_00229823.1; *P. micra*, ZP_02093865.1; *R. intestinalis*, ZP_03483057.1 and ZP_03485669.1; *R. inulinovorans*, ZP_03752747.1; *R. obeum*, ZP-01964421.1 and ZP_01962871.1; *S. equi*, CAP20357; *S. pneumoniae* AP200 (Tn*1806*), EF469826; *S. pyogenes*, CAQ56294.1; *S. variabile*, ZP_03776736.1; *U. urealyticum* serovar 9, ZP_03079552.1; *U. urealyticum* serovar 13, ZP_02696018.1; ColIbP-9, NP_052501.1; pAH82, NP_862611.1; pAsal1, CAD48422.1; pAsal2, NP_710170.1; pC221, CAA26106; pC223, NP_943089; pCI1528, AAB28188.1; pFN1, NP_862674.1; pIP1629, AAD02378.1; pIP1630, AAD02405.1; pLME300, CAD59910; pMD136, NP_037562.1; pNZ4000, NP_053037; pRAS3, NP_387457.1; pRS01, AAB06502.1; pS194, NP_976272; pSK639, NP_976278; RP4, Q00191; R64, B38529; Tn*5252*, NP_358551.

certain RCR plasmids of Gram-positive bacteria. The detection of sequences identical to $oriT_{Tn1549}$ just upstream from *mobC*-like and *rlx* genes allows us to propose a putative transfer origin for various transposons, such as the mobile elements carrying the erythromycin resistance determinant *erm*(TR) in *S. pneumoniae* (Tn*1806*) (8) and *S. pyogenes* (ICE 10750-RD.2) (4) or involved in iron acquisition in *S. equi* (ICESe2) (26). Interestingly, these elements are known to be mobilizable, a feature that could be shared by other elements which encode relaxases closely related to Rlx_{Tn1549} and carry this common transfer origin. The relationship between plasmid relaxases has been extensively described, and relaxases have been recently proposed as suitable markers for plasmid evolution (21). In contrast, relaxases from ICEs have not been classified and, in addition, the data banks have been recently enriched with numerous genomic sequences from anaerobic bacteria. The phyletic tree drawn in Fig. 6 shows that, in a similar manner, relaxases from ICEs represent a useful tool to understand lateral gene transfer mediated by these elements, which contributes to the diversification and adaptation of microorganisms. The relaxases related to rlx_{Tn1549} were mostly confined to the *Firmicutes* except those found in *Bacteroidetes* (*Bacteroides capillosus*), proteobacteria (*H. somnus*) and *Tenericutes* (*U. urealyticum*), which likely resulted from genetic exchange between phyla.

Trans-Gram genetic exchanges have occurred under natural conditions, as exemplified by the acquisition by Gram-negative bacteria of genes from Gram-positive bacteria (49, 51). Evidence that transfer can be mediated by conjugation was demonstrated by the use of shuttle vectors (48). In these models the *tra* functions were provided with their cognate *oriT*. However, mobilization by heterologous systems can be the result of interplay between compatible transfer mechanisms. Mobilization of *Bacteroides* plasmids by IncP plasmids in *E. coli* was reported in the late 1980s (42); nevertheless, mobilization of a transposon from Gram-positive bacteria such as Tn*1549* by RP4, as obtained from constructs in *E. coli*, remains an intriguing feature. The interaction between the relaxosome of mobilizable plasmids and the transfer apparatus of bacteria is thought to be mediated by type IV family coupling proteins (6). These proteins, by their capacity to direct this interaction, are crucial for the specificity of mobilization. It has been shown with the IncW plasmid R388 that membrane protein TrwB is responsible for coupling the relaxosome with the DNA transport apparatus (6, 47). In general, the coupling proteins are specific (31). However, several interactions in heterologous mobilization systems have been reported; for example, the RP4 *traG* gene can replace the R388 *trwB* gene for mobilization of plasmid ColE1 (7). Similarly, the Tn*4555* $mobA_{Tn}$ gene and the adjacent $oriT_{Tn}$ from *Bacteroides* are mobilized by IncPα plasmids such as RK2 (44). The *oriT* of this element is related to a plasmid family from Gram-positive bacteria. This plasmid family also includes pMV158 of *S. agalactiae*, pT181 of *S. aureus*, pIP823 of *L. monocytogenes*, and the mobilizable transposon Tn*4451* of *C. difficile* (13, 16). Interestingly, mobilization of pMV158 and pIP823 by functions provided by RP4 has been reported (19). Similarly, the *oriT*-relaxase complex of Tn*1549* is likely able to interact in *E. coli* with the TraG coupling protein for mobilization. This is an indirect but consistent argument, suggesting that conjugative transfer of Tn*1549* from *C. symbiosum* to *Enterococcus* spp. occurred by mobilization by a helper element. If this hypothesis is true, conjugative transfer of Tn*1549* obtained from an *E. faecium* transconjugant harboring Tn*916* is due to the latter transposon. In the same manner, transfer of Tn*1549* from anaerobes could be associated with the presence in the donors of resident ICEs or conjugative plasmids. It remains to be established if the mobilization systems related to the $oriT_{Tn1549}$ family are also mobilizable by the RP4 transfer apparatus or by other elements.

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