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Exploration of DNA processing features unravels novel properties of ICE conjugation in Gram-positive bacteria

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ABSTRACT

Integrative and conjugative elements (ICEs) are important drivers of horizontal gene transfer in prokaryotes. They are responsible for antimicrobial resistance spread, a major current health concern. ICEs are initially processed by relaxases that recognize the binding site of oriT sequence and nick at a conserved nic site. The ICESt3/Tn916/ICEBs1 superfamily, which is widespread among Firmicutes, encodes uncanonical relaxases belonging to a recently identified family called MOBT. This family is related to the rolling circle replication initiators of the Rep trans family. The nic site of these MOBT relaxases is conserved but their DNA binding site is still unknown. Here, we identified the bind site of RelSt3, the MOBT relaxase from ICESt3. Unexpectedly, we found this bind site distantly located from the nic site. We revealed that the binding of the RelSt3 N-terminal HTH domain is required for efficient nicking activity. We also deciphered the role of RelSt3 in the initial and final stages of DNA processing during conjugation. Especially, we demonstrated a strand transfer activity, and the formation of covalent DNA-relaxase intermediate for a MOBT relaxase.

INTRODUCTION

Horizontal gene transfer (HGT) plays an important role in the rapid evolution of bacterial genomes by driving gene exchanges between cells and therefore leading to genetic variability of bacteria. This allows a better adaptation of bacterial populations to their environment and colonization of new ecological niches (1,2). These HGT have contributed to the emergence of new pathogenic bacteria and to the spread of antibiotic resistance.

Bacterial conjugation is considered to be one of the most important mechanisms of HGT and is defined as the transmission of genetic material from one bacterium to another by direct cell contact (1–3). During conjugation, DNA is transported from a donor to a recipient cell through a conjugation machinery, a type IV secretion system (T4SS) (4). Conjugation is mediated by mobile genetic elements. There are two types of autonomous conjugative elements: conjugative plasmids which are extra-chromosomal elements, and integrative and conjugative elements (ICEs) which are integrated in bacterial chromosomes (5,6).

Genome analyses indicate that ICEs are more widespread than conjugative plasmids in most bacterial phyla, especially in Firmicutes (5,7,8). A major superfamily of ICEs in Gram-positive bacteria is the ICESt3/Tn916/ICEBs1 superfamily (9). Tn916 and its relatives are involved in the spread of various antimicrobial resistance genes, especially resistance to tetracyclines, macrolides and bacitracin (10–12). These elements were found in a large broad host range among Firmicutes, including severe multi-resistant pathogens such as Streptococcus pneumoniae, Clostridium difficile or Staphylococcus aureus (10,13). Thus, the molecular mechanisms of conjugative transfer are worth to be investigated in this widespread superfamily of ICEs to gain future insights aiming to control its dissemination.

After excision from the bacterial chromosomes, ICEs transfer to other cells by using mechanisms similar to those of conjugative plasmids. Thus, ICE conjugation is performed in two distinct steps: (i) processing of the DNA molecule to be transferred by a multi-protein complex called relaxosome, and (ii) translocation of the DNA through a T4SS apparatus from the donor to the recipient cells (4,5). The key enzyme of the relaxosome complex is the relaxase. It initiates conjugation by nicking the DNA at...
a specific sequence located on the origin of transfer (oriT) of the conjugative element, called the nic site. This enables its rolling-circle replication (RCR) starting from the 3′OH end of the nicked DNA (14). As relaxases are transenzymes, they usually remain covalently attached to the 5′ end of the nic site by their catalytic tyrosine (15,16). The nascent DNA-relaxase complex is then recognized and recruited by the coupling protein encoded by the element which directs it to the conjugative T4SS (17). In the recipient cell, the incoming single-stranded DNA is re-circularized by the relaxase (16). The complementary strand is then replicated by host enzymes, and in the case of ICEs, the DNA is then integrated in the chromosome of both recipient and donor strains (5,14).

Conjugative relaxases are classified into different MOB families according to the presence of conserved motifs, phylogenetic analysis and structural data (16,18,19). The ICESt3/Tn916/ICEBs1 HTH superfamily of ICEs encodes non-canonical relaxases that are unrelated to the canonical HUH endonucleases (20). They belong to the MOBT family (7), which is distantly related to the Rep_trans RCR initiators encoded by the well-studied pT181 plasmid family (19,21,22). The nic site was previously identified for ICEBs1 and contains a conserved CAAT sequence located within the loop of a G/C rich hairpin (23). This nic sequence is conserved among ICEs of the ICESt3/Tn916/ICEBs1 superfamily and is identical to the dso (double-stranded origin) sequence used by Rep_trans proteins to initiate RCR (24,25), strengthening their relationship with MOBT relaxases. Indeed, beyond their role in conjugation initiation, MOBT relaxases encoded by Tn916 and ICEBs1 (Orf20 and NicKr, respectively) are also involved in the autonomous RCR initiation of the ICE after excision (26,27).

In contrast to those of conjugal plasmids, only few relaxases from ICEs have been experimentally characterized. So far, three MOBT relaxases have been studied. In 2006, the Orf20 relaxase was surprisingly found to require the cognate Tn916 integrase for its nicking specificity (28). At that time, 19 putative nicking sites were detected within oriT_{Tn916}, without occurrence of the conserved CAAT nic site among them. It is now known that, due to misidentification of the initiator codon, the protein used in the 2006 study was a truncated version of Orf20, deprived from its N-terminal HTH (Helix-Turn-Helix) domain. Therefore, this HTH domain of Orf20 could be required for identification of the nic site and for nicking at the correct and unique position, questioning the role of the integrase in this process. Subsequently, this HTH domain was shown to be critical for the RCR initiation activity of Orf20 (27). In 2007, the CAAT nic site of ICEBs1 was clearly identified within the loop of a conserved GC-rich hairpin by Lee and Grossman (23), as previously shown for the dso of Rep_trans proteins. The MOBT NicK relaxase from ICEBs1 was involved in this nicking activity, but it was not characterized biochemically. Recently, we characterized another MOBT representative relaxase, RelSt3 encoded by ICESt3 from Streptococcus thermophilus (19,29). RelSt3 is dimeric and harbors a catalytic PF02486 domain which is also present in Rep_trans proteins. We demonstrated that RelSt3 active site, as for Rep_trans proteins, contains three conserved acidic residues for the coordination of a cationic cofactor (19). However, little is known about how MOBT proteins interact with DNA, and whether a covalent adduct is formed between the cut DNA and a catalytic tyrosine residue of their active site. Several canonical HUH relaxases encoded by conjugal plasmids were studied in some details, revealing that they recognize a bind site located close to the nic site on the oriT sequence. Such examples include the TrwC relaxase (MOBp) from Escherichia coli R388 plasmid (30), and the TraA relaxase (MOBv) from Enterococcus faecalis pP501 plasmid (31), both of which bind to a sequence located upstream of the nic site. This bind site usually contains inverted repeats. In other cases, the relaxase is assisted by auxiliary proteins, which can favor the binding and the positioning of the relaxase at oriT. For example, the relaxase PcfG (MOBF) from the E. faecalis plasmid pCF10 is unable to bind DNA directly, but relies on its PcfF partner to do so (32). These auxiliary proteins often harbour a RHH (Ribbon-Helix-Helix) domain responsible for their interaction with DNA (32–35). Instead, Rep_trans proteins do not need auxiliary proteins to bind to their dso sequence (36,37). Their bind site contains an inverted repeat (ICRIII) located immediately downstream of the nic site, that allows discrimination of the cognate Rep_trans protein (22,37).

Interestingly, most of the MOBT relaxases from ICEs harbour an HTH domain in their N-terminal part. It is thus expected that this HTH domain could bind DNA at oriT. In this study, we present how the relaxase RelSt3 interacts with its target binding site in the oriT region through its HTH domain. Mapping of the oriT sequence required for ICESt3 conjugative transfer revealed that the RelSt3 bind site was unusually distant from the nic site. We also reported that the binding of the HTH domain was important for conjugative transfer and for full nicking activity in vitro. By using structural modeling, we identified an alpha-helix of the HTH domain involved in the interaction with DNA, which was experimentally confirmed. In addition, we investigated the joining activity of RelSt3 and its cognate binding to the 5′ end of the cleaved DNA. All these data provide significant novel information enabling a better comprehension of how MOBT relaxases are involved in DNA processing during conjugative transfer.

**MATERIALS AND METHODS**

**Bacterial strains, cloning and mutagenesis**

The bacterial strains and plasmids used in this work are listed in Supplementary Tables S1 and S2. Streptococcus thermophilus strains were grown in Lactose (0.5% w/v) M17 broth at 42°C with, when necessary, chloramphenicol (4 µg/ml) and erythromycin (5 µg/ml). In case of mobilization experiments with poriT, spectinomycin was used at 500 µg/ml. S. thermophilus LMG18311 harboring ICESt3 labelled with a chloramphenicol resistance gene was used as a donor strain in conjugation experiments, whereas an isogenic strain without ICESt3 but harboring the pMGS36e plasmid carrying erythromycin resistance was used as recipient cell. Escherichia coli EC101 was used for cloning procedures with plasmid pG"host9. Deletion of the 5′ part of the orfJ relaxase gene encoding the N-terminal HTH domain was done as already described in ref (19), leading to the LMG18311(ICESt3orfJΔHTHcat) strain. Plasmid
constructs for protein over-expression were built into the *E. coli* DH5α strain and over-expression was performed in *E. coli* BL21(DE3).

For mobilization experiments, poriT plasmids containing various fragments of the ICESt3 oriT sequence cloned into the pORI180 plasmid were constructed using EcoR1 and *ApaI* restriction sites as described in (19). For production of recombinant proteins, the kanamycin resistant pSKB3 vector was used (gift from Stephen K. Burley), introducing a 6 His-tag and a TEV protease cleavage site in frame at the N-terminal end. The pSKB3-RelSt3 vector encoding ICESt3 oriT*J* gene (19) was used as a template for the cloning of RelSt3 sub-domains using *NdeI* and *HindIII* restriction sites. This way, pSKB3 derivatives were generated to produce RelSt364-410 (depleted of its HTH domain) and RelSt31-63 (HTH domain) variants. For the generation of point substitution variants, site-directed mutagenesis was performed by overlap PCR using oligonucleotides described in Supplementary Table S3. For all constructs, the sequence of both strands of the recombinant plasmids was verified by sequencing (Genewiz Inc., Germany).

**Mating experiments**

Mating experiments were done as described previously (38). Briefly, *S. thermophilus* LMG18311-derived donor (containing ICESt3 and harboring a poriT construct for mobilization experiments) and recipient strains were grown overnight in the presence of the appropriate antibiotics. Overnight cultures were diluted to 1:100 and further grown without any antibiotic for the recipient strain, and if appropriate, with spectinomycin (500 μg/ml) for the donor strain to maintain the poriT construct. After growth reached an OD₆₀₀ of 0.4, cells were mixed (1:1) and concentrated 30 times in LM17 broth. For each mating experiment, two 150 μl aliquots of cells were spread separately on 0.45/0.8% LM17 plates and then incubated overnight at 37°C. Transconjugant (TC) cells were recovered from the plates with nitrocellulose filters (Millipore) deposited on soft agar (0.8%) LM17 plates and then incubated overnight at 37°C. Transconjugant (TC) cells were recovered from the filters with 10 ml of LM17, directly spread or concentrated 10 times before plating onto LM17 plates containing chloramphenicol and erythromycin (ICE transfer), or spectinomycin and erythromycin (poriT mobilization). After a 24 h-incubation at 37°C, mating frequency was calculated as the ratio of TC per recipient cell. At least three independent biological replicates were done.

**Protein expression and purification**

Over-expression and purification of recombinant RelSt3 (full-length), RelSt364-410, RelSt31-63 and RelSt3 point variant proteins were done as previously described for RelSt3 (19).

**Electrophoretic mobility shift assays (EMSA)**

Electrophoretic mobility shift assays (EMSA) were performed in agarose and in acrylamide gels. For agarose EMSAs, 400 ng of each oriT DNA produced by PCR were incubated with the indicated amount of RelSt3 protein in a final volume of 20 μl. After incubation for 15 min at 37°C, the mixture was resolved in 1.5% agarose gels in 1× Tris-acetate–EDTA for 1.5 h at 11 V cm⁻¹. Gels were then stained with ethidium bromide and images captured with a Bio-Rad XR+ system (Bio-Rad Laboratories, California). Each experiment was reproduced at least three times.

For EMSAs performed with acrylamide gels, DNA substrates were oligonucleotides labelled at their 5’ end with 6-FAM (Eurogentec, France), except for the complementary oligonucleotides that were unlabeled (see Supplementary Table S3 for the list of oligonucleotides). Reactions performed in final volume of 18 μl contained 2 pmol of labelled DNA and increasing amounts of the respective proteins (RelSt3 WT or variants, see figure legends). Reaction buffer contained 20 mM Tris–HCl, 500 mM NaCl and 100 μg/ml of salmon sperm DNA as competitor. The mixtures were incubated for 15 min at 37°C prior addition of 2 μl of loading buffer (2.5 mg/ml bromophenol blue and 0.4 g/ml sucrose). Prior loading samples, 5% (w/v) native polyacrylamide gels (PAGE) were pre-run using miniPROTEAN Bio-Rad cells at 4°C for 1 h at 84 V in 0.5× Tris-Borate-EDTA buffer. Samples were then run at 4°C for 1h 20 min at 84 V. Labelled DNA was visualized using the ChemiDoc XRS system (Bio-Rad) and band intensity was quantified with the Image Lab software (Bio-Rad). At least three replicates were performed for each experiment.

**Hairpin structure of IR2**

In order to investigate the putative IR2 hairpin structure, we first submitted the IR2 sequence to the RNAfold web server (39), using DNA parameters. We next performed S1 nuclease assays with the double-stranded ori41 oligonucleotide labelled at the 5’ end of its plus strand with 6-FAM (Eurogentec, France). Reactions were performed in a final volume of 18 μl with 2 pmol of DNA and S1 nuclease at increasing concentrations (see figure legend). Mixtures were incubated for 30 min at 37°C before addition of the loading buffer. The samples were resolved in a 5% (w/v) native PAGE in 0.5× Tris–borate–EDTA buffer for 75 min at 84 V. Labelled DNA was visualized as described for EMSAs.

**Relaxase modeling**

The predicted RelSt3 model, as well as the confidence score (pLDDT score) were produced with AlphaFold v2.0 (40) using Colabfold (https://github.com/sokrypton/ColabFold). All 3D models were handled using VMD (41) and VMD-python (https://github.com/Eigenstate/vmd-python). The electrostatic potential was computed using the APBS server (server.poissonboltzmann.org/) (42).

**Construction of a DNA-HTH domain model**

The HTH domain from the best scored Alphafold prediction model was extracted and screened against the protein databank (RCSB PDB) with FATCAT 2.0 (43) to search for structural homolog. The resulting hits were further filtered with the RCSB PDB in order to keep only those containing both the HTH domain and the double-stranded DNA (dsDNA). In the best hit structure (PDB ID: 5J2Y), the HTH-like domain was replaced by the Alphafold model of RelSt3.
HTH, and optimized by energy minimization with NAMD (41) using CHARMM36 force-field (44).

Nicking-closing assays

Nicking-closing assays were performed in 18 µl final volume reaction, with the indicated oligonucleotides (2 pmol each) and the protein (RelSt3 WT or variants, see figure legends). We used the same reaction buffer as described for EMSAs to which 5 mM MnCl2 was added. Mixtures were incubated for 15 min at 37°C, and treated with proteinase K (1 mg/ml, final concentration) for a second 15 min incubation at 37°C. The resulting oligonucleotides were then run in 5% native PAGE as described for EMSAs. At least three replicates were performed for each experiment.

In order to confirm that the recombinant DNA was generated by religation of ori50 and ori57 oligonucleotides, PCR amplification was performed using a forward primer located on the 5’ end of ori57 oligonucleotide and a reverse primer located on the 3’ end of ori50 oligonucleotide. Amplification was done according to standard procedures provided by the manufacturer using the Phusion High-Fidelity DNA polymerase (Thermoscientific), and the nicking-closing product as template. A 10 sec extension step at 72°C was programmed to obtain a 136-bp PCR product. Electrophoresis of PCR products was performed on 2% agarose gel in 1× Tris–borate–EDTA for 1 h at 110 V. Gels were then stained with ethidium bromide and images captured with a Bio-Rad XR + system (Bio-Rad Laboratories, California).

Circular UV dichroism analysis

Far-UV circular dichroism (CD) spectra were recovered from 190 to 260 nm with a Chirascan Plus spectrophotometer (Applied Photophysics, Ltd, UK) equipped with a Peltier temperature control unit (20°C). RelSt3 WT and variants proteins were dialyzed in CD buffer (50 mM sodium phosphate pH 7.0, 500 mM NaF) and loaded on a flat quartz cell of 0.1 mm path length. Spectra were recorded at a scan speed of 60 nm min–1 and 1 nm spectral bandwidth. The average of three scans were converted to mean residue ellipticity (deg cm2 dmol–1 per residue) using ProDara Viewer software (Applied Photophysics, Ltd, UK).

DNA-relaxase covalent complex formation

To visualize the formation of DNA-RelSt3 covalent complex, 3′ labelled (6-FAM) ori50 DNA (50 µM) was incubated for 15 min at 37°C with 5 µM RelSt3 (WT or variants) in the reaction buffer containing 5 mM MnCl2 (except in the control where 1 mM EDTA was added). When indicated, samples were subsequently treated by nuclease S1 (150 units, ThermoScientific) or proteinase K (1 mg/ml, final concentration). Samples were then resolved in denaturing SDS-PAGE (12%) for 1 h at 180 V in miniPROTEAN Bio-Rad cells. The gel was analyzed using a ChemiDoc XRS system (Bio-Rad) to detect fluorescent labelled DNA and in the case of gel Figure 7A, the same gel was also stained with Coomassie blue to detect proteins.

RESULTS

Two distant DNA sequences of the oriTICESt3 are required for in vivo mobilization by ICESt3

In a previous study, we showed that RelSt3 functions as a relaxase, displaying a relaxation activity on a supercoiled plasmid harboring the oriTICESt3 sequence localized within the orfJ/orfK intergenic region of ICESt3 (19). We also demonstrated that a plasmid harboring this oriTICESt3 sequence (corresponding to poriT1 below) was successfully mobilized in trans by ICESt3. Indeed, this sequence encompasses a conserved nic site similar to the one previously identified experimentally for ICEBs1 (23).

To determine more precisely the nucleotide sequence of the oriTICESt3 essential for DNA transfer, 4 other overlapping segments with similar size (≈300 bp) located in the oriTICESt3 and adjacent sequences were cloned into the non-mobilizable vector pOri1180, carrying a spectinomycin resistance marker (19). The recombinant plasmids each containing a different segment of oriT (oriT1 to oriT5, Figure 1A), were tested for their ability to be mobilized in trans by the transfer apparatus of ICESt3 in Streptococcus thermophilus LMG18311 (Table 1). In these assays, conjugation of ICESt3 was monitored in parallel and was found to occur at similar frequencies (about 1–2 × 10–3 TC per recipient cell), regardless the oriT sequence present on the poriT of the donor strain. This indicates that the various oriTICESt3 sequences carried by the plasmids did not impair ICESt3 transfer. As expected, no mobilization of poriT2 and poriT3 plasmids, both lacking the full nic site, was observed (below detection level of 10–8). Interestingly, while poriT1 and poriT5 plasmids were successfully mobilized (1–2 × 10–4 TC per recipient cell), only very few transconjugants were obtained with poriT4 (Table 1). When normalized to ICESt3 transfer, poriT4 mobilization frequency was found to be 50 times lower than that of poriT1. Yet, all three plasmids (poriT1, poriT4 and poriT5) harbor the entire nic site. Thus, these results suggested that poriT4 lacks another sequence required for full functional mobilization in vivo, which is present in poriT1 and poriT5 and therefore located downstream of the nic site. The oriTICESt3 sequence present in poriT4 contains 51 bp downstream of the nic site, a sequence that is usually long enough to encompass the bind site (ICRIII) in the homologous RC replication driven by Rep_trans proteins (37). We deduced from these results that the in vivo mobilization by ICESt3 requires an additional sequence, which is surprisingly distant from the nic site.

To determine the location of RelSt3 bind site on oriTICESt3, EMSA experiments were conducted on dsDNA substrates corresponding to the five sequences cloned in the poriT plasmids (Figure 1B), produced by PCR amplifications. No shifted band was observed with poriT2 and poriT4 sequences, indicating that RelSt3 does not interact in a stable manner with the sequences located upstream nor immediately adjacent to the nic site. In contrast, strongly shifted DNA–protein complexes were observed with poriT1, poriT3 and poriT5 sequences. This localizes the bind site for RelSt3 at the 3′-end of the poriT5 segment, within the 78 bp fragment that is absent in oriT4 sequence (hatched box in Figure 1A). Altogether, these results indicated that two sequences
of the oriTICEST3 are required for DNA transfer: the nic site, and a downstream region required for the stable binding of RelSt3. The latter corresponds to an unusually bind site distantly located from nic, since relaxases from other families usually bind sequences situated in proximity to the nic site.

IR2 within oriTICEST3 is the binding site of RelSt3

Data from the literature indicate that relaxases usually bind to inverted repeat structures located close to the nic site. Inspection of the oriTICEST3 sequence allowed the detection of two inverted repeats: IR1 and IR2 (Figure 2A). IR1 is a 26-bp sequence, which includes a perfect 7-bp GC-rich inverted repeat (in grey) and an intervening 12 bp containing the conserved CT’AA nic site (in red and bold, Figure 2A). IR2 is located 68 bp downstream of IR1 and contains two 13-bp inverted sequences separated by 9 bp. Considering these structures, we divided the oriTICEST3 sequence into five regions: ori46 containing IR1, ori41 containing the full IR2, ori47 containing the IR1-IR2 68-bp spacer, and ori34 and ori43 encompassing one of the two parts of IR2 (IR2a and IR2b, respectively). These dsDNA sequences (labelled in 5′ with 6-FAM) were used in EMSA experiments to determine which regions could interact with RelSt3.

As expected, RelSt3 was able to bind the full IR2 (ori41) (Figure 2B) in a dose-dependent manner. Instead, no specific binding was observed with IR1 (ori46) (Figure 2B) nor with the spacer region between IR1 and IR2 (ori47) (data not shown). At high RelSt3:DNA ratio, almost all of the ori41 dsDNA shifted to a DNA–protein complex. The apparent Kd was estimated to be about 1.6 μM (Supplementary Figure S1). No or very poor binding was observed with ori34 and ori43 (Figure 2B) indicating that the whole IR2 is necessary for RelSt3 binding. When EMSA
Figure 2. Identification of IR2 as the RelSt3 binding sequence. (A) Map of IR and oligonucleotides used in EMSA assays. The position of the IR1 and IR2 are shown on the oriT ICESt3 DNA sequence. The nuc site is indicated as a short and red arrowhead within IR1. Solid horizontal lines materialize the dsDNA oligonucleotides used in EMSA experiments, labelled with 6-FAM at the 5' end. (B) EMSA assays illustrating the specific binding of IR2 by RelSt3. The dsDNA-relaxase mixtures were electrophoresed as described in Materials and Methods. Increasing concentrations of RelSt3 were applied as follows: lane 1, 0 μM; lane 2, 0.1 μM; lane 3, 0.2 μM; lane 4, 0.4 μM; lane 5, 0.8 μM; lane 6, 1.6 μM; lane 7, 3.2 μM; lane 8, 6.4 μM. The dsDNA oligonucleotides concentration was 0.11 μM.

Experiments were conducted with single stranded ori41 DNA, or with another non-specific dsDNA of similar size used as a negative control, no binding was observed in both cases, even at high RelSt3 concentrations (Supplementary Figure S2). These data demonstrate that IR2 in a double-stranded form, is the bind site for RelSt3 on oriT ICESt3.

Intact IR2 sequence is required for RelSt3 binding

The importance of each individual repeat from IR2 was analyzed by performing EMSA using oligonucleotides harboring mutations modifying one repeat or both (Figure 3A). No binding of RelSt3 was observed when either IR2a (ori41M5) or IR2b (oriM6) sequence was modified (Figure 3B). These results indicated that RelSt3 binding is sequence dependent, and requires both of IR2a and IR2b parts, possibly through the formation of a hairpin structure. The structuration of ori41 ssDNA was investigated by RNAfold using DNA parameters confirming the possible formation of a stem-loop structure at the IR2 (Supplementary Figure S3A) that could result in a cruciform structure of the ori41 dsDNA (Supplementary Figure S3B). To examine further this hypothesis, we incubated ori41 dsDNA with increasing amounts of nuclease S1, an endonuclease that degrades single-stranded DNA. As observed in Supplementary Figure S3C, cleavage products are indeed observed, whose intensity increases with the concentration of S1 nuclease. This result is consistent with the formation of a cruciform structure at IR2.

RelSt3 binding was also tested with a modified sequence harboring an IR of same length and same GC% as IR2 (ori41M11), thus able to form a similar putative hairpin structure. No binding was observed with this ori41M11 substrate, suggesting that RelSt3 binding to IR2 does not rely on the formation of a hairpin structure but also requires the presence of a specific sequence.

The HTH domain of RelSt3 is required for oriT binding in vitro and is essential for conjugation

At its N-terminal end, from position 8 to 57, RelSt3 displays a predicted Helix-Turn-Helix domain (HTH-XRE, cd00093) that likely functions as a DNA binding domain (Figure 4A). To confirm this hypothesis, we produced a truncated variant of RelSt3 deprived of its HTH domain (RelSt364–410) and used it in EMSA experiments in the presence of ori41 dsDNA. No binding was found even when the RelSt364–410 protein was used at high concentration (Figure 4B). To further assess the role of the HTH domain in oriT DNA binding, the variant RelSt31–63, encompassing the HTH domain, was produced and used in EMSA experiments. Even at low concentration of RelSt31–63, DNA–protein complexes were observed indicating the HTH domain of RelSt3 is responsible for its stable binding to oriT ICESt3.

To test the importance of this recognition between the HTH domain and DNA in the context of in vivo conjugative transfer, a mutant strain of *S. thermophilus* LMG18311 harboring ICESt3 with a *relSt3 (orfJ)* gene depleted from its HTH domain was constructed. Mating experiments were carried out using as donor strains, either a *S. thermophilus* LMG18311 strain with the wild-type ICESt3 (control) or the mutated orfJΔHTH strain. While transconjugants were obtained in the control experiment, none were observed using the orfJΔHTH strain (with a detection limit <1.0 × 10^-8 TC/recipient). Altogether, these results demonstrate that the HTH domain of RelSt3 is essential for the conjugative transfer of ICESt3 since it ensures the binding of RelSt3 to its cognate oriT region.
Figure 3. Substitutions in IR2 sequence impair RelSt3 binding. (A) Sequence of each substitution mutants used for EMSA assays. IR2a and IR2b are in bold, and substitutions are indicated in bold red and underlined characters. In the upper yellow part, arrows correspond to IR2a and IR2b. Stars indicate the 6-FAM labeling positions. (B) EMSA experiments were done as described in Figure 2 with the indicated ds oligonucleotides.

Modeling of RelSt3 and of the HTH-DNA complex

The structure of the full RelSt3 protein was predicted using AlphaFold (40) (Supplementary Figure S4). Five distinct models were obtained, with a highest RMSD between backbones of 5.4 Å. This denotes that these models are close to each other, providing a high degree of confidence. In all these RelSt3 models, the HTH domain was clearly separated from the PF02486 domain by a flexible 10-residues linker. Looking at the electrostatic surface potential of RelSt3 (Supplementary Figure S4), two main positively charged areas were identified: one in the region surrounding the catalytic tyrosine that ensures DNA processing (19), and a second one in the RelSt3 HTH domain that interacts with the oriT DNA.

To get further information on the RelSt3 HTH-DNA complex, structural homologs of RelSt3 were recovered from databases (2560 hits, see Materials and Methods) and later filtered to keep only those which consist of protein structures bound to double-stranded DNA (149 hits). The best confidence score (p-value of $2.27 \times 10^{-08}$) was found with the PDB ID 5J2Y, a structure of the DNA-bound RsaL repressor of Pseudomonas aeruginosa (45). In the absence of an experimental RelSt3-oriT complex structure, we investigated which amino acids of the RelSt3 HTH domain could interact with oriT. A model was constructed by replacing the HTH structure of 5J2Y by the HTH model of RelSt3 obtained by AlphaFold. The superimposition of the RelSt3 HTH with RsaL HTH domain was carried out by aligning their respective H_9251 helices (Figure 4C). It is important to notice that the DNA structure used in our modeling corresponds to the DNA from the 5J2Y and it is only used as a structural reference to predict the putative amino acids closest to the DNA. The H_9251 helix encompassing residues 37–45 in the 5J2Y structure was found facing the DNA major groove. This region corresponds to the residues 30–38 in the RelSt3 HTH model. Among these amino acids, two arginine residues (R32 and R35) harbor their side-chains towards the major groove of the DNA, and thus could play a role in DNA binding (Figure 4C).

To experimentally assess the potential implication of R32 and R35 in DNA-binding, the variant protein R32A–R35A was produced and tested in EMSAs. Substitution of these residues completely abolished ori41 binding (Figure 4D), indicating that this alpha-helix, and more precisely these arginine residues are important for the recognition of IR2 by the HTH domain.

Binding to IR2 is required for full RelSt3 nicking activity

We previously demonstrated that RelSt3 relaxes supercoiled DNA by introducing a single-stranded nick at the nic site of oriT (19). This nicking reaction requires a divalent metal ion as cofactor such as Mn$^{2+}$. To assess whether RelSt3 binding to IR2 was required for its nicking activity, we designed the chimeric ori50 substrate (Figure 5A). This ori50 DNA substrate contains (i) the nic region as a single-stranded DNA to allow easy visualization of the cleavage and (ii) the binding region (IR2) as a double-stranded DNA to ensure the proper fixation of RelSt3 through its HTH domain. We beforehand checked that RelSt3 could bind to ori50 by EMSA
Figure 4. RelSt3 binds to IR2 by its N-terminal HTH domain. (A) Organization of the different domains of RelSt3 protein. (B) EMSA assays were performed with increasing amounts of RelSt3<sub>64-410</sub> (left panel) or RelSt3<sub>1-63</sub> (right panel) incubated with ds ori41 DNA. DNA-protein complexes are observed with the HTH domain of RelSt3 (RelSt3<sub>1-63</sub>) but not with RelSt3 depleted from this N-terminal domain (RelSt3<sub>64-410</sub>). Lane 1, 0 μM; lane 2, 0.5 μM; lane 3, 1 μM; lane 4, 1.25 μM; lane 5, 1.75 μM of protein. (C) Model of RelSt3 HTH-DNA complex based on 5J2Y PDB structure. The α-helix extending from residues 30 to 38 of RelSt3 HTH Alphafold model is found in the DNA major groove. The R32 and R35 side chains are shown in yellow and red, respectively. (D) EMSA assay performed as described in Figure 2B using the RelSt3 R32A-R35A variant.

experiments (Figure 5B). We found that RelSt3 was able to bind ori50 with an apparent $K_d$ of 3.2 μM (Supplementary Figure S1), in a similar range to that obtained for ori41 binding.

To examine RelSt3 nicking activity, the wild-type protein was incubated with ori50 in the presence of Mn<sup>2+</sup>. After treatment with proteinase K (thus eliminating DNA-protein complexes), the sample was electrophoresed in native condition. With RelSt3 present at concentrations above 0.4 μM, we obtained a 22 nt DNA, corresponding to the cleavage product at the nic site by RelSt3 (Figure 5C). As expected, proportion of this cleavage product increased with concentration of RelSt3. When the RelSt3<sub>64-410</sub> variant was used in this nicking assay, no cleavage product was observed even at high protein concentrations (Figure 5C). Because this truncated version of RelSt3 lacks the HTH domain, this suggests a direct link between binding and nicking processes. Nicking experiments were also carried out using the ori50M11 as the substrate DNA. Like ori41M11, ori50M11 carries the same modified IR2 (Figure 3A), which was shown to prevent RelSt3 binding. Although a residual nicking activity was observed (Figure 5D), the use of ori50M11 caused a severe decrease of RelSt3 nicking activity. Overall, these results indicate that binding of RelSt3 to IR2 is required for an efficient nicking activity at the nic site of ori<sub>ICE/st3</sub>.

RelSt3 catalyzes strand transfer reaction

During the conjugation process, relaxases not only initiate DNA transfer in the donor cell but are also thought to re-circularize the transferred single-stranded DNA in the recipient cell (4,46). This occurs through a strand transfer reaction mediated by the catalytic tyrosine. In order to explore the putative closing activity of RelSt3, strand transfer reaction experiments were conducted in the presence of two labeled oligonucleotides, ori50 (described in the previous section) and ori57. The ori57 is a 59-mer oligonucleotide corresponding to the sequence upstream of the nic position (Figure 6A), so that the 3′ end of this ori57 corresponds to the 22 nt sequence located in 3′-end of ori50 (left hand of nic site). Data from these experiments indicate that RelSt3 was able to generate recombinant ori57-ori50 molecules in the presence of Mn<sup>2+</sup> (red box, lanes 6 and 7, Figure 6B), whereas such product was not observed in the absence of Mn<sup>2+</sup> cofactor (lanes 8 and 9). We ver-
Tyrosine 252 is involved in nicking-closing reactions

Nicking and strand transfer reactions are transesterifications usually involving catalytic tyrosine residues in relaxases active sites (except for MOBγ relaxases for which a histidine residue is involved) (16,47). In a previous study, we have assigned the conserved tyrosine 252 (Y252) as the catalytic residue of RelSt3 through plasmid relaxation assays (19). Here, we assessed the involvement of Y252 in the nicking but also in the strand transfer activity of RelSt3 using oligonucleotides. As expected, the replacement of the Y252 by a phenylalanine residue (RelSt3-Y252F) fully abolished the RelSt3 nicking activity, and as a consequence its strand transfer activity (Figure 6D). Unexpectedly, the variant RelSt3-Y252A still showed a low nicking activity, but did not exhibit any strand transfer activity. This result raised the possibility that another catalytic residue could be responsible for the residual nicking activity of the RelSt3-Y252A mutant, and/or possibly participate in the strand transfer activity. In RelSt3, Y252 is enclosed within a conserved motif (motif III) found in both MOBT relaxases and Rep_trans proteins. Careful analysis of protein sequence alignments allowed us to identify another conserved tyrosine residue (Y256 for RelSt3) in motif III of MOBT proteins (Supplementary Figure S6, Supplementary Table S4).

Even if this residue was not conserved in Rep_trans proteins, the motif YxxY is typical for the Rep proteins of the superfamily I (Rep_I) (48,49), whose prototype is the protein A of E. coli virus ΦX174 (50). Indeed, viral Reps of this superfamily I use two adjacent tyrosine residues to initiate RCR, alternatively for each replication cycle. In the case of RelSt3, variant Y256F exhibited the same activity profile as the wild type protein (Figure 6BD), indicating that the Y256 is not involved in strand transfer activity. We also used a
RelSt3 variant affected on the two tyrosine residues, RelSt3 Y252A-Y256F, to investigate a possible role of Y256 in the low nicking activity observed in the RelSt3 Y252A variant. However, the RelSt3 Y252A-Y256F variant displayed the same nicking-closing activity as the Y252A variant (Figure 6D), indicating that Y256 would not be involved in catalysis. Thus, this residual nicking activity observed with Y252A variant could be due to another residue of the active site, or alternatively, by the nucleophilic attack of a water molecule activated by the metallic cofactor (see Discussion section). This latter hypothesis would also be consistent with the absence of strand transfer activity in RelSt3 Y252A and Y252A-256F variants. We exclude that activity differences found with our variants could be due to major incorrect folding as they displayed similar circular dichroism spectra compared to that of the WT protein (Supplementary Figure S7).

Trapping the covalent RelSt3-DNA complex

It is generally assumed that relaxases generate covalent protein-DNA complexes that constitute the substrate for conjugative transfer through T4SS apparatus (4,16). However, in many cases this protein-DNA complex has never
been identified experimentally. We thus aimed to capture such a complex formed by RelSt3 and its cognate oriT. As the protein is supposed to bind covalently at the 5′ end of the nick, we used an ori50 substrate labelled with 6-FAM at its 3′-end, allowing detection of the protein-DNA complex by fluorescence. Analysis of the gel by fluorescence imaging followed by a Coomassie blue staining would allow both DNA and protein detection to verify the nucleoprotein nature of the complex. As shown in Figure 7, a DNA–RelSt3 complex (red stars in Figure 7A, lane 4) was detected by denaturing SDS-PAGE in the presence of RelSt3 and high concentrations of ori50 (50 μM). The band corresponding to this complex was found to increase with DNA concentration (Supplementary Figure S8). When assays were performed with subsequent incubations with nuclease S1 or with proteinase K, this led to the disappearance of the band corresponding to the nucleoprotein complex (Figure 7A, lanes 5 and 6). This complex appeared only in the presence of the cofactor (Mn2+) but not in the presence of EDTA, confirming that its formation relies on the transesterase activity of RelSt3. As expected, when our catalytic tyrosine Y252 variants were used in similar experiments, no complex was detected (Figure 7B). Instead, the relaxase-oriT covalent complex was formed with the Y256F variant, where the Y252 tyrosine is intact.

**DISCUSSION**

In this work, we have demonstrated that binding of RelSt3 to oriT relies on its N-terminal HTH domain, and that RelSt3 binding is a prerequisite for efficient cleavage of the nic site in vitro. Conjugation experiments also indicated that (i) the binding sequence is required for functional mobilization, and (ii) the HTH domain of RelSt3 is essential for conjugation. These results altogether led us to propose that the binding of RelSt3 through its HTH domain (19,27). Our EMSA experiments showed that RelSt3 was not able to stably bind neither to its nic site nor to closely adjacent sequences. We demonstrated instead that RelSt3 only bound to its bind site located 68 nt further downstream of its nic site. Noteworthy, when we compared ICESt3 oriT sequence with those of other related ICEs, we found similar IR2 sequences located at a similar distance downstream from nic site.
understand the interplay between RelSt3 and oriT. In the F plasmid model, even if the mapped relaxase active site towards the IR1-HTH and IR2-active site interfaces. This loop corresponds to the so called ‘spacer’ DNA segment of 68 bp in oriT (see our proposed model in Figure 8). We do not exclude that other relaxosome partners could be recruited to bend this DNA region to assist in correct positioning of the IR1 hairpin loop (the CT’AA nic site) into the relaxase active site in vivo, although they were not strictly required for nicking-closing activities in vitro. These putative partners remain to be identified, and could be encoded by the ICE and/or from the bacterial genome.

In turn, the Rep_trans RCR initiators from the pT181 family are known to recognize their IRIII bind site located next to their IRII nic site, via a short string of residues in the C-terminal part of the protein (61,62). This Rep_bind site recognition was shown to be responsible for the specificity between the Rep protein and the cognate origin of replication (37). In a seminal study, Carr et al solved the 3D crystallographic structure of three Rep_trans proteins. Notably, in the RepDE and RepDN structures, it was found that this C-terminal DNA-binding domain was formed by four α-helices arranged similarly to HTH domains (63). These proteins are also dimeric, and surprisingly, the symmetry axis of the DNA-binding domain is tilted 128° with respect to the symmetry axis of the PF02486 catalytic domain. This tilt led the authors to propose a RepDE model interacting with its DNA substrate considering the proximity of the bind and the nic sites in the dso. This is quite different from our presented data. As previously indicated, this DNA-binding domain is located at the C-terminal part of the Rep_trans proteins whereas the HTH domain is located at the N-terminal part of the MOB7 relaxases. Taken together, all these data are indicative of two different evolutionary pathways leading to the DNA binding function of these transesterases.

Unlike RelSt3, some MOB7 relaxases lack the HTH domain, such as the NicK relaxase from ICEBs1 (64). As no obvious DNA binding domain has been identified in the genetic context of nick gene on ICEBs1 genome (65), further studies are needed to understand how these proteins recognize oriT DNA. Interestingly, the Rep_trans protein RepSTK1 from Geobacillus stearothermophilus harbour a C-terminal extended loop in place of the DNA-binding domain of the staphylococcal proteins of the pT181 family (22). The DNA binding interface of RepSTK1 has never been investigated so far, however, this loop could indeed functionally replace the HTH-like DNA-binding domain of its counterparts as it is predicted to be located at a similar position in the 3D structure, and to be positively charged. It is therefore tempting to speculate that MOB7 relaxases lacking a HTH domain could bind DNA using a similar loop located in the C-terminal part of these relaxases. With the hypothesis of the presence of such a loop in the 3D structure of NicK in mind, we used Alphafold to obtain a model of NicK (Supplementary Figure S10). Interestingly, a positively charged loop is indeed found for this model, in the same location of the loop found in the RepSTK1 structure and the HTH-like DNA-binding domain of RepDE. Even though this loop is much smaller, this result provides clues to the putative DNA-binding domain of MOB7 relaxases lacking an HTH domain like NicK, but this remains to be verified experimentally.
Figure 8. Proposed schematic model for RelSt3 interaction with its oriT DNA substrate. The RelSt3 model was obtained using AlphaFold. DNA strands are materialized with red and orange lines. The first contact of the relaxase with oriT would be mediated by the HTH domain (upper part of the dimer, yellow and light blue), which binds IR2 (bind site) with high affinity. Then putative relaxosome partners could bind the DNA in the spacer region (pink and purple), and help bending it in such a way that the induced DNA twists result in IR1 hairpin protrusion inside the relaxase active site. Thus, the active tyrosine of the catalytic domain of RelSt3 (red) would nick the oriT at the conserved nic site.

When we analysed which catalytic tyrosine residue of RelSt3 were important for nicking-closing activities, a residual nicking activity was unexpectedly found with the Y252A variant. This raised the hypothesis of another intervening residue. As the conserved motif YxxxY had already been described in the active site of some transesterases using two active tyrosine residues (Rep SFI) (48–50), we tested the putative role of the Y256 residue. This was reinforced by the fact that the YxxxY motif was found to be conserved among MOB T proteins. However, the Y256F variant was not altered in its nicking-closing activities, ruling out a catalytic role of Y256 residue in these reactions. This was confirmed with the analysis of the Y252A-Y256F variant, which exhibited the same residual activity observed for the Y252A variant. The residual nicking activity found with the Y252A variant could either be explained by the involvement of another residue in this process, or alternatively, by the action of a water molecule. Indeed, it is possible that in the Y252A variant, the slightly modified folding of the active site would allow the positioning of a metal-activated water molecule in front of the nic site. This water molecule could trigger nucleophilic attack when the nic site is correctly positioned in the active site. Such metal induced activation of a water molecule has already been described for several types of enzymes, including nucleases (66–69). In line with this hypothesis, we observed no formation of covalent protein-DNA adduct nor religation activity with the Y252A variant. Indeed, such religation can only occur in the case of energy conservation of the ester bond, which is not the case if the nick is catalysed by a water molecule. Taken together, these
data argue for a single catalytic tyrosine in MOB T proteins, as previously observed for Rep\textsubscript{trans} proteins of the pT181 family (22,24).

Our biochemical data demonstrated that the conserved catalytic tyrosine Y252 of RelSt3 was essential for its nicking-closing activities, and for the formation of a stable protein-DNA covalent adduct. These characteristics are shown here for the first time for a MOB T relaxase, and they are likely to be generalizable to other relaxases of the same family. Given that conjugative transfer requires the formation of a stable covalent ssDNA-relaxase adduct, the reaction activity described here could be an obstacle to the conjugation process, favouring instead the RCR in the donor cell (26,27). Thus, as yet unidentified regulatory factors may be involved in maintaining the covalent relaxase-DNA complex to promote its recruitment to the T4SS apparatus. Such factors could take part, even transiently, in formation of the relaxosome complex.

During the 90's, a peculiar regulation mechanism for DNA replication was uncovered for RepC encoded by the pT181 staphylococcal plasmid. The initially active RepC was shown to be converted into a new form after a round of RCR, the inactive RepC\textsuperscript{*} (61,70,71). The latter is still covalently bound through its active tyrosine to a 10–12 oligonucleotide corresponding to the sequence immediately downstream of the nic site. Thus, each dimer of RepC can only perform one cycle of pT181 replication, participating in the copy number regulation. Such regulation has never been observed for conjugative relaxases. Given that Rep\textsubscript{trans} and MOB T proteins share conserved protein sequence motifs and similar 3D folding and as they target identical sequence at nic sites (19,22,72,73), a similar regulation of ICE DNA copy number could also be reasonably postulated for MOB T relaxases. Furthermore, as they have already been implicated in autonomous RCR (26,27), several circular copies of ICE could accumulate in the cell, and thus this copy number has to be controlled in some way.

DATA AVAILABILITY

Source data as well as plasmids and strains are available upon request.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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