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Mobilization Functions of the Bacteriocinogenic Plasmid pRJ6 of *Staphylococcus aureus*

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Plasmid pRJ6 is the first known bacteriocinogenic mobilizable (Mob) plasmid of *Staphylococcus aureus*. Its Mob region is composed of four *mob* genes (*mobCDAB*) arranged as an operon, a genetic organization uncommon among *S. aureus* Mob plasmids. $oriT_{pRJ6}$ was detected in a region of 431 bp, positioned immediately upstream of *mobC*. This region, when cloned into pCN37, was able to confer mobilization to the recombinant plasmid only in the presence of pRJ6. The entire Mob region, including $oriT_{pRJ6}$, is much more similar to Mob regions from several coagulase-negative staphylococci plasmids, although some remarkable similarities with *S. aureus* Mob plasmids can also be noted. These similarities include the presence within $oriT_{pRJ6}$ of the three *mcb* (MobC binding sites), firstly described in pC221 and pC223, an identical *nick* site also found in these same plasmids, and a nearly identical sra_{pC223} site (sequence recognized by MobA). pRJ6 was successfully transferred to *S. epidermidis* by conjugation in the presence of the conjugative plasmid pGO1. Altogether these findings suggest that pRJ6 might have been originally a coagulase-negative staphylococci plasmid that had been transferred successfully to *S. aureus*.

***Keywords*:** mobilization, bacteriocin, *oriT*, *Staphylococcus*, plasmid pRJ6, conjugal transfer

Mobilization is an opportunistic way by which a non-conjugative (mobilizable) plasmid can be transferred between bacteria, using the transfer machinery of a conjugative (self-transmissible) donor plasmid (Francia *et al.*, 2004). All mobilizable (Mob) plasmids that have been examined in sufficient detail show, at least, two well-conserved elements essential for DNA transfer: (i) a site-specific endonuclease, relaxase or MobA, and (ii) a *cis*-acting DNA sequence, the origin of transfer (*oriT*). The mobilization process starts when the relaxase cleaves one of the *oriT* DNA strands by a reversible transesterification to generate a protein-DNA complex at *oriT*, termed relaxosome. After relaxosome formation, the transfer process is performed by the conjugative plasmid transfer machinery (Francia *et al*., 2004). This step is performed by the type IV-like secretion system proteins encoded by the conjugative plasmids (Backert and Meyer, 2006; Abajy *et al*., 2007). In Gram-positive bacteria, this system is very well characterised for the broad host range conjugative plasmid pIP501 (Abajy *et al*., 2007).

Although relaxase and *oriT* seem to be the basic mobilization elements encoded by Mob plasmids, there are other accessory functions that may be present in different combinations, creating a great diversity of Mob plasmids that can be found in both Gram-negative and Gram-positive bacteria (Nomura *et al*., 1996; Perwez and Meyer, 1996; Llosa *et al*., 2002; Grohmann *et al*., 2003; Caryl and Thomas, 2006). The Mob proteins, from which relaxese is the centrally important component, is a family of proteins that helps the correct assemblage of the relaxosome in Mob plasmids in which relaxase is not able to promote mobilization by itself.

Relaxase is the most conserved Mob protein. Amino acid sequence comparison among these proteins is nowadays the most accepted method to construct a phylogenetic classification criterion (Francia *et al*., 2004). Relaxase structure, in a broad overview, can be divided into two functionally distinct domains: an N-terminal catalytic domain, responsible for *oriT* cleavage, and a C-terminal domain that may present a primase activity (for several MOB_Q relaxases) or even a helicase function (in most MOB_F and in a few MOB_Q relaxases) (Grohmann *et al*., 2003; Francia *et al*., 2004). The N-terminal domain typically carries three conserved amino acid sequence motifs: motif I contains the catalytic Tyr residue which nicks the DNA and typically forms a phosphodiester linkage with the nucleotide at the 5' end of the *nick*; motif II is a broadly hydrophobic region generally implicated in maintaining the stability of the protein-DNA interaction; and motif III is proposed to help the nucleophilic activity of the Tyr residue in motif I by coordinating Mg^{2+} or Mn^{2+} ions needed for cleavage of the DNA substrate (Grohmann *et al*., 2003; Francia *et al*., 2004).

The other Mob proteins are quite different from each other and may vary in number (1∼4) in different mobilization systems (Projan and Archer, 1989; Grohmann *et al*., 2003; Francia *et al*., 2004; Smith and Thomas, 2004; Caryl and Thomas, 2006). Their functions are not known for all Mob plasmids, but well characterised examples are available.

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Based on studies performed with the Gram-positive plasmids pC221 and pC223, functions have been proposed for the MobB and MobC proteins of plasmids belonging to the MOB_P relaxase family. MobB is proposed to interact with MobA and to stimulate plasmid mobilization. On the other hand, MobC was found to bind to conserved sequences present on the *oriT* region, increasing the MobA-mediated *oriT* cleavage and, thus, the relaxosome formation (Smith and Thomas, 2004; Caryl and Thomas, 2006). For the remaining Mob proteins, such as MobD and MobE, just a few and indirect information is available. These proteins seem to participate in the mobilization process of some plasmids but performing non-essential roles. Deletion of these proteins barely affects the plasmid transfer frequency (Francia *et al*., 2004).

Despite the examples shown here, there are relatively few ones of well-characterised mobilizable plasmids from Gram-positive bacteria, when compared to the existing data from Gram-negative ones (Grohmann *et al*., 2003; Francia *et al*., 2004). pRJ6 is a small (~8.0 kb) bacteriocinogenic plasmid found in the *S. aureus* strain A70 isolated from commercial milk, in Brazil (Giambiagi-deMarval *et al*., 1990). It codes for aureocin A70, a broad spectrum bactericidal bacteriocin with potential biotechnological applications. pRJ6 has been shown to be mobilizable between *S. aureus* strains by the staphylococcal conjugative plasmid pGO1 (Oliveira *et al*., 1998). Plasmids similar to pRJ6, coding for aureocin A70-like antimicrobial substances, have also been found in both coagulase-positive and -negative *Staphylococcus* spp. strains isolated from cows suffering from bovine mastitis, either from Brazilian or Argentinean herds (Nascimento *et al*., 2002; Nascimento *et al*., 2005a). Genomic fingerprinting of the *S. aureus* strains hosting pRJ6-like plasmids revealed that pRJ6 is found in genetically distinct *S. aureus* strains, suggesting the horizontal spread of this plasmid among the staphylococcal population (Nascimento *et al*., 2005b).

The genes involved in aureocin A70 expression and in plasmid maintenance functions have already been shown by DNA sequencing to be present on pRJ6 *Hin*dIII-A and B fragments (Netz *et al*., 2001). In the present work, we carried out the DNA sequencing of the remaining *Hin*dIII-C and D fragments of plasmid pRJ6 in order to localize and study its Mob region, including *oriT*.

Materials and Methods

Bacterial strains and plasmids

Plasmids and bacterial strains used in this study are described

Table 1. Strains, plasmids, and relevant phenotypes

Strains	Plasmids and relevant phenotypes	References
Staphylococcus aureus		
A70	pRJ6; Bac^+	Giambiagi-deMarval *et al*. (1990)
MB154	pRJ6::Tn*917-lac* (pRJ49); Em^R Bac^+	Oliveira *et al*. (1998)
MB362	pRJ49, pGO1; Em^R Gm^R Bac^+	This study
MB407	pCN37, pGO1; Em^R Gm^R	This study
MB408	pRJ6, pCN37, and pGO1; Em^R Gm^R Bac^+	This study
MB409	pCN*oriT*; Em^R	This study
MB410	pCN*oriT*, pGO1; Em^R Gm^R	This study
MB411	pRJ6, pCN*oriT*, and pGO1; Em^R Gm^R Bac^+	This study
RN2677	*recA*$^-$; Nov^R Rif^R	Novick (1967)
RN4220	*hsdR*$^-$	Novick (1967)
RN7242	pGO1 (52 kb); Gm^R	Projan and Archer (1989)
RN9590	pCN37 (in RN4220 genetic background); Em^R	Charpentier *et al*. (2004)
Staphylococcus spp.		
CN23	*S. warneri*; Tc^R	[a]
CN30	*S. epidermidis*; Tc^R	[a]
Escherichia coli		
DH5α	*lacZ*αM15; *hsdR*$^-$	Sambrook *et al*. (1989)
Topo10	*lacZ*αM15; *hsdR*$^-$	Invitrogen
EC30	pUC18 (2,686 bp); Ap^R	Yanisch-Pérron *et al*. (1985)
EC43	pUC18::*Hin*dIII-C pRJ6; Ap^R	This study
EC72	pUC18::*Hin*dIII-D pRJ6; Ap^R	This study
EC77	pRJ76 (pCR-BluntII TOPO::$oriT_{pRJ6}$); Km^R	This study
Other		
Corynebacterium fimi NCTC7547	Bacteriocin indicator	Giambiagi-deMarval *et al*. (1990)

Ap, ampicillin; Bac, bacteriocin; Em, erythromycin; Gm, gentamicin; *hsdR*$^-$, restriction deficiency; Km, kanamycin; *lacZ*, β-galactosidase gene; Nov, novobiocin; *recA*$^-$, recombination deficiency; Rif, rifampicin

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in Table 1. *Staphylococcus aureus* strains were grown in either Triptic Soy Broth (TSB; Difco, USA), for the mobilization assays, or Brain-Heart Infusion (BHI; Difco), for the bacteriocin production assays. *Escherichia coli* strains were grown in Luria-Bertani broth (Sambrook *et al*., 1989). *Corynebacterium fimi* was grown in an enriched BHI-derived medium containing 10 g/L of *Lab-Lemco* (Difco), 10 g/L of peptone (Difco), and 5 g/L of yeast extract (Difco). When necessary, the media were supplemented with agar (Merk KGaA, Germany) at either 0.7% or 1.5% (w/v) and the following antimicrobials: gentamicin (Sigma Chemical Co., USA; 10 μg/ml), erythromycin (Sigma; 10 μg/ml), novobiocin (Sigma; 5 μg/ml), and rifampicin (Sigma; 5 μg/ml), for *S. aureus*, and ampicillin (Sigma; 75 μg/ml), for *E. coli*.

Plasmid DNA isolation and manipulations

The isolation of plasmid DNA from *S. aureus* was performed by the method described previously (Giambiagi-deMarval *et al*., 1990). Preparation of *E. coli* plasmid DNA was done with Qiagen-tip 20 columns (QIAGEN Inc., Germany) according to the manufacturer's instructions. Restriction enzymes (Invitrogen Ltd., USA), T4 DNA-ligase (Invitrogen), and *Taq* DNA-polymerase (Promega Corp., USA) were each used according to the manufacturer's recommendations. Oligonucleotides were purchased from Promega. PCR products were purified with the Wizard® SV Gel and PCR Clean-Up System (Promega) and DNA fragments were isolated from agarose gels using the same kit.

Transcription analysis of the *mob* genes

Isolation of total RNA from *S. aureus* strain A70 was performed with the RNeasy Mini kit (QIAGEN). Cultures were harvested after 18 h of growth at 37°C. The cells were lysed by incubation with lysozyme (Sigma; 40 mg/ml) and lysostaphin (Sigma; 10 U) in Tris/HCl-EDTA (TE) buffer [prepared in diethylpirocarbonate (DEPC; Sigma) treated water] for 30 min at 37°C. After this step, the procedure followed the manufacturer's recommendations. RNA preparations were quantified by using the NanoDrop-ND1000-Spectrophotometer (Thermo Scientific, USA). Before the RT-PCR analysis, RNA samples were freed from contaminating DNA by extensive incubation with RNase-free DNase I (Invitrogen). DNase I-pretreated RNA (500 ng) was subjected to cDNA synthesis using SuperScript™ III Reverse-Transcriptase (Invitrogen) as described by the manufacturer. cDNA was quantified as described above for RNA. RT-PCR reactions were performed with primers (Table 2) MobCF and MobCR; MobDF and MobDR; MobAF and MobAR, and MobBF and MobBR. Different combinations of these primers were also used, such as: MobCF and MobDR; MobDF and MobAR; MobAF and MobBR, and MobCF and MobBR. An internal control was performed using the primer GyrF and GyrR targeting the housekeeping gene *gyrA*, that codes for the DNA-gyrase subunit A. Yet, a DNA contamination control was performed using this same pair of primers. This control is a PCR reaction using the same conditions of the RT-PCR process without the RT step and it demonstrates the purity of the RNA preparation. The PCR mixtures consisted of 1× reaction buffer, 1 U of DyNAzyme™ II DNA-polymerase (Finnzymes Oy, Finland), 2.5 mM $MgCl_2$, 10 mM concentration of each dNTP (Promega) and 20 pmol of each primer. Amplification was carried out in a MyCycler™ Thermal Cycler (BioRad, USA). After an initial denaturation step at 98°C for 60 sec, 30 cycles of amplification were performed as follows: denaturation at 98°C for 30 sec, annealing at 50°C for 60 sec, and extension at 72°C for 60 sec. The reaction was completed with a final extension at 72°C for 60 sec. The samples were analyzed on a 1% (w/v) agarose gel.

Construction of plasmid pCN*oriT*

Plasmid pCN*oriT* (6.7 kb) was constructed by cloning of a 431 bp DNA fragment of pRJ6, corresponding to the region immediately upstream of the *mobC* gene, into pCN37 (6.3 kb), which is an *E. coli-S. aureus* shuttle vector, harboring the multiple cloning site (MCS) of pUC19. pCN37 contains

Table 2. Primers used in this study

Primer	Sequence	Amplicon size (bp)
MobCF	5'-GGCTAGCGATACGGTTAATGG-3'	350
MobCR	5'-GTCGTCCAGCCTTTGTCG-3'	
MobDF	5'-GGCAAAGGCTAAATTAACAATAC-3'	200
MobDR	5'-TGCCATTTTTATCACGATCTTTC-3'	
MobAF	5'-CGCAACTAAATCAACGTCTCG-3'	500
MobAR	5'-GAATACTGGGCTGTTGATTTCG-3'	
MobBF	5'-GATAGAGACGACGGCTACG-3'	450
MobBR	5'-CGCTTATATGCGTTTAAAATGCC-3'	
GyrF	5'-GCGTGAATCATTTTTAGATTATGCG-3'	447
GyrR	5'-AAGTTAGGGAATCGAGCAG-3'	
Ori1	5'-GTATTCTTTATAAGAATATGGGGTGTCTA-3'	431
Ori2	5'-CATCCCACCACGCTTTCTAC-3'	

the pT181 basic replicon for stable maintenance in *S. aureus*, the erythromycin resistance cassette from pE194 and the *amp* ColE1 *ori*, conferring ampicillin resistance and stable maintenance in *E. coli* (Charpentier *et al*., 2004). This fragment was PCR-amplified using primers Ori1 and Ori2 (Table 2), ligated to pCR-Blunt II-TOPO (Invitrogen) according to the manufacturer's instructions, and transformed into thermal-competent *E. coli* DH5α. Five kanamycin-resistant transformants were chosen for plasmid DNA extraction and sequencing to confirm the correct amplicon sequence prior to digestion with *Eco*RI to liberate $oriT_{pRJ6}$. This fragment was then ligated into pCN37 digested with the same enzyme to generate pCN*oriT*. The recombinant plasmid was transformed into electrocompetent RN4220 cells as described elsewhere (Schenk and Laddaga, 1992). Ten erythromycin-resistant transformants were chosen for plasmid DNA isolation and the presence of $oriT_{pRJ6}$ was confirmed by PCR using primers Ori1 and Ori2. The strain carrying pCN*oriT* was named MB409.

Construction of heteroplasmid strains

All heteroplasmid strains used in this study (Table 1) were constructed by either transductions (Bastos *et al.*, 1980) or filter matings (Projan and Archer, 1989), as previously described. To create a strain harboring pCN*oriT* and pGO1 (named MB410), MB409 (pCN*oriT*) was conjugated with RN7242 (pGO1) and transconjugants were selected for Em^R and Gm^R. To create strain MB407 (pCN37 and pGO1), RN9590 (pCN37) was conjugated with RN7242 and transconjugants were selected for Em^R and Gm^R. To create strains MB408 (pRJ6, pCN37, and pGO1) and MB411 (pRJ6, pCN*oriT*, and pGO1), transductions with phage 80α, involving either strains RN9590 (pCN37) and A70 (pRJ6) or strains MB409 (pCN*oriT*) and A70 were performed. In each case, the resulting Em^R transductants were then conjugated with RN7242 and selections were performed for Em^R and Gm^R transconjugants. Finally, strain MB362 (pRJ49 and pGO1) was created by a filter mating involving strains MB154 (pRJ49) and RN7242, selecting for Gm^R and Em^R transconjugants.

Mobilization experiments

These experiments were performed to access the mobilization transfer of pCN*oriT* and pCN37, either alone or in the presence of pRJ6. The mobilization was accessed by the filter-mating technique, as described elsewhere (Projan and Archer, 1989), using strain RN2677 as recipient and strains MB407, MB408, MB410, and MB411 as donors. Transconjugants were selected in the presence of either Gm (for pGO1) or Em (for either pCN37 or pCN*oriT*), both at 10 μg/ml, and always in the presence of novobiocin and rifampicin, both at 5 μg/ml, to counterselect the donor strains. Em^R transconjugants were checked for the presence of plasmids, Gm^R and bacteriocin production, as described elsewhere (Giambiagi-deMarval *et al*., 1990). The bacterial strain used to detect bacteriocin production was *Corynebacterium fimi* NCTC 7547 (Giambiagi-deMarval *et al*., 1990).

DNA sequencing and sequence analysis

pRJ6 *Hin*dIII-C and D fragments were individually cloned into the unique *Hin*dIII site of pUC18 and sequenced using the universal sequencing primers M13 forward and M13 reverse (Yanisch-Pérron *et al*., 1985). Plasmid DNA templates were prepared following strictly the protocol suggested by Applied Biosystems (USA). The automated sequencing was performed using the ABI Prism 3100 System and the Terminator Chemistry Big Dyes, version 3.1 (Applied Biosystems). Analysis of the sequence and ORF prediction were performed using the program Web Map Preferences (http://pga.mgh.harvard.edu/web_apps/web_map/start). Sequence homology searches were carried out using Basic Local Alignment Search Tool (BLAST) analysis. Multiple sequence alignment was done using the BioEdit software (Hall, 1999). Protein transmembrane domains were predicted by using the TMpred Server (http://www.ch.embnet.org/ software/TMPRED_form.html). The complete nucleotide sequence of the *Hin*dIII-C and D fragments of plasmid pRJ6 was deposited in the GenBank database as an update of the accession number AF241888.

Results

*Hin*dIII C-D fragments sequence analysis

A 6,332 bp region of pRJ6, encompassing both *Hin*dIII-A and B fragments, was previously sequenced. DNA sequence analysis of this region revealed the presence of four transcriptional units involved in either bacteriocin expression or plasmid replication (Netz *et al*., 2001). Moreover, one complete open reading frame (ORF) and a partial ORF, that could be involved in pRJ6 mobilization, were found on the left end of the *Hin*dIII-B fragment. To identify any other possible ORF involved in pRJ6 mobilization, sequencing of the *Hin*dIII-C and D fragments was required (Supplementary Fig. 1). pRJ6 size was thus determined as 7,904 bp [1,513 bp from *Hin*dIII-CD fragments plus 6,391 bp from *Hin*dIII-AB (Netz *et al*., 2001)] and its genetic map could finally be generated (Fig. 1).

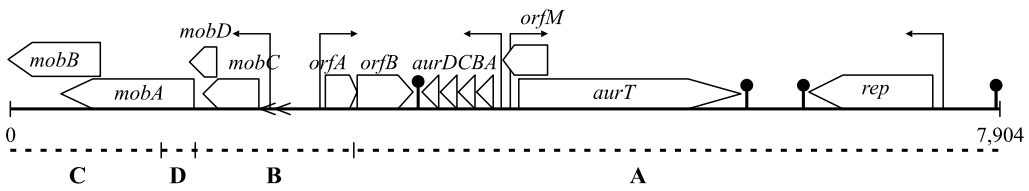

Fig. 1. Genetic organization of the bacteriocinogenic plasmid pRJ6 (7,904 bp). The map is oriented with the restriction site corresponding to the beginning of the *Hin*dIII-C fragment at the +1 position. The arrows indicate putative promoters; lollipop symbols represent putative transcription terminators; '<<' represents the *oriT* region. The bar below the figure represents the *Hin*dIII fragments.

Searching for mobilization genes within the *Hin*dIII-B, C, and D fragments

To search for the mobilization genes in this region, program WebMap was used to identify any possible ORF. This analysis revealed four putative open reading frames resembling an operon. Since all four pRJ6 functions encoded by this region exhibited strong similarity to functions associated to plasmid mobilization, they will be referred to as *mobC*, *D*, *A*, and *B*, respectively.

The first gene, *mobC*, starts at the left end of fragment *Hin*dIII-B. Its predicted product, MobC, is a protein of 127 amino acids (14.5 kDa), with an estimated p*I* of 9.67, and a high level identity with MobC-like proteins from several coagulase-negative *Staphylococcus* (CNS) plasmids, such as pSHaeC (85%), pSERP (84%), and pIP1629 (81%) (see Fig. 2 of Supplementary material).

The second gene, *mobD*, is the smallest ORF found. It overlaps *mobC* by 19 bp and encodes a polypeptide with only 65 amino acids (7.5 kDa) and an estimated p*I* of 9.15. $MobD_{pRJ6}$ exhibited a low identity with MobD-like proteins from six CNS plasmids [pSE-12228-06 (32%), pSHaeC (23%), pSERP (23%), pIP1629 (21%), pIP1630 (16%), and pSK639 (10%)] (Fig. 2A).

mobA overlaps the 3' end of *mobD* by 13 bp and codes for a putative protein of 325 amino acids (37.5 kDa) with an estimated p*I* of 8.76. It shows a high identity with MobA-like proteins of staphylococcal plasmids, such as pSERP (86%), pIP1629 (85%), and pSHaeC (85%), and a significant identity (47%) with MobA of pC221. $MobA_{pRJ6}$ sequence alignment with the other MobA proteins reveals a strong sequence conservation within the first 125 amino acids (corresponding to relaxase motifs I, II and III; Fig. 2B) with a

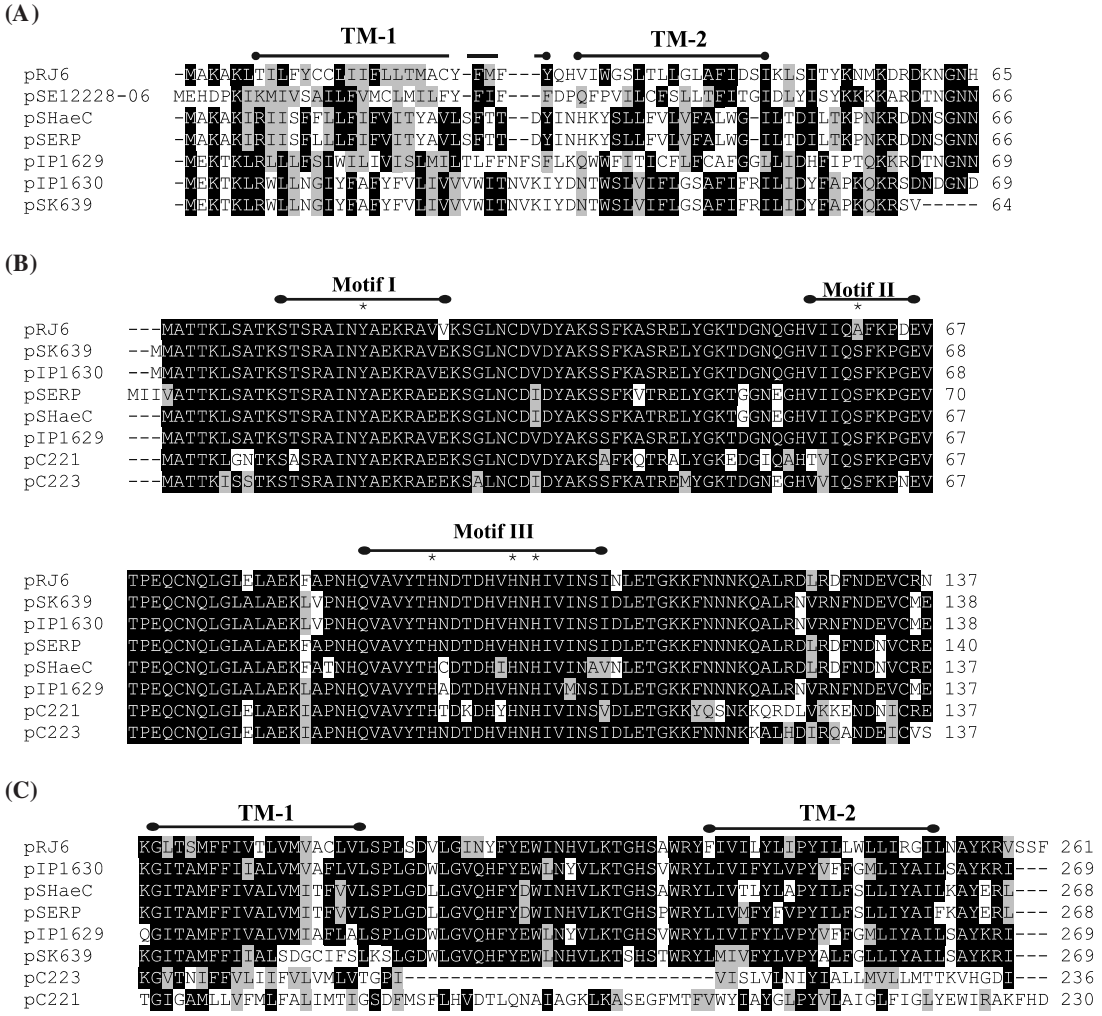

Fig. 2. Informative alignments between pRJ6 Mob proteins and other *Staphylococcus* spp. plasmids Mob proteins. (A) MobD showing its putative transmembrane regions (TM-1 and TM-2; indicated by dot-ended bars above sequences), (B) MobA (residues 1 to 137) showing the three conserved N-terminal motifs (indicated by dot-ended arrows) and functional amino acid residues in each motif (marked with an asterisk), (C) MobB (residues 182 to 261) showing its putative C-terminal transmembrane regions (TM-1 and TM-2; indicated by dot-ended bars above sequences). Dark shaded sequences indicate identical amino acids, while grey shaded ones indicate conservative substitutions. Regions with low or no conservation are indicated in white; (-) indicates the gaps between the sequences.

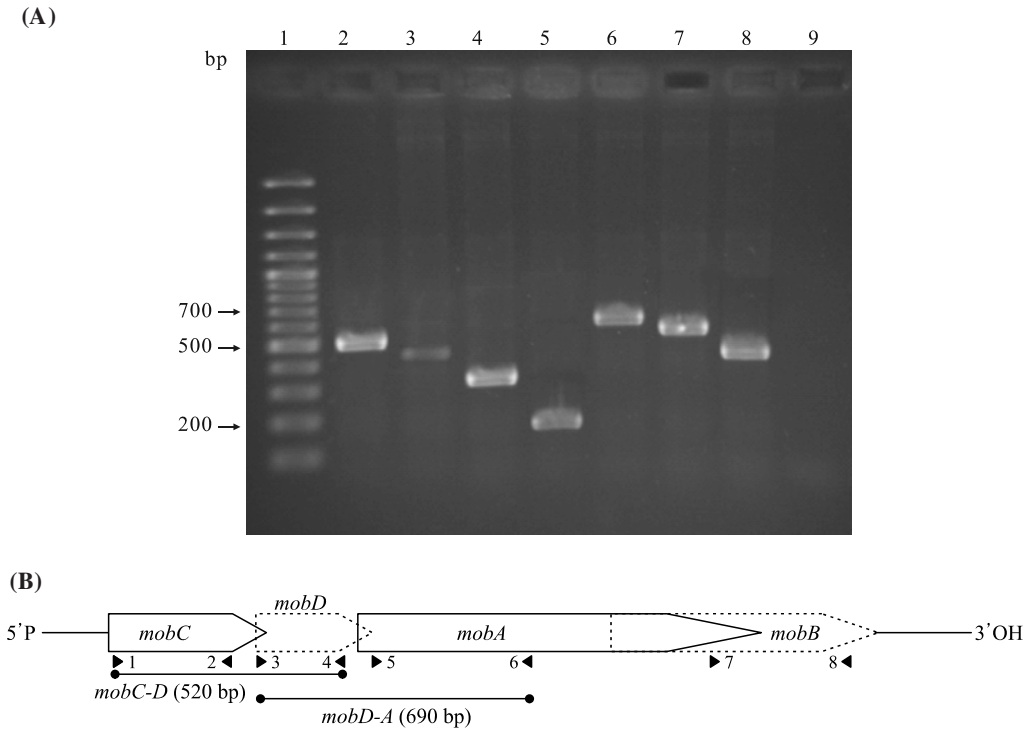

Fig. 3. (A) RT-PCR analysis of the pRJ6 *mob* genes. 1, 100 bp DNA ladder (New England Biolabs); 2, *mobA* (500 bp); 3, *mobB* (450 bp); 4, *mobC* (350 bp); 5, *mobD* (200 bp); 6, *mobD-mobA* (690 bp); 7, *mobC-mobD* (520 bp); 8, *gyrA* (447 bp; internal control); 9, DNA contamination control. (B) Schematic view of the RT-PCR amplicons. 1 to 8, oligonucleotides used in the analysis, following the order of Table 2. The dotted bars indicate the amplicons involving two adjacent genes and their sizes.

less pronounced conservation in the remaining two-thirds of the proteins. This appears to be a common feature of all relaxases.

The last orf, *mobB*, exhibits an extensive overlapping region (269 bp) with the 3' end of *mobA* and codes for a protein of 261 amino acids (30.8 kDa) with an estimated p*I* of 8.42. It shows a significant identity with MobB-like proteins from several staphylococcal plasmids, such as pIP1630 (67%), pSHaeC (67%), pSERP (65%), and pC223 (31%). However, MobB of pRJ6 carries a 10-residue deletion in its N-terminal region when compared to other plasmids. Hydrophobicity analysis (TMpred Server; data not shown) of all these proteins predicted the presence of two highly hydrophobic alpha-helices (residues 183 to 213 and 232 to 252, in relation to pRJ6) in the C-terminal region of the protein (Fig. 2C), suggesting that $MobB_{pRJ6}$ is probably a membrane-bound protein, potentially locating the relaxosome in the cell membrane prior to transfer. The membrane insertion model predicted for MobB points its N-terminal portion towards the protoplasm (data not shown).

Putative Shine-Dalgarno (SD) sequences (Shine and Dalgarno, 1974) could be detected upstream of *mobC*, *mobD*, and *mobB* but no recognizable one could be predicted for *mobA* (Supplementary Fig. 1).

mob Genes are transcribed as an operon

A putative SigA-dependent promoter is located upstream of *mobC*. A-10 region, with five matches to the consensus sequence (TATAAT), is found 49 bp upstream of the start codon ATG, and a -35 region, with four matches to the consensus sequence (TTGACA), is located 70 bp upstream of the start codon ATG (Supplementary Fig. 1). In between, there is a spacer of 15 bp. Since no obvious promoters were found upstream of the remaining *mob* genes, these data suggested that the pRJ6 *mob* genes are arranged as on operon. An inverted repeat resembling a putative *rho*-independent stem-and-loop transcriptional terminator (ΔG -18.4 Kcal/mol) was detected 225 bp downstream of the last gene, *mobB*, and may be involved in termination of the operon transcription (Supplementary Fig. 1).

RT-PCR analysis detected the transcription of the four genes contained in the *mob* region of pRJ6 (Fig. 3). It was also possible to detect amplicons corresponding to co-transcription of either *mobC* and *mobD*, or of *mobD* and *mobA*, confirming that theses genes are coordinately transcribed. However, it was not possible to detect co-transcription of either *mobA* and *mobB* or *mobC* and *mobB*.

Searching for an *oriT* region

A 431 bp region immediately upstream of *mobC* was chosen for search of $oriT_{pRJ6}$. Homology analysis involving this region and the well characterised *oriT* regions from *S. aureus* plasmids pC221 and pC223 (Fig. 4) led to detection of a 210 bp region with high identity among these plasmids, which includes three inverted repeats and two identical mcb_{pC223} sites, among other similar features (Smith and Thomas, 2004;

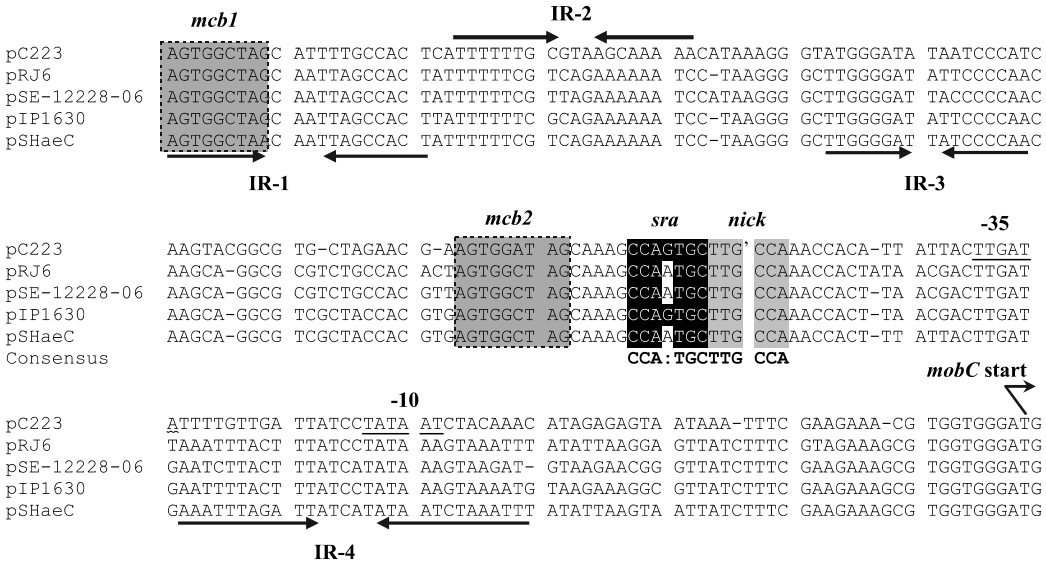

Fig. 4. Alignment of the final 210 bp from the putative *oriT* region of pRJ6 and the *oriT* regions from different staphylococcal plasmids. Dark grey boxes represent homologous MobC binding site sequences (*mcb*; agtggctag) identified primarily in plasmids pC221 and pC223. Solid arrows represent inverted-repeated sequences found in all plasmids analyzed. The black shadow shows the MobA recognition site (*sra*) from pC223 and other *sra*-like sequences found in the other plasmids, including pRJ6 (non-conserved bases are shown in white). pC223 MobA *nick*-like sequences are indicated in light grey and the exact nicking position in pC223 is shown by a comma. -35 and -10 *mobC* putative promoter regions are underlined. The putative *mobC* start codon is indicated by an arrow. All information about pC223 can be found elsewhere (Caryl and Thomas, 2006).

Caryl and Thomas, 2006). pRJ6 *mcb1* and *mcb2*, which contain conserved asymmetric 9 bp direct repeats, are located 106 bp (*mcb1*) and 15 bp (*mcb2*) upstream of the pRJ6 putative *nick* site. A third direct repeat, designated *mcb3*, located within the N-terminal coding region of *mobC* and containing 7 bp in common with the other *mcb*s, was not included in the 431 bp selected region, although it is present in pRJ6 (Fig. 1 of Supplementary material).

A nearly identical *sra* sequence from pC223, that serves as the MobA recognition site prior to cleavage, could also be detected in $oriT_{pRJ6}$, possessing only one base substitution (G→A). An identical pC223 cleavage (*nick*) site was also found on $oriT_{pRJ6}$, that might be the exact point where $MobA_{pRJ6}$ cleaves its cognate *oriT*.

Table 3. Analyses of either plasmid conjugation or mobilization

Donor strains (plasmids)	Conjugation or mobilization frequency*	
	Gm	Em
RN7242 (pGO1)	2.6×10^{-6}	NT
MB362 (pGO1; pRJ49)	2.2×10^{-6}	1.6×10^{-6}
MB407 (pGO1; pCN37)	1.1×10^{-6}	$<1.2\times10^{-10}$
MB410 (pGO1; pCN*oriT*)	1.9×10^{-6}	$<1.5\times10^{-10}$
MB411 (pGO1; pCN*oriT*; pRJ6)	2.2×10^{-7}	1.6×10^{-6}
MB408 (pGO1; pCN37; pRJ6)	1.2×10^{-7}	$<2.0\times10^{-10}$

Strain RN2677, a strain with no plasmid DNA, was used as recipient in all experiments. The frequencies represent the number of transconjugants per donor cells and are the means of two independent experiments; NT, not tested; Gm, selection using gentamicin (for pGO1); Em, selection using erythromycin (for pRJ49, pCN37, and pCN*oriT*); <, no transconjugants could be detected in the experiments performed.

Mobilization assays to test *oriT* functions

To test if the putative *oriT* region found on pRJ6 was functional, the 431 bp region was amplified and cloned into pCR-Blunt II-TOPO. Correct DNA sequence amplification was confirmed by DNA sequencing. This fragment was then sub-cloned into pCN37, creating plasmid pCN*oriT* (Supplementary Fig. 3).

Mobilization experiments, in the presence of the staphylococcal conjugative plasmid pGO1, were then performed with the donor strains RN7242, MB362, MB407, MB408, MB410, and MB411 and the recipient strain RN2677. Transconjugants harboring pCN*oriT* or pCN37 were selected in the presence of Em, while those carrying pGO1 were selected in the presence of Gm. Em^R transconjugants were also tested for Gm^R and for the presence of pGO1. Detection of transconjugants carrying only Em^R but not Gm^R was indicative of transfer of either pCN*oriT* or pCN37 alone and, therefore, of mobilization. In each case, DNA extraction of randomly selected transconjugant colonies, followed by agarose gel electrophoresis, was used to ensure the presence of the expected plasmids. The results of these crosses are summarized in Table 3. It was observed that plasmid pCN*oriT* could be mobilized by the conjugative plasmid pGO1 only in the presence of the Mob functions of pRJ6. The mobilization frequencies of pCN*oriT* were similar to those of pRJ49, a derivative of plasmid pRJ6 tagged with transposon Tn*917-lac* and not affected in mobilization (Oliveira *et al*., 1998). Transfer of pCN*oriT* alone was confirmed by agarose gel electrophoresis of plasmid DNA preparations obtained from randomly selected Em^R transconjugants (Fig. 5). Besides, no bacteriocin production could be detected in these selected

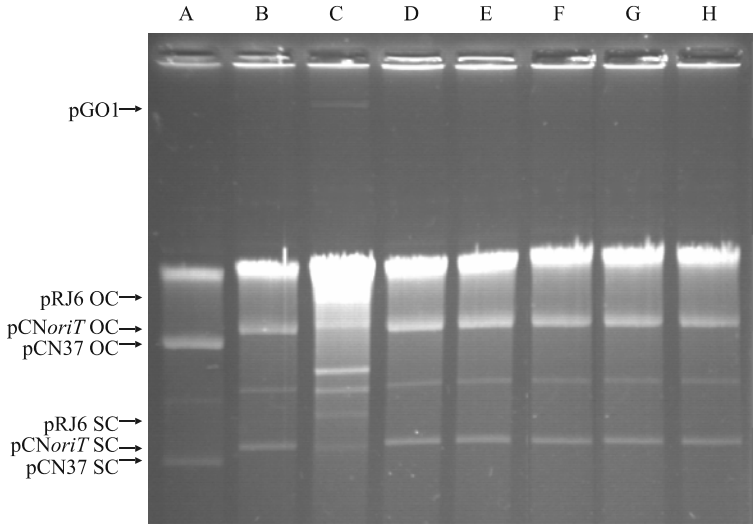

Fig. 5. Agarose gel electrophoresis of whole cell lysates from transconjugants from matings between MB411 and RN2677, confirming the mobilization of pCN*oriT* alone. A, RN9590 (pCN37); B, MB409 (pCN*oriT*); C, MB411 (pGO1, pRJ6, and pCN*oriT*); D to H, transconjugants carrying only the pCN*oriT* plasmid. Plasmid forms are indicated on the left. OC, open circle; SC, supercoiled. The bands between the SC and OC forms are the linear forms of the plasmids.

transconjugants, confirming the absence of plasmid pRJ6.

Transfer of pRJ6 to coagulase-negative *Staphylococcus* spp. strains

To test if pRJ6 could be successfully transferred to other *Staphylococcus* species, strains of CNS, *S. epidermidis* and *S. warneri*, which were Em^S and not susceptible to aureocin A70 produced by the donor strain, were used as recipients in mating experiments. Mobilization of the pRJ6 derivative, pRJ49, could only be detected to *S. epidermidis* in frequencies of 2.0×10^{-9}. Despite the low number of CNS strains used as recipients, which was due to an unavailability of additional CNS strains sensitive to erythromycin and resistant to aureocin A70, these results suggest that pRJ6 can be transferred to other staphylococcal species rather than only to *S. aureus*.

Discussion

pRJ6 was shown to possess four genes (*mobCDAB*) as the only candidates to be involved in its mobilization. The proposed organization of these genes as an operon is supported by the presence of a putative promoter only upstream of the first gene (*mobC*), suggesting that the putative *mob* operon is most probably transcribed by the housekeeping SigA-dependent RNA-polymerase, and by the presence of a putative Rho-independent transcription terminator located downstream of the last gene, *mobB*. SD sequences could be detected preceding *mobC*, *mobD*, and *mobB*, but no recognizable one could be detected preceding *mobA*, which encodes the key protein in plasmid mobilization. It has already been reported that not all genes contain an SD sequence and that the lack of an SD sequence might substantially reduce the expression level of a given gene (Ma *et al*., 2002). If it occurs with *mobA*, it could help to regulate plasmid mobilization.

RT-PCR analysis showed that all four genes are transcribed and that *mobC*, *mobD*, and *mobA* are coordinately transcribed. Unfortunately, it was not possible to prove that *mobB* is co-transcribed with the other genes. Bacterial mRNAs generally have a short half-life. Their degradation may involve activity of endonucleases which attack the RNA being transcribed, even before the transcription reaches its end, which may result in lacking of a template region for primers annealing with either *mobA* or *mobC*, which are located upstream of *mobB*. Therefore, the lack of detection of *mobA-mobB* and *mobC-mobB* amplicons may be due to mRNA degradation reducing the amount of the full-length mRNA of nearly 2.3 kb.

A DNA single strand is thought to be transferred between donor and recipient cells during conjugation. The only locus required *in cis* for generation of the single-stranded plasmid intermediate is the *oriT*, where relaxase exerts its cleavage. In *Staphylococcus*, the better characterized *oriT*s are those from plasmids pC221 and pC223. These *oriT*s contain a series of conserved features that include many inverted repeats, three MobC binding sites (*mcb*), a site recognized by MobA (*sra*), and the cleavage site (*nick*) (Caryl and Thomas, 2006).

All three MobC binding sites within $oriT_{pRJ6}$ are highly conserved in terms of both sequence and location within the *oriT* of a number of staphylococcal plasmids. Based on studies performed with the pC221-family *oriT*s, the presence of three MobC binding sites has the potential for DNA looping or other high-order structures with a core of MobC-mediating interactions between these positions (Caryl and Thomas, 2006).

The *sra* site located in the putative $oriT_{pRJ6}$ is nearly identical to that found in pC223, presenting only a base pair substitution. If this sole base substitution is enough to promote $MobA_{pRJ6}$ site specificity, it is not possible to infer. For pC221 and pC223, the difference of 4 bp (mainly tran-

sitions) between both *sra* sites is enough to promote site-specificity. In this case, experiments performed with both pC221 and pC223 variants, possessing interchangeable *sra*, demonstrated that $MobA_{pC221}$ is capable of cleaving, although with less efficiency, the pC221 variant carrying sra_{pC223}, while $MobA_{pC223}$ is more specific, being unable to cleave the pC223 variant carrying sra_{pC221} (Caryl and Thomas, 2006). Here, the tiny difference found between sra_{pRJ6} and sra_{pC223} might result in both MobA proteins being interchangeable without loss of MobA cleavage capability, once both *nick* sites are nearly identical. Such assumption, however, needs to be tested.

The high similarities between $MobC_{pRJ6}$ and the MobC proteins from plasmids pC221 and pC223 suggest that these proteins might be homologues. It is proposed that pC221 and pC223 MobC function at several levels: primarily, to initiate nicking by MobA, presumably via interaction at *mcb2*; and subsequently, by mediating a potential high-order complex formation, involving *mcb1* and *mcb3*, to yield a mobilizable substrate. Although experimental evidence is lacking for the role of $MobC_{pRJ6}$, the high identity (73%) exhibited by this protein and the $MobC_{pC221}$ encourages us to believe that they may function similarly. Moreover, the conserved motif (L/FxxxG/SxNxNQxAxxxN), suggested to be present in all MobC proteins (Apisiridej *et al*., 1997), is also found in $MobC_{pRJ6}$ (Supplementary Fig. 2).

In contrast with MobC, MobD is the most divergent Mob protein found among all mobilizable staphylococcal plasmids, described so far, that possess four Mob proteins. Interestingly, it is absent from the *S. aureus* plasmids pC221, pS194, and pC223, but seems to play an important role in the CNS mobilizable plasmids, being present in all of them characterised so far. Despite the low similarities amongst the MobD, the presence of two putative transmembrane domains in all of them could point to a possible conserved function of these proteins.

The relaxases of the staphylococcal plasmids can be allocated in three different families, based on the classification scheme proposed by Francia and co-workers (Francia *et al*., 2004). This grouping is based on the homologies between their N-termini (first 125 amino acids). $MobA_{pRJ6}$ can be placed in the MOBp family of relaxases, which also includes the RP4 relaxase (TraI), the prototype protein of this family. $MobA_{pRJ6}$ motifs I and III present the key conserved amino acid residues (Y18, H94, H101, and H103) described for relaxases of the MOBp family, which are involved in the transesterification reaction and coordination of Mg^{2+} and Mn^{2+} ions (Grohmann *et al*., 2003; Francia *et al*., 2004). In relation to motif II of $MobA_{pRJ6}$, an amino acid substitution (S61→A) was observed when compared to the MOBp family motif II consensus (IIQSFKPGE). An identical substitution (S74→A) was experimentally introduced into RP4 TraI, which resulted in a destabilizing effect in the relaxosome formation and in an increasing detection of plasmid relaxed forms in RP4 DNA preparations (Pansegrau *et al*., 1994). The C-terminal domain of $MobA_{pRJ6}$ shows no sequence motifs corresponding to helicase or primase functions; its role in mobilization may involve interaction with other Mob proteins.

The last mob protein found in pRJ6 is MobB. In the well-studied staphylococcal plasmids pC221 and pC223, MobB is proposed to interact with the C-terminal half of MobA. Disruption of MobB in both plasmids did not alter dramatically relaxosome formation, but completely abolished the mobilization process (Smith and Thomas, 2004). Being a membrane protein, $MobB_{pRJ6}$, which shares similarity with $MobB_{pC221}$ and $MobB_{pC223}$, could either act as a bridge, or modify the conformation of $MobA_{pRJ6}$ to better fit the conjugal machinery.

The mobilization frequency of pCN*oriT* was comparable to that of the pRJ6 derivative, pRJ49. Since pCN*oriT* carries a 431 bp fragment from pRJ6, which is supposed to carry $oriT_{pRJ6}$, and since no mobilization of pCN37 could be detected either in the presence or in the absense of pRJ6, the mobilization of pCN*oriT* can only be attributed to the presence of the $oriT_{pRJ6}$. Moreover, transfer of pCN*oriT* alone in the presence of pRJ6 was also indicative of the functionality *in trans* of the Mob proteins encoded by pRJ6.

In conclusion, the Mob region of the bacteriocinogenic plasmid pRJ6 has been characterized, revealing that the Mob regions of the staphylococcal plasmids are quite similar. The *oriT* region of these plasmids show a high degree of conservation that includes regions expected to be different, such as the MobA binding site and the *nick* site, regarding the plasmid-*oriT* specificity attributed to MobA. This similarity was even higher among the *S. epidermidis* plasmids and pRJ6. Based on these observations, one could speculate that the MobA proteins from the different *S. epidermidis* Mob plasmids could be interchangeable without the plasmids losing their mobilization capability. Altogether these findings also suggest that pRJ6 might have been originally a CNS plasmid that had been transferred successfully to *S. aureus.*

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