Specificity determinants in the relaxase binding site

Relaxase DNA binding and cleavage are two distinguishable steps in conjugative DNA processing that involve different sequence elements of the nic site

Maria Lucas1,2, Blanca González-Pérez1,3, Matilde Cabezas1, Gabriel Moncalian1, Germán Rivas4 and Fernando de la Cruz1 *

1 Departamento de Biología Molecular (Universidad de Cantabria) and Instituto de Biomedicina y Biotecnología de Cantabria (CSIC-UC-IDICAN), C. Herrera Oria s/n, 39011 Santander, Spain
2 Present Address, Department of Chemistry and Biochemistry, Gene Center, University of Munich, Feodor-Lynen-Str. 25, 81377 Munich, Germany
3 Present Address, Department of Biochemistry, University of Cambridge, 80 Tennis Court Road, Cambridge CB2 1GA, UK
4 Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, 28040 Madrid, Spain

Address correspondence to: Fernando de la Cruz, PhD * Corresponding author.
Telephone: +34 942 201942. Fax: +34 942 201945. E-mail: delacruz@unican.es

RUNNING TITLE: Specificity determinants in the relaxase binding site

Abbreviations: scDNA, supercoiled DNA; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA.

TrwC, the relaxase of plasmid R388, catalyzes a series of concerted DNA-cleavage and strand transfer reactions on a specific site (nic) of its origin of transfer (oriT). nic contains the cleavage site and an adjacent inverted repeat (IR2). Mutation analysis in the nic-region indicated that recognition of the IR2 proximal arm and the nucleotides located between IR2 and the cleavage site were essential for supercoiled DNA processing, as judged either by in vitro nic-cleavage or by mobilization of a plasmid containing oriT. Formation of the IR2 cruciform and recognition of the distal IR2 arm and loop were not necessary for these reactions to take place. On the other hand, IR2 was not involved in TrwC single-stranded DNA processing in vitro. For ssDNA nic-cleavage, TrwC recognized a sequence embracing six nucleotides upstream the cleavage site and two nucleotides downstream. This suggests that TrwC DNA binding and cleavage are two distinguishable steps in conjugative DNA processing and that different sequence elements are recognized by TrwC in each step. IR2 proximal arm recognition was crucial for the initial scDNA binding. Subsequent recognition of the adjacent ssDNA binding site was required to position the cleavage site in the active center of the protein so that the nic-cleavage reaction could take place.

Bacterial conjugation is an efficient and sophisticated DNA transport mechanism, genetically encoded by self-transmissible plasmids. The transfer of DNA by bacterial conjugation plays an important role in the genetic variability of bacteria as well as in the propagation of antibiotic resistance and virulence factors (1). In order to avoid the spread of antibiotic resistance genes via bacterial conjugation one promising strategy is the use of anti-conjugation-based antimicrobial agents (2,3). Our group identified unsaturated fatty acids as conjugation inhibitors (4). Their target is unknown, although membrane-associated ATPases could be good candidates. As the relaxase is the key catalytic enzyme in the conjugative process it is, a priori, a better target for a specific inhibitor. Potts et al. (5) found that bisphosphonates inhibited the activity of plasmid

Copyright 2010 by The American Society for Biochemistry and Molecular Biology, Inc.
F relaxase TraI. Their effect on conjugation inhibition was small although, surprisingly, they could specifically kill relaxase-containing cells. Moreover, bacterial relaxases might find a use as tools for site-specific DNA delivery to target eukaryotic cells for gene therapy (6). Thus, a detailed study of the specificity determinants of the reaction performed by relaxases could lead to the a la carte design of relaxases able to act on any potentially interesting sequence (7).

Conjugative DNA processing is carried out by the relaxosome, composed by the enzyme relaxase and auxiliary proteins that act on the oriT region (see (8) for review). It starts by a site- and strand-specific DNA cleavage reaction that occurs at a specific oriT site called nic. The nic-cleavage reaction is mediated by a tyrosine residue that catalyzes a transesterification reaction. After cleavage, the relaxase remains covalently bound to the 5’ end of the cleaved strand via a phosphotyrosyl linkage while the 3’ hydroxyl is sequestered by tight non-covalent interaction with the relaxase. The cleavage reaction is reversible since the free DNA 3’ hydroxyl group can attack the 5’ phosphotyrosyl bond. However, when the relaxase-DNA complex releases the 3’-OH portion of the DNA (as when it is transported to the recipient cell), a second tyrosine can attack a second nic site positioned at the protein active site. This type of reaction takes place at the end of conjugation for regenerating the oriT in the recipient cell and it is known as strand-transfer reaction (9,10).

TrwC is a multidomain protein of 966 amino acids that forms dimers in solution (11). The N-terminal part of the protein contains the relaxase domain (amino acids 1-300) (12), while the C-terminal region (amino acids 192-966) is responsible for dimerization and DNA-helicase activity, required for unwinding the transferring DNA (13,14). TrwC specifically nicks oriT-containing supercoiled plasmids in vitro in the absence of accessory proteins and remains covalently bound to the 5’ end of the cleaved DNA strand (15). The nicking activity of TrwC allows intermolecular site-specific recombination between two plasmids containing oriT in the absence of conjugation (13). Two specific tyrosyl residues in TrwC, Tyr18 and Tyr26, are involved in the DNA strand-transfer reactions (9,10,12). Tyr18 catalyzes the first strand cleavage, while Tyr26 is involved in the strand transfer reaction that terminates the DNA processing. Between these two steps in conjugation, the DNA strand which was first cleaved is displaced by the helicase activity of TrwC. Similar reactions occur during processing of F plasmid oriT by the related relaxase TraI_F. The relaxases of F and R100 plasmids also act as bifunctional relaxases, with relaxase and helicase domains in the same protein (16-18).

Conjugative and mobilizable plasmids of the same MOB family show conservation of the DNA sequence of oriT (19,20). Nevertheless, the oriT sequences specifically involved in the so-called initiation and/or termination reactions are unknown for the vast majority of plasmids. The initiation reaction is the first cleavage reaction performed by Y18 in TrwC. The termination reaction is the second cleavage and strand transfer reaction performed by Y26 in TrwC. In most analyzed oriT regions, an inverted repeat (IR, named IR2 in R388) is located upstream the nic site (20,21), which is recognized either by the relaxase or by some auxiliary relaxosomal protein (8). The proximal arm of the IR and the region surrounding the nic site are sufficient for the initiation reaction in plasmids R64 and R1162, while a larger DNA substrate that includes the complete IR is required in the termination reaction. Conversely in F, initiation demands a larger DNA substrate than the termination reaction (22).

The three-dimensional crystal structure of the relaxase domain of TrwC (TrwC_R), has been solved in complex with its cognate 25-base oligonucleotide substrate, folded in a DNA hairpin (23). The DNA is firmly held by the relaxase by two identifiable binding sites. The hairpin forms an almost perfect B-DNA that is bound by two different motifs through its major and minor grooves. The nic-proximal ssDNA is housed in a deep narrow cleft that contains the relaxase catalytic site. Nucleotides involved in that “frozen” interaction with the relaxase were established. But the 3D structure could not reveal which nucleotides participate in the enzymatic reactions of cleavage and strand-transfer. In this work, we characterize the biochemical and biophysical properties of the TrwC-DNA complex. Besides, we study the elements involved in DNA sequence recognition in the independent reactions catalyzed by TrwC during conjugative DNA processing. We present evidence that TrwC recognizes its target nic region in two steps: an initial scDNA binding involving the proximal arm of IR2, following by
specificity determinants in the relaxase binding site 3
recognition of the adjacent ssDNA binding site that situates the cleavage site in the right position to be cleaved.

Experimental Procedures

Bacterial strains, plasmids and oligonucleotides

*Escherichia coli* K12 strains used were DH5α (F⁻, endA1 recA1 gyrA96 thi-1 hsdR17 supE44 relA1 Δ(argF lacZYA) U169 φ80d lacZ ΔM15 gyrA96) (24), YJ1020 (lon-510 Δarg malPp::Iq rpl) (25) and C43(DE3) (F⁻ dcm ompT hsdS (rB⁻ mB⁻) gal λ (DE3)) (26). Plasmids used are listed in Table 1, together with details of their construction. Oligonucleotides were purchased from MWG and are listed in Table 2.

Protein purification

For TrwC purification, plasmid pSU1588 was used and the *E. coli* BL21 derivative strain C43-DE3 was employed as overexpression host. TrwC was purified as described (27) and stored at -80°C.

Sedimentation equilibrium

The experiments were performed in a Optima XL-A analytical ultracentrifuge (Beckman-Coulter) equipped with absorbance optics, using an An50Ti rotor. TrwC (ranging in concentration from 0.1 to 10 μM) in 10 mM Tris-HCl pH 7.6, 110 mM NaCl, 0.02 mM EDTA was centrifuged at sedimentation equilibrium using short columns (70 μL) at two successive speeds (13 and 15 Krpm) in the absence or in the presence of 1.5 μM oligonucleotide R(25+0) (Table 2). The equilibrium scans were taken at 20°C and three wavelengths (250, 255, and 280 nm) using either standard 12mm-double sector or six-channel centerpieces of charcoal-filled Epon. High-speed sedimentation was conducted afterwards for baseline correction. Cell average molar masses were determined by fitting a sedimentation equilibrium model for a single sedimenting solute to individual datasets with the programs XLAEQ and EQASSOC (supplied by Beckman; see (28)). The partial specific volume of the oligonucleotide was taken as 0.55 ml/g and the corresponding one of the protein was 0.727 ml/g at 20°C, calculated from the amino acid composition of the TrwC fragment (13) using the program SEDNTERP (29).

Sedimentation velocity

Experiments were carried out at 50,000 rpm and 20°C in the same XL-A instrument, using 12 mm double-sector centerpieces. Apparent sedimentation coefficients were calculated using the programs SVEDBERG (30) and SEDFIT (31), which gave comparable results. The latter program was used to generate apparent sedimentation coefficient distributions, g*(s), by least-squares boundary modeling of sedimentation velocity data (32).

Electrophoresis Mobility Shift Assay (EMSA)

TrwC binding to the oligonucleotides listed in Figure 1 and Table 2 was analyzed by EMSA. Binding reactions contained 1 nM radiolabeled oligonucleotide, 1 μM competitor oligonucleotide and increasing concentrations of TrwC in buffer A (10 mM Tris-HCl pH 7.6, 110 mM NaCl, 0.02 mM EDTA). The competitor oligonucleotide was a mixture of the following three non-labeled oligonucleotides: 5’-CCAGGTACCTGAGCTGGCCGAAAA, 5’-GCATGCGGATCCGTCGACCTGCAGGG and 5’-CCAGGATCCCCTTCACGCGATTGGAGCCT. Reaction mixtures were incubated for 20 min at 20°C and were loaded onto a 12% non-denaturing polyacrylamide gel. Binding constants were calculated as described before (27). Binding assays with the oligonucleotides listed in Figure 3 were performed in the same conditions as described before but using a lower concentration of NaCl (50 mM instead of 110 mM).

Oligonucleotides cleavage and strand-transfer assays

Cleavage reaction mixtures contained 50 nM fluorescein labeled oligonucleotide and variable concentrations of protein TrwC in 10 mM Tris-HCl pH 7.6, 5 mM MgCl₂, 110 mM NaCl and 20 μM EDTA. After incubation for 30 min at 37°C, digestion with 0.6 mg/ml proteinase K and 0.05 % (w/v) SDS was carried out for 20 min at 37°C. For the oligonucleotide strand-transfer reactions, after the incubation of 50 nM 3’ fluorescein...
labeled R(12+18) with 1 µM TrwC\textsubscript{R} for 30 min at 37°C, 250 nM of R(25+8) or the modified mut oligonucleotides (Figure 3) were added to the reaction mixture. Reactions were incubated for 1 h at 37 °C and then digested with 0.6 mg/ml proteinase K and 0.05 % (w/v) SDS. Samples were injected in the capillary system BioFocus\textsuperscript{®}2000 (BioRad). Oligonucleotide separation and quantification was performed as described previously (9,27).

Supercoiled DNA nicking assay

Reaction mixtures (40 μl) contained 10 nM scDNA of plasmid pSU4910 (or each of the mutants) and 300 nM TrwC\textsubscript{R} in 10 mM Tris-HCl pH 7.6, 50 mM NaCl, 0.02 mM EDTA and 5 mM MgCl\textsubscript{2}. After incubation for 30 min at 37°C, 20 μl of the reactions mixtures were digested with 1 mg/ml Proteinase K (Roche) in 0.5 % (w/v) SDS for 15 min at 37°C. The other 20 μl were precipitated with KCl in the presence of SDS (33). SDS was added to a final concentration of 0.2 % (w/v) and EDTA was added to a final concentration of 10 mM. The samples were heated at 70°C for 10 min. Subsequent addition of KCl to a final concentration of 100 mM followed by 15 min incubation at 0°C induced SDS-KCl precipitation. Separation was carried out by centrifugation at 4°C for 15 min in a microcentrifuge. The supernatant was removed and the pellet resuspended in 20 μl of 10 mM Tris-HCl pH 8.0, 1 mM EDTA. Reaction mixtures were applied to 0.8 % (w/v) agarose gels containing 0.5 μg/ml ethidium bromide and electrophoresed at 100 V in 45 mM Tris-borate 0.5 mM EDTA buffer (pH 8.2). Bands were visualized in a BioRad Gel Doc system and quantified using Quantity One software.

Conjugation experiments

Conjugation experiments were carried out by the plate-mating procedure as described (34). Derivatives of DH5\textalpha containing plasmid pSU2007 (a Km\textsuperscript{r} derivative of R388 (35)), and a second plasmid contributing R388-\textit{oriT} (the wild type \textit{oriT} or each of the mutants when indicated) were mated with strain UB1637. Conjugation frequencies were expressed as the number of transconjugants per donor cell.

RESULTS

TrwC \textit{nic}-cleavage activity on single stranded DNA.

TrwC\textsubscript{R} cleaves oligonucleotides containing the \textit{nic} site, resulting in two products that can be analyzed by capillary electrophoresis. Experiments were carried out with protein TrwC\textsubscript{R}, which lacks the helicase domain, to avoid non-specific interactions between oligonucleotides and the helicase. TrwC\textsubscript{R} cleaves both ssDNA and scDNA substrates containing \textit{nic} as efficiently as full length TrwC (9), and therefore is suitable for binding and \textit{nic}-cleavage analysis.

A series of oligonucleotides that varied in the number of nucleotides 5’ and 3’ to \textit{nic} (Figure 1 and Table 2) were used to map the sequence that is essential for the \textit{nic}-cleavage reaction. Cleavage was carried out by incubating each oligonucleotide with increasing concentrations of TrwC\textsubscript{R} and digesting the protein that remains covalently attached to the oligonucleotide to release the two cleavage products. These products were subjected to capillary electrophoresis under the conditions described in Experimental Procedures. There was always a molar excess of protein to guarantee that all the oligonucleotide is complexed with the protein. To compare the different cleavage ratios, we used 5μM TrwC\textsubscript{R}, which allowed saturation in cleavage for all the samples. Figure 1 shows the dissociation constants and \textit{nic}-cleavage activity of TrwC\textsubscript{R} using different oligonucleotides ranging from 6 to 35 nucleotides 5’ of the \textit{nic} site and 0 to 18 nucleotides 3’ of the \textit{nic} site. Oligonucleotides R(12+18), R(12+4) and R(6+4) did not form complexes with TrwC\textsubscript{R}, which allowed saturation in cleavage for all the samples. Figure 1 shows the dissociation constants and \textit{nic}-cleavage activity of TrwC\textsubscript{R} using different oligonucleotides ranging from 6 to 35 nucleotides 5’ of the \textit{nic} site and 0 to 18 nucleotides 3’ of the \textit{nic} site. Oligonucleotides R(12+18), R(12+4) and R(6+4) did not form complexes with TrwC\textsubscript{R} in the analyzed concentration range. Nevertheless, TrwC\textsubscript{R} was able to efficiently cleave these oligonucleotides at the same protein concentrations (Figure 1). In fact oligonucleotides with the highest \textit{nic}-cleavage activity resulted to be R(12+18) (93%), R(16+17) (83%) and R(19+14) (62%), all of them with poor binding constants, moreover, a tendency to increase \textit{nic}-cleavage efficiency correlated with a reduction of the length of the sequence located 5’ of the cleavage site (from 25 to 12 nucleotides) if the sequence 3’ to the cleavage site was longer than 7 nucleotides (Figure 1). In the same way, an inverse relationship between binding and nicking efficiency was observed. Oligonucleotides
Specificity determinants in the relaxase binding site 5

R(35+8), R(25+8) and R(25+4), showed the highest binding constants (Kd < 100 nM), but poor cleavage. Decreased binding was observed for oligonucleotides R(25-6) and R(25-3) compared to R(25-0) (23), despite all three oligonucleotides contained a perfect IR2. Oligonucleotides from related plasmids, like Fnic(29+10) and R46nic(31+8), or oligonucleotide R388-33comp (Table 2), containing the complementary strand of plasmid R388 nic, were not cleaved at all. No cleaved product was observed with these oligonucleotides even at high (10 µM) TrwC R concentration (data not shown).

Biochemical characterization of TrwC-DNA complex.

Guasch et al., determined the crystal structure of the complex formed by TrwC R and oligonucleotide R(25+0) (23). This structure showed a 1:1 complex. This result was in apparent contradiction with a previous observation that TrwC was a dimer in solution (11). Moreover, the transposase TnpA of insertion sequence IS608, that exhibits a common structural topology with TrwC relaxase domain, was shown to act as a dimer (36,37). Thus, it seemed important to elucidate if the structure of TrwC:R(25+0) showed the physiological stoichiometry of the complex in solution.

To analyze TrwC R binding to a radiolabeled R(25+8) oligonucleotide, electrophoretic mobility shift assays (EMSA) were carried out (Experimental Procedures). TrwC R binding to this oligonucleotide produced a shifted band (Figure S1A). Such complex results from rapid association/dissociation equilibrium, which is achieved in less than 1 min. Increasing the incubation temperature from 20 ºC to 37 ºC had little effect in binding affinity (data not shown).

By plotting the EMSA data, the dissociation constant of the protein-DNA complex was calculated to be 30 nM (Figure S1B). The TrwC R:R(25+8) complex could be isolated by gel filtration. After high resolution gel filtration column chromatography of the binding mixture, fractions were analyzed by non-denaturating PAGE and the fluorescent label of the oligonucleotide was detected (Supplementary Experimental Procedures). The major peak corresponded to a TrwC R:oligonucleotide complex (Figure S2). The complex was stable, with a half life of 11 hours (23).

Sedimentation equilibrium analysis of TrwC R showed that, under the experimental conditions, the protein sedimented as a single species with average molecular mass 32,900 ± 3,000 Da (Figure 2A), essentially identical to the theoretical monomer mass derived from its sequence (32,924 Da). The protein had no tendency to self-associate in the analyzed concentration range (0.1 – 10 µM). The sedimentation coefficient of TrwC R monomer was 2.68 ± 0.05 S (data not shown). From the combined data, a translational frictional coefficient ratio of 1.27 ± 0.07 was calculated, which is compatible with TrwC R being a globular monomeric protein in solution.

The oligonucleotide R(25+0) at 1.5 µM sedimented also as a single species with molar mass 8,300 ± 1,000 Da (Figure 2B), which essentially corresponds to the monomer (8,290), with a sedimentation coefficient of 1.78 ± 0.05 S (inset of Figure 2B) and a frictional ratio of 1.36. Upon incubation of oligonucleotide R(25+0) with 2.0 µM TrwC R, the mixture sedimented faster (3.91 S) and the equilibrium gradient was steeper (apparent molecular mass 44,000 Da) than the oligonucleotide alone (Figure 2B) which suggested the formation of a 1:1 protein:oligonucleotide complex.

The specific nic sequence required for TrwC function in vivo.

To analyze in detail the role of specific nic nucleotides recognized by TrwC in vivo, we carried out site-directed mutagenesis (Figure 3). Mutations were introduced on plasmid pSU4910, carrying a functional 264 bp-size oriT, systematically changing nucleotides from position 2 to position 29 of the nic site (Figure 3). As summarized in the first column of Figure 3, mutations from position 13 to 27 decreased plasmid mobilization drastically (to 0.04% or less). On the other hand, mutations in the IR2 loop (nucleotides 8-11) had almost no effect (2-fold), while mutations in the distal arm of IR2 (nucleotides 4-7), which abolish pairing with the proximal arm and would not allow hairpin formation, had quite a small effect in mobilization frequency (10-fold reduction). Conversely, the DNA sequence of the proximal arm of IR2 seemed to be critical for oriT conjugative processing, since mutations in positions 17 and 13-16 dropped mobilization to 0.038 and 0.0002%, respectively. Mutations in
both arms of the hairpin (mutIR), which maintained the secondary structure but changed the nucleotide sequence, promoted a drastic reduction of the mobilization frequency (2 x 10^5-fold). All these results taken together indicated that the proximal arm was the only essential component of IR2 for in vivo recognition of R388 nic, while the hairpin structure only slightly improved recognition.

Besides, the 8 nucleotides located between IR2 and the cleavage site were crucial for mobilization, which decreased 10^5 to 10^6-fold in the oriT variants mut18-19, mut20-22 and mut23-25. At the right side of the cleavage site, the first four nucleotides were analyzed. While the first two nucleotides were found to be essential (mut26-27), mutation of nucleotides 28-29 had a relatively small effect (7-fold decrease).

**Relaxase reactions in vitro on mutant nic sites.**

To complement the data obtained by mobilization, the oriT mutants were studied in vitro using two types of DNA substrates: scDNA (plasmid DNAs carrying the oriT mutations) and ssDNA (33-mer oligonucleotides with the mutations shown in Figure 3). Mutated oriT-containing scDNA was used to test the relaxation ability of the protein on different oriT variants, and ssDNA oligonucleotides to dissect binding, cleavage and strand-transfer reactions.

Relaxation of scDNA was analyzed as described in Experimental Procedures, using the same pSU4910 derivatives than those used for mobilization (Figure 4). Three different outcomes were observed in the relaxation of scDNA by TrwC_R: wild-type or fully relaxed DNA (mut4-7), partially relaxed DNA (mut8-11 and mut28-29), and non-relaxed DNA (mut13-16, mut17, mut18-19, mut20-22, mut23-25, mut26-27 and mutIR) (Figure 4 and last column of Figure 3). These data indicated that the in vitro requirements for scDNA recognition by TrwC_R were the same as those for in vivo mobilization. The critical region coincided in both in vivo and in vitro processes, and comprised nucleotides 13-27 (Figure 3).

Furthermore, these results indicate that scDNA processing might be the limiting step in plasmid R388 mobilization.

Electrophoretic mobility shift assays with oligonucleotides (Experimental Procedures) allowed the determination of the region that was specifically recognized for TrwC_R binding.

High-specificity binding to these oligonucleotides required a larger DNA sequence than that needed for scDNA relaxation or in vivo mobilization. The region involved was comprised from the cleavage site to the end of the distal arm of IR2, with the exception of the hairpin loop. The nucleotides located 3’ to the nic site seemed not to be specifically recognized for binding (Figure 3, column 2). Thus, high-affinity binding is not a basic requirement for mobilization ability.

Finally, cleavage and strand transfer of ssDNA oligonucleotides did not require IR2, and occurred efficiently with oligonucleotides containing wt positions 20 to 27 (see Figure 3, column 3). Remarkably, mutations in positions 13 to 19 resulted in increased cleavage, suggesting that these positions were important for complex stability. In all cases, the nic-cleavage products corresponded to the length expected for cleavage at the canonical site (data not shown).
DISCUSSION

The interaction between a conjugative relaxase and its target site is the initial step for conjugative DNA processing. Recognition of the nic site has to be specific enough so that a single sequence can be selected out of a complete bacterial genome (in fact out of a number of genomes of potential bacterial hosts). As we show in this paper, this exquisite recognition is brought about by separating it in two different steps. TrwC binds to a palindromic DNA sequence formed in a double stranded region of the DNA (binding sequence), and then cleaves in an adjacent sequence if a second specific sequence is found (cleavage sequence). TrwC binding to the palindromic sequence IR₂ was previously defined by protein crystallography. The present results indicate that TrwC binds IR₂ with high affinity. Moreover, the stoichiometry of the complex was found to be 1:1 molar ratio. This oligomerization state is consistent with the data presented in (9).

Although this perfect palindromic IR was recognized and bound by TrwC with high affinity, shorter oligonucleotides not containing the entire IR were effectively cleaved by TrwC. When binding and cleavage of oligonucleotides R(25+4), R(14+4), R(12+4) and R(6+4) was compared, we observed that the absence of the distal repeat of the IR₂ deteriorated TrwC binding ability (Figure 1). However, nic-cleavage activity remained intact in the oligonucleotides without IR₂ distal arm, indicating that IR₂ is dispensable for cleavage, but essential for high-affinity binding to the relaxase. The relaxase binds these oligonucleotides poorly but sufficiently well to recognize the sequence required for nic-cleavage. These results suggest that TrwCₐ has to recognize one sequence for binding and another for nic-cleavage, although both are required for proper binding and both are required for a proper nic-cleavage.

Nic-cleavage efficiency was increased by reduction of the length of the sequence located 5’ of the cleavage site (from 25 to 12 nucleotides). In the same way, we observed an inverse relationship between binding and nic-cleavage efficiency. This apparent contradiction was explained by experiments using suicide nucleotides (9). These nucleotides displaced the reaction equilibrium to the formation of products, therefore reducing the reverse joining reaction. In this way, R(25s+4) did not show reduced nic-cleavage activity but rather increased rejoining efficiency, due to better TrwC binding that positions the 3’-OH in a better place to attack the phospho-tyrosyl bond and religate the oligonucleotide. In the same line of thought, we observed that increasing the incubation time produced higher nic-cleavage yields in all cases. In fact, after 48h incubation, all oligonucleotides were cleaved to a similar amount. Therefore, different cleavage yields are due to the different dissociations rates of the cleaved product and not to different recognition or cleavage efficiency. Unstable binding could provoke dissociation of the 5’ product that normally remains captured by the relaxase. Consequently, the equilibrium of the cleavage-joining reaction would be displaced towards the nic-cleavage products.

To further analyze the role of the different DNA residues on TrwC binding and cleavage, we performed the mutagenesis analysis whose results are summarized on Figure 3. According to them, we can dissect TrwC binding site in two regions: the IR₂ binding site (comprising the distal and proximal arms) and the single-stranded binding site IR₃ distal arm. As mentioned above, IR₃ is essential for oligonucleotide binding but not for scDNA cleavage. Thus, mutations in the distal arm, which affect ssDNA but not scDNA binding, only slightly affect mobilization. As expected, binding of the oligonucleotide containing this mutation is impaired, but not its cleavage. Strikingly, the mobilizable scDNA was cleaved by TrwC with the same efficiency as wt-oriT. These results are surprising considering that the DNA sequence bound by TrwC starts at -25 according to the 3D structure of the TrwC-nic complex. Thus, it seems that the role of the IR₃ distal arm is to allow cruciform formation (that probably only occurs during the termination reaction on the transported T-strand), since specific interactions with TrwC do not play a crucial role.

IR₃ loop. Mutations in the IR₃ loop did not affect substantially any of the properties analyzed (see Rm8-11 results in figure 3). This is consistent with TrwC:nic crystal structure, where no direct interaction between TrwC and any of the four nucleotides of the loop was observed.

IR₃ proximal arm. This segment is essential for mobilization, binding and cleavage of scDNA (but not ssDNA cleavage). The specific interactions of TrwC with these residues are
Specificity determinants in the relaxase binding site

abundant in the crystal structure. Thus, modification of these residues abrogates TrwC$_R$ binding to this site. TrwC$_R$ recognizes not only the B-DNA form of IR$_2$ (that is, its proximal arm on dsDNA) but also the nitrogenated bases of the nucleotides forming the IR, as observed in the mutant that changes the nucleotides but maintains an IR at the same position than IR$_3$. In this case, mobilization and binding activity are both lost. Since the specific sequence of the distal arm or the loop is not essential, but the specific sequence of the hairpin is essential, we can conclude that the interactions of this DNA region with the protein are crucial in the recognition. These data allow us to present a model for the role of IR$_2$ in R388 conjugation (Figure 5). According to this model, TrwC recognizes the dsDNA containing the proximal arm of IR$_2$ in the donor cell (Figure 5A). This is consistent with the fact that TrwC recognizes and cleaves scDNA containing mutations in IR$_2$ distal arm. It is also consistent with the crystal structure of the TrwC::nic complex if we understand that the hairpin bound in the structure is a representation of the proximal arm dsDNA bound by the relaxase in vivo. In fact, the absence of involvement of the loop in recognition makes a ss cruciform containing the distal and proximal arms of IR$_2$ indistinguishable from a scDNA containing both strands of the proximal arm. High affinity binding to the proximal arm allows local melting of the DNA around the cleavage site and the generation of a U-shape turn in the transferred ssDNA strand that positions the nic site in the TrwC active site (Figure 5B). The specific requirements of the nucleotides that form the U-shape turn will be discussed later. After cleavage, the displaced ssDNA in the donor DNA molecule is transported to the recipient cell being piloted by the relaxase, where the ssDNA is recircularized. In this step, the reaction requires TrwC to recognize the nic site after one round of replication. However, since the DNA is transported in a single stranded form, the new binding site will not be dsDNA this time, but ssDNA. It is in this second recognition step where both arms of the IR$_2$ are needed (Figure 5C).

An interesting result was obtained with the mutants in G17. This nucleotide should interact with its counterpart C2. Instead, according to the available crystal structures, G17 interacts with TrwC residues R81 and D183. Due to this interaction, G17 is the first nucleotide of the ssDNA region and it seems that the interaction of G17 with R81 and D183 is essential for the extension of the ssDNA segment up to the nic-site. This structural observation could explain why the mutant oligonucleotide is bound and cleaved by the protein, but nevertheless the corresponding plasmid cannot be mobilized.

**Single-stranded binding site** Using oligonucleotides lacking IR$_2$ (R(14+4), R(12+4) and R(6+4)) we observed that IR$_3$ is dispensable for cleavage, but essential for high-affinity binding to the relaxase (Figure 1). The relaxase binds the above oligonucleotides poorly but sufficiently to recognize and cleave the nic-site. Even oligonucleotide R(6+4) seemed to contain enough sequence information to position the scissible phosphate in the catalytic centre so that the oligonucleotide could be cleaved.

As observed when binding to oligonucleotides R(25-6), R(25-3) and R(25-0) was compared, the ssDNA binding site also contributes to TrwC stable binding (23). These results suggest that TrwC$_R$ is recognizing two different sequences, one for high-affinity binding and a second one for nic-cleavage.

The effect of the mutations between IR$_2$ and the nic-cleavage site corresponded to what could have been predicted from the crystal structure. Inside this core region (nucleotide positions 13 to 27), two phenotypes could be distinguished. Mutations in the segment from positions 20 to 27 resulted in oligonucleotides inactive for cleavage. Nucleotides 20 to 27 form the U-shaped turn necessary to localize the nic site at the catalytic center. Mutations in any of these nucleotides affect the interaction with several residues within the TrwC$_R$ cleft were the U turn is bound. Moreover, the base interaction between T25 and G22 stabilizes the U-turn that drives the nic site to the close proximity of the catalytic tyrosine. This three-base intrasand interaction to form the U-turn was also observed in the crystal structure of the TraI relaxase (39,40).
On the other hand, mutations in the region 19 to 13 resulted in oligonucleotides that were cleaved with enhanced efficiency. A similar result occurred when oligonucleotide R(12+18) was used, suggesting that the lack of appropriate interactions in this region could be affecting: (i) the stability of the bound oligonucleotide, and thus its off rate (unlikely because Kd is not grossly affected, and complex half life is 11 hours), or (ii) the positioning of the oligonucleotide with respect to the cleavage site. Perhaps binding to this region is modulating the cleavage efficiency of the protein. In fact, Williams and Schildbach also found that similar mutations in the nic site of plasmid F resulted in enhanced cleavage at high relaxase concentration (41).

In summary, TrwC recognizes dsDNA and specifically binds the proximal arm of IR₂. Upon binding, the bound DNA is distorted so that local DNA melting is created around the nic-cleavage site and the DNA can be cleaved by TrwC. For this second step, recognition of specific nucleotides is required to allow the formation of a U-shape turn that locates the nic site at the catalytic centre of TrwC. Finally, both the distal and proximal arms of IR₂ are necessary for hairpin formation in the recipient cell. Thus, there are two distinguishable recognition sites, each for a different step of the processing reaction, both required for efficient conjugation. As all the reported nic sites are located between 5 and 10 nucleotides from a more or less perfect inverted repeat (20), we propose that the above mechanism is a general mechanism shared by all the conjugative relaxases. As a consequence, we hope our results and the two-step model in TrwC-target recognition will have an application in the search and characterization of relaxase inhibitors that inhibit plasmid conjugation. Besides, they could help in the design of relaxase variants that can insert in specific genomic sequences, thus providing new tools for genomic engineering.

**FUNDING**

This work was supported by grants BFU2008-00995/BMC and CIT-010000-2008-4 (Spanish Ministry of Education). M.L. was a recipient of a predoctoral fellowship from the Public Foundation "Marqués de Valdecilla".

**FIGURE LEGENDS**

**Figure 1.** TrwC-mediated cleavage of oligonucleotides embracing R388 nic site. The figure shows the dissociation constant (Kd) and percentage cleavage (nic) of the oligonucleotides represented below the sequence by horizontal lines. The values shown are the averages of at least 3 independent experiments. The DNA sequence of R388 nic site is shown on top. The inverted repeat IR₂ is symbolized by horizontal arrows under the DNA sequence. The nic site is represented by a slash in the sequence. Kd and % cleavage for each oligonucleotide are represented in the right columns. N.a. not applicable. Most of the dissociation constants were published by (23), but are included in the figure for clarity.

**Figure 2.** Stoichiometry of TrwCₚ:R(25+0) complexes in solution. (A) Sedimentation equilibrium gradient (15,000 rpm, 20°C) of 6 μM TrwCₚ. The symbols represent the experimental data and the solid line shows the best fit gradient, with an average molar mass of 32,900 ± 3,000, which is essentially the monomer mass. (B) The symbols represent the sedimentation equilibrium gradient (13,000 rpm, 20°C) of a mixture of 2.0 μM TrwCₚ with 1.5 μM R(25+0) oligonucleotide. The solid line is the best fit gradient to a single sedimenting species of 44,000 molar mass. For comparative purposes the theoretical gradients of monomer TrwCₚ (dashed line) and R(25+0) oligonucleotide (dotted line) are also shown. Inset: Apparent sedimentation coefficient distributions, g*(s), at 50,000 rpm and 20°C for 1.5 μM R(25+0) alone (dashed line) and in the presence of 2.0 μM TrwCₚ (solid line).
Figure 3. Analysis of TrwC binding site by mutagenesis. The DNA sequence of R388 nic site is shown at the top of the figure. A slash in the sequence indicates the position of the nic-cleavage site. Horizontal bars represent the oligonucleotides named in the left column. Mutant nucleotides are indicated over the horizontal bar. For the mobilization experiments, derivatives of strain DH5α containing plasmid pSU2007 plus either pSU4910 or any of its mutants (Table 1), were mated with strain UB1637 and transconjugants selected as explained in Experimental Procedures. Mobilization frequencies (column 1) were calculated as the number of transconjugants (CmNxR) divided by the number of donors (CmRSmR). The first value corresponds to the mean value, while standard deviations (assuming a log-normal distribution) appear in parentheses. The figures are averages of 5 independent experiments. Kd, are the dissociation constants calculated with the binding data as represented in figure 1, by non-linear regression fit of the data using GraphPad Prism™ 3.02. % nic, is the cleavage ratio at 1 μM TrwC R. ND, not determined. The nucleotides within the extension that are critical for mobilization (yellow) TrwC binding (blue), nic cleavage (pink) and strand-transfer (green) are marked with a box over the DNA sequence.

Figure 4. Relaxation reaction of protein TrwC R with different plasmid DNAs containing mutant oriTs. (A) 20 μl of plasmids (10 nM). Lanes: 1) pSU18, 2) pSU4910, 3) pSU1671 (mut28-29), 4) pSU1672 (mut26-27), 5) pSU1673 (mut23-25), 6) pSU1674 (mut20-22), 7) pSU1675 (mut18-19), 8) pSU1676 (mut13-17), 9) pSU1677 (mut8-11), 10) pSU1678 (mutIR) and 11) pSU1679 (mut4-7). (B) Relaxation products of the same plasmids as in the A panel. TrwC R (300 nM) was incubated with DNA (10 nM) in the presence of 10 mM Tris pH 7.6, 50 mM NaCl, 0.02 mM EDTA and 5 mM MgCl₂ for 30 min at 37 °C. Then, the reaction mixture was digested with proteinase K. (C) DNA-protein covalent complexes precipitation in the presence of KCl. The lanes correspond to the plasmids indicated in (A). sc: supercoiled. oc: Open circle.

Figure 5. Model of TrwC oriT recognition in the conjugation process. In red are colored the bases T20-T25, that are recognized in the ssDNA processing, in cyan the additional bases (G12-A19) relevant for scDNA relaxation, in dark green the complementary sequence of the proximal arm of IR₂, in yellow the IR₂ loop, in light green the distal arm of IR₂ and in orange the DNA generated by rolling circle replication. The position of TrwC tyrosine Y18 is indicated in magenta on the protein surface. The sequence of the dsDNA or ssDNA recognized by TrwC is shown below with the same color code as in the model.
TABLES

Table 1. Plasmids

| Plasmid | Description       | Phenotype | Size | Reference |
|---------|-------------------|-----------|------|-----------|
| pET3a  | Expression vector | Ap<sup>R</sup>, Rep(pMB8) | 4.6  | (42)      |
| pSU1186| pUC8::oriT (R388) | Ap<sup>R</sup>, Rep(pMB8) | 3.1  | (34)      |
| pSU1501| pKK223-3::trwC   | Ap<sup>R</sup>, Rep(pMB8) | 7.7  | (11)      |
| pSU1588| pET3a:: trw<sub>C</sub><sub>R</sub> | Ap<sup>R</sup>, Rep(pMB8) | 5.5  | (9)       |
| pSU1671| pSU18::oriT mut28-29 | Cm<sup>R</sup>, Rep(p15A) | 4.9  | This work |
| pSU1672| pSU18::oriT mut26-27 | Cm<sup>R</sup>, Rep(p15A) | 4.9  | This work |
| pSU1673| pSU18::oriT mut23-25 | Cm<sup>R</sup>, Rep(p15A) | 4.9  | This work |
| pSU1674| pSU18::oriT mut20-22 | Cm<sup>R</sup>, Rep(p15A) | 4.9  | This work |
| pSU1675| pSU18::oriT mut18-19 | Cm<sup>R</sup>, Rep(p15A) | 4.9  | This work |
| pSU1676| pSU18::oriT mut13-16 | Cm<sup>R</sup>, Rep(p15A) | 4.9  | This work |
| pSU1677| pSU18::oriT mut8-11 | Cm<sup>R</sup>, Rep(p15A) | 4.9  | This work |
| pSU1678| pSU18::oriT mutIR  | Cm<sup>R</sup>, Rep(p15A) | 4.9  | This work |
| pSU1679| pSU18::oriT mut4-7  | Cm<sup>R</sup>, Rep(p15A) | 4.9  | This work |
| pSU1680| pSU18::oriT mut17  | Cm<sup>R</sup>, Rep(p15A) | 4.9  | This work |
| pSU2007| Km<sup>R</sup> derivative of R388 | Km<sup>R</sup>Tp<sup>R</sup>IncWtra<sup>+</sup> | 32.0 | (35)      |
| pSU4910| pSU18::oriT (R388) | Cm<sup>R</sup>, Rep(p15A) | 4.9  | This work |

<sup>a</sup> Variants of plasmid R388 <i>oriT</i> were constructed from plasmid pSU4910, which contains a fully functional R388 <i>oriT</i> segment of 264 bp (GenBank accession no. X51505.1, coordinates 59 to 322). Primers orit322EcoRI and orit322HindIII were used to PCR-amplify the <i>oriT</i> segment of plasmid pSU1186. The amplified product was digested with <i>EcoRI</i> and <i>HindIII</i> and cloned at the equivalent sites of plasmid pSU18 resulting in plasmid pSU4910. Plasmids with mutant <i>oriTs</i> were built using the megaprimer site-directed mutagenesis method (43). A first PCR reaction was carried out on template pSU1186 with primer orit322HindIII and the oligonucleotide with the desired mutation (either R(12+18)mut28-29, R(12+18)mut26-27, Rm(23-25), Rm(20-22), Rm(18-19), Rm(13-16), Rm(8-11), Rm(IR), Rm(4-7) or Rm(17)) to obtain plasmids pSU1671, pSU1672, pSU1673, pSU1674, pSU1675, pSU1676, pSU1677, pSU1678, pSU1679 and pSU1680 respectively. The resulting PCR products together with oligonucleotide orit322EcoRI were used as primers for a second PCR using pSU1186 as template. This fragment, digested with <i>EcoRI</i> and <i>HindIII</i>, was cloned in pSU18 digested with <i>EcoRI</i> and <i>HindIII</i>. Ligation products were used to transform <i>E.coli</i> strain DH<sub>5</sub><sub>a</sub>. The identity of all plasmids was verified by DNA sequencing.
Table 2. Oligonucleotides

| Name           | Oligonucleotide sequence<sup>a</sup>                                                                 |
|----------------|-------------------------------------------------------------------------------------------------------|
| TrwCNdel       | TCACCTATATGCTCAGTCACATGTTATTGACC                                                                     |
| TrwC293END     | GGGGGATTCCTAGCTGAAATCTATGCGG                                                                        |
| oriT332EcoRI   | GGGCAATTCTGATGGTTAAGTTGAGG                                                                         |
| oriTR388HindIII| TGACATCTTGAGCTTGATAAACCAGTTAAGGA                                                                     |
| R(12+18)mut26-27| TGGGTATTGATCGACCCAGATTTAAGGA                                                                      |
| R(12+18)mut28-29| TGGGTATTGATCGACCCAGATTTAAGGA                                                                      |
| R(35+8)mutIR   | AATGACTTACGCGTGGGCCACAGTTTCTAGAGGA                                                               |
| R388-33comp    | TGGGCTATAGACAATACGCACCTTTCGCGCG                                                                   |
| R46nic(31+8)   | ATAGCGTATAGTTGCGGTTAGGTGT/ATAGCGG                                                              |
| Fnic(29+10)    | CAGCCCCACACTTGATGCGTGAGGTGGTG/ATAGGTTTTTG                                                        |
| R(35+8)       | AATGACTTACGCGTGGGCCACAGTTTCTAGAGGA                                                               |
| R(25+8)       | GGGCAACCAGAGGTTGCTATTGTC/ATAGGCC                                                               |
| R(22+11)      | CACCGAAAAGGTTGCTATTGTC/ATAGGCCGAGAG                                                              |
| R(19+14)      | GGACAAAGGTTGCTATTGTC/ATAGGCCAGATTAGGA                                                            |
| R(16+17)      | AAGGTGCGTATTGTC/ATAGGCCAGATTAGGA                                                                |
| R(12+18)      | TGGGTATTGATCGACCCAGATTTAAGGA                                                                      |
| R(14+4)       | TGGGTATTGATCGACCCAGATTTAAGGA                                                                      |
| R(12+4)       | TGGGTATTGATCGACCCAGATTTAAGGA                                                                      |
| R(6+4)        | TGGGTATTGATCGACCCAGATTTAAGGA                                                                      |
| R(25+4)       | GGGCAACCAGAGGTTGCTATTGTC/ATAGGCC                                                               |
| R(25+0)       | GGGCAACCAGAGGTTGCTATTGTC/ATAGGCC                                                               |
| R(25-3)       | GGGCAACCAGAGGTTGCTATTGTC/ATAGGCC                                                               |
| R(25-6)       | GGGCAACCAGAGGTTGCTATTGTC/ATAGGCC                                                               |
| Rm(28-29)     | GGGCAACCAGAGGTTGCTATTGTC/ATAGGCC                                                               |
| Rm(26-27)     | GGGCAACCAGAGGTTGCTATTGTC/ATAGGCC                                                               |
| Rm(23-25)     | GGGCAACCAGAGGTTGCTATTGGA/ATAGGCC                                                               |
| Rm(20-22)     | GGGCAACCAGAGGTTGCTATTGTC/ATAGGCC                                                               |
| Rm(18-19)     | GGGCAACCAGAGGTTGCTATTGTC/ATAGGCC                                                               |
| R3m(17)       | GGGCAACCAGAGGTTGCTATTGTC/ATAGGCC                                                               |
| Rm(13-16)     | GGGCAACCAGAGGTTGCTATTGTC/ATAGGCC                                                               |
| Rm(8-11)      | GGGCAACCAGAGGTTGCTATTGTC/ATAGGCC                                                               |
| Rm(4-7)       | GGGCAACCAGAGGTTGCTATTGTC/ATAGGCC                                                               |
| Rm(1IR)       | GGGCAACCAGAGGTTGCTATTGTC/ATAGGCC                                                               |

<sup>a</sup>The sequence that corresponds to the inverted repeat IR<sub>2</sub> of R388 nic is underlined. Nucleotides which are different from R388 wildtype sequence are shown in bold. The / symbol indicates the position of the nic-cleavage site.
Specificity determinants in the relaxase binding site

1. de la Cruz, F., and Davies, J. (2000) *Trends Microbiol* 8(3), 128-133
2. Filutowicz, M., Burgess, R., Gamelli, R. L., Heinemann, J. A., Kurenbach, B., Rakowski, S. A., and Shankar, R. (2008) *Plasmid* 60(1), 38-44
3. Potts, R. G., Lujan, S. A., and Redinbo, M. R. (2008) *Future microbiology* 3, 119-123
4. Fernandez-Lopez, R., Machon, C., Longshaw, C. M., Martin, S., Molin, S., Zechner, E. L., Espinosa, M., Lanka, E., and de la Cruz, F. (2005) *Microbiology* 151(Pt 11), 3517-3526
5. Lujan, S. A., Guogas, L. M., Ragonese, H., Matson, S. W., and Redinbo, M. R. (2007) *Proc Natl Acad Sci U S A* 104(30), 12282-12287
6. Llosa, M., and de la Cruz, F. (2005) *Res Microbiol* 156(1), 1-6
7. Gonzalez-Perez, B., Carballera, J. D., Moncalian, G., and de la Cruz, F. (2009) *Biotechnology journal* 4(4), 554-557
8. Zechner, E. L., de la Cruz, F., Eisenbrandt, R., Grahn, A. M., Koraimann, G., Lanka, E., Muth, G., Pansegrau, W., Thomas, C. M., Wilkins, B. M., and Zatyka, M. (2000) Conjugative DNA transfer processes In: Thomas, C. M. (ed). *The horizontal gene pool: bacterial plasmids and gene spread*, Harwood Academic Publishers, Amsterdam
9. Gonzalez-Perez, B., Lucas, M., Cooke, L. A., Vyle, J. S., de la Cruz, F., and Moncalian, G. (2007) *Embo J* 26(16), 3847-3857
10. Garcillan-Barcia, M. P., Jurado, P., Gonzalez-Perez, B., Moncalian, G., Fernandez, L. A., and de la Cruz, F. (2007) *Mol Microbiol* 63(2), 404-416
11. Grandoso, G., Llosa, M., Zabala, J. C., and de la Cruz, F. (1994) *Eur J Biochem* 226(2), 403-412
12. Grandoso, G., Avila, P., Cayon, A., Hernando, M. A., Llosa, M., and de la Cruz, F. (2000) *J Mol Biol* 295(5), 1163-1172
13. Llosa, M., Bolland, S., Grandoso, G., and de la Cruz, F. (1994) *J Bacteriol* 176(11), 3210-3217
14. Llosa, M., Grandoso, G., Hernando, M. A., and de la Cruz, F. (1996) *J Mol Biol* 264(1), 56-67
15. Llosa, M., Grandoso, G., and de la Cruz, F. (1995) *J Mol Biol* 246(1), 54-62
16. Reygers, U., Wessel, R., Muller, H., and Hoffmann-Berling, H. (1991) *Embo J* 10(9), 2689-2694
17. Matson, S. W. (1991) *Prog Nucleic Acid Res Mol Biol* 40, 289-326
18. Fukuda, H., and Ohstubo, E. (1995) *J Biol Chem* 270(36), 21319-21325
19. Garcillan-Barcia, M. P., Francia, M. V., and de la Cruz, F. (2009) *FEBS Microbiol Rev* 33(3), 657-687
20. Francia, M. V., Varsaki, A., Garcillan-Barcia, M. P., Latorre, A., Drainas, C., and de la Cruz, F. (2004) *FEBS Microbiol Rev* 28(1), 79-100
21. Parker, C., Becker, E., Zhang, X., Jandle, S., and Meyer, R. (2005) *Plasmid* 53(2), 113-118
22. Gao, Q., Luo, Y., and Deonier, R. C. (1994) *Mol Microbiol* 11(3), 449-458
23. Guasch, A., Lucas, M., Moncalian, G., Cabez, M., Perez-Luque, R., Gomis-Ruth, F. X., de la Cruz, F., and Coll, M. (2003) *Nat Struct Biol* 10(12), 1002-1010
24. Grant, S. G., Jessee, J., Bloom, F. R., and Hanahan, D. (1990) *Proc Natl Acad Sci U S A* 87(12), 4645-4649
25. Jubete, Y., Maurizi, M. R., and Gottesman, S. (1996) *J Biol Chem* 271(48), 30798-30803
26. Miroux, B., and Walker, J. E. (1996) *J Mol Biol* 260(3), 289-298
27. Boer, R., Russi, S., Guasch, A., Lucas, M., Blanco, A. G., Perez-Luque, R., Coll, M., and de la Cruz, F. (2006) *J Mol Biol* **358**(3), 857-869
28. Minton, A. P. (1994). In: Schuster, T. M., and Laue, T. M. (eds). *Modern Analytical Ultracentrifugation*, Birkhauser, Boston, MA
29. Laue, T. M., Shah, B. D., Ridgeway, T. M., and Pelletier, S. L. (1992). In: Harding, S. E., Rowe, A. J., and Horton, J. C. (eds). *Analytical Ultracentrifugation in Biochemistry and Polymer Science*, Royal Society of Chemistry, Cambridge, UK
30. Philo, J. S. (1997) *Biophys J* **72**(1), 435-444
31. Schuck, P. (1998) *Biophys J* **75**(3), 1503-1512
32. Schuck, P., and Rossmanith, P. (2000) *Biopolymers* **54**(5), 328-341
33. Trask, D. K., DiDonato, J. A., and Muller, M. T. (1984) *Embo J* **3**(3), 671-676
34. Llosa, M., Bolland, S., and de la Cruz, F. (1991) *Mol Gen Genet* **226**(3), 473-483
35. Martinez, E., and de la Cruz, F. (1988) *Mol Gen Genet* **211**(2), 320-325
36. Barabas, O., Ronning, D. R., Guynet, C., Hickman, A. B., Ton-Hoang, B., Chandler, M., and Dyda, F. (2008) *Cell* **132**(2), 208-220
37. Guynet, C., Hickman, A. B., Barabas, O., Dyda, F., Chandler, M., and Ton-Hoang, B. (2008) *Mol Cell* **29**(3), 302-312
38. Becker, E. C., and Meyer, R. J. (2000) *J Mol Biol* **300**(5), 1067-1077
39. Datta, S., Larkin, C., and Schildbach, J. F. (2003) *Structure* **11**(11), 1369-1379
40. Hekman, K., Guja, K., Larkin, C., and Schildbach, J. F. (2008) *Nucleic Acids Res* **36**(14), 4565-4572
41. Williams, S. L., and Schildbach, J. F. (2006) *Nucleic Acids Res* **34**(2), 426-435
42. Rosenberg, A. H., Lade, B. N., Chui, D. S., Lin, S. W., Dunn, J. J., and Studier, F. W. (1987) *Gene* **56**(1), 125-135
43. Sarkar, G., and Sommer, S. S. (1990) *Biotechniques* **8**(4), 404-407
Figure 1

Table:

| Region   | Kd (nM)  | nic (%) |
|----------|----------|---------|
| R(35+8)  | 34 ± 5   | 17 ± 1  |
| R(25+8)  | 66 ± 8   | 10 ± 2  |
| R(22+11) | 77 ± 10  | 15 ± 2  |
| R(19+14) | 134 ± 17 | 62 ± 3  |
| R(16+17) | 270 ± 25 | 83 ± 5  |
| R(12+18) | >2000    | 93 ± 3  |
| R(14+4)  | 320 ± 31 | 31 ± 1  |
| R(12+4)  | >2000    | 34 ± 1  |
| R(6+4)   | >2000    | 20 ± 2  |
| R(25+4)  | 84 ± 7   | 12 ± 2  |
| R(25+0)  | 70 ± 12  | n.a     |
| R(25-3)  | 167 ± 22 | n.a     |
| R(25-6)  | 482 ± 37 | n.a     |
Figure 2
Figure 3
Figure 4
Figure 5

5'-ACGCGCACCAGGAGTGCATATTGTCTATA-3'
3'-TGCGCCTGGCTTTCAGCACTACAGATAT-5'

5'-GGTGCGTATTGTCTATA-3'
3'-CCACGC-5'
Relaxase DNA binding and cleavage are two distinguishable steps in conjugative DNA processing that involve different sequence elements of the nic site

Maria Lucas, Blanca Gonzalez-Perez, Matilde Cabezas, Gabriel Moncalian, German Rivas and Fernando de la Cruz

J. Biol. Chem. published online January 8, 2010

Access the most updated version of this article at doi: 10.1074/jbc.M109.057539

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2010/01/08/M109.057539.DC1