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Interaction between relaxase MbeA and accessory protein MbeC of the conjugally mobilizable plasmid ColE1

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This work is dedicated in memory of Professor Constantin Drainas, who passed away in an untimely car accident, on the 5th of July 2011, while this work was in progress. He is missed immensely.

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ABSTRACT

MbeA and MbeC are two key proteins in plasmid ColE1 conjugal mobilization. Isothermal titration calorimetry was used to detect and quantify an interaction between MbeA and MbeC. As a result of this interaction, the affinity of MbeA for single stranded DNA increased. The interaction was confirmed in vivo using a bacterial two-hybrid system, which revealed that MbeA–MbeC complexes are formed through the amino-terminal region of MbeA and the carboxy-terminal region of MbeC. To the best of our knowledge, this is the first report of direct interactions between conjugative proteins encoded by a mobilizable plasmid.

Structured summary of protein interactions:
mbeA and **mbeC** physically interact by two hybrid (View interaction)
mbeA and **mbeC** bind by isothermal titration calorimetry (View interaction)

1. Introduction

ColE1 is a colicinogenic factor discovered in *Escherichia coli* and is extensively used in the construction of cloning vectors [1]. It is mobilized by a wide array of conjugal plasmids [2] and reports on its conjugal mobilization were already published in the late '60s [3]. Despite this early start, studies on ColE1 mobilization proteins were temporarily neglected. Nevertheless, the development of bacterial multi-drug resistance at the end of the 20th century and its connection with horizontal gene transfer, revived the scientific interest in conjugal mobilization, and thereby re-ignited interest in ColE1.

ColE1 is the prototype of the ColE1-superfamily (MOB_{P5}) of mobilizable plasmids [4]. Its *mob* region is well characterised and consists of a cluster of five genes (Fig. 1 and [5]). Studies confirmed the location and functionality of its *nic* site, *oriT* region, its relaxase (MbeA) and an accessory protein (MbeC) [6,7]. MbeB is proposed to be part of the relaxosome [8], while MbeD is probably an "entry exclusion" protein [9]. ColE1 therefore is the best known mobilizable plasmid and provides an excellent system to unravel the functions of and interactions between the mobilization proteins themselves, with DNA and with proteins of helper type IV secretion systems (T4SS).

Throughout the past 50 years, scientists focused largely on defining the mechanisms of a few model plasmid conjugation systems and data for the construction of the T4SS models have been derived from protein–protein interaction studies [10,11]. These studies revealed the importance of the physical interface between the relaxosome and the coupling proteins (T4CP) in both gram-negative and gram-positive bacteria. The most important molecule in that interface is the relaxase, as it plays a key role in two types of protein–protein interactions; interactions with the T4CP and with accessory DNA transfer and replication (Dtr) processing factors, both involved in essential steps during conjugation. The

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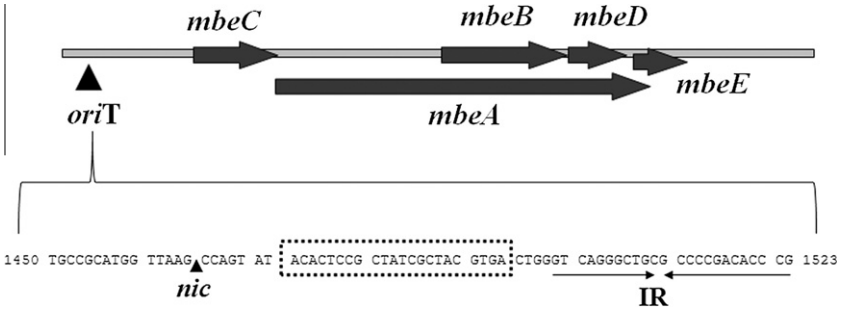

Fig. 1. Genetic structure of the mobilization region of ColE1 plasmid. The DNA sequence is of the *oriT* region. The characteristic IR is illustrated by horizontal arrows below the nucleotide sequence. The putative MbeC binding site is indicated by a dotted-line square. The position of the *nic* (cleavage occurs in the complementary DNA-strand) is indicated by a vertical arrowhead. Numbers refer to coordinates in the ColE1 sequence (GenBank accession No. NC_001371).

interactions with the T4CP deal with the process of secretion of the relaxase attached to the T-DNA strand through the T4SS and the export of the remaining DNA molecule which couples the plasmid replication machinery to the export system in the membrane. The interactions with the Dtr-accessory proteins stimulate the recognition of *oriT* by the relaxase and the initial DNA-processing reaction. The Dtr-accessory proteins probably function as molecular wedges to melt dsDNA and facilitate the access of the relaxase to the *nic* site [12]. Several Dtr-accessory proteins have been characterised [7,13–19] and they are proposed to interact with their cognate relaxase, although these interactions were not proven experimentally. In contrast, interactions with the T4CPs were confirmed in vitro and/or in vivo, both in gram-negative and in gram-positive bacteria and involved interactions with their cognate relaxase [20–22] and, in some cases, with the Dtr-accessory proteins [14,23–26].

Although information about interactions that involve proteins from conjugative plasmids are constantly generated, our knowledge about the interactions of proteins from mobilizable plasmids is limited. The only proven interactions involve the relaxases Mob_pBHR1 and MbpB_pLV22a with the TraG_RP4 [27,28]. Here we report the interaction between the relaxase MbeA_ColE1 and the accessory protein MbeC_ColE1, detected and quantified in vitro by isothermal titration calorimetry (ITC) and confirmed in vivo by a bacterial two-hybrid (2HB) system [29].

2. Materials and methods

2.1. Bacterial strains

Escherichia coli DH5α [30] was used for all cloning procedures and for plasmid standard maintenance; BL21::DE3 [31] for protein over-expression; the *cya*-deficient strain BTH101 [29] as host in the 2HB assays. Growth media were supplemented, when required, with antibiotics at concentrations: ampicillin (Ap), 100 μg/ml; kanamycin (km), 50 μg/ml. Bacterial transformations were carried out as described in [32].

2.2. Plasmid constructs and oligonucleotides

Plasmids and oligonucleotides used are listed in Table 1SM and Table 2SM, respectively, provided in the Supplementary data. PCR-generated fragments of ColE1 were synthesized using pSU4601 [2] as template and checked by DNA sequencing. Plasmids were constructed following standard recombinant methods [32]. The construction of fusions with the T18 and T25 domains of adenylate cyclase (AC) used in the 2HB assays, were obtained by placing in-frame either *mbeA* or *mbeC* with T18 and T25 into the *Pst*I/*Kpn*I sites of plasmids pUT18C, pUT18 and pKT25 [29].

2.3. Protein purification

Proteins MbeA and MbeC were purified as previously described [6,7] with the following modifications that improved the yield. MbeA was purified from cell free sonicated extracts of BL21::DE3/pUIV205 by sequential chromatography on SP Sepharose and Sephacryl S300 columns. 25 g of cells yielded approximately 150 mg of MbeA protein at greater than 95% purity. MbeC was purified from sonicated cell free extracts of BL21::DE3/pUIV239 by sequential chromatography on Probond (Invitrogen) and MONO-S HR 10/10 FPLC (GE Healthcare) columns. 25 g cells yielded approximately 33 mg of MbeC protein at greater than 95% purity.

2.4. Isothermal titration calorimetry (ITC)

ITC experiments were performed at 25 °C using a high precision VP-ITC system (Microcal Inc.), as previously described [33,34]. The heat evolved following each 10 μl injection was obtained from the integral of the calorimetric signal. The heat due to the binding reaction was obtained as the difference between the heat of reaction and the corresponding heat of dilution (not shown). Analysis of data was performed using Microcal Origin software.

2.4.1. Study of MbeA–MbeC interactions

MbeA–MbeC interactions were studied by titrating MbeC (25–55 μM) in the calorimetric cell (1.4 ml) with MbeA (228–323 μM) in the injection syringe. Both MbeA and MbeC proteins were dialysed against Buffer A (0.1 M Tris–HCl, 1.0 mM DTT, 0.7 M NaCl pH 8.0). A high salt buffer (0.7 M NaCl) was necessary to keep MbeA in solution at the concentrations required for ITC.

2.4.2. Interactions of MbeA–MbeC complex with ssDNA

Interactions of MbeA with ssDNA were studied in the presence and absence of MbeC. Proteins were dialysed against Buffer B (50 mM Tris–HCl, 0.5 mM DTT, 0.01 mM EDTA, 0.35 M NaCl pH 8.0) prior to the ITC experiments and *#ColE1-nic* (Table 2SM) was dissolved in Buffer B. MbeA and MbeC were placed in the calorimetric cell in a 1:5 M ratio respectively, conditions in which most of the MbeA was in a binary complex with MbeC, and the complex was then titrated with ssDNA in the injection syringe. The lower salt concentration (0.35 M) in Buffer B compared to Buffer A was to ensure that MbeA could interact with its ssDNA target. As the concentration of MbeA used in these experiments was around six-fold less than that used for the MbeA–MbeC interaction, 0.7 M salt was not required to maintain solubility.

2.5. Bacterial two-hybrid system (2HB)

The 2HB used is based on the reconstitution of AC activity in a *cya* mutant of *E. coli* mediated by the interaction between different