Investigating the basis of substrate recognition in the pC221 relaxosome

Jamie A. Caryl and Christopher D. Thomas*
Astbury Centre for Structural Molecular Biology, Institute of Molecular and Cellular Biology, University of Leeds, Leeds LS2 9JT, UK.

Summary
The nicking of the origin of transfer (oriT) is an essential initial step in the conjugative mobilization of plasmid DNA. In the case of staphylococcal plasmid pC221, nicking by the plasmid-specific MobA relaxase is facilitated by the DNA-binding accessory protein MobC; however, the role of MobC in this process is currently unknown. In this study, the site of MobC binding was determined by DNase I footprinting. MobC interacts with oriT DNA at two directly repeated 9 bp sequences, mcb1 and mcb2, upstream of the oriT nic site, and additionally at a third, degenerate repeat within the mobC gene, mcb3. The binding activity of the conserved sequences was confirmed indirectly by competitive electrophoretic mobility shift assays and directly by Surface Plasmon Resonance studies. Mutation at mcb2 abolished detectable nicking activity, suggesting that binding of this site by MobC is a prerequisite for nicking by MobA. Sequential site-directed mutagenesis of each binding site in pC221 has demonstrated that all three are required for mobilization. The MobA relaxase, while unable to bind to oriT DNA alone, was found to associate with a MobC–oriT complex and alter the MobC binding profile in a region between mcb2 and the nic site. Mutagenesis of oriT in this region defines a 7 bp sequence, sra, which was essential for nicking by MobA. Exchange of four divergent bases between the sra of pC221 and the related plasmid pC223 was sufficient to swap their substrate identity in a MobA-specific nicking assay. Based on these observations we propose a model of layered specificity in the assembly of pC221-family relaxosomes, whereby a common MobC:mcb complex presents the oriT substrate, which is then nicked only by the cognate MobA.

Introduction
The initial events in mobilization of bacterial plasmids are characterized by two distinct cellular and molecular processes: DNA processing reactions, involved with preparing the plasmid for transfer; and utilization of the conjugative transfer apparatus involved in the mating-pair formation between donor and recipient cells.

The complex most frequently associated with conjugal DNA processing is the relaxosome, a nucleoprotein structure that includes a relaxase, which is a site- and strand-specific transesterase, typically associated with accessory proteins. These protein components interact with the origin of transfer (oriT) DNA and result in a phosphodiester bond cleavage from which transfer of a single-stranded DNA is initiated. The relaxosome thus provides a substrate for the membrane-spanning conjugal transfer apparatus.

Much of the current understanding of these processes has been derived from work on plasmids in Gram-negative bacteria (reviewed by Zechner et al., 2000). In contrast, the transfer regions of conjugal and mobilizable plasmids in Gram-positive bacteria have received comparatively little attention (Grobmann et al., 2003). Staphylococcal plasmid pC221 presents a simple system embodying the DNA processing reaction and is one of a number of closely related small (<5 kb), mobilizable antibiotic-resistance plasmids found in the staphylococci, which also include pC223 (Smith and Thomas, 2004), pS194 (Projan et al., 1988), pRJ6 (Netz et al., 2001) and pRJ9 (Netz et al., 2002) of Staphylococcus aureus; and pIP1629, pIP1630 (Aubert et al., 1998) and pSK639 (Apisiridej et al., 1997) of Staphylococcus epidermidis.

Typically for a small plasmid, pC221 encodes only those functions required for DNA processing and contains four such loci: a cis-acting oriT; a DNA relaxase, MobA; and the accessory proteins MobB and MobC. It is thus mobilizable only in the presence of a compatible, co-resident conjugative plasmid such as pGO1, which encodes the required conjugal transfer apparatus (Projan and Archer, 1989). The minimal requirements for nicking have been defined using purified components in vitro (Caryl et al., 2004). The MobA relaxase makes a reversible site- and strand-specific nick at a highly conserved sequence within oriT (5′-GCTTG’CCAAA-3′), whereupon it forms a
phosphodiester bond with the 5’ end of the nicked strand. Nicking of supercoiled plasmid DNA substrate by MobA is dependent on the presence of the dimeric accessory protein MobC and divalent metal ions such as Mg$^{2+}$; however, the process by which MobC mediates the cleavage by the relaxase is not yet understood.

While relaxase proteins themselves tend to share a core set of conserved functions, such as the presence of N-terminally encoded catalytic motifs and the requirement for a single-stranded DNA substrate and divalent metal cations, accessory proteins have more diverse lineages. This may reflect the origin of these proteins as components sequestered from the cellular background within which the respective plasmids have evolved (Parker et al., 2005). Almost all relaxases characterized to date are able to cleave single-stranded DNA substrates containing a nick site sequence in the absence of accessory proteins (Zechner et al., 2000); thus one role for the accessory proteins may be to provide a relaxase substrate with sufficient single-stranded character within a supercoiled DNA context. This may be achieved by enhancing strand denaturation at nic. In the case of plasmid R1162, the MobC protein extends MobA-induced DNA strand melting at oriT up to the nick site, potentially revealing a single-stranded substrate for cleavage (Zhang and Meyer, 1995; 1997).

Alternatively, accessory proteins may act as recognition complexes to initiate relaxosome assembly, either by direct binding proximal to nic or indirectly by binding elsewhere and inducing significant bending at the cleavage site. In plasmid RP4, the TraJ accessory protein binds to the proximal arm of an inverted repeat upstream from the nick site (Ziegelin et al., 1989), and is believed to act as a recognition complex for the TraI relaxase. The same is true for the NikA accessory protein of plasmid R64: it has been proposed that binding of NikA bends the DNA at that point with the resulting conformational change playing a role in nicking (Furuya and Komano, 1997). The accessory proteins TraYp and IHF bind sequentially to oriT of plasmid F to facilitate binding by the Tralp relaxase. Such binding may present a suitable nicking substrate via possible protein–protein distortion of the DNA at the nick site (Howard et al., 1995; Byrd and Matson, 1997).

In addition, accessory proteins may also act in a regulatory manner: F plasmid TraYp binds a second site at the P$^{+}$ promoter and is required for upregulation of tra gene expression (Silverman and Sholl, 1996). In R388, the TrnA accessory protein acts as both a transcriptional repressor of the trwABC operon and an enhancer of TrnC relaxase-mediated nicking (Moncalian et al., 1997). Both TraJ and TraK of RP4 repress their traJp and traKp promoters respectively (Zatyka et al., 1994).

The MobC proteins of pC221 and pC223 are interchangeable with regard to nicking, with the MobA proteins displaying specificity towards their respective substrates despite the highly conserved nature of the oriT region (Caryl et al., 2004). The pC221 MobA relaxase represents one of three unrelated relaxases from Gram-positive bacterial plasmids that have been subjected to detailed characterization to date. The remaining two are TraA of the enterococcal plasmid pIP501 (Wang and Macrina, 1995; Kurenbach et al., 2003; Kopec et al., 2004), and MobM of the streptococcal rolling-circle replicon pMV158 (Guzman and Espinosa, 1997; Grohmann et al., 1999; de Antonio et al., 2004). These relaxases are grouped into the MOB$\alpha$ and pMV158/Pre families respectively (Francia et al., 2004), which account for the majority of relaxases identified in Gram-positive bacterial plasmids (Grohmann et al., 2003). Indeed, the transfer functions of large conjugative staphylococcal plasmids pGO1 and pSK41 are also grouped within the MOB$\alpha$ family of relaxases. In contrast, the pC221-family of relaxases are grouped within the MOB$\beta$ family, most commonly associated with the plasmids of Gram-negative bacteria. This family also includes TraI of the conjugative plasmid RP4 and VirD2 of the agrobacterium pTi plasmid pTiC58 (Francia et al., 2004).

Of the mobilization functions of Gram-positive plasmids listed above, those of the pC221 family represent the only examples among the staphylococci for which detailed biochemical analysis has been reported. Elucidation of the mechanism of relaxosome formation and DNA processing in pC221 will provide a useful comparison to the well-known MOB$\alpha$ family member RP4, as well as providing the model for all pC221-related mobilization functions.

In this work we present data regarding the formation and specificity of the pC221 relaxosome, with the objective of differentiating the respective roles of the MobC and MobA proteins in substrate recognition. We report the use of DNase I footprinting to identify the sites of interaction of both MobC and MobA proteins with oriT, and confirm the sequence specificity of MobC through both electrophoretic mobility shift assay (EMSA) and Surface Plasmon Resonance (SPR) techniques. By mobilization studies in vivo and cleavage assays in vitro we have identified a minimal, functional oriT which itself contains a minimal nicking region. Site-directed mutagenesis of the latter has revealed elements essential for plasmid-specific cleavage by the cognate MobA relaxases of pC221 and pC223. Based on these observations we present a model of layered specificity that governs the assembly of the pC221 relaxosome.

**Results**

MobC protein binds to a specific sequence at three positions within oriT

The propensity of MobC to bind linear oriT DNA has been described previously (Caryl et al., 2004); a supercoiled
DNA substrate is required for the nicking reaction. An alternative DNase I footprinting methodology that permitted the probing of supercoiled substrates was employed. The technique, described by Gralla (1985), uses end-labelled oligonucleotide primers in a primer extension reaction to map strand discontinuities introduced within the target region by DNase I. As the DNase I cleavage products are not isotopically labelled, they can be re-probed a number of times with the same or different primers, or stored indefinitely. In addition, the ability to use large templates increases the site-specificity of protein–DNA interactions by providing abundant non-specific binding sites. Furthermore, under appropriate conditions the influence of secondary structure, promoted in the context of a supercoiled template, can be assessed. Finally, the method offers a high degree of sensitivity, even at relatively low protein concentrations, due to multiple rounds of primer extension on the template strand (Gralla, 1985).

Three control samples were used in all gels. First, a lane containing a sample of untreated supercoiled substrate DNA subjected to primer extension (Fig. 1, lane 1), thus revealing natural strand discontinuities resulting from the native DNA sequence/structure. Second, a lane containing a sample of supercoiled substrate DNA incubated only with the Mob protein prior to primer extension (Fig. 1, lane 2) was used to control for any strand discontinuities introduced by the proteins, or contaminants thereof, in the absence of DNase I cleavage. Finally, a lane that consists of a naked supercoiled substrate DNA treated with DNase I and subjected to primer extension, thus providing the control with which to assess potential footprints (Fig. 1, last lane). The observed strand discontinuities in all examples presented were independent of the observed patterns of DNase I protection and hypersensitivity observed in the protection experiments, thus the profiles observed can be attributed to the effect of Mob protein binding.

![Fig. 1. Analysis of GSH–MobC binding at oriT and mobC by DNase I footprinting. DNase I footprinting was performed on both the upper (A) and lower (B) strands upstream of oriT, and (C) the upper strand of mobC located downstream of oriT. A supercoiled pC221cop903 template was used and the resulting fragments probed by primer extension using primers NIC+98, NIC−178 and NIC+203 respectively. Lane identity is as follows: lane 1, DNA only (no DNase I); lane 2, GSH–MobC only (no DNase I); lanes 3–6, GSH–MobC (nM): 1.2, 12, 120, 372; lane 7, DNase I only. Sequencing ladder standards G, A, T, C are as indicated. Solid bars indicate protection, designated mcb1, mcb2, mcb3. Diminished and increased DNase I hypersensitivity are indicated by dotted bars and arrowheads respectively. The position of nic is indicated.](image)
DNase I footprinting analysis was performed on pC221cop903 DNA, incubated with varying concentrations of GSH–MobC (Fig. 1A and B). GSH–MobC is a thrombin-cleaved variant of the purified (His)_6-tagged MobC, containing only the additional N-terminal residues GSH, thus reducing the possibility of any steric effects of the full tag on DNase I footprinting. The strands were probed by primer extension, initially using a primer that binds 98 nucleotides (nt) (NIC+98) upstream of nic in the upper strand (Fig. 1A), and 178 nt (NIC−178) upstream from the nic complement in the lower strand (Fig. 1B). Footprinting on both strands revealed two corresponding regions of MobC–mediated DNase I protection. These regions, designated mcb1 (MobC binding) and mcb2 in the upper strand, were located 101 bp and 9 bp upstream from the oriT nic site and encompassed 16 bp and 17 bp of DNase I protected DNA respectively. The regions of protection were comparable on both strands, not being significantly asymmetric, and were flanked by sites of DNase I hypersensitivity. Both mcb1 and mcb2 were protected with comparable affinity, apparent at 120 nM GSH–MobC, and clearer still at 372 nM. The ratio of MobC:DNA at 120 nM is also comparable to that used in plasmid nicking assays (Caryl et al., 2004). At the higher concentration of MobC used, a pattern of hyper- and diminished-DNase I hypersensitivity with a periodicity of 10–12 bp was more evident between mcb1 and mcb2.

A 9 bp sequence (5′-AGTGGCTAG-3′), conserved in both mcb1 and mcb2 was identified. Searches for this sequence elsewhere identified a match of 7 bp located 115 bp downstream from nic, designated mcb3, which is located within the mobC gene itself. The upper strand of this region was re-probed using an alternate primer (NIC−203) and although less distinct, demonstrated evidence of protection by MobC, flanked by DNase I hypersensitivity (Fig. 1C). Similar searches were performed in a selection of related plasmids, which identified similarly conserved sequences in comparable positions, with only a few instances of base substitutions (Fig. 2). In each of the seven plasmids aligned, mcb1 was separated from the corresponding nic by 103–108 bp; however, in each case the mcb2 was 15 bp upstream from nic.

The juxtaposition of mcb1 and mcb2 with respect to nearby inverted repeats was not conserved (Fig. 5), with mcb1 corresponding to the left arm of IR1 and mcb2 to the loop region adjacent to the right arm of IR4 (Smith and Thomas, 2004). Thus we are unable to correlate the implied secondary structure with binding in the double-stranded context. An identical footprinting experiment using a linearized pC221cop903 substrate displayed an identical protection and hypersensitivity profile to that observed with an ostensibly supercoiled substrate (data not presented). This, together with sequence conservation between the binding sites, suggests a sequence-based interaction; although involvement of these potential hairpins in other processes cannot be ruled out.

The conserved 9 bp mcb sequence contains the MobC binding site

To differentiate the specific MobC binding site sequence from those flanking sequences protected due to steric hindrance, we performed competitive EMSA experiments. The conservation of the 9 bp sequence within the DNase I protected regions was suggestive of it being active in
MobC binding, especially given the lack of conservation in the flanking regions of mcb1 and mcb2. To confirm the extent to which the respective sequences were involved in binding by MobC, a 20 bp oligoduplex DNA (21P) containing the mcb2 sequence and wild-type flanking DNA was synthesized and used as a competitor DNA by titrating it into a pre-formed MobC–oriT complex (Fig. 3A). The MobC–oriT complex represents isoform 3, the third and largest MobC–oriT complex observed before non-specific protein–DNA interactions are observed (Caryl et al., 2004); and which likely represents saturation of all the binding sites by MobC.

The 21P substrate demonstrated efficient competition with the 494 bp oriT fragment for binding by GSH–MobC, indicating that the sequence is active in MobC–oriT interaction. As a control, an identical assay was performed using a control competitor (21Pmut) a 20 bp oligoduplex in which the 9 bp consensus sequence was randomized, maintaining identical base composition; while retaining the mcb2 flanking sequences (Fig. 3B). This 21Pmut substrate was unable to compete for binding by MobC, confirming that it is the conserved 9 bp sequence that contains crucial elements constituting the binding target.

In addition to these indirect studies, SPR was used to measure direct interaction between MobC and mcb. MobC was immobilized to the sensor chip surface via an NTA–His interaction and subjected to buffer containing competitor and control oligoduplexes as analytes. Binding was evident between 21P and MobC, with a Kd in the order of 1.8 µM, but not with 21Pmut, thus confirming the observed effect of randomization at mcb2 (Fig. 3C and D). The term mcb is therefore used to describe the 9 bp containing the MobC binding site.

MobA alters the footprint profile of MobC binding at mcb2 and nic

Unlike MobC, specific DNA binding by MobA was not apparent in earlier EMSA experiments (Caryl et al., 2004). Given the requirement for MobC for nicking by MobA, we assessed the potential of MobA to protect oriT DNA from DNase I in the presence and absence of MobC. As with the earlier DNase I protection assays, control samples containing untreated supercoiled DNA in the presence and absence of Mob proteins confirmed that the observed pattern of strand discontinuities and hypersensitivity in experimental sample lanes were not due to innate DNA structure or contaminating endonucleases. A DNase I only treated supercoiled DNA was used to assess the extent to which MobA protected the substrate DNA. MobAH6 was titrated against supercoiled pC221cop903 and probed with primer NIC+98, which extends along the upper strand (Fig. 4A). No discernable protection or hypersensitivity was observed, relative to the DNase I only control.

To assess whether MobC facilitates binding or interaction of the MobA with substrate DNA, the titration was repeated using a supercoiled pC221cop903 substrate pre-incubated with GSH–MobC (120 nM) (Fig. 4B). The purified template was probed as above and the resulting observations summarized (Fig. 5). A control lane containing MobC alone presented an identical profile to that previously observed (Fig. 1A, also summarized in Fig. 5). With the addition of MobAH6, the observed profile was modified, such that there was a loss of distinct MobC-induced DNase I hypersensitivity within a short (∼7 bp) region located between mcb2 and the nick site (nic). This suggests that the addition of MobA may modulate the binding of the MobC proximal to nic, such that the MobC-induced DNase I hypersensitivity is lost. Alternatively, MobA may be associated with and positioned over this region, thus protecting these sites from DNase I cleavage. MobA also appeared to induce DNA bending or kinking downstream of the nic site, as indicated by the appearance of MobA-induced DNase I hypersensitivity 5 bp and 18 bp downstream from nic. The MobA-induced modifications were not apparent at the corresponding region downstream of mcb1, despite the presence of comparable MobC-induced hypersensitivity.

Derivation of a minimal target susceptible to nicking

One functional region defining oriT of pC221 corresponds to a cloned 514 bp fragment (roughly corresponding to the pC221 MboI–ClaI fragment) referred to as oriTA, which encompasses all three mcb sites, nic and the N-terminal coding region of mobC. When a comparable fragment is cloned in a pUC19-derivative (pCER21T) this construct can be nicked in vitro (Caryl et al., 2004). When present within the shuttle vector, pCERoriTA, the fragment is seen to be nicked in whole cell lysate analysis (data not presented) and can be mobilized at a comparable frequency to the wild-type pC221 plasmid (Fig. 6A).

To determine a minimal target susceptible to nicking, we cloned alternative regions of pC221 oriT DNA that encompassed either mcb2 and mcb3 (386 bp), mcb1 and mcb2 (256 bp), or mcb2 alone (139 bp and 77 bp). Constructs, prepared in the context of shuttle vector pCΔ (see Table 1), were initially tested using an in vitro nicking assay (Fig. 6A). In all cases the cloned oriT fragments were susceptible to nicking, thus the two smallest regions cloned demonstrated that a fragment containing only mcb2 is sufficient for nicking.

While an interaction between MobC and oriT at mcb2 has been identified, this does not confirm that binding by the MobC at this site is a prerequisite for nicking by
Substrate recognition in the pC221 relaxosome

Fig. 3. A and B. Competition assay between pre-formed oriT–MobC complex and an increasing concentration of a synthetic oligoduplex DNA. Pre-formed oriT–MobC complex consisted of 10.75 nM BstXI/HindIII-digested pCER21T and 300 nM GSH–MobC. The complex was titrated with a 0–5-fold molar excess of 21P, an oligoduplex corresponding to the mcb2 (sequence highlighted in light grey) and wild-type flanking sequence; or 21Pmut, an oligoduplex containing a mutated 9 bp mcb2 (sequence highlighted in light grey), but maintaining wild-type flanking sequences. Short arrows indicate position of the free 20-mer oligoduplex DNA. Long arrows indicate free oriT DNA and the three oriT–MobC complex isoforms. Lane values refer to concentration of oligoduplex (nM).

C and D. SPR using an NTA-chip bound H6MobC221 ligand and analytes 21P and 21Pmut at concentrations of 1000 nM (black), 100 nM (dark grey), buffer only control (light grey).

© 2006 The Authors
Journal compilation © 2006 Blackwell Publishing Ltd, Molecular Microbiology, 60, 1302–1318
MobA. The minimal 77 bp target identified as being susceptible to nicking by the MobA and MobC proteins of pC221, although not mobilized in vivo, was referred to as oriT\textsubscript{nict}. This oriT\textsubscript{nict} was cloned into pCER19, as pCER\textsubscript{21wt} (Fig. 7A), and employed as a control to examine the effect of mutation within the mcb2 site. Using this substrate as a basis, we designed a variant, pCER\textsubscript{77mcb} (Fig. 7A), with the 9 bp mcb2 randomized to a sequence previously shown to demonstrate no binding (Fig. 3). This substrate was not susceptible to nicking in vitro by 21MobA and GSH–MobC (Fig. 7B). Barring any pleiotropic effects of mutating mcb2, this suggests that MobC binding at mcb2 is indeed a requirement for nicking by the MobA relaxase.

The nic sites of the pC221-family oriT regions are identical, suggesting a strict sequence requirement at this region. This was investigated by mutation of the pCER\textsubscript{21wt} substrate at the 5′ nucleotide of the nic dinucleotide from 5′-GC-3′ to 5′-TC-3′ to create the substrate pCER\textsubscript{77nic} (Fig. 7A). This substrate was also not susceptible to nicking in the in vitro assay (Fig. 7B).

All three binding sites are required for mobilization of pC221

The presence of multiple MobC binding sites within oriT suggests a potential for a higher-order structure formation between MobC and more than one mcb site. While the presence of mcb2 alone is sufficient for nicking, mcb1 and mcb3 may be involved in the subsequent mobilization of oriT. The pC\textDelta-based shuttle plasmid constructs described above were assayed for mobilization in staphylococcal filter-mating experiments. This involved introducing them into a donor S. aureus RN4220 strain containing pGO1, encoding the conjugative transfer apparatus, and wild-type pC221 as a helper plasmid to provide mob functions in trans. Recipients were scored for their ability to grow on erythromycin, conferred by pC\textDelta, and both novobiocin
Substrate recognition in the pC221 relaxosome

1309

© 2006 The Authors
Journal compilation © 2006 Blackwell Publishing Ltd, Molecular Microbiology, 60, 1302–1318

and rifampicin, conferred by the recipient strain RN2677. Within the sensitivity of the experiment, these assays demonstrated that the gross deletion of regions containing mcb1 or mcb3 resulted in an 18-fold reduction in mobilization efficiency, suggesting all three mcb sites are required to constitute a fully functional oriT.

To more fully investigate the requirement for each MobC binding site in isolation, we used overlap extension PCR to generate mutations in either mcb1, mcb2, mcb3 or mcb1 + mcb3, which were then cloned in the context of pC221 (see Table 1). This yielded four mutant plasmids that differ from the wild type only at those mcb sites mutated. Binding sites mcb1 and mcb2 were mutated to the same randomized sequences described previously (Fig. 3). In order to mutate mcb3 within the mobC gene, it was necessary to encode a silent mutation to maintain the amino acid sequence, although 9 bp were still altered. The resulting pC221 mutants were introduced into donor RN4220 cells containing pGO1, filter-mated with RN2677 and the transconjugant cells scored for resistance to chloramphenicol, as well as the novobiocin and rifampicin encoded by the recipient RN2677. It was observed that mutation of mcb1, mcb2, mcb3 or mcb1 + mcb3, in the context of pC221, resulted in a loss of mobilizable phenotype (Fig. 6A). Pleiotropic effects aside, this suggests that all three MobC binding sites are required for mobilization.

To assess whether the loss of mobilization was due to abolishment of nicking, whole cell lysate analysis was performed on the donor strains containing each of the pC221 mutants (Fig. 6B). No nicking was observed on the plasmid containing mutation at mcb2, which confirmed the earlier in vitro data from pCER77mcb1 (Fig. 7B). In all the other mutants a nicked open-circular complex, comparable to wild-type pC221, was observed, signifying successful relaxosome formation. This represents the first time in vivo nicking has been observed in the absence of mobilization in pC221 containing wild-type Mob proteins.

The sra sequence is the basis of substrate recognition

An alignment between the oriT regions of the three closely related plasmids pC221 (and cop903), pC223 and pS194 highlights the clustering of divergent sequences within oriT_{308}, which could bear functional significance (Fig. 7A).
We have previously shown that the MobA proteins of pC221 and pC223 encode the plasmid-specific sequence specificity, such that these proteins recognize only the cognate nic (Caryl et al., 2004). In a comparison of MobA activities against an additional cloned substrate we found that MobA of pC221 will also nick the pS194 oriT (pCER194T) at an identical position, whereas the pC223 MobA does not (Fig. 7A).

The alteration of the MobC-induced DNase I hypersensitivity in the region between mcb and nic on addition of MobA suggests this sequence is a specific site for interaction with MobA. This 7 bp sequence was randomized (maintaining wild-type base composition) in the oriT nic mutant pCER77sra (Fig. 7A), and was found to not be susceptible to nicking by 21MobA in the in vitro nicking assay (Fig. 7B). It is therefore likely that this sequence, designated sra (site recognized by MobA), plays a role in the interaction of the relaxosome at oriT.

The sra sequence of different plasmids may offer a means of substrate-specific interaction with the cognate MobA protein. Therefore, the four bases within sra that are not conserved between pC221 and pC223 were exchanged (Fig. 7A), and the resultant substrates assessed for nicking by MobA of either pC221 or pC223, in the presence of pC221 MobC protein. Four oriT nic substrates were compared: two controls consisting of...
wild-type pC221 (pCER21wt) and pC223 (pCER23wt) sequences; and two sra-substrate, pCER21sra23 and pCER23sra21. In vitro nicking assays demonstrated that exchange of these 4 bp within the sra resulted in swapping of substrate identities and consequently preferential cleavage by MobA (Fig. 7C); with only residual nicking of pCER21sra23 by 21MobA (14%), compared with 23MobA (39%); and no detectable nicking of pCER23sra21 by 23MobA (0%), compared with 21MobA (40%). The use of MobC derived from pC223, rather than pC221, yielded comparable results (data not presented). Thus the four exchanged base pairs represent the determinants within sra of pC221 and pC223 MobA specificity.

Discussion

The MobA relaxases of pC221 and pC223 display specificity towards their cognate origins of transfer. This is in contrast to the MobC accessory proteins essential for the site- and strand-specific transesterification reaction mediated by the relaxase, which are interchangeable between the two related relaxosomes (Caryl et al., 2004). In the present study we have distinguished the respective roles of the MobA and MobC proteins in substrate recognition and subsequent nicking specificity.

The pC221 MobC protein is a dimeric DNA-binding protein able to bind oriT DNA of either pC221 or pC223 (Caryl et al., 2004). We have used DNase I footprinting, mutational analysis and SPR to investigate the interaction of MobC at oriT and identified three distinct binding sites within the oriT region. Two of the binding sites, designated mcb1 and mcb2, which contain a conserved asymmetric 9 bp direct repeat, are located 106 bp (mcb1) and 15 bp (mcb2) upstream of nic. A third, designated mcb3, is encoded within the N-terminal coding region of mobC, and contains 7 bp in common with the direct repeat sequence.
Thus the binding sites appear to conform to direct repeats despite the presence of local inverted repeats (Fig. 5). SPR analysis suggests a $K_D$ of 1.8 µM for the interaction between MobC and the single mcb2 site in isolation; however, given the presence of three such sites within the oriT region, it is quite possible that cooperativity may enhance this affinity.

All three binding sites are highly conserved in terms of both sequence and location within the mobilon regions of a number of plasmids from Staphylococcus previously identified. The conservation of these sequences between pC221 and pC223 explains the interchangeable nature of the respective MobC proteins with regard to binding observed in earlier EMSA experiments (Caryl et al., 2004); a single base pair difference between the pC221 mcb2 and the corresponding site on pC223 appears to have no effect on this activity. This is in contrast to plasmid RP4, where the accessory protein TraJ is able to distinguish between its cognate binding site, sri, and that of the related plasmid R751, which differ in 4 of 10 bp. Heterologous nicking and transfer of a cloned RP4 oriT by R751 proteins is only possible if RP4 traJ is encoded in cis (Ziegelin et al., 1989). Thus in this latter example, in addition to initiating assembly of the relaxosome at oriT, the accessory protein is able to fulfil a role in substrate specificity.

The presence of three binding sites within the pC221-family oriT's has the potential for DNA looping or other high-order structures with a core of MobC mediating interactions between these positions. This possibility is further supported by the pattern of diminished- and hypersensitivity to DNase I cleavage between mcb1 and mcb2, evident at higher concentrations, indicative of increased DNase I access to the minor groove within such a bend (Hochschild and Ptashne, 1986). However, given the excess of MobC over binding substrate, it remains possible that such a pattern results from non-specific DNA interactions.

A minimal oriT, functional in mobilization, in trans, is defined by a 524 bp fragment of pC221. Deletion of 97 bp (including mcb1) from the 5' end and 253 bp (including mcb3) from the 3' end of this fragment does not diminish nicking susceptibility, but compromises transfer efficiency. This bears similarity to the plasmid RP4, which has a functional oriT comprising ~350 bp; however, a 194 bp oriT is also mobilizable but with 100–200-fold reduced mobilization efficiency. This was found to result from the loss of the TraK binding site, the binding of which is stimulatory to nicking and is proposed to alter the superhelical density at nic (Ziegelin et al., 1992).

Sequential site-directed mutagenesis of the three mcb sites, in the context of pC221, indeed confirms that an oriT containing all three mcb sites is required for wild-type levels of mobilization. The loss of mcb2 abolishes in vivo nicking activity and therefore subsequent mobilization. This parallels observations where mutagenesis of accessory protein binding sites reduces nicking and transfer frequency in RP4 (via TraJ; Waters et al., 1991) and R64 (via Nika; Furuya and Komano, 1997). The loss of mcb1 or mcb3, while having no impact on nicking, effectively abolishes mobilization. Thus MobC may function at several levels: primarily to initiate nicking by MobA, presumably via interaction at mcb2; but furthermore by mediating a potential high-order complex formation, additionally involving mcb1 and mcb3, to yield a mobilizable substrate. Such a complex may even regulate the transcription of mobC.

In pC221, the binding of MobC induces DNase I hypersensitivity in the DNA flanking the protected sequence, indicative of a localized bending of DNA often observed on binding of a protein to DNA. Such bending could serve to stabilize subsequent relaxosome formation. On addition of MobA to a MobC-oriT complex, the observed pattern of DNase I protection and hypersensitivity is altered, revealing MobA protection over a 7 bp region (sra) between mcb2 and nic. In addition, MobA contributes to increased DNase I hypersensitivity downstream from the nic, indicating that distortion of DNA structure may be important within the relaxosome.

Mutation of sra abolished susceptibility of a minimal 77 bp oriTnic substrate to cleavage, which taken together with footprinting data identifies this as a site of interaction with MobA. This conclusion is supported by several other observations. First, the region coincides with a region of sequence divergence between the pC221 and pC223 oriTnic (Fig. 7A), which, despite their similarity, can only be nicked by their cognate relaxases.

Second, comparison of the pC221 oriTnic and a comparable region of the IncP plasmid RP4 demonstrates the similar juxtaposition of the RP4 TraJ accessory protein binding site upstream of nic. Mutations within a 10 bp sequence upstream of nic, comparable to the position of sra, in the identical plasmid RK2 result in complete loss of nicking activity (Waters et al., 1991). A 6 bp core TraI recognition sequence within this region, sri, was subsequently determined in the context of RP4 using oligonucleotide cleavage assays (Pansegrau et al., 1993).

Finally, conservation of the mcb2 site and interchangeable nature of heterologous MobC proteins suggests that while necessary for nicking activity, this interaction is unable to account for the observed nicking specificity. The interaction of MobA at sra does explain such specificity.

Mutation of the region proposed as the sra results in a loss of nicking activity. By swapping those bases within sra that are divergent between pC221 and pC223 we were able to turn a substrate of the pC221 MobA protein into one preferentially cleaved by that of pC223 and vice
versa. Thus sra represents not only a site of interaction between oriT and MobA, but also provides differentiation between pC221 and pC223.

The oriT<sub>nic</sub> of the pC221 mobilon family thus consists of at least three sequence domains: mcb, sra and nic, each of which is required for correct nicking activity. The current model of pC221 relaxosome assembly could be considered one of layered specificity. The MobC proteins of pC221 and pC223 are interchangeable with respect to nicking; this is borne out by the conservation of the binding sequence between the respective substrates, with a single base exception in mcb2 of pC223. A substrate encoding a mutated mcb2 is not nicked, thus, barring any pleiotropy due to such mutation, MobC binding is a prerequisite for subsequent nicking by MobA, and can be considered the first layer of relaxosome specificity.

The role of MobC is not clear cut, but apparently facilitates the recruitment of MobA to the nick site. In this regard, MobC may perform a structural or positional role where it could mediate MobA recruitment by structurally altering the substrate, perhaps to present a recognition complex. Alternatively, it could act to position the MobA over its recognition sequence, which may be presented as single-stranded DNA. Evidence suggests that a higher-order structure between MobC and mcb1, 2 and 3 is important for subsequent mobilization of a nicked plasmid; the nature of such a complex is yet to be elucidated. Each of these roles are not mutually exclusive and not dissimilar in principle to the binding of DnaA at oriC prior to the recruitment of the DnaBC helicase complex in the initiation of bacterial DNA replication (Messer, 2002), or the recruitment of ParF into the DNA partition complex via interaction with sequence-specific ParG protein (Barilla and Hayes, 2003).

In addition to recruitment, specific recognition of sra is required for subsequent cleavage by MobA. Whether recognition is mediated through extrusion of a single-stranded DNA as a potential result of MobC binding is yet to be established. Single-stranded DNA recognition and binding has, however, been demonstrated for the relaxase domain, TraI<sub>36</sub>, of the unrelated F-plasmid (Stern and Thomas, 2004). Standard nucleotide sequencing was performed commercially (Lark Technologies, Essex, UK). For electroporation, recovery was in B2 broth (Schenk and Laddaga, 1992) but using 0.5% (w/v) NaCl rather than their stated 2.5% (A. O’Neill, pers. comm.). When appropriate, antibiotics were used at the following concentrations: Ampicillin (Ap) 100 µg ml<sup>−1</sup>; Chloramphenicol (Cm) 10 µg ml<sup>−1</sup>; Erythromycin (Em) 5 µg ml<sup>−1</sup>; Gentamicin (Gm) 5 µg ml<sup>−1</sup>; Novobiocin (Nv) 5 µg ml<sup>−1</sup>; Rifampicin (Rf) 5 µg ml<sup>−1</sup>. Plasmid DNA preparations were performed as described (Smith and Thomas, 2004). Standard nucleotide sequencing was performed commercially (Lark technologies, Essex, UK). Oligonucleotides were prepared by commercial synthesis (MWG Biotech, Ebersberg, Germany).

### Experimental procedures

#### Bacterial strains, growth conditions and manipulation of DNA

Bacterial strains, plasmids and primers are listed in Table 1. *E. coli* strain DH5<sup>α</sup> was used for the preparation and maintenance of plasmid constructs. *S. aureus* strain RN4220 was used as a donor in all filter mating experiments; RN2677 (kindly provided by T.J. Foster) was used as the recipient. *Escherichia coli* DH5<sup>α</sup> was cultured at 37°C using 2YT broth or Luria–Bertani (LB) agar (Sambrook and Russell, 2001). *S. aureus* was cultured at 30–37°C using Brain-Heart Infusion (BHI) broth or agar (Oxoid, Oxford, UK). For electroporation, recovery was in B2 broth (Schenk and Laddaga, 1992) but using 0.5% (w/v) NaCl rather than their stated 2.5% (A. O’Neill, pers. comm.). When appropriate, antibiotics were used at the following concentrations: Ampicillin (Ap) 100 µg ml<sup>−1</sup>; Chloramphenicol (Cm) 10 µg ml<sup>−1</sup>; Erythromycin (Em) 5 µg ml<sup>−1</sup>; Gentamicin (Gm) 5 µg ml<sup>−1</sup>; Novobiocin (Nv) 5 µg ml<sup>−1</sup>; Rifampicin (Rf) 5 µg ml<sup>−1</sup>. Plasmid DNA preparations were performed as described (Smith and Thomas, 2004). Standard nucleotide sequencing was performed commercially (Lark technologies, Essex, UK). Oligonucleotides were prepared by commercial synthesis (MWG Biotech, Ebersberg, Germany).

#### Preparation of plasmid constructs

All plasmid constructs were prepared using *E. coli* DH5<sup>α</sup>. The shuttle vector pC<sub>Δ</sub> was constructed by digestion of pCER19, a pUC19-derivative containing the cer resolution sequence (Caryl et al., 2004), and the staphylococcal plasmid pE194 with restriction endonucleases EcoRI and BspEI respectively. Overhanging ends were filled in with DNA polymerase I Klenow fragment and the fragments ligated such that pCER19 bla was in series with pE194 repF. The pE194 pre (plasmid recombinase) was subsequently excised via an Acc651–BsrGI digestion and self-ligation of the compatible ends. For constructs based on the pC<sub>Δ</sub> shuttle vector, oriT fragments were generated by PCR from a pC221 template using standard methods and *Pfu* turbo polymerase (Stratagene). All PCR products contained PstI and BamHI restriction tags, which were digested for directional cloning into similarly digested pC<sub>Δ</sub>. The primer sequences used for preparation of oriT shuttle constructs are listed in Table 1. oriTA was pro-
**Table 1.** Bacterial strains, plasmids and oligonucleotides.

| Strain, plasmid or oligonucleotide | Relevant characteristic(s) | Reference or source |
|------------------------------------|-----------------------------|---------------------|
| E. coli F−, φ80dlacZ M15, endA1, recA1, HsdR17 (r−, m−), supE44, thi-1, gyrA96, relA1, Δ(lacZYA−argF)U169, λ | | BRL |
| S. aureus DH5x Attenuated, mecA, TSSE+, rfbU, agr, restriction deficiency, modification proficient | | Kreiswirth et al. (1983) |
| S. aureus RN220 derivative of strain 8325–4. | | |
| S. aureus RN2677 8325 derived s11, s12, s13 lysogens, agr+, sak+, rfbU, hib+, restriction deficient, spontaneous Nv+ and Rv+ mutant, recipient strain for conjugation. | | Kornblum et al. (1986), T.J. Foster |
| **Plasmids** | | |
| pC221 4555 bp: Cm+, repD, mobCAB | | Novick and Bouanchaud (1971) |
| pC221cop903 4168 bp: Cm+, inc4, repD, mobCAB (Δs06-1185: copy no. mutation) | | Projan et al. (1985) |
| pCER19 3063 bp: pUC19: Ap+, lacZ, cer (dimer resolution) | | Caryl et al. (2004) |
| pCER21T 3575 bp: pCER19 containing pC221cop903 oriT | | Caryl et al. (2004) |
| pCER23T 3584 bp: pCER19 containing pC223 oriT | | Caryl et al. (2004) |
| pCER194T 3560 bp: pCER19 containing pS194 oriT | | This work |
| pCER21wt 3127 bp: wild type 77 bp pC221 oriT | | This work |
| pCER21wt 3127 bp: 77 bp pC221 oriT mutated at mcb | | This work |
| pCER77sra 3127 bp: 77 bp pC221 oriT mutated at sra | | This work |
| pCER77nic 3127 bp: 77 bp pC221 oriT mutated at nic | | This work |
| pCER21sra23 3127 bp: 77 bp pC221 oriT mutated with pC223 sra swap | | This work |
| pCER23sra21 3127 bp: 77 bp pC223 oriT mutated with pC223 sra swap | | This work |
| pCER23wt 3127 bp: wild type 77 bp pC23 oriT | | This work |
| pΔ 5625 bp: pE194: pUC19 based shuttle vector. | | This work |
| pCA21wt 5689 bp: Shuttle vector containing wt 77 bp pC221 oriT | | This work |
| pCA1oriTA 6128 bp: Shuttle vector containing 514 bp oriTA | | This work |
| pCA1oriTB 5880 bp: Shuttle vector containing 256 bp oriTB | | This work |
| pCA1oriTC 5753 bp: Shuttle vector containing 139 bp oriTC | | This work |
| pCA1oriTD 6000 bp: Shuttle vector containing 386 bp oriTD | | This work |
| pC221.mcb1 4555 bp: pC221 with a 9 bp mutation of mcb1 | | This work |
| pC221.mcb2 4555 bp: pC221 with a 9 bp mutation of mcb2 | | This work |
| pC221.mcb3 4555 bp: pC221 with a 9 bp mutation of mcb3 | | This work |
| pC221.mcb1–3 4555 bp: pC221 with a 9 bp mutations of mcb1 and mcb3 | | This work |
| pG01 52 314 bp: Gm+, Tr+, Qam+, tra | | T.J. Foster |

**Oligonucleotides**

| Oligonucleotides | Sequence (5′–3′)* | Description |
|------------------|-------------------|-------------|
| PforORITA AACTGCAAAATTATGAAACGGTTAGA | Psfl forward oriT | |
| BrevORITA CCGGGATCCCGGTGGAATTGCGC | BamHI reverse oriT | |
| PforORITC AACTGCAAAATTATGAAACGGTTAGA | Psfl forward oriT | |
| PrevoRITC CCGGGATCCCGGTGGAATTGCGC | BamHI reverse oriT | |
| NIC−203 CATATGGGATCCCGGTGGAATTGCGC | Primer extension + strand | |
| NIC−198 CACTATGGGATCCCGGTGGAATTGCGC | Primer extension + strand | |
| NIC−178 GAAACTATGGGAACCCAC | Primer extension − strand | |
| HPA2− AAGTTTATTGAAACCCAC | Primer extension + strand | |
| 21P+ AACAAGGTGCTAGAATATAATA | mcb oligoduplex + strand | |
| 21P− TATAT TATGCTACCGGTTAGAATATTAC | mcb oligoduplex − strand | |
| 21Pmut+ AAGCTGACGATGCGTAGAATATTAC | mutagenic mcb + strand | |
| 21Pmut− TATATGCTACCGGTTAGAATATTAC | mutagenic mcb − strand | |
| ORIT−MCB+ GGGTTGGGTGGAATTTATGCAAGCCCGTTAGAAGCGGTCGA | pC221 oriT with | |
| ORIT−MCB− GAGTACGCTGTTTGGTGAATTTATGCAAGCCCGTTAGAAGCGGTCGA | pC221 oriT with | |
| ORIT−SRA+ GATCTGTTTGGTGAATTTATGCAAGCCCGTTAGAAGCGGTCGA | pC221 oriT with | |
| ORIT−SRA− GATCTGTTTGGTGAATTTATGCAAGCCCGTTAGAAGCGGTCGA | pC221 oriT with | |
| ORIT−NIC+ GGGTTGGGTGGAATTTATGCAAGCCCGTTAGAAGCGGTCGA | pC221 oriT with | |
| ORIT−NIC− GATCTGTTTGGTGAATTTATGCAAGCCCGTTAGAAGCGGTCGA | pC221 oriT with | |
| 21WT+ GGGTTGGGTGGAATTTATGCAAGCCCGTTAGAAGCGGTCGA | pC221 oriT with | |
| 21WT− GATCTGTTTGGTGAATTTATGCAAGCCCGTTAGAAGCGGTCGA | pC221 oriT with | |
| 21SRA23+ GGGTTGGGTGGAATTTATGCAAGCCCGTTAGAAGCGGTCGA | pC221 oriT with | |
| 21SRA23− GATCTGTTTGGTGAATTTATGCAAGCCCGTTAGAAGCGGTCGA | pC221 oriT with | |

*Sequence (5′–3′) refers to the oligonucleotide sequence oriented for transcription from the promoter region.*
Mutant plasmids were confirmed by sequencing and transformed by electroporation as described above. Ligated with a similarly digested reciprocal part of the pC221 releasing the mutagenized strands were annealed and ligated with BamHI–PstI-digested pC223 and BstXI forward.

Oligonucleotides Sequence (5′–3′) Description

23SRA21+ GGATGGATCCCTGGATGATAAATAATCCATCAAGTACGGCGTGCTAGAACGA pC223 orTnic with

23SRA21− GAGGCTGCAAGTACGGCGTGCTAGAACGA pC223 orTnic with

23WT+ GGATGTATGCTGTATG Forward

23WT− TGTGGTTTGGCAAGC Reverse

PST–BSTXI+ AGGCTGCAAGTACGGCGTGCTAGAACGA PstI and BstXI forward

BAM–CLAI− CGGATCCATCGATTGCTATGTTTTTG PsI + strand

MCB1A+ AGCTTGATCCCTGGATGATAAATAATCCATCAAGTACGGCGTGCTAGAACGA

MCB1A− AAATGGGACCGTCATCGATTGCTATGTTTTTG Reverse mcb1 mutation

MCB2A+ ACTATGGGAGATTGCTACCTTTTCTCATAGTTCAAGACCTAATTC Reverse mcb2 mutation

MCB2B+ GAACGCAAGTACGGCGTGCTAGAACGA Forward mcb2 mutation

MCB3A+ CAAACCTGTACGCAAGTACGGCGTGCTAGAACGA Reverse mcb3 mutation

MCB3B+ AATAATCTGGTCAGTACGCTGGATGAAAGAACCTAATTC Forward mcb3 mutation

a. T.J. Foster, Department of Microbiology, Trinity College Dublin.
b. Underlined nucleotides indicate restriction tags, whilst those emboldened denote nucleotides that are mutated with respect to the wild type.

For orTnic constructs based on pCER19, complementary strands corresponding to either wild-type or specifically mutated 77 bp orTnic were synthesized incorporating PsI and BamHI cohesive ends for directional cloning. Complementary strands were annealed and ligated with BamHI–PstI-digested pCER19. Selection of recombinant plasmids was made as above, and confirmed by sequencing.

Proteins

MobA and MobC from pC221 and pC223 were purified as described previously (Caryl et al., 2004). Where cited in text, 21MobA and 23MobA represent the C-terminally hexahistidine-tagged MobA proteins derived from pC221 and pC223 respectively. The N-terminal (His)_6-tag was removed from the MobC proteins by thrombin cleavage, yielding GSH–MobC. Cleavage was performed in batches of 1 mg protein using 40 U thrombin (Pharmacia) in a total volume of 5 ml containing 50 mM KCl and 20 mM Tris.HCl (pH 8.0), 2 mM CaCl_2 and 0.37 pmol supercoiled DNA with orTnic

DNase I footprinting

Footprinting experiments were performed using the methodology of Leblanc and Moss (2001) and Gralla (1985). Puriﬁed MobC (and/or MobA) and 0.37 pmol supercoiled DNA were mixed in a volume of 50 μl containing 50 mM KCl and 20 mM Tris.HCl (pH 8.0). Reaction mixes were incubated at 30°C for 30 min whereupon an equal volume (50 μl) of cofactor solution (10 mM MgCl_2 and 5 mM CaCl_2) was added and the temperature adjusted to 25°C. 0.5 Kunitz units of DNase I (D-4263, Sigma) were added to each sample and incubated for 3 min. Reactions were stopped by addition of 100 μl reaction stop buffer (SDS 1%, v/v, 200 mM

© 2006 The Authors
Journal compilation © 2006 Blackwell Publishing Ltd, Molecular Microbiology, 60, 1302–1318
NaCl, 20 mM EDTA pH 8.0) containing 40 µg ml⁻¹ tRNA (Sigma) as a carrier. The digested fragments were purified by phenol extraction (Sambrook and Russell, 2001) and 160 µl of the aqueous phase was precipitated with ethanol and resuspended to a concentration of 20 fmol µl⁻¹. Control reactions consisted of a sample lacking protein but treated with DNase I and a sample containing protein, but without DNase I treatment.

Primer extension analysis
Primer extensions (NIC-98 and NIC-203) were designed to anneal 98 nt and 203 nt respectively, from the pC221 oriT nic on the plus-strand; primer NIC-178 binds 178 nt upstream from the position of nic on the complementary strand (Table 1). The DNase I-digested templates were probed by primer extension using an AmpliCycle® Sequencing Kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Briefly, oligonucleotide (10 pmol) was 5’ end-labelled in a forward reaction by incubation with T4 polynucleotide kinase (30 units) (Invitrogen) and 10 pmol γ-[³²P]-ATP (3000 Ci mmol⁻¹) (MP Biomedicals, Irvine, CA).

Primer extension reactions contained 0.07 pmol of DNA-digested plasmid DNA, 1.2 pmol 5’-end-labelled oligonucleotide primer, 0.25 units of AmpLTaq DNA polymerase CS and 160 pmol of each deoxyribonucleoside triphosphate (dNTP) (Invitrogen) in 8 µl 50 mM Tris.HCl pH 8.9, 10 mM KCl, 2.5 mM MgCl₂, 0.025% v/v Tween 20. Dideoxy nucleotide sequencing ladders were prepared according to the manufacturer’s instructions using 0.02 pmol supercoiled pC221cop903 plasmid DNA as a template. An additional control reaction was prepared in a similar manner to the sequencing ladder, but using 160 pmol of each dNTP in place of ddNTP.

All reactions (8 µl) were overlaid with 20 µl mineral oil (USB, Cleveland, OH) and were subjected to the following thermocycling parameters: 1× (95°C, 2 min); 25× (95°C, 1 min; 45–50°C, 1 min; 72°C, 1 min); 1× (72°C, 5 min). The reactions were stopped by addition of a formamide dye solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) and primer extension products were resolved on a standard 6% polyacrylamide sequencing gel (Sambrook and Russell, 2001). Radiolabelled gels were imaged using Fuji BAS plates and a FujiBas-1000 phosphorimager (Fuji, Japan).

Competitive EMSA
Pre-formed MobC–oriT complexes were prepared in a manner similar to that previously reported (Caryl et al., 2004). BstXI/HindIII digestion of pCER21T DNA (0.22 pmol), yielded fragments of 2789 bp, 494 bp (containing pC221 oriT) and 292 bp, which were mixed with purified GSH–MobC (6 pmol). To equal aliquots of the MobC–oriT complex, 20 bp synthetic competitor or control DNA was added at concentrations of 25, 50, 100, 200, 400, 800 and 1600 nM. The reactions were assembled in a total volume of 20 µl 100 mM KCl, 20 mM Tris.HCl pH 8.0 and incubated at 30°C for 30 min. Reaction mixtures were separated by electrophoresis using 0.8% agarose gels in TBE as running buffer, limiting at 5 V cm⁻¹ for 100 min, then stained with ethidium bromide (1 µg ml⁻¹) for 30 min and destained for 20 min in 1× TBE. Gel images were captured digitally using a GDS-8000 gel documentation system (UVP, Cambridge).

Surface Plasmon Resonance
Surface Plasmon Resonance was performed using a BIACORE 2000 unit with NTA sensor chip (Biacore AB, Uppsala). Prior to loading with protein the sensor chip was prewashed with a 1 min pulse of EDTA (0.35 M) at 40 µl min⁻¹ to remove loose metal ions. The NTA was charged with a 1 min pulse of NiSO₄ (500 µM) at 5 µl min⁻¹. H₄MoBCO1 at a concentration of 100 nM in running buffer 50 mM KCl, 20 mM Tris.HCl (pH 8.0), 50 µM EDTA, 0.005% P20 was injected over the NTA chip at a flow rate of 5 µl min⁻¹ to an immobilization of 358 RU.

Flow cell 1 was treated with Ni³⁺ only and served as a reference surface to correct for refractive index changes and other bulk effects. Flow cell 4 was left untreated as a control for non-specific association of protein or DNA with the carboxymethylated dextran to which the NTA is immobilized.

Binding experiments were performed at a flow rate of 5 µl min⁻¹ in running buffer at 25°C. Injections of two concentrations of each oligoduplex, 100 nM and 1000 nM, were performed over all four flow cells, separated by buffer only controls, for 3 min pulses at a flow rate of 40 µl min⁻¹. After each injection the chip was regenerated with a 3 min pulse of high-salt buffer [300 mM KCl, 20 mM Tris.HCl (pH 8.0), 50 µM EDTA, 0.005% P20] to displace any remaining DNA, and rinsed prior to injection of the next analyte/concentration.

Nicking assays
Assembly of relaxosomes in nicking assays, and where appropriate the quantification of nicking, was performed as previously reported (Caryl et al., 2004).

Whole cell lysate analysis
Whole cell lysate analysis was performed as previously reported (Smith and Thomas, 2004).

Filter mating experiments
The mobilizable phenotype of pC221 and pCa shuttle constructs containing alternate oriT fragments was assessed using a modification of the filter mating methodology reported previously (Smith and Thomas, 2004). RN4220 containing pGO1 and pC221 was used as a donor strain. Overnight cultures of donor and recipient (RN2677) were grown in BHI broth and diluted into fresh media, in the presence of appropriate antibiotics, to an OD₆₀₀ equivalent of 0.1 and incubated at 30°C until mid-log phase.

Volumes of donor and recipient cultures equivalent to ~3 × 10⁶ cells (1 ml culture at OD₆₀₀ of 0.6) were harvested and resuspended separately in 1 ml fresh BHI broth. The washed donor and recipient cells were mixed and a further 1 ml fresh BHI broth added before filtering through a sterile 13 mm diameter, 0.2 µm pore nitrocellulose filter (Millipore).
The filter was extracted and placed bacteria side up on a BHI agar plate, without selection, and incubated at 30°C for 18 h. Cells were recovered from filters by vortexing in 1 ml sterile phosphate-buffered saline and serial dilutions were plated onto separate BHI agar plates containing appropriate selection for donors or transconjugants. The efficiency of transfer was calculated as a ratio of the mobilization frequency (erythromycin- or chloramphenicol-resistant transconjugants per donor) to the conjugative frequency (gentamicin-resistant transconjugants per donor cell). In those assays where no transconjugants were observed, the resolution of experimental sensitivity is given. Average values from three independent experiments, performed in duplicate, are presented.

Acknowledgements

We are grateful to T.J. Foster (Dublin, Eire) for the kind donation of S. aureus strain RN2677 and plasmid pGO1. We also acknowledge Andy Baron (Centre for Biomolecular Interactions, University of Leeds) for technical advice on SPR. The BIAcore 2000 machine was provided by a grant from the Wellcome Trust. This work was supported by Grant BBSB04927 from the British Biotechnology and Biological Sciences Research Council (BBSRC).

References

de Antonio, C., Farias, M.E., de Lacoba, M.G., and Espinosa, M. (2004) Features of the plasmid pMV158-encoded MobM, a protein involved in its mobilization. J Mol Biol 335: 733–743.

Apisiridej, S., Leelaporn, A., Scaramuzzli, C.D., Skurray, R.A., and Firth, N. (1997) Molecular analysis of a mobilizable theta-mode trimethoprim resistance plasmid from coagulase-negative staphylococci. Plasmid 38: 13–24.

Aubert, S., Dyke, K.G., and Solh, N.E. (1998) Analysis of two Staphylococcus epidermidis plasmids coding for resistance to streptogramin A. Plasmid 40: 238–242.

Barilla, D., and Hayes, F. (2003) Architecture of the ParF+ParG protein complex involved in prokaryotic DNA segregation. Mol Microbiol 49: 487–499.

Byrd, D.R., and Matson, S.W. (1997) Nicking by transesterification: the reaction catalysed by a relaxase. Mol Microbiol 25: 1011–1022.

Caryl, J.A., Smith, M.C.A., and Thomas, C.D. (2004) Reconstitution of a staphylococcal plasmid-protein relaxation complex in vitro. J Bacteriol 186: 3374–3383.

Dagert, M., and Ehrlich, S.D. (1979) Prolonged incubation in calcium chloride improves the competence of Escherichia coli cells. Gene 6: 23–28.

Francia, M.V., Varsaki, A., Garcia-Ban,Bacías, M.P., Letorre, A., Drainas, C., and de la Cruz, F. (2004) A classification scheme for mobilization regions of bacterial plasmids. FEMS Microbiol Rev 28: 79–100.

Furuya, N., and Komano, T. (1995) Specific binding of the NikA protein to one arm of 17-base-pair inverted repeat sequences within the oriT region of plasmid R64. J Bacteriol 177: 46–51.

Furuya, N., and Komano, T. (1997) Mutational analysis of the R64 oriT region: requirement for precise location of the NikA-binding sequence. J Bacteriol 179: 7291–7297.

Gralla, J.D. (1985) Rapid ‘footprinting’ on supercoiled DNA. Proc Natl Acad Sci USA 82: 3078–3081.

Grohmann, E., Guzman, L.M., and Espinosa, M. (1999) Mobilisation of the streptococcal plasmid pMV158: interactions of MobM protein with its cognate oriT DNA region. Mol Gen Genet 261: 707–715.

Grohmann, E., Muth, G., and Espinosa, M. (2003) Conjugative plasmid transfer in Gram-positive bacteria. Microbiol Mol Biol Rev 67: 277–301.

Guausch, A., Lucas, M., Montcalian, G., Cabezás, M., Perez-Luque, R., Gomis-Ruth, F.X., et al. (2003) Recognition and processing of the origin of transfer DNA by conjugative relaxase TrwC. Nat Struct Biol 10: 1002–1010.

Guzman, L.M., and Espinosa, M. (1997) The mobilization protein, MobM, of the streptococcal plasmid pMV158 specifically cleaves supercoiled DNA at the plasmid oriT. J Mol Biol 266: 688–702.

Hochschild, A., and Ptashne, M. (1986) Homologous interactions of lambda repressor and lambda Cro with the lambda operator. Cell 44: 925–933.

Howard, M.T., Nelson, W.C., and Matson, S.W. (1995) Stepwise assembly of a relaxosome at the F plasmid origin of transfer. J Biol Chem 270: 28381–28386.

Kopeck, J., Bergmann, A., Fritz, G., Grohmann, E., and Keller, W. (2004) TraA and its N-terminal relaxase domain of the Gram-positive plasmid pIP501 show specific oriT binding and behave as dimers in solution. Biochem J 387: 401–409.

Kornblum, J., Hartman, B.J., Novick, R.P., and Tomasz, A. (1986) Conversion of a homogeneously methicillin-resistant strain of Staphylococcus aureus to heterogeneous resistance by Tn551-mediated insertional inactivation. Eur J Clin Microbiol 5: 714–718.

Kreiswirth, B.N., Lofdahl, S., Betley, M.J., O’Reilly, M., Schlievert, P.M., Bergdoll, M.S., and Novick, R.P. (1983) The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. Nature 305: 709–712.

Kurenbach, B., Bohn, C., Prabhu, J., Abudukerim, M., Szwazyk, U., and Grohmann, E. (2003) Intergeneric transfer of the Enterococcus faecalis plasmid pIP501 to Escherichia coli and Streptomyces lividans and sequence analysis of its tra region. Plasmid 50: 86–93.

Leblanc, B., and Moss, T. (2001) DNase I footprinting. In Methods in Molecular Biology – DNA–Protein Interactions: Principles and Protocols. Moss, T. (ed.). Totowa, NJ: Humana Press, volume 148, pp. 31–38.

Messer, W. (2002) The bacterial replication initiator DnaA. DnaA and oriC, the bacterial mode to initiate DNA replication. FEMS Microbiol Rev 26: 355–374.

Montcalian, G., Grandos, G., Lisoa, M., and de la Cruz, F. (1997) oriT-processing and regulatory roles of TrwA protein in plasmid R388 conjugation. J Mol Biol 270: 188–200.

Netz, D.J., Sahi, H.G., Marcelino, R., dos Santos Nascimento, J., de Oliveira, S.S., Soares, M.B., et al. (2001) Molecular characterisation of aureocin A70, a multi-peptide bacteriocin isolated from Staphylococcus aureus. J Mol Biol 311: 939–949.

Netz, D.J., Pohl, R., Beck-Sickinger, A.G., Selmer, T., Pierik, A.J., Bastos Mdo, C., and Sahi, H.G. (2002) Biochemical characterisation and genetic analysis of aureocin A53.
a new, atypical bacteriocin from Staphylococcus aureus. J Mol Biol 319: 745–756.

Novick, R.P., and Bouanchaud, D. (1971) The problems of drug-resistant pathogenic bacteria: extrachromosomal nature of drug resistance in Staphylococcus aureus. Ann N Y Acad Sci 182: 279–294.

Pansegrau, W., Schroder, W., and Langa, E. (1993) Relaxase (TraI) of IncP alpha plasmid RP4 catalyzes a site-specific cleaving-joining reaction of single-stranded DNA. Proc Natl Acad Sci USA 90: 2925–2929.

Parker, C., Becker, E., Zhang, X., Jandle, S., and Meyer, R. (2005) Elements in the co-evolution of relaxases and their origins of transfer. Plasmid 53: 113–118.

Projan, S.J., and Archer, G.L. (1989) Mobilization of the relaxable Staphylococcus aureus plasmid pC221 by the conjugative plasmid pGO1 involves three pC221 loci. J Bacteriol 171: 1841–1845.

Projan, S.J., Kornblum, J., Moghazeh, S.L., Edelman, I., Gennaro, M.L., and Novick, R.P. (1985) Comparative sequence and functional analysis of pT181 and pC221, cognate plasmid replicons from Staphylococcus aureus. Mol Gen Genet 199: 452–464.

Projan, S.J., Moghazeh, S., and Novick, R.P. (1988) Nucleotide sequence of pS194, a streptomycin-resistance plasmid from Staphylococcus aureus. Nucleic Acids Res 16: 2179–2187.

Relic, B., Andjelkovic, M., Rossi, L., Nagamine, Y., and Hohn, B. (1998) Interaction of the DNA modifying proteins VirD1 and VirD2 of Agrobacterium tumefaciens: analysis by subcellular localization in mammalian cells. Proc Natl Acad Sci USA 95: 9105–9110.

Sambrook, J., and Russell, D.W., Jr (2001) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schenk, S., and Laddaga, R.A. (1992) Improved method for electroporation of Staphylococcus aureus. FEMS Microbiol Lett 94: 133–138.

Silverman, P.M., and Sholl, A. (1996) Effect of traY amber mutations on F-plasmid traY promoter activity in vivo. J Bacteriol 178: 5787–5789.

Smith, M.C.A., and Thomas, C.D. (2004) An accessory protein is required for relaxosome formation by small staphylococcal plasmids. J Bacteriol 186: 3363–3373.

Stern, J.C., and Schildbach, J.F. (2001) DNA recognition by F factor Tra36: highly sequence-specific binding of single-stranded DNA. Biochemistry 40: 11586–11595.

Wang, A., and Macrina, F.L. (1995) Streptococcal plasmid pIP501 has a functional oriT site. J Bacteriol 177: 4199–4206.

Waters, V.L., Hirata, K.H., Pansegrau, W., Langa, E., and Guiney, D.G. (1991) Sequence identity in the nick regions of IncP plasmid transfer origins and T-DNA borders of Agrobacterium Ti plasmids. Proc Natl Acad Sci USA 88: 1456–1460.

Zatyka, M., Jagura-Burdzyl, G., and Thomas, C.M. (1994) Regulation of transfer genes of promiscuous IncP alpha plasmid RK2: repression of Tra1 region transcription both by relaxosome proteins and by the Tra2 regulator TrbA. J Bacteriol 176: 4256–4262.

Zechner, E.L., de la Cruz, F., Eisenbrandt, R., Grahn, A.M., Koraimann, G., Langa, E., et al. (2000) Conjugative-DNA transfer processes. In The Horizontal Gene Pool: Bacterial Plasmids and Gene Spread. Thomas, C.M. (ed.). Amsterdam: Harwood Academic Publishers, pp. 87–174.

Zhang, S., and Meyer, R.J. (1995) Localized denaturation of oriT DNA within relaxosomes of the broad-host-range plasmid R1162. Mol Microbiol 17: 727–735.

Zhang, S., and Meyer, R. (1997) The relaxosome protein MobC promotes conjugal plasmid mobilization by extending DNA strand separation to the nick site at the origin of transfer. Mol Microbiol 25: 509–516.

Ziegelin, G., Fürste, J.P., and Lurz, R.A. (1989) TraJ protein of plasmid RP4 binds to a 19-base pair invert sequence repetition within the transfer origin. J Biol Chem 264: 11989–11994.

Ziegelin, G., Pansegrau, W., Lurz, R., and Langa, E. (1992) TraK protein of conjugative plasmid RP4 forms a specialized nucleoprotein complex with the transfer origin. J Biol Chem 267: 17279–17286.