Genetic and biochemical characterization of MbeA, the relaxase involved in plasmid ColE1 conjugative mobilization

Athanasia Varsaki,1,2 María Lucas,2 Amalia S. Afendra,1 Constantin Drainas1 and Fernando de la Cruz*1

1 Sector of Organic Chemistry and Biochemistry, Department of Chemistry, University of Ioannina, 451 10 Ioannina, Greece.
2 Departamento de Biología Molecular (Unidad asociada al CIIB, CSIC), Universidad de Cantabria, C/Herrera Oria s/n, 39011 Santander, Spain.

Summary

MbeA is a 60 kDa protein encoded by plasmid ColE1. It plays a key role in conjugative mobilization. MbeA*, a slightly truncated version of MbeA, was purified for in vitro analysis. MbeA* catalysed DNA cleavage and strand-transfer reactions using oligonucleotides embracing the ColE1 nic site, which was mapped to 5’-(1469)CTGG/CTTA(1462)-3’. Thus MbeA is the relaxase for ColE1 conjugal mobilization, in spite of the fact that it lacks a three histidine motif considered the invariant signature of conjugative relaxases. Amino acid sequence comparisons suggest MbeA is nevertheless related to the common relaxase protein family. For instance, MbeA residue Y19 could correspond to the invariant tyrosine in Motif I, whereas H97, E104 and N106 may constitute the equivalent residues to the histidine triad in Motif III. This hypothesis was tested by site-directed mutagenesis. MbeA amino acid residues Y19, H97, E104 and N106 were changed to alanine. MbeA mutant N106A showed reduced oligonucleotide cleavage and strand-transfer activities, whereas mutation in the other three residues resulted in proteins without detectable activity, suggesting they are directly implicated in catalysis of DNA-cleavage and strand-transfer reactions. A double substitution of E104 and N106 by histidines, therefore reconstituting the canonical histidine triad, restored relaxase activities to 1% of wild type. Thus, MbeA is a variant of the common relaxase theme with a HEN signature motif, which has to be added to the canonical three histidine motif of previously reported relaxases.

Introduction

Bacterial conjugation (reviewed in Zechner et al., 2000) is a specialized gene transfer process that involves unidirectional transfer of DNA from donor to recipient bacteria by a mechanism that requires cell-to-cell contact. Plasmids spread both within and between bacterial species primarily through conjugal transfer. Plasmids are classified according to their conjugative ability as conjugative (auto-transmissible), mobilizable (transmissible only when in the presence of a helper conjugative plasmid) or non-mobilizable. Conjugative plasmids contain the necessary genetic information to catalyse conjugal DNA processing and DNA transport. Mobilizable plasmids lack part of this machinery. Typically they contain an oriT, the only DNA sequence required in cis for conjugation, and a set of proteins to process oriT and make it available to the transport complex.

The colicinogenic factor ColE1 (6.65 kb) is a mobilizable plasmid. It is well-known because of its massive use for the construction of bacterial cloning vectors. It is mobilized at widely different frequencies by an ample set of conjugative plasmids, including IncIa, IncFI, IncW and IncP incompatibility groups (Finnegan and Sherratt, 1982; Willetts and Wilkins, 1984; Cabezón et al., 1997). ColE1 occurs in vivo as ‘relaxation complex’, a specific association of proteins with oriT (Clewell and Helinski, 1969) that produces a relaxed (open circular) form separable from its covalently closed circular topoisomer. The ColE1 relaxation complex was some time ago reported to contain three proteins, which were only identified for their molecular masses: 60, 16 and 11 kDa (Lovett and Helinski, 1975). They were later supposed to be the products of genes mbeA, mbeB and mbeC, respectively, although this assignment was not rigorously proven (Boyd et al., 1989).

According to genetic studies, ColE1 mobilization required oriT (Bastia, 1978), the mob region (Boyd et al., 1989) and the tra-gene products of a conjugative plasmid...
(Willets and Wilkins, 1984). Four mob genes (mbeA, mbeB, mbeC and mbeD) were essential for mobilization (Broyd et al., 1997). Surprisingly for us, none of the four encoded proteins contained the relaxase signature, assigned as invariant in conjugative relaxases (Zechner et al., 2000). The term ‘relaxase’ is used to define proteins involved in initiation and termination of DNA conjugative transfer (Byrd and Matson, 1997). Relaxases recognize a specific oriT sequence called nic and cleave it in a strand-and-site-specific fashion. The product of the reaction is a protein–DNA complex in which the relaxase binds covalently to the 5’-end of nic in the DNA strand that is transferred to the recipient cell. An interesting aspect of relaxase-catalysed transesterification reactions is that, despite the loss of the covalent bond between the 3’-OH and the 5’-PO4 of the cleaved sugar-phosphate backbone, the 3’-OH remains sequestered in the complex and there is no loss of superhelicity unless protein-denaturing agents are present. Traf, the P-family relaxase of plasmid RP4, is one of the best-analysed relaxases in biochemical terms (Pansegrau and Lanka, 1996). Another well-described relaxase is the F-family relaxase TrwC, from the IncW plasmid R388 (Grandoso et al., 2000). Most relaxases show a significant degree of sequence similarity as well as three conserved amino acid sequence motifs (de la Cruz and Lanka, 1998; Zechner et al., 2000). Motif I contains one or two tyrosines, which are considered the catalytic sites of the relaxase (Pansegrau et al., 1993; 1994; Grandoso et al., 2000). Motif II contains a serine implicated in the interaction of the relaxase with the 3’-end of the nic DNA (Pansegrau et al., 1994). Motif III contains a histidine triad and has been used as a relaxase diagnostic signature (Pansegrau et al., 1994; Zechner et al., 2000).

Eye inspection of a CLUSTAL multiple alignment of MbeA-like proteins based on an objective search, suggested putative homologues of Motifs I and III, as shown in Fig. 1. Noteworthy, only one histidine (H97) exists in the putative Motif III, where glutamate E104 and asparagine N106 substitute for the pair of invariant histidines in the 3H motif. In order to ascertain if our hypothesis was correct we mutated the amino acids mentioned above by site-directed mutagenesis and we checked the effect of the mutations on MbeA activity. Our results confirm that MbeA is CoIE1 relaxase, support the involvement of four essential amino acids in relaxase function and provide evidence for the existence of a new variant within the relaxase family bearing a HEN diagnostic signature.

**Motif I**

**Motif II**

**Motif III**

Fig. 1. Sequence alignment of MbeA and related proteins. The figure shows a CLUSTALW alignment of the 150 N-terminal amino acids of each protein, and points out the location of the three motifs conserved in conjugative relaxases. The four MbeA residues that have been mutated in this work are shown by small vertical arrows. Colour codes: white on black background = invariant residues; black on dark grey = residues identical in at least four proteins; black on medium grey = strongly conserved residues; black on pale grey = weakly conserved residues; black on white = non-conserved residues. The protein sequences shown, and their Accession Numbers are: MbeA of plasmid CoIE1 (JQ0390), MobA of ColA (S04790), MobB of pHE1 (Q9K591), MobB of pLO510 (AA61811), Rel of pC221 (QQSAA2), Rel of pSK639 (AAC18947), VirD2 of the Ti plasmid (B29826) and Traf of RP4 (S87583).
Results

Does ColE1-encoded protein MbeA belong to the common conjugal relaxase protein family?

Most plasmid relaxases belong to a protein family characterized by a diagnostic signature of three invariant histidines, the so-called 3H motif. MbeA protein was assumed to be the ColE1 relaxase because a protein with an approximate similar mass remained specifically associated with the nicked strand of the open circular DNA after SDS treatment (Lovett and Helinski, 1975). The fact that none of the ColE1 Mob proteins showed the 3H motif made the provisional assignment of MbeA as ColE1 relaxase somewhat doubtful. Nevertheless, a BLAST search for similar sequences using MbeA amino acid sequence retrieved homologues containing the 3H motif, so that MbeA could be unequivocally aligned to the IncP-family relaxases, as shown in Fig. 1. There is some apparent conservation in the three relaxase motifs. The equivalence is suggested between Y19 of MbeA and Y22 of RP4 Tral, the residue known to be involved in catalysis of the DNA-strand transfer reactions (Pansegrau and Lanka, 1996). Furthermore, motif III in MbeA-like proteins could be aligned to the IncP-like relaxase family as a result of conservation of several residues, especially with respect to the relaxases of the Gram-positive plasmids pC221 and pSK639. However, as can be observed in Fig. 1, RP4_Tral residues H116 and H118, which are known to be essential for relaxase activity (Pansegrau et al., 1994), are not conserved in MbeA-like proteins. Instead, E104 and N106 occupy the equivalent positions. This result prompted us to verify that MbeA is indeed a relaxase protein and if so, that E104 and N106 play the same role as the invariant histidines of motif III in RP4_Tral.

Mapping the 5’-end of ColE1 nic site

The ColE1 nic site position was originally determined by Bastia (1978), although a subsequent review casted some doubt on its precise location (Willett and Wilkins, 1984). To clarify this essential issue, we mapped ColE1 nic site 5’-end by primer extension analysis, using plasmids pUIV208 and pUIV209 as templates and oligonucleotide #plus as a primer. pUIV208 carries the complete ColE1 DNA cloned at the SalI site of pUC19, whereas pUIV209 derives from pUIV208 by insertion of a 489 bp at the CiaI site thus inactivating mbeA. A single polynucleotide chain resulted when the MbeA+ plasmid pUIV208 DNA was used in the extension reaction (Fig. 2A, track 2), whereas no interruption occurred when the MbeA- plasmid pUIV209 DNA was used (Fig. 2A, track 3). In order to find out the exact nucleotide at which strand interruption occurred, sequencing reactions were carried out on the nicked pUIV208 DNA template using oligonucleotide #plus as a primer. Results are shown in Fig. 2A, track 1. The exact site of interruption is at the first N in the figure, which corresponds to 5’-(1469)CTGG/CTTA(1462)-3’ in the cleaved strand (Fig. 2B). The second band in all sequencing lanes corresponds to a nucleotide added by the terminal transferase activity of Thermo Sequenase (Llosa et al., 1995; Núñez and de la Cruz, 2001), and coincides with the primer extension termination product (lane 2). The precise assignation of the nic site was later confirmed by an in vitro cleavage reaction (see Fig. 4). Thus, the unique interruption in the template was exactly at the site reported by Bastia (1978). Oligonucleotides can thus be unequivocally designed that contain ColE1 nic site.

MbeA+ purification and in vitro activities

Escherichia coli strain BL21(DE3) containing plasmid pUIV205 was used to overproduce a truncated but yet functional MbeA protein (MbeA*, Fig. 3), as full length MbeA could not be overproduced, and MbeA* was later shown to be a fully functional relaxase both in vivo and in vitro. MbeA* was purified by a three-step procedure, using two ion exchange steps (phosphocellulose P11 and Mono-S HR 5/5 columns) and a final gel filtration chroma-
tography (Superdex 75 HR 10/30 column). In gel filtration, MbeA* behaved as a monomer of approximately 60 kDa (data not shown). oriT-specific cleavage and strand-transfer activities were analysed by gel electrophoresis using purified MbeA* protein and oligonucleotide #ColE1-nic [32P]-labelled at its 5'-end (Fig. 4). MbeA* cleaves the labelled 23-mer oligonucleotide #ColE1-nic producing a 14-mer labelled product (Fig. 4, track 3 versus 2). Similarly, MbeA* cleaves the related 29-mer oligonucleotide #ColE1-str giving a 17-mer labelled product with similar efficiency (not shown). If the #ColE1-nic cleavage reaction is repeated but adding an excess of unlabelled oligonucleotide #ColE1-str giving a 17-mer labelled product with similar efficiency (not shown). If the #ColE1-nic cleavage reaction is repeated but adding an excess of unlabelled oligonucleotide #ColE1-str, a new 26-mer labelled oligonucleotide is produced by DNA-strand transfer reaction (Fig. 4, track 5 versus 4). Analogous products are obtained by the in vitro activities of other relaxases (Pansegrau et al., 1993; Llosa et al., 1996). Cleavage products are not produced when labelled oligonucleotide #R388–33nic, that contains R388 nic site, is included instead of #ColE1-nic (Fig. 4, tracks 7 versus 6). This result shows that the cleavage reaction is sequence-specific. Concentration-dependence curves showed that reactions saturated at 1 μM MbeA*, both for cleavage and strand-transfer at the conditions used (Fig. 5A and B). The cleavage reaction reached equilibrium rapidly (in less than 10 min) when 70% of the initial oligonucleotide was cleaved (Fig. 5C). Maximal cleavage occurred at a temperature of 45°C (Fig. 2D); nevertheless, all reactions were performed at 37°C, the optimal growth temperature for E. coli. Both reactions showed an absolute requirement for divalent cations. Mg2+, Co2+ and Ni2+ were accepted with similar efficiencies, whereas no detectable cleavage occurred in the presence of Mn2+ (not shown). In summary, MbeA* shows the biochemical activities expected for a conjuga-

Biochemical analysis of MbeA* mutants

MbeA* mutant proteins were purified as described for MbeA*. In order to ascertain that the mutations introduced were not significantly affecting the structure of the mutant proteins, CD spectra were obtained for all of them. The results, shown in Fig. 6, demonstrate that no significant differences in the circular dichroism spectra were found between the wild-type protein and any of the mutants used.

Fig. 3. Purification of protein MbeA*. Fractions from the different purification steps were analysed by SDS-PAGE 12%. Lane 1, BL21(DE3)/pU1V205 before induction. Lane 2, BL21(DE3)/pU1V205 after 4 h induction. Lane 3, 6 μg sample after P11 phosphocellulose column. Lane 4, 5 μg sample after Mono-S HR 5/5 ion exchange chromatography. Lane 5, 3 μg sample after Superdex 75 HR 10/30 gel filtration chromatography. Lane 6, BL21(DE3)/pET29c(+)- before induction. Lane 7, BL21(DE3)/pET29c(+)- after 4 h induction. Lane 8, molecular weight markers. Sizes are shown in the margin.

Fig. 4. In vitro cleavage and strand-transfer reactions catalysed by MbeA*. Reactions (10 μl) contained 3 pmol of either the 23-mer 5'-end [32P]-labelled #ColE1-nic oligonucleotide (Lanes 2–5) or end-labelled oligonucleotide #R388–33nic (Lanes 6 and 7). Lanes 4 and 5 also contained 30 pmol of unlabelled oligonucleotide #ColE1-str. Lanes 3, 5 and 7 contained 50 pmol MbeA*, while lanes 2, 4 and 6 did not contain protein. Reactions were carried out for 1 h as described in Experimental procedures. Lane 1 contains three [32P]-labelled oligonucleotides whose sequences correspond to potential MbeA cleavage products: 13-mer (5'-GGA GTG TAT ACT G-3'), 14-mer (5'-GGA GTG TAT ACT GG-3') and 15-mer (5'-GGA GTG TAT ACT GGC-3').
in this work. Thus, the presently analysed mutations do not affect the overall folding of the protein, and their effects can be considered to be essentially local. oriT-specific cleavage and strand-transfer activities of the mutant proteins were tested with oligonucleotides #ColE1-nic and #ColE1-str, as shown above for MbeA*. Results are summarized in Table 1. Assays showed that out of the four single mutations tested, only N106A maintained significant cleavage and strand-transfer activities (about one-third of wild type). Mutations Y19A, H97A and E104A completely lost biochemical activity. This result indicates that these three amino acids are essential for the referred reactions. As a consequence, it is likely that Y19 is the equivalent residue to the conserved Tyr in motif I, whereas

![Fig. 5. Some properties of the in vitro reactions catalysed by MbeA.](image)

A. Per cent DNA-strand transfer versus MbeA* concentration.
B. Per cent DNA cleavage versus MbeA* concentration.
C. Per cent cleavage versus time (min).
D. Per cent cleavage versus temperature. All reactions were carried out as described in Experimental procedures, using oligonucleotides #ColE1-nic. Reaction (A) contained oligonucleotides #ColE1-nic and #ColE1-str. Curves were fitted by non-linear regression using the GRAPHPAD PRISM™ 3.02 program.

Table 1. Binding ability, cleavage and strand-transfer activities of truncated MbeA and the derived mutants.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mutation</th>
<th>Cleavage</th>
<th>Strand-transfer</th>
<th>Binding Kd (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MbeA*</td>
<td>none</td>
<td>65%</td>
<td>70%</td>
<td>146 ± 12</td>
</tr>
<tr>
<td>MbeA*(Y19A)</td>
<td>Y19A</td>
<td>&lt;2%</td>
<td>&lt;2%</td>
<td>296 ± 49</td>
</tr>
<tr>
<td>MbeA*(H97A)</td>
<td>H97A</td>
<td>&lt;2%</td>
<td>&lt;2%</td>
<td>128 ± 76</td>
</tr>
<tr>
<td>MbeA*(E104A)</td>
<td>E104A</td>
<td>&lt;2%</td>
<td>&lt;2%</td>
<td>112 ± 28</td>
</tr>
<tr>
<td>MbeA*(N106A)</td>
<td>N106A</td>
<td>15%</td>
<td>25%</td>
<td>156 ± 59</td>
</tr>
<tr>
<td>MbeA*(E104H-N106H)</td>
<td>(E104H + N106H)</td>
<td>&lt;2%</td>
<td>&lt;2%</td>
<td>222 ± 60</td>
</tr>
</tbody>
</table>
H97, E104 and N106 are probably equivalent to the invariant residues in Motif III Histidine triad. Following this thread of reasoning, it seemed logical to substitute E104 and N106 for two histidines, thus reconstructing the canonical 3H signature. Surprisingly, results showed that MbeA*(N106H-E104H) was devoid of cleavage and strand-transfer activities (Table 1).

In vitro binding of MbeA* and derived mutants to oligonucleotides containing nic was tested by electrophoresis mobility shift assays, using radiolabelled oligonucleotide #ColE1-nic. Figure 7 shows that MbeA* binds oligonucleotide #ColE1-nic, even in the presence of a large excess of unlabelled non-specific oligonucleotides. Binding experiments were repeated with the MbeA* mutant proteins. Results are summarized in Table 1. All four single mutants bound #ColE1-nic as well as MbeA* itself, indicating that the mutations considered above are not affecting oligonucleotide binding, and thus DNA recognition, but are specifically affecting the cleavage reactions and thus belong to the catalytic centre of the enzyme.

Cleavage of scDNA containing ColE1 oriT
Plasmid pUIV201 contains ColE1 oriT cloned in vector pUC19. This plasmid was used to test the ability of MbeA* to relax scDNA in vitro. The relaxation reaction requires only the reactants and Mg\(^{2+}\), and can be detected after protein denaturation, as described in Experimental procedures. Figure 8A shows that pUIV201 scDNA was almost quantitatively converted to the OC form after incubation with purified MbeA*. On the other hand, pUC19 DNA did not show any nicked form when incubated under the same conditions. To confirm that the observed pUIV201 relaxation was indeed catalysed by MbeA*, the nicked DNA was analysed by primer extension. Nicked DNA from plasmid pUIV201 was used as template and oligonucleotide #plus as primer. A single polynucleotide chain resulted when nicked pUIV201 was used in the extension reaction (Fig. 8B, Lane 3). On the other hand, no interruption was shown in the reaction when pUIV201 scDNA without added MbeA* was used (Fig. 8B, Lane 2). The strand interruption was found to be at the same nucleotide position \([5'-\text{CTGG}/\text{CTTA}(1462)-3']\), as the nic site assigned \textit{in vivo} by primer extension of plasmids pUIV208 and pUIV209 (relaxation \textit{in vivo}, Fig. 2A), except that this time Thermo Sequenase did not show an efficient terminal transferase activity. Variation in the terminal transferase activity of Thermo Sequenase has been previously

<table>
<thead>
<tr>
<th>Complementing plasmid</th>
<th>Mobilization frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUIV216 (mutant Y19A)</td>
<td>(&lt;1 \times 10^{-7})</td>
</tr>
<tr>
<td>pUIV218 (mutant H97A)</td>
<td>(&lt;1 \times 10^{-7})</td>
</tr>
<tr>
<td>pUIV220 (mutant E104A)</td>
<td>(&lt;1 \times 10^{-7})</td>
</tr>
<tr>
<td>pUIV222 (mutant N106A)</td>
<td>(3 \times 10^{-4})</td>
</tr>
<tr>
<td>pUIV224 (mutant E104H-N106H)</td>
<td>(2 \times 10^{-4})</td>
</tr>
<tr>
<td>pUIV206 (mbeA gene)</td>
<td>(1 \times 10^{-3})</td>
</tr>
<tr>
<td>pUIV205 (mbeA* gene)</td>
<td>(2 \times 10^{-4})</td>
</tr>
<tr>
<td>pSU4601 (ColE1::Km)</td>
<td>(2 \times 10^{-3})</td>
</tr>
</tbody>
</table>

\(a.\) Derivatives of \textit{E. coli} strain BL21(DE3) carrying pUIV229 and each of the plasmids shown in the first column were separately used as donors in triparental matings using DH5\(\alpha/R64\text{drd11 as helper intermediate strain and HMS174 as the final recipient, as described in Experimental procedures. After filter mating for 1 h at 37°C, bacteria were plated on selective media containing Rif to counterselect donors and Cm to select for pUIV229 mobilization. Transfer frequencies are expressed as the number of transconjugants per recipient cell. Figures shown represent the average of at least three separate experiments.}

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reported (Núñez and de la Cruz, 2001). These results indicate that MbeA* is capable of specific in vitro relaxation of scDNA without a requirement for relaxase accessory proteins.

Complementation in vivo

Plasmid pUIV229 is MbeA– and can be rendered mobilizable in the presence of plasmids contributing MbeA, such as pUIV205 or pUIV206 (Table 2). Complementation by protein MbeA* (pUIV205) is only fivefold less efficient than complementation by wild-type MbeA (pUIV206). Thus, MbeA* can be considered as a functional relaxase. Complementation in vivo of plasmid pUIV229 mobilization was analysed in the presence of the MbeA* mutants pUIV216, pUIV218, pUIV220, pUIV222, pUIV224 using triparental matings. Results are shown in Table 2. Mutations Y19, H97 and E104 resulted in proteins completely incapable of complementation and thus non-functional. On the other hand, the Mob+ phenotype was fully recovered with pUIV222 (mutation N106A) and partially recovered with pUIV224 (double mutation E104H-N106H). The first result indicates that the mutation N106A is not very damaging, and the reduced in vitro relaxase activities shown by the corresponding mutant protein are not significant in vivo. According to the second result, it is very important to see that the protein containing the double mutation E104H-N106H was capable of significant complementation (to about 1% of wild type), in spite of the fact that we were not able to detect cleavage or strand transfer in vitro with the mutant protein. This is not surprising, as our in vitro methods do not allow to detect less than 2% cleavage or strand-transfer.

Discussion

Relaxase biochemistry dates back more than three decades when Clewell and Helinski (1969) demonstrated that mobilizable plasmid ColE1 could be isolated from cells as a DNA–protein complex that was converted to a relaxed form upon exposure to protein denaturants. Despite that early start, this is the first report on the biochemical characterization of protein MbeA, the relaxase of plasmid ColE1. MbeA deserved the interest for deeper analysis not only because of historical reasons, but also because it does not contain the histidine triad, a signature shared by most previously reported relaxases (Zechner et al., 2000). We propose that the alignment shown in Fig. 1 represents a true homology relationship between 3H and HEN relaxases, and not chance similarity. The alignment is objective, and derives from a BLAST search using default settings and MbeA as a query. In the first iteration it retrieves most or all HEN relaxases, as well as a number of 3H relaxases including for instance the MobA proteins of Aeromonas salmonicida plasmids pAsal1 and pAsal3 (accession numbers CAD48428.1 and CAD48422.1), with highly significant scores, well above the default cut-off value. These are 25–27% identical to MbeA, and furthermore, they are 32% identical to the HEN relaxase of another A. salmonicida plasmid.
(CAD48436.1). Besides, the first BLAST iteration also retrieves the relaxase protein NikA of the IncI plasmid R721, a well-known member of the 3H relaxase family. These levels of sequence identity undoubtedly reflect a true evolutionary relationship, indicating that HEN and 3H are two alternative versions of a common protein sequence signature.

In this study we report on the purification of MbeA*+, a truncated version of MbeA, lacking 100 amino acids from its C-terminus. By a complementation experiment we demonstrate that MbeA* is functional in vivo and we describe the in vitro activities of the purified protein. We could characterize nic cleavage, DNA-strand transfer reactions and cleavage of supercoiled DNA. In all these reactions MbeA*+ behaved very much like previously reported relaxases such as TraI of plasmids F and R100 (Matson and Morton, 1991; Reygers et al., 1991; Fukuda and Ohtsubo, 1995), TrwC of plasmid R388 (Llosa et al., 1995; Grandoso et al., 2000), MobM of plasmid pMV158 (Guzmán and Espinosa, 1997), TraI of plasmid RP4 (Pansegrau et al., 1993) and VirD2 of the Ti plasmid (Vogel and Das, 1992; Vogel et al., 1995). A 23-mer oligonucleotide (#ColE1-nic) contained enough sequence information to be specifically recognized and cleaved by the relaxase.

Perhaps the most important contribution of this work is to describe a relaxase that does not contain the 3H signature that up to now was thought to be invariant in plasmid conjugative relaxases. We achieved this objective by studying DNA binding, cleavage and strand-transfer reactions catalysed by MbeA*+ and comparing these activities to those of a set of mutants in those invariant amino acids. Results summarized in Tables 1 and 2 indicate that MbeA*+ residue Y19 plays a key catalytic role during conjugative DNA processing. Replacement of this amino acid to alanine, resulted in a complete loss of in vitro cleavage activity and of in vivo functionality. Besides, Y19 apparently does not play an important role in DNA recognition, as the mutant protein showed almost wild-type binding ability in vitro. According to Fig. 1 MbeA residue Y19 is equivalent to residue Y22 of RP4 protein TraI and to residue Y29 of protein VirD2 from A. tumefaciens plasmid pTiA6, and thus is the one that forms the covalent linkage to the DNA during relaxase operation (Vogel and Das, 1992; Pansegrau and Lanka, 1996). Mutation analysis of virD2 revealed that VirD2 Y29 was essential for cleavage and could not be substituted by any other amino acid (Vogel and Das, 1992).

The only histidine present in MbeA putative motif III is H97. Mutation of H97 to alanine resulted in total loss of in vitro cleavage activity accompanied by loss of functionality in vivo while, as observed with mutant Y19, it remained capable of in vitro oligonucleotide binding. This indicates normal folding of the protein and retention of nic binding specificity. In the case of RP4_TraI, the effects of an equivalent mutation within Motif III (H109S) were much less spectacular. H109S resulted in only threefold decrease in TraI in vivo functionality and almost wild type in vitro relaxase properties (Balzer et al., 1994; Pansegrau et al., 1994).

Instead of the other two histidines of Motif III, MbeA carries a glutamic acid (E104) and an asparagine (N106). The E104A mutation had no effect on in vitro nic binding, but resulted in loss of in vitro nic cleavage activity and in loss of in vivo functionality. Similarly, the equivalent mutation in RP4_TraI (H116S) resulted in undetectable relaxase activities and a 10 000-fold reduction in the in vivo functionality. On the other hand, MbeA mutation N106A resulted in a protein with wild type complementation ability and only reduced effects on the in vitro reactions. Once again, these results were paralleled by RP4_TraI mutation H118S, which showed reduced cleavage and nicking, and 10% complementation activity compared to wild type (Balzer et al., 1994; Pansegrau et al., 1994).

In summary, it is clear that MbeA residues Y19, H97 and E104 play an important role in the catalysis of DNA processing during plasmid ColE1 mobilization. As in the case of TraI (Pansegrau et al., 1994), it is proposed that motifs I and III might be in close contact in the folded protein, forming together part of MbeA catalytic centre. From the above results we conclude that MbeA shares the basic protein fold presented by RP4_TraI and the 3H family of conjugative relaxases.

The fact that MbeA shows only one histidine (H97) in the 3H motif triggered our curiosity to find out what would happen if we changed E104 and N106 to histidines. It was expected that the protein would still be active. The result only partially fulfilled our expectation, since MbeA*(E104H+N106H) had only 1% of the in vivo activity of the wild-type protein, and no detectable in vitro relaxase activities. However, it maintained wild-type DNA binding capacity, indicating that the protein was properly folded. Taking the above into consideration, the properties of the double mutant protein confirm the equivalence of E104 and N106 with H116 and H118 of RP4_TraI. Any hypothesis on the biochemical pathway of the relaxase-catalysed DNA processing mechanism has to take into account that E104 and N106 of ColE1_MbeA can catalyse similar reactions as H116 and H118 of RP4_TraI. In the proposed mechanism for RP4 relaxase TraI, H116 is postulated to act by abstracting a proton from the aromatic hydroxyl group of Y22 thus converting Y22 in an efficient nucleophile that can attack the phosphodiester bond at nic. The same role would have to be played by Glutamate E108 of MbeA. Furthermore, most of the relaxases reported so far are active in the presence of Mn2+ (Pan-
segrau et al., 1993; Llosa et al., 1995; Guzmán and Espinosa, 1997). The peculiar inability of MbeA* to react in the presence of this cation may be considered as evidence of the divergence of MbeA catalytic centre.

In conclusion, the results presented here suggest that MbeA belongs to the common family of plasmid relaxases. It constitutes an interesting variant that, instead of possessing a 3H triad, shows a novel motif (HEN), which might be used as a diagnostic sequence for the detection of putative relaxases in other plasmids.

**Experimental procedures**

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

Plasmids used are listed in Table 3. *Escherichia coli* strains used were DH5α (F− endA1 hsdR17 supE44 thi-1 recA1 (argF-lacZYA) u160–80d lacZΔ M15 gyrA96) (Grant et al., 1990), BL21(DE3) (F− ompT r−m−) (Studier and Moffatt, 1986) and HMS174 (recA1 hsdR rif) (Campbell et al., 1978). Bacteria were grown at 37°C in Luria–Bertani (LB) broth (Sambrook et al., 1989), supplemented with 2% (w/v) agar for growth on plates. When indicated, growth media were supplemented with antibiotics at the following concentrations: sodium ampicillin (Am) 100 μg ml−1; kanamycin sulphate (Km) 50 μg ml−1; chloramphenicol (Cm) 25 μg ml−1; tetracycline (Tc) 5 μg ml−1; rifampicin (Rif) 100 μg ml−1.

**Standard genetic experiments**

Bacterial transformation was carried out according to the methods of Chung and Miller (1988) or Kushner et al. (1978). When a high frequency was required, highly efficient competences were prepared (Hanahan, 1983) and transformed by electroporation (Dower et al., 1988).

**Plasmid methodology, enzymes and oligonucleotides**

Plasmid DNA was purified in a small scale according to Sambrook et al. (1989) and in large scale using the JETSTAR plasmid maxiprep kit (Genomed). DNA fragments larger than 1 kb were purified from agarose gels with silica as described by Boyle and Lew (1995). For the purification of DNA fragments smaller than 100 bp, the Mermaid Kit (Bio 101) was used. DNA fragments of intermediate size (200 bp–1 kb) were purified by the GFX™ DNA and Gel Band Purification kit (Amersham). The PCR-amplification of small DNA fragments (up to 400 bp) was carried out with Taq DNA polymerase (Promega). For PCR-amplification of larger fragments the Expand High Fidelity PCR System (Roche) was used. Cloning techniques were carried out by standard methodology (Sambrook et al., 1989). Phage T4 Polynucleotide kinase and T4 DNA ligase were from New England Biolabs. Restriction endonucleases were purchased from Amersham. Oligonucleotides used are listed on Table 4.

**Plasmid constructions**

The truncated mbeA gene was cloned into pET29c(+) vector by a three-step procedure. First, the DNA fragment corresponding to ColE1 bp 2179–2580 was PCR-amplified with #plus-1 and #minus-1 oligonucleotide primers. The resulting DNA was digested with endonucleases Ndel and EcoRI and cloned into the same sites of plasmid pUC19, leading to plasmid pUV202. Second, the 1123 bp CiaI/PvuII ColE1 fragment was cloned at the CiaI/Smal sites of pUV202, leading to plasmid pUV203. Third, the 1254 bp Ndel/BamHI fragment of pUV203 was cloned at the corresponding sites of plasmid pET29c(+), producing pUV205, the vector used for MbeA* overexpression and purification. Vectors pUV203 and pUV205 carry a truncated mbeA gene lacking the last

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Table 3. Plasmids.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Phenotype</th>
<th>Description</th>
<th>Size (bp)</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>pET29c(+)</td>
<td>Km</td>
<td>expression vector</td>
<td>5372</td>
<td>Novagen</td>
</tr>
<tr>
<td>pUC19</td>
<td>Ap</td>
<td>cloning vector</td>
<td>2686</td>
<td>Bioline</td>
</tr>
<tr>
<td>pSU18</td>
<td>Cm</td>
<td>cloning vector</td>
<td>2300</td>
<td>Bartolomé et al., 1991</td>
</tr>
<tr>
<td>pUV201</td>
<td>Ap</td>
<td>pUC19::oriT(ColE1)</td>
<td>2886</td>
<td>This study</td>
</tr>
<tr>
<td>pUV202</td>
<td>Ap</td>
<td>pUC19::400 bp mbeA segment*</td>
<td>2873</td>
<td>This study</td>
</tr>
<tr>
<td>pUV203</td>
<td>Km</td>
<td>pET29c(+)::mbeA*</td>
<td>6574</td>
<td>This study</td>
</tr>
<tr>
<td>pUV206</td>
<td>Km</td>
<td>pET29c(+)::mbeA</td>
<td>6974</td>
<td>This study</td>
</tr>
<tr>
<td>pUV208</td>
<td>Ap</td>
<td>pUC19::ColE1</td>
<td>9332</td>
<td>This study</td>
</tr>
<tr>
<td>pUV209</td>
<td>Ap</td>
<td>pUV208::mbeA*</td>
<td>9821</td>
<td>This study</td>
</tr>
<tr>
<td>pUV216</td>
<td>Km</td>
<td>mbeA*(Y19A)</td>
<td>6574</td>
<td>This study</td>
</tr>
<tr>
<td>pUV218</td>
<td>Km</td>
<td>mbeA*(H97A)</td>
<td>6574</td>
<td>This study</td>
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<tr>
<td>pUV220</td>
<td>Km</td>
<td>mbeA*(E104A)</td>
<td>6574</td>
<td>This study</td>
</tr>
<tr>
<td>pUV222</td>
<td>Km</td>
<td>mbeA*(N106A)</td>
<td>6574</td>
<td>This study</td>
</tr>
<tr>
<td>pUV224</td>
<td>Km</td>
<td>mbeA*(E104H→N106H)</td>
<td>6574</td>
<td>This study</td>
</tr>
<tr>
<td>pUV227</td>
<td>Cm</td>
<td>pSU18::(1.2 kb fragment ColE1)</td>
<td>3484</td>
<td>This study</td>
</tr>
<tr>
<td>pUV229**</td>
<td>Cm, Mob-</td>
<td>pSU18:: mob(ColE1)::mbeA</td>
<td>4395</td>
<td>This study</td>
</tr>
<tr>
<td>pSU4601</td>
<td>Km</td>
<td>ColE1::kan</td>
<td>7930</td>
<td>Cabezón et al., 1997</td>
</tr>
<tr>
<td>R64drd-11</td>
<td>Tc, Sm</td>
<td>R64 derepressed for transfer</td>
<td>56 700</td>
<td>Komano et al., 1990</td>
</tr>
</tbody>
</table>

a. The 400 bp mbeA segment corresponds to ColE1 bp 2179–2580 bp
b. pUV229 is Mob- because mbeA is partially deleted (see plasmid construction in Experimental procedures); mbeB and mbeD are still functional.
300 bp of the coding region. The complete mbeA gene was cloned into pET29c(+) after PCR amplification of ColE1 region 2169–3769 bp using oligonucleotides #plus-1 and #minus-ab. The resulting DNA was digested with endonucleases NdeI and BamHI and cloned at the corresponding sites of plasmid pSU18, leading to pUIV201. Plasmid pUIV201 was constructed by cloning the ColE1 oriT region (1370–1573 bp) in the EcoRI site of vector pUC19 after PCR-amplification using primer oligonucleotides #plus and #minus.

**Purification of in vivo nicked plasmids**

Cleared lysates of CoIE1 derivatives were obtained from 400 mL cultures of strain DH5a containing the appropriate plasmid, as described by Avila *et al.* (1996). CsCl/EtBr gradients and extraction of OC DNA forms were performed as described previously (Avila *et al.*, 1996).

**DNA sequencing and analysis**

DNA fragments were sequenced using Thermo Sequenase cycle sequencing Kit (Amersham) and 7% Long Ranger gels in TBE buffer in an automatic Vistra DNA sequencer (Amersham). Data were collected and analysed by the Vistra DNA sequencer 725 version 2.0 software. Oligonucleotide primers (HPLC degree) were labelled with Texas red 5¢-end of nic by primer extension. pUIV208 and pUIV209 SC and OC plasmid DNAs were used as templates for sequencing with Thermo Sequenase DNA polymerase (Amersham). Extension reactions (10 μL) contained 250 ng of DNA, 1 pmol Texas red-labelled primers, 0.2 mM dNTPs and Thermo Sequenase (2 units). Reaction mixtures were subjected to cycle extension (first, 2 min at 95°C; then 30 cycles of 30 s at 95°C, 30 s 56°C and 30 s

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**Table 4. Oligonucleotides.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Oligonucleotide sequence</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>#plus</td>
<td>5’-CCT GGA CTT GAA TCT CTC TCT TAC GC</td>
<td>1370–1395</td>
</tr>
<tr>
<td>#minus</td>
<td>5’-AGC GAA TTC CGG GAG CTG ACG AGC</td>
<td>1573–1550</td>
</tr>
<tr>
<td>#plus-1</td>
<td>5’-GAA CAG GAG GCC CCA TAT GAT GT T</td>
<td>2164–2188</td>
</tr>
<tr>
<td>#minus-1</td>
<td>5’-GTC TGC CAA GCA TGA ATT CTA GGC CT</td>
<td>2589–2564</td>
</tr>
<tr>
<td>#mutY</td>
<td>5’-CCA CTT GAT CCA CCG AAG CTG C</td>
<td>3769–3748</td>
</tr>
<tr>
<td>#minus-BamHI</td>
<td>5’-TCC TGT TGC GGA TCC TCT AAG</td>
<td>3445–3425</td>
</tr>
<tr>
<td>#mutU</td>
<td>5’-CTT GTC CTG GCC CTC CAC C</td>
<td>2461–2479</td>
</tr>
<tr>
<td>#mutE</td>
<td>5’-ATT CAG CGC AAG CCG TCC</td>
<td>2480–2497</td>
</tr>
<tr>
<td>#mutN</td>
<td>5’-GTT CCG AAT GAC AAA CTA G</td>
<td>2529–2552</td>
</tr>
<tr>
<td>#mut2his</td>
<td>5’-GAA AAC GCG TCG ACC CGT ACT AC</td>
<td>1483–1454</td>
</tr>
<tr>
<td>#ColE1-nic</td>
<td>5’-GGA GTG TAT ACT GG C TTA ACC AT</td>
<td>1312–1334</td>
</tr>
<tr>
<td>#ColE1-sto</td>
<td>5’-AGC GGA GTG TAT ACT GG C TTA ACC ATG CG</td>
<td>2227–2213</td>
</tr>
<tr>
<td>#comp-ColE1-plus</td>
<td>5’-CTC GCT GTA GTG CCG AAG CGA CCG AG</td>
<td>2529–2552</td>
</tr>
<tr>
<td>#mut2his-2</td>
<td>5’-GTT CGG AAT GAC AAA CTA G</td>
<td>1312–1334</td>
</tr>
<tr>
<td>#plus-comp-sal</td>
<td>5’-GAA AAC GCG TCG ACC GTT ACT AC</td>
<td>2529–2552</td>
</tr>
<tr>
<td>#R388-33nic</td>
<td>5’-GCGCACCAGAAGGGCGCGTGATAGCGTATAGGCGCCA</td>
<td></td>
</tr>
</tbody>
</table>

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a. Oligonucleotides used for site-directed mutagenesis and mutant nucleotides are in bold.
b. Numbers refer to co-ordinates in ColE1 DNA sequence (GeneBank Acc n° NC_001371). Base position 1 corresponds to the first T in the single EcoRI site.
c. The slash shows the position of MbeA-dependent cleavage in ColE1.

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Oligonucleotides and labelling

Unlabelled oligonucleotides were purchased from MWG-Biotech. Oligonucleotides were labelled at their 5′-end using [γ-32P]ATP (3000 Ci mmol−1) and polynucleotide kinase (New England Biolabs) (Sambrook et al., 1989). Unbound [γ-32P]-ATP was eliminated from the mixture by MicroSpin™ G-25 column (Amersham) purification.

Purification of MbeA* and derivative mutants

Escherichia coli BL21(DE3)-pET29c(+) host-vector expression system was used to overproduce MbeA*. Cells containing MbeA*-overexpressing plasmids were incubated in 2 l LB broth containing Km (50 μg ml−1). The culture was grown with aeration in a micro-DCU fermentation system (B. Biotech International). Cultures were induced at A600 = 0.6 by adding 0.5 mM IPTG and incubation continued for four additional hours. Cells were harvested, resuspended in 50 mM Tris-HCl (pH = 7.6), 10 mM EDTA, 10% (w/v) sucrose, lysozyme 0.5 mg ml−1, 10 mM benzamidine, 1 mM phenylmethylsulphonylfluoride and incubated 45 min in ice. An equal volume of solution 50 mM Tris-HCl (pH = 7.6), 400 mM NaCl, 1 mM DTT, 0.1 mM EDTA and 0.5% (v/v) Triton X-100 was added and the lysate was centrifuged at 138 000 g for 30 min at 4°C. The supernatant was collected and applied to P11 phosphocellulose column (Whatman) in Buffer A (Tris-HCl 50 mM pH = 7.6, 0.1 mM EDTA) containing 200 mM NaCl. Proteins eluted in a 0.2–1.3 M NaCl gradient of Buffer A at a flow rate of 1 ml min−1. Fractions containing MbeA* (which eluted at around 0.5 M NaCl) were collected and diluted in Buffer A, so that the NaCl concentration was set to 200 mM. MbeA* was further purified by Mono-S S HR 5/5 column chromatography (Pharmacia) using a 0.2–1 M NaCl gradient in Buffer A at a flow rate 0.5 ml min−1. Finally, protein was purified by gel-filtration through Superdex 75 HR 10/30 (Pharmacia) using Buffer A containing 500 mM NaCl at a flow rate of 0.5 ml min−1. When required, fractions containing MbeA* were concentrated after Mono-S ion exchange with Ultrafree-MC Centrifugal Filter Units (Millipore). The resulting protein was more than 95% pure. The yield was 8 mg protein from 1 l culture. The purified protein was stored in 20% glycerol at −80°C. No loss of activity was observed after 6 months in these conditions.

Oligonucleotide cleavage and strand-transfer reactions using 5′-labelled [32P]-ATP-oligonucleotides

For oligonucleotide cleavage reactions, 50 pmol MbeA* were added to 3 pmol 5′-labelled ColE1-nic oligonucleotide in a total volume of 10 μl cleavage buffer (10 mM Tris-HCl, pH = 7.6, 5 mM MgCl2, 50 mM NaCl, 0.02 mM EDTA). After 1 h incubation at 37°C, reaction products were processed by proteinase K/SDS treatment as described by Grandoso et al. (2000) and analysed by polyacrylamide slab gel electrophoresis followed by autoradiography as previously described (Sambrook et al., 1989). Oligonucleotide strand-transfer reactions were carried out and analysed as the cleavage reactions, except that reaction samples contained 3 pmol 5′-labelled oligonucleotide #ColE1-nic and 30 pmol unlabelled #ColE1-str oligonucleotide.

In vitro nicking of scDNA containing plasmid ColE1 oriT region

Plasmid pUV201 carries a 200 bp ColE1 segment, containing oriT cloned in vector pUC19. The standard reaction mixture (20 μl) contained 100 ng pUV201 scDNA and 50 pmol MbeA* protein in nicking buffer (10 mM Tris-HCl (pH 7.6), 5 mM MgCl2, 100 mM NaCl and 0.02 mM EDTA). After 30 min incubation at 37°C, the mixture was digested by addition of 1 μl Proteinase K (15 mg ml−1) and 1 μl of 1% (w/v) SDS. Reaction products were applied to 0.8% (w/v) agarose gels containing 0.5 μg ml−1 ethidium bromide and electrophoresed at 6 V cm−1 in 0.5 × Tris-Borate-EDTA buffer.

Specificity of the in vitro relaxation of scDNA containing ColE1 oriT

The specificity of relaxation was tested by primer extension. At first, relaxation reactions were performed using a reaction mixture (200 μl) containing 1 μg pUV201 DNA and 500 pmol MbeA* in nicking buffer. The reaction was incubated for 30 min at 37°C. The Proteinase K digestion step was skipped, as it could hydrolyse the DNA-polymerase at the step of primer extension. The portion of MbeA* that did not react was removed from the reaction mixture by phenol extractions and DNA was recovered following Na-acetate/ethanol precipitation. The precipitated DNA was used for primer extension as described for 5′-end mapping.

DNA electrophoresis mobility shift assay (EMSA)

Binding reactions (10 μl) contained 1 nM radionlabelled oligonucleotide #ColE1-nic, 1 μM unlabelled oligonucleotide mixture (acting as non-specific inhibitor) and increasing protein concentrations (wild-type or mutant MbeA*) in 10 mM Tris-HCl (pH = 7.6), 100 mM NaCl, 0.02 mM EDTA and 4% glycerol. Reaction mixtures were incubated for 30 min at 20°C. Samples were loaded onto a 10% polyacrylamide Bio-Rad minigel (0.75 mm × 6.5 cm) and run for 60 min at 100 V in 90 mM Tris-Borate buffer, pH = 8.2. Gels were dried for 2 h at 80°C and radioactivity quantified in a Molecular Imager FX system (Bio-Rad). The amount of shifted and unshifted oligonucleotide in each lane was determined using Quantity One Software (Bio-Rad). Protein-oligonucleotide dissociation constants (Kd) were calculated by fitting the data (considering primarily the amount of unshifted oligonucleotide) to a one-site binding hyperbole with GRAPHPAD PRISM™ 3.02.

Conjugation experiments

Cultures were grown overnight without shaking at 37°C in LB with appropriate antibiotics for plasmid selection until A600 = 0.3–0.4. Escherichia coli strain BL21(DE3) carrying the appropriate plasmids was used as donor, DH5α/R64/str-1.
as helper and HMS174 as recipient. BL21(DE3) (0.5 ml) carrying the appropriate plasmids and 1.5 ml DH5 α/R64drd-11 were centrifuged separately at 5000 r.p.m. for 5 min and washed from the antibiotics. Then cells were mixed and placed onto a GS Millipore filter (0.22 μm pore size) on a prewarmed LB agar plate for 1 h at 37°C. Then 3 ml HMS174 were centrifuged at 5000 r.p.m. for 5 min, washed from the antibiotics and placed on the filter mentioned above. After 1 h at the 37°C, bacteria were washed from the filter with LB broth, diluted and plated on selective media. Transfer frequencies are expressed as the number of transconjugants per recipient cell. The plasmid content of transconjugants was verified electrophoretically when appropriate.

Protein analysis

Proteins were visualized by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and stained with Coomassie brilliant blue R-250. Protein quantification was carried out with Bio-Rad Protein Assay dye reagent, using bovine serum albumin as a standard.

Circular dichroism spectra

Circular dichroism spectra were recorded in a JASCO J-810 spectropolarimeter. Far UV-spectra were recorded in 0.02 cm path length quartz cells at protein concentration of around 0.4 mg ml\(^{-1}\). The observed elipticities were converted to mean residue elipticities [6] by using mean molecular masses per residue.

Acknowledgements

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References


MbeA protein, the relaxase of ColE1 plasmid


