Two atypical mobilization proteins are involved in plasmid CloDF13 relaxation

Belen Nuñez and Fernando de la Cruz*
Departamento de Biología Molecular (Unidad asociada al CIB, CSIC), Universidad de Cantabria, C/Herrera Oria s/n, 39011 Santander, Spain.

Summary
The mobilization region of plasmid CloDF13 was localized to a 3.6 kb DNA segment that was analysed by transposon mutagenesis and DNA sequencing. Analysis of the DNA sequence allowed us to identify two mobilization genes and the CloDF13 origin of conjugative transfer (oriT), which was localized to a 661 bp segment at one end of the mobilization (Mob) region. Thus, the overall organization was oriT–mobB–mobC. Plasmid CloDF13 DNA was isolated mainly as a relaxed form that contained a unique strand and site-specific cleavage site (nic). The position of nic was mapped to the sequence 5‘-GGTG/GTCGGG-3‘ by primer extension and sequencing reactions. Analysis of Mob− insertion mutants showed that mobC was essential for CloDF13 relaxation in vivo. The sequence of mobC predicts a protein (MobC) of 243 amino acids without significant similarity to previously reported relaxases. In addition to MobC, the product of mobB was also required for CloDF13 mobilization and for oriT relaxation in vivo. mobB codes for a protein (MobB) of 653 amino acids with three predicted transmembrane segments at the N-terminus and the NTP-binding motifs characteristic of the TraG family of conjugative coupling proteins. Membership of the TraG family was confirmed by the fact that CloDF13 mobilization by plasmid R388 was independent of TrwB and only required PILW. However, contrary to the activities found for other coupling proteins, MobB was required for efficient oriT cleavage in vivo, suggesting an additional role for this particular protein during oriT processing for mobilization. Additionally, the cleavage site produced by the joint activities of MobB and MobC was shown to contain unblocked ends, suggesting that no stable covalent intermediates between relaxase and DNA were formed during the nic cleavage reaction. This is the first report of a conjugative transfer system in which nic cleavage results in a free nicked-DNA intermediate.

Introduction
Mobilizable plasmids are not able to promote their own transfer unless an appropriate conjugation system is provided by a co-resident helper plasmid. Mobilizable plasmids have an origin of conjugative transfer (oriT) and code for proteins (Mob) involved in DNA conjugative processing. Among Mob proteins are the oriT-specific relaxase and nicking-accessory proteins that, through specific oriT interactions, form the relaxosome, an intermediate in DNA transfer. Available evidence suggests an ‘idling reaction’ in the relaxosome, with cleavage and rejoining of the strands without loss of superhelicity. A hypothetical mating signal is thought to disrupt this equilibrium and start conjugative DNA processing. After complete displacement of the DNA strand to be transferred, a termination reaction takes place, in which the transferred strand is recircularized (Zechner et al., 1999). Moreover, a third class of Mob proteins, named ‘coupling proteins’, as well as the proteins responsible for the synthesis and assembly of the conjugative pilus (Pil or Mpf) are required for mobilization. These latter functions must be provided by a helper conjugative system. Examples of mobilizable plasmids are ColE1, ColE3, RSF1010 and CloDF13 (Cabezón et al., 1997).

CloDF13 is a 9.9 kb bacteriocinogenic plasmid that originated from Enterobacter cloacae. It is stably inherited in Escherichia coli at about 10 copies cell−1 (Tieze et al., 1969). Its genetic organization, gene expression and DNA replication have attracted attention during the past three decades. In particular, CloDF13 mRNAs and proteins synthesized in vivo by purified minicells or in vitro have been analysed extensively (Kool et al., 1974; Konings et al., 1976). Transposon insertion mutagenesis experiments assigned the Mob region to one-third of the plasmid DNA (van de Pol et al., 1978). Minicell analysis of proteins produced by a series of Tn901 insertion mutants led to the proposal that the CloDF13 Mob region directed the synthesis of polypeptides of 61 kDa (MobB) (van de Pol et al., 1978) and 24 kDa (MobC) (Andreoli et al., 1978). Support for this came from the DNA sequence of CloDF13 Mob (van Putten et al., 1987). Two open reading frames (ORFs) were found coding for proteins of 57.9 kDa and

Accepted 4 December, 2000. *For correspondence. E-mail delacruz@unican.es; Tel. (+34) 42 201 942; Fax (+34) 42 201 945.
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15.8 kDa, presumed to correspond to MobB and MobC respectively. CloDF13 cop3, a copy mutant of CloDF13, inhibited F transfer and reduced the efficiency with which the host cells plated F-specific RNA phages, but not single-strand DNA phages (van de Pol et al., 1979). Studies by van de Pol et al. (1979) with both insertion and deletion mutants of CloDF13 cop3 showed that these effects resulted from the large amount of two plasmid gene products, MobB and additional polypeptide D. It was proposed that both polypeptides were membrane bound and that a large amount of them would inhibit passage of F lac or RNA phages.

It has been reported recently that CloDF13 exhibits a broad host range for mobilization. It can be mobilized efficiently by plasmids from many incompatibility groups, including IncF, I, N, P and W (Cabezón et al., 1997). CloDF13 mobilization displayed a unique property. Unlike other Mob plasmids, such as ColE1, ColE3 or RSF1010, mobilization of CloDF13 was independent of the TraG-like protein (coupling protein) encoded by the helper conjugative plasmid and only required the Mpf (genes involved in mating pair formation) gene products. Thus, it was suggested that CloDF13 codes for its own TraG homologue (Cabezón et al., 1997). However, although the published sequence of MobB contained the putative ATP-binding motifs characteristic of TraG-like proteins (Cabezón et al., 1994), it lacked the N-terminal hydrophobic stretches, thought to represent α-helical transmembrane segments. Moreover, although the complete sequence of CloDF13 was known (Nijkamp et al., 1986), no protein-encoding sequence with the conserved relaxase signature motifs could be identified. In all these respects, CloDF13 seemed to be rather atypical for a mobilizable plasmid, so it deserved further analysis.

The present work re-examines the CloDF13 Mob region. Revision of the previously published DNA sequence resulted in changes in all encoded ORFs within Mob. Analysis of the new sequence indicated that CloDF13 encodes two mobilization proteins, MobB and MobC, that are atypical when compared with previously characterized plasmid Mob proteins in both sequence and biochemical properties. For instance, CloDF13 DNA was isolated as a specifically relaxed molecule with non-blocked 5' and 3' ends of the nic site. This atypical nic cleavage reaction required both MobB and MobC. Thus, CloDF13 DNA processing reactions for conjugation are rather different from previously described Mob plasmids. Consequently, CloDF13 represents a new class of mobilizable elements.

**Results**

* **Tn5τac1 mapping and DNA sequencing of the CloDF13 Mob region**

Plasmid pSU4628 is a CloDF13 derivative obtained by insertion of the ampicillin resistance transposon TnA and subsequent deletion of the transposition functions (Cabezón et al., 1997). Tn5τac1 insertion mutagenesis (Experimental procedures) was used to map the pSU4628 Mob region genetically. A collection of 27 different insertions was obtained and mapped (Núñez, 1998). It should be

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**Fig. 1.** Gene organization of the CloDF13 Mob region. The gene organization shown summarizes the data contained in the CloDF13 EMBL sequence AJ224861 (see also supplementary data on the journal website http://www.blackwell-science.com/mmi). On top of the gene boxes (box arrowheads point in the direction of translation), we represent the map position of the Tn5τac1 insertion mutants (the pSU prefixes have been omitted for clarity). In parenthesis, the phenotype of each mutant is shown: +, mobilization proficient; -, mobilization deficient. Below the gene boxes, some key sequence co-ordinates (in bp) are shown. The black arrowhead indicates the position of the nic site in the complementary DNA strand. The lower part of the figure shows the localization of additional ORFs deduced from the DNA sequence.

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noted that plasmid pSU4628 was remarkably refractory to Tn5 mutagenesis, compared with plasmids R388 (Llosa et al., 1994a) or R6K (Nuñez et al., 1997), and the few insertions that were obtained within Mob appeared repeatedly in independent experiments. Tn5tac1 mutants were tested for their ability to be mobilized by pSU1424, a helper plasmid that provided the functions necessary for W-pilus formation. The five insertions that showed a Mob− phenotype were located between the insertions in plasmids pSU4790 and pSU4795 (Fig. 1). This assignment was further confirmed by construction of plasmid pSU4814. This plasmid carries a 3.6 kb DNA segment of CloDF13 and was Mob+ (Fig. 2). Sequence analysis of this DNA segment resulted in the genetic map shown in Fig. 1. The upper strand of Mob carried four ORFs that were named orfA1, orfA2, mobB and mobC. The lower strand carried three additional ORFs, as shown in Fig. 1.

**Localization of the oriT and nic sites in CloDF13**

In order to localize CloDF13 oriT, a series of pSU4814 deletion derivatives was constructed (Fig. 2). When mobilization was tested in the presence of both pSU1456 and a second helper plasmid providing CloDF13 Mob functions (either pSU4628 or pSU4814), some of the derivatives were mobilized (Fig. 2). The smallest mobilizable fragment was 661 bp long (pSU4824). Thus, this fragment should contain oriT. Surprisingly, pSU4822 (Fig. 2), which contains the same

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**Fig. 2.** Functional analysis of plasmid pSU4628 and derivatives. The central, long horizontal line represents the map of the pSU4628 mobilization region. The positions of the Tn5tac1 insertion mutants (the pSU prefixes have been omitted for clarity) are shown above the map. The small horizontal arrows point in the direction of transcription of the P_tac promoter present in Tn5tac1. The information over the grey background refers to some properties of the Tn5tac1 mutants. The arrowhead in oriT indicates the position of the nic site. Below the pSU4628 map, horizontal lines show the DNA segments remaining in several pSU4628 derivatives. The name of each plasmid is shown at the lefthand side. The DNA segment cloned in pSU4824 was also obtained in the opposite orientation, resulting in pSU4825. pSU4824 and pSU4825 showed the same oriT and Mob phenotype. pSU4839 contains the same CloDF13 DNA segment as in pSU4815. Functional properties of deletion derivatives (right): Mob, mobilization frequencies of each derivative in the presence of pSU1456; nic, relative amount of plasmid DNA recovered as the nicked form from CsCl/EtdBr gradients of cleared lysates; +, 50–80% of the DNA was recovered as the nicked form; −, 10–30%; −, undetectable nicked form. ND, not determined. Restriction enzyme sites: H, HpaII; K, KpnI; N, NaeI; P, PstI; S, SmaI.

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DNA fragment proposed by Snijders et al. (1983) to contain the CloDF13 oriT, was not mobilized. Poor yields of supercoiled (SC) pSU4814 DNA (and of other Mob \(^1\) derivatives of CloDF13) were usually obtained when plasmid DNAs were isolated using either SDS/alkaline or Triton X-100 lysis procedures. This was not the case for Mob \(^2\) derivatives such as pSU4815 and pSU4824 (data not shown). When Triton X-100 lysates of pSU4814 and pSU4628 were subjected to cesium chloride (CsCl)–ethidium bromide (EtdBr) density gradient centrifugation, most of the plasmid DNA appeared in the upper band (corresponding to the open circular form (OC) (Fig. 3), suggesting that CloDF13 was mainly isolated in the relaxed form. CloDF13 relaxation could not be increased further by adding EtdBr before CsCl, a treatment applied successfully to induce relaxation in the case of plasmid R6K (Ávila et al., 1996). In order to find out whether CloDF13 OC DNA molecules were randomly or specifically nicked, they were treated with snake venom phosphodiesterase as described previously (Ávila et al., 1996). This enzyme selectively cleaves the DNA strand opposite a nick. When the OC form of pSU4814 plasmid DNA was digested with phosphodiesterase, it was converted to a linear DNA molecule of 5.95 kb. Further digestion of this molecule with endonuclease EcoRI resulted in two fragments of 3.3 kb and 2.65 kb, indicating that phosphodiesterase was cleaving in a specific site (Fig. 3). Accordingly, the nic site should be located at a distance of 3.3 kb from the EcoRI site. As CloDF13 oriT was previously assigned to a particular 661 bp sequence (Fig. 2), nic should be located approximately in the middle of this DNA fragment, that is around bp 300 in Fig. 2.

**Precise localization of the CloDF13 nic site**

Mapping the 5’ end of nic in pSU4628 DNA was carried out by primer extension using oligonucleotide CloDF13 #11 as a primer. A single polynucleotide chain of 85 nucleotide residues resulted when the extension reaction used the OC DNA band purified from CsCl/EtdBr gradients, indicating that the extension product was

![Fig. 3.](image-url) pSU4814 DNA is nicked at a specific site. Left. Photograph of a CsCl/EtdBr density gradient from a cleared lysate of DH5\(\alpha\)(pSU4814) obtained as described in Experimental procedures. Arrowheads indicate the OC and SC DNA bands. Right. Agarose slab gel electrophoresis of pSU4814 DNA nicked in vivo. SC and OC DNAs were obtained from CsCl/EtdBr gradients as shown in the photograph on the left. Lanes: 1, SC pSU4814 DNA; 2, OC DNA; 3, OC DNA incubated with snake venom phosphodiesterase; 4, OC DNA digested with EcoRI; 5, OC DNA incubated with phosphodiesterase and subsequently digested with EcoRI; M, size markers (DNA digested with EcoRI + HindIII). DNA bands were visualized using a Bio-Rad Gel Doc video system.

![Fig. 4.](image-url) Mapping of the 5’ end of nic in pSU4628 and its derived insertion mutants. OC DNA of plasmid pSU4628 from a CsCl/EtdBr gradient was used as a substrate for sequencing reactions primed by oligonucleotide CloDF13 #11. ACGT, sequencing lanes using ThermoSequenase and a Texas Red-labelled primer in a Vistra 725 DNA sequencer. Lanes 1–7, extension reactions primed by Texas Red-labelled oligonucleotide CloDF13 #11 and extended by Sequenase DNA polymerase using OC DNAs as template. Lane 1, pSU4817; lane 2, pSU4818; lane 3, pSU4823; lane 4, pSU4826; lane 5, pSU4828; C+ and C−, positive and negative controls used pSU4628 OC and pSU4841 SC DNA as templates respectively. On the left, the sequence around nic is depicted. The long vertical arrow indicates the direction of deoxynucleotide incorporation and finishes in the last deoxynucleotide incorporated. The two small vertical arrows represent the extra nucleotides added by Sequenase and ThermoSequenase (Llosa et al., 1995). The template strand shows the proposed site of interruption indicated as a line between nucleotides G and G.

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stopping at nic (lane C+ in Fig. 4). No product was obtained when using the SC DNA band (lane C– in Fig. 4). Besides, extension of an oligonucleotide hybridizing to the complementary DNA strand did not show any interruption in this region (not shown). The exact nucleotide at which the strand interruption occurred in the template DNA could be determined by inspection of a sequencing ladder obtained with the same oligonucleotide. Sequenase was shown previously (Llosa et al., 1995) to display terminal transferase activity, adding an extra nucleotide once it reaches the end of the template DNA. Thus, the exact site of interruption had to be corrected by one nucleotide, suggesting that the interruption in the template was at 5′-GGGTG/GTCGGG-3′.

As a consequence, it is not so surprising that the pSU4814 derivative pSU4822, formerly proposed to contain a functional oriT, was not mobilized, as it does not include the nic site.

**CloDF13 nic site ends are not blocked**

To verify the integrity of nic site ends, we end-labelled 5′ ends of a 627 bp DNA fragment obtained after digestion of pSU4814 DNA with Hpal and PvuII. The experiment was carried out with DNA samples obtained from either the OC or the SC bands of a CsCl/EtdBr density gradient. The products were separated by electrophoresis in a denaturing polyacrylamide gel. The technique would allow us to determine whether the 5′ end-labelling of nic was or was not blocked as a result of a covalent linkage to the relaxase (Fig. 5A). The results are shown in Fig. 5B. 5′ End-labelling of the SC DNA band resulted in a unique labelled fragment of 630 bp. 5′ End-labelling of the OC DNA band resulted in the expected 630 bp and 420 bp bands, as well as an additional band of 200 bp, indicating that the 5′ end of nic was accessible to phosphorylation.

Further confirmation of the presence of free 5′-terminal phosphate in nic, in addition to a 3′-OH end, was obtained using pSU4814 OC DNA as a substrate and T4 DNA ligase. We incubated pSU4814-nicked DNA with T4 DNA ligase and analysed the products by electrophoresis on a non-denaturing agarose gel (Fig. 5C). The results indicate that most of the OC DNA is converted to circular covalently relaxed DNA (CCC). All these data taken together allowed us to conclude that relaxation of CloDF13 results in a nic site with both ends free.

**MobC is absolutely required for oriT relaxation**

We wanted to know which of the inferred ORFs within Mob was involved in CloDF13 relaxation. First, we deleted the internal EcoRI fragments from Tn5tat1 in the five insertions that exhibited a Mob− phenotype, so that the resulting mutants contained just a 72 bp insertion of known sequence (Fig. 2). Inspection of these sequences could predict polar effects on genes distal from each of the insertion sites (Llosa et al., 1991). For instance, the 72 bp insertion in pSU4817 within orfA1 did not introduce translation stop codons, whereas the insertions in mobB (pSU4818 and pSU4828) did. From the insertions in

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Table 1. Complementation analysis of *mobB* and *mobC*.

<table>
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<tr>
<th>Mobilizable plasmid</th>
<th>Mutant genes</th>
<th>Complementing plasmid</th>
<th>Complementing gene products</th>
<th>Mobilization frequency</th>
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<td>–</td>
<td>–</td>
<td>MobB</td>
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<tr>
<td>pSU4814</td>
<td>–</td>
<td>–</td>
<td>MobB</td>
<td>$6 \times 10^{-3}$</td>
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<td><em>mobB</em></td>
<td>–</td>
<td>MobB</td>
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<tr>
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<td><em>mobB</em></td>
<td>–</td>
<td>MobB</td>
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<td><em>mobB</em></td>
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<td>MobB</td>
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<td><em>mobB</em></td>
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<td>pSU4811</td>
<td>Old MobB(^b)</td>
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<td>MobC</td>
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<td>$4 \times 10^{-3}$</td>
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\(\text{a. Derivatives of DH}5\alpha\text{ carrying pSU1456 and the mobilizable and complementing plasmids indicated were mated with UB1637 for 1 h at 37°C on solid medium as described in Experimental procedures. Transfer frequency of the helper plasmids was not detected (< 1 \times 10^{-5}). Transfer frequencies are expressed as the number of transconjugants per donor cell and are the mean of at least three experiments.}\)

\(\text{b. Old mobB; formerly proposed amino acid sequence of MobB (van Putten et al., 1987) (Fig. 2).}\)

*mobC*, the insertion in pSU4823 generated a translation stop, whereas the insertion in pSU4826 did not. In order to study the *nic* cleavage activities of the constructed Tn\(^{5}\)ac11EcoRI derivatives, cleared lysates from plasmids pSU4817, pSU4818, pSU4823, pSU4826 and pSU4828 (and pSU4628 as a positive control) were ultracentrifuged in CsCl/EtdBr density gradients. Bands corresponding to the OC forms were collected and used as templates in extension reactions with primer CloDF13 #11 to detect whether there was an interruption in the *nic* site. Figure 4 shows that pSU4817, pSU4818 and pSU4828 displayed a *nic*\(^-\) phenotype (lanes 1, 2 and 5 respectively), whereas pSU4823 and pSU4826 were *nic*\(^+\) (lanes 3 and 4). The two new plasmids were *mobC* mutants, suggesting that MobC was absolutely required for plasmid relaxation. The faint bands observed in pSU4818 and pSU4828 (*mobB* insertions; lanes 2 and 5) could result from a polar effect of their corresponding 72 bp insertion on *mobC*. To evaluate this possibility, mobilization of several Mob\(^-\) mutants was analysed in the presence of different cloned CloDF13 DNA segments and a helper plasmid providing the W pilus system. Results are shown in Table 1. *mobC* mutant plasmids pSU4823 and pSU4826 recovered wild-type mobilization frequencies when complemented by pSU4835, which expresses MobC (Table 1 and Fig. 2). The *nic*\(^-\) phenotype was also recovered, as inferred from positive primer extension of oligonucleotide CloDF13 #11 using OC forms isolated as described in Experimental procedures (not shown). These data indicate that the presence of MobC is required for the production of relaxed forms from CloDF13 DNA, in turn pointing to MobC as the CloDF13 relaxase. *mobB* mutant plasmids pSU4818 and pSU4828 recovered the parental mobilization frequency only partially when complemented with pSU4839 (containing *mobB*; Table 1 and Fig. 2). Only when pSU4835 (providing MobC) was also present did they display a wild-type mobilization frequency. In contrast, wild-type mobilization frequency was restored in pSU4833 (in phase deletion within *mob*; Fig. 2) by complementing with pSU4834 (containing *mobB*). The results derived from the complementation analysis again point to the existence of a polar effect of the *mobB* insertions in pSU4818 and pSU4828 (but not of that in pSU4833) on *mobC*.

*MobB* participates in CloDF13 relaxation

According to the results shown in Table 1, the reason for the faint *nic*\(^+\) phenotype exhibited by pSU4818 and pSU4828 (*mobB* insertion mutants) could be the lack of a sufficient expression level of *mobC*. This possibility was checked by examining the *nic* phenotypes of these plasmids in the presence of pSU4835 (which expresses MobC) using the cleared lysate and primer extension method. Unexpectedly, the *nic* phenotypes of both insertion mutants remained as faint as they were without the addition of MobC (data not shown). This result suggested that MobB plays a more direct role in the relaxation event. Besides, MobB should play an additional role in mobilization, as pSU4818 and pSU4828, containing insertional mutations in *mobB*, produced relaxed forms (Fig. 4, lanes 2 and 5) but were completely Mob\(^-\)
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Phenotype</th>
<th>Description</th>
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<td>4.9 kb EcoRI–BamHI fragment from pSU4788 (Fig. 1) cloned at the</td>
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<td>12.4</td>
<td>This work (Fig. 2)</td>
</tr>
<tr>
<td>pSU4819</td>
<td>Cm Rep(p15A) orfA1+</td>
<td>0.4 kb fragment of pSU4814 cloned into pSU19 using PCR amplificationb</td>
<td>2.7</td>
<td>This work (Fig. 2)</td>
</tr>
<tr>
<td>pSU4822</td>
<td>Cm Rep(p15A)</td>
<td>0.14 kb Nael fragment deleted from pSU4819</td>
<td>2.6</td>
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</tr>
<tr>
<td>pSU4824</td>
<td>Ap Rep(CloDF13)</td>
<td>4.6 kb EcoRI fragment internal to Tn9ac1 deleted from pSU4790 (Fig. 1)</td>
<td>12.4</td>
<td>This work (Fig. 2)</td>
</tr>
<tr>
<td>pSU4825</td>
<td>Ap Rep(CloDF13)</td>
<td>0.7 kb fragment cloned into PCR-Script SK+ using PCR² amplification</td>
<td>3.6</td>
<td>This work (Fig. 2)</td>
</tr>
<tr>
<td>pSU4826</td>
<td>Ap Rep(CloDF13)</td>
<td>4.6 kb EcoRI fragment internal to Tn9ac1 deleted from pSU4791 (Fig. 1)</td>
<td>12.4</td>
<td>This work (Fig. 2)</td>
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<tr>
<td>pSU4827</td>
<td>Ap Rep(p15A)</td>
<td>0.1 kb Hpal–Sal fragment deleted from pSU4815</td>
<td>3.1</td>
<td>This work (Fig. 2)</td>
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<tr>
<td>pSU4830</td>
<td>Ap Rep(pMB1) MobC+</td>
<td>pET3a::mobC²</td>
<td>5.3</td>
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</tr>
<tr>
<td>pSU4831</td>
<td>Ap Rep(pMB1)</td>
<td>0.6 kb fragment (corresponding to 5′ region of mobB) cloned into pET3a by PCR amplificationb</td>
<td>5.2</td>
<td>This work</td>
</tr>
<tr>
<td>pSU4832</td>
<td>Cm Rep(p15A) Mob(CloDF13) Deletion of the XbaI–HindIII sites at the polylinker of pSU4814 in order to eliminate the Psl site between them</td>
<td>5.9</td>
<td>This work</td>
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<tr>
<td>pSU4833</td>
<td>Cm Rep(p15A) MobB–</td>
<td>0.8 kb Psl fragment deleted from pSU4832</td>
<td>5.1</td>
<td>This work (Fig. 2)</td>
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<tr>
<td>pSU4834</td>
<td>Cm Rep(p15A) MobB+</td>
<td>1.7 kb KpnI fragment of pSU4814 (containing the 3′ region of mobB) cloned into the KpnI site of pSU4831</td>
<td>7.0</td>
<td>This work (Fig. 2)</td>
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<tr>
<td>pSU4835</td>
<td>Cm Rep(p15A) MobC+</td>
<td>0.7 kb BgIII–BamHI fragment from pSU4830 cloned in the BamHI site of pSU19</td>
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<td>pSU4839</td>
<td>Km Rep(pMB1) MobB+</td>
<td>2.3 kb Ndel–BamHI fragment from pSU4834 cloned into the Ndel–BamHI sites of pET29c</td>
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<td>This work</td>
</tr>
<tr>
<td>pSU4841</td>
<td>Ap Rep(CloDF13)</td>
<td>2.1 kb Smal fragment deleted from pSU4628</td>
<td>10.2</td>
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</table>

a. A 1640 bp fragment from pSU4628 was amplified by PCR using oligonucleotides NdelMobB, TCTGGCCATATGGCTTTTGTTG (co-ordinates 816–841 bp in EMBL sequence AJ224861) and FinMobB, CAGCCGCCGCCGCCGCATCA (co-ordinates 2473–2455 bp in EMBL sequence AJ224861). The fragment was digested with BamHI and Ndel and ligated into the BamHI–Ndel sites of pET3a.b. A 497 bp fragment of pSU4814 was amplified by PCR using primers M13 reverse (−48) and 4814orIT, TCTAGATTCGCAGATGAG (co-ordinates 414–394 bp in EMBL sequence AJ224861). The fragment was digested with EcoRI and HindIII and ligated to the EcoRI–HindIII sites of pSU19.c. A 748 bp fragment from pSU4814 was amplified by PCR using primers M13 reverse (−48) and 8citoDFN, TCTGCTCTTTCGACGAGC (co-ordinates 663–645 bp in EMBL sequence AJ224861). The fragment was ligated to the SfiI site of PCR-Script SK+ in the same orientation regarding the vector oriV as in pSU4814.d. A 755 bp fragment from pSU4814 was amplified by PCR using oligonucleotides NdelORF4, CGTACATGCGCTGAGAC (co-ordinates 2621–2641 bp in EMBL sequence AJ224861) and BamHlORF4, CAGCCGCCAGATCCTACT (co-ordinates 3376–3355 in EMBL sequence AJ224861). The fragment was digested with BamHI and Ndel and ligated into the BamHI–Ndel sites of pET3a.e. A 676 bp fragment of pSU4814 was amplified by PCR using oligonucleotides NewNdelMobB, AGAGAGTACATGTTTATTAGG (co-ordinates 657–680 bp in Fig. 3) and BamHIMobB, ATCGCGCCACGCCTGCCGCTCCCGG (co-ordinates 1333–1360 bp in EMBL sequence AJ224861). The fragment was digested with BamHI and Ndel and ligated into the BamHI–Ndel sites of pET29a.
or pSU4841 + pSU4835 + pSU4839. Figure 6A shows that the upper band of the gradients was significantly more intense when mobB and mobC genes were present in addition to oriT (lane 4). To confirm that the increase in intensity was caused by specific relaxation of pSU4841, the upper bands were collected and analysed. Primer extension of oligonucleotide CloDF13 #11, using these DNAs as templates, showed one interruption at the CloDF13 nic site for the lane corresponding to the upper band of pSU4841 + pSU4835 + pSU4839 (Fig. 6B, lane 4). From these results, we infer that MobB and MobC together, but neither MobC nor MobB on their own, are able to induce the relaxation of CloDF13 oriT. Besides, no intact orf6, orf7 or orf8 was contained within the complementing plasmids, so we can rule out a significant contribution from those orfs to CloDF13 mobilization. It should be pointed out that the oriT-containing plasmid pSU4841 includes the DNA sequences corresponding to orfA1 and orfA2 and, in consequence, participation of these putative products in CloDF13 relaxation cannot be excluded.

Discussion

We have characterized the 3623 bp DNA segment that contains the CloDF13 Mob region. The main genetic elements on this region are oriT, mobB and mobC. The smallest CloDF13 DNA region that possesses oriT activity is contained within a 661 bp fragment located at one edge of MOB. The oriT displays nine inverted repeats of variable length containing the sequence GCTG (see supplementary data on the journal website http://www.blackwell-science.com/mmi). This structural feature could have implications in the specific interactions with CloDF13 Mob proteins. Snijders et al. (1983) proposed that oriT was located in a 264 bp fragment in the vicinity of the unique HpaI site. This fragment does not confer mobilization ability (Fig. 2). Conjugal and mobilizable plasmids have their transfer regions organized in such a way that oriT maps asymmetrically with regard to the transfer genes, such that the tra genes enter the recipient last with the 5’ end leading. This set-up is maintained in CloDF13 candidate Mob genes: orfA1, orfA2, mobB and mobC. Moreover, mobB and mobC, in contrast to the sequences proposed formerly, showed translational coupling, suggesting that they form part of an operon. The potential orf5 starts immediately after mobC (Fig. 1) but does not seem to play a significant role in CloDF13 mobilization, as it is truncated in plasmid pSU4814, which is mobilized at wild-type frequency.

The amino acid sequence deduced from mobB predicts a protein of 653 amino acids. A protein of 64 kDa was observed by PAGE after overexpression of the cloned mobB (B. Núñez and F. de la Cruz, unpublished results).
Our data obtained from overexpression of MobB and MobC are in agreement with previously reported minicell results (Konings et al., 1976). Insertions within mobB were strongly polar on mobC, supporting the notion that both genes form part of an operon. Consequently, the nic phenotype of mobB insertions could have been affected by altered mobC expression levels. However, a wild-type nic phenotype was not recovered when MobC was provided in trans, suggesting that MobB, like MobC, participates in oriT-specific nicking. We have shown that MobB and MobC together are required to relax CloDF13 oriT (Fig. 6). According to the data from Flashner et al. (1996), it was deduced that, in addition to TaxC, TaxA was also required for the plasmid R6K in vivo nicking reaction (Núñez et al., 1997). Similarly, Tral and TraJ of RP4 fulfilled the requirements for nicking specificity in vitro (Pansegrau et al., 1990). Therefore, MobB could be considered as an accessory protein for MobC nicking activity and is probably part of the CloDF13 relaxosome.

The proposed MobB amino acid sequence contains three potential transmembrane segments, as predicted by the algorithm of Klein et al. (1985), and has the consensus NTP-binding Walker boxes A and B. These characteristics are common to TraG-like proteins, suggesting that MobB could act as a coupling protein, connecting the relaxosome to the membrane-based transport apparatus (Cabezon et al., 1997). Supporting this hypothesis was the observation that CloDF13 mobilization was independent of the TraG-like protein when mobilized by several conjugative systems, in contrast to other mobilizable plasmids (Cabezon et al., 1997). Altogether, these results suggest a dual participation of MobB in CloDF13 mobilization, both as a coupling protein and as a nicking accessory protein. Interestingly, MobB is only very distantly related to other relaxosome coupling proteins of the TraG family. In fact, objective search parameters applied to protein sequence alignment algorithms failed to pair MobB with the otherwise well defined TraG family (F. de la Cruz, unpublished results). Therefore, a word of caution is warranted before assignment of MobB to the TraG family.

Analysis of the nic phenotype of several Mob-deficient CloDF13 insertion mutants allowed us to identify the mobC gene as absolutely required for relaxation, suggesting that MobC is the CloDF13-specific relaxase. The mobC sequence predicts a basic protein of 243 amino acids. A protein with an electrophoretic mobility corresponding to a protein of 30 kDa was produced when mobC was cloned and overexpressed (B. Núñez and F. de la Cruz, unpublished results). Database searches did not reveal any significant similarity of MobC to other protein sequences. MobC has a tyrosine in the amino-terminal region (Tyr-5) that could act as a nucleophile in the cleaving–joining reaction. However, it lacks the three histidine motifs present in most conjugative relaxases (de la Cruz and Lanka, 1998). This motif is apparently essential for relaxase activity and could activate the aromatic hydroxyl group of the catalytic Tyr, as proposed for Tral protein (Pansegrau et al., 1994). Thus, how MobC exerts its function is presently unknown. In addition, MobC was not able to relax CloDF13 DNA in vivo in the absence of MobB.

van Putten et al. (1987) reported that relaxation complexes could not be isolated for CloDF13. In particular, treatment of CloDF13 cleared lysates obtained by gentle lysis with Triton-X-100 with denaturing agents such as SDS or with pronase did not induce relaxation of SC DNA (Veltkamp et al., 1975). However, such complexes were isolated from plasmid ColE1, which shares a similar gene organization and replication properties with CloDF13 (Lovett et al., 1974; Veltkamp and Stuitje, 1981). When we isolated CloDF13 DNA by the cleared lysate method, we observed that most of the plasmid DNA was already present in a relaxed form (Fig. 3). Relaxed molecules contained a strand-specific nick in oriT. Based on this, we assume that this is the CloDF13 nic site.

Results presented in this work show that the 5′ end of the CloDF13 nic site is not blocked to the action of polynucleotide kinase and, furthermore, both ends can be resealed by DNA ligase, indicating that protein is not covalently bound to the nicked CloDF13 DNA. This result suggests that phosphodiester bond transesterification at nic can proceed in the absence of a covalent DNA intermediate. Alternatively, this covalent intermediate could be relatively unstable and be lost during sample preparation. In all conjugative systems analysed in biochemical detail, transesterification by relaxases involves two steps and the generation of a covalent bond between a catalytic tyrosyl residue and the generated 5′ end. The protein–DNA intermediate has a long life and, thus, can be isolated (for a review, see Byrd and Matson, 1997). Although this is also the case for most rolling circle replication (RCR) phages and plasmids, filamentous phages, such as M13 and RC-replicating plasmids of the pMV158 family, are significant exceptions. In these cases, the complex is very short lived and could be inferred when using suitable substrates and short reaction times (Moscoso et al., 1997; Asano et al., 1999). This could also be the case for CloDF13.

A second mechanism for transesterification of phosphodiester bonds implies a one-step transesterification in which covalent DNA–protein intermediates are not required. This mechanism has been reported for Mu transposition and the integration of HIV-1 DNA (Mizuuchi, 1992). Studies on the stereochemistry of the phosphate group involved in the strand transfer reaction demonstrated that the chirality is inverted in these cases, as
expected for a one-step transesterification mechanism (Engelman et al., 1991; Mizuuchi and Adzumak, 1991). Further analysis of the chirality of the phosphate involved in the CloDF13 relaxase-catalysed reaction should shed light on how the nicking process occurs. To our knowledge, this is the first report of a free (non-protein bound) nicked-DNA molecule as an intermediate in a conjugation or mobilization system.

**Experimental procedures**

**Bacterial strains, phages, plasmids and bacterial growth conditions**

*E. coli* strains used were DH5α (Grant et al., 1990) and UB1637 (de la Cruz and Grinsted, 1982). Plasmid DNA was usually transformed to and stored in strain DH5α. For conjugation experiments, the *recA* strains DH5α and UB1637 were used as donor and recipient strains respectively. Mutagenesis was carried out in strain UB1637 using phage λTn5·tac1 (Chow and Berg, 1988).

Plasmids are listed in Table 2. Luria–Bertani (LB) broth (Sambrook et al., 1989) was used for liquid bacterial growth. LB agar was used for growth on plates. Plasmids were selected for their antibiotic resistance using the following concentrations: ampicillin (Ap), 100 mg ml⁻¹; chloramphenicol (Cm), 25 mg ml⁻¹; kanamycin (Km), 25 mg ml⁻¹; nalidixic acid (Nx), 25 mg ml⁻¹; streptomycin (Sm), 300 mg ml⁻¹; trimethoprim (Tp), 20 mg ml⁻¹.

**Genetic experiments**

Bacterial transformations were performed according to the method of Chung and Miller (1988). When a high frequency was required, highly efficient competent cells were prepared (Hanahan, 1983) and transformed by electroporation (Dower et al., 1988). Conjugation experiments were carried out by the plate-mating procedure as described previously (Llosa et al., 1991) using 100 μl of both donor and recipient cultures in stationary phase. Transfer frequencies are expressed as the number of transconjugants per donor cell. The plasmid content of transconjugants was verified electrophoretically when appropriate. *In vivo* insertional mutagenesis with transposon Tn5·tac1 was carried out as described by de Bruijn and Lupski (1984). Strain UB1637 harbouring the plasmid to be mutagenized was infected with phage λ b221 cl857 Oam29 Pam80·Tn5·tac1 (Chow and Berg, 1988).

**Plasmid methodology**

Plasmid DNA was purified at different scales using appropriate techniques (Martinez and de la Cruz, 1988; Sambrook et al., 1989). When required, DNA fragments were purified with silica gel as described by Boyle and Lew (1995). Molecular cloning was performed using T4 DNA ligase (New England Biolabs), according to the method of Sambrook et al. (1989). Polymerase chain reaction (PCR) experiments used BIO-X-ACT low-error-frequency DNA polymerase (Bioline).

Snake venom phosphodiesterase was from Boehringer Mannheim.

**Purification of in vivo nicked plasmids**

Cleared lysates of CloDF13 derivatives were obtained from 400 ml cultures of DH5α containing the appropriate plasmid, as described by Avila et al. (1996). CsCl/EtdBr gradients and extraction of OC DNA forms were performed as described previously (Avila et al., 1996). Photographs of gradients were taken using a Bio-Rad Fluor Analyser system.

**DNA sequencing and analysis**

The 3623 bp CloDF13 segment contained in pSU4814 was sequenced using Amersham ThermoSequenase cycle sequencing kit and 7% Long Ranger (FMC)—6.1 M urea gels in TBE buffer in an automatic Vistra sequencer (Amersham). Data were collected and analysed by the Vistra DNA sequencer 725 version 2.0 software. Oligonucleotide primers (high-performance liquid chromatography degree; Boehringer Mannheim) were labelled with Texas Red using an iodoacetyl Texas Red 5'-labelling kit (Amersham).

The BLAST algorithm (Altschul et al., 1990) was used for protein database searches. These sequence data have been submitted to the EMBL database under accession number AJ224861.

**Mapping the 5’ end of nic by primer extension**

Oligonucleotide primer CloDF13 #8 spans co-ordinates 663–645 of sequence AJ224861 and corresponds to the nicked strand, whereas primer CloDF13 #11 spans co-ordinates 233–251 and corresponds to the unnicked strand. Template OC DNA was obtained from CsCl/EtdBr gradients of pSU4814 cleared lysates. SC DNA was used as a control. Primer extension reactions were carried out using either Sequenase version 2.0 (USB) or ThermoSequenase as DNA polymerases. Sequenase extension reactions (15 min at 37°C) contained 1 μg of OC DNA, 2 pmol of Texas Red-labelled primer and Sequenase (13 units) in 10 μl of commercial buffer. ThermoSequenase extension reactions contained 1 μg of OC DNA, 0.5 pmol of Texas Red-labelled primer and ThermoSequenase (2 units). Reaction mixtures were subjected to cycle extension (first, 5 min at 95°C, then 20 cycles of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C). Both types of reaction products were analysed by electrophoresis in a Vistra DNA sequencer.

**5’ end labelling of pSU4814 DNA fragments**

Samples (3 μg) of either pSU4814 OC or SC DNA were digested with enzymes Hpal and PvuII. In each case, the resulting 627 bp fragment was isolated from an agarose gel, heat denatured for 5 min at 95°C, chilled on ice and treated with alkaline phosphatase as specified by the supplier (Boehringer Mannheim). DNA was recovered with silica gel, denatured again and incubated for 30 min at 37°C with 1 unit of T4 polynucleotide kinase (BioLabs) and [γ-32P]-ATP.

(30 μCi) in a final volume of 20 μl. Reactions were stopped by adding 6 μl of USB loading buffer and boiled for 5 min. Aliquots (5 μl) were loaded in a 7% polyacrylamide gel containing 6 M urea and electrophoresed for 30 min at 300 V. The gel was scanned in a GS-505 Molecular Imager (Bio-Rad).

Nic ligation assays

pSU4814 OC DNA (0.5 μg) was incubated with 200 units of T4 DNA ligase (Biolabs) in a 20 μl volume for 8 h at 16°C. A control reaction containing DNA and enzyme buffer but no enzyme was incubated under the same conditions. Reaction products were electrophoresed in 1% agarose gel in Tris borate—EDTA buffer containing EtBr (0.5 μg ml⁻¹).

Phosphodiesterase assays

These were carried out as described by Ávila et al. (1996).

Acknowledgements

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References


