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Investigating the basis of substrate recognition in the pC221 relaxosome

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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.

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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 conjugative 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 conjugative 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 conjugative and mobilizable plasmids in Gram-positive bacteria have received comparatively little attention (Grohmann *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 conjugative 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 TraIp 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_Y promoter and is required for upregulation of *tra* gene expression (Silverman and Sholl, 1996). In R388, the TrwA accessory protein acts as both a transcriptional repressor of the *trwABC* operon and an enhancer of TrwC 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_Q 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_Q family of relaxases. In contrast, the pC221-family of relaxases are grouped within the MOB_P 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_P 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 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.

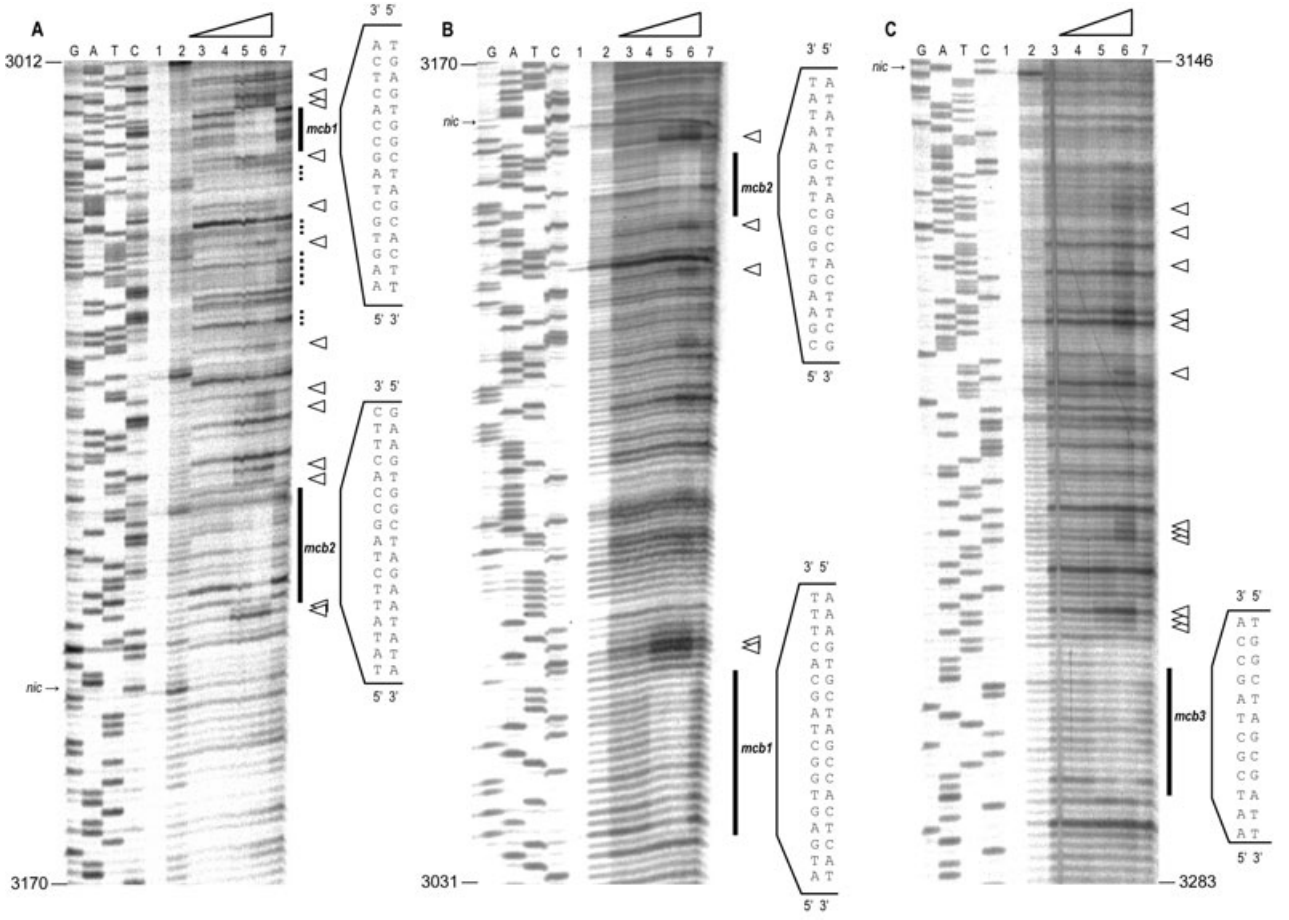

Fig. 1. Analysis of GSH–MobC binding at *oriT* and *mobC* by DNase I footprinting. DNase I footprinting was performed on both the upper (A) and lower (B) strands upstream of *oriT*, and (C) the upper strand of *mobC* located downstream of *oriT*. A supercoiled pC221*cop903* template was used and the 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* and *mcb3*. Diminished and increased DNase I hypersensitivity are indicated by dotted bars and arrowheads respectively. The position of *nic* is indicated.

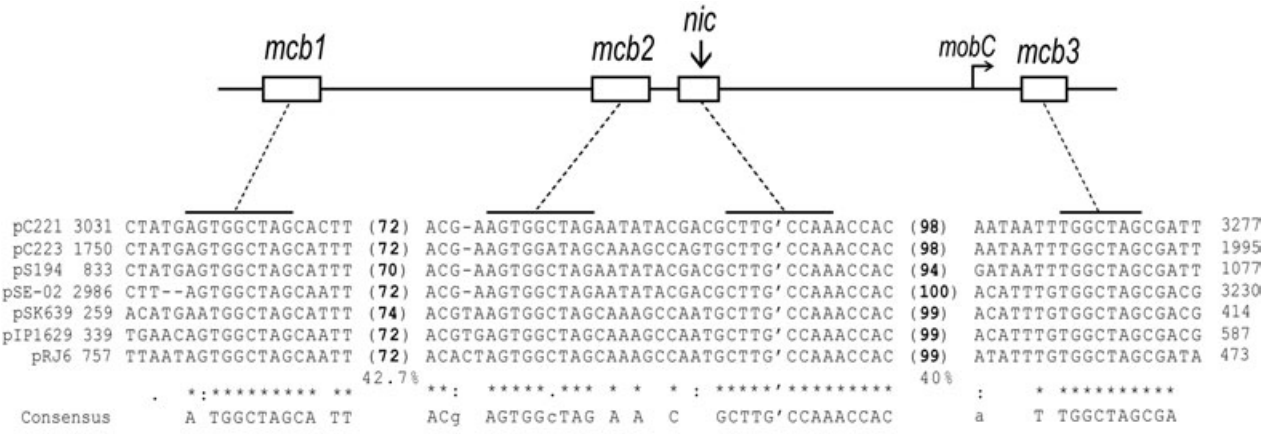

Fig. 2. Sequence alignment of seven related *oriT* regions containing conserved motifs: pC221 [GenBank Accession No. X02166, as modified by Smith and Thomas (2004): nucleotides 3019–3280], pC223 (GenBank Accession No. NC_005243: nucleotides 1737–1998), pS194 (GenBank Accession No. X06627: nucleotides 820–1080), pSE-12228-02 (GenBank Accession No. NC_005007: nucleotides 2973–3233), pSK639 (GenBank Accession No. U40259: nucleotides 246–417), pIP1629 (GenBank Accession No. AF045240: nucleotides 326–590), pRJ6 (GenBank Accession No. AF241888: nucleotides 744–476 on the complementary strand). Length (bp) of intervening sequences are indicated in brackets with percentage identity of those sequences below.

DNase I footprinting analysis was performed on pC221*cop903* 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 pC221*cop903* 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 K_D 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. $MobAH_6$ was titrated against supercoiled pC221*cop903* 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 pC221*cop903* 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 $MobAH_6$ 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, pCΔ*oriTA*, 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

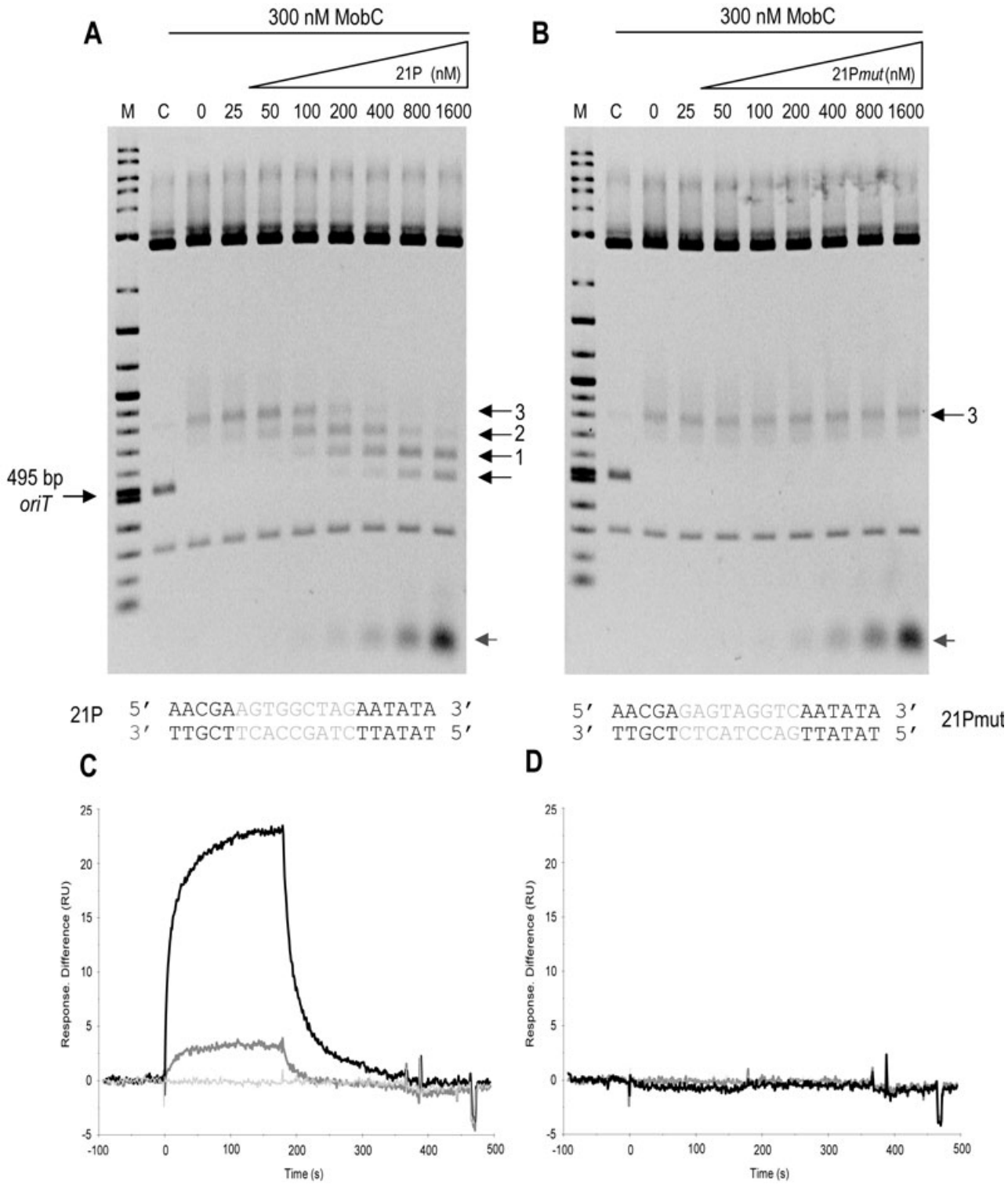

Fig. 3. A and B. Competition assay between pre-formed *oriT*–MobC complex and an increasing concentration of a synthetic oligoduplex DNA. Pre-formed *oriT*–MobC complex consisted of 10.75 nM BstXI/HindIII-digested pCER21T and 300 nM GSH–MobC. The complex was titrated with a 0–5-fold molar excess of 21P, 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 H_6MobC^{221} ligand and analytes 21P and 21Pmut at concentrations of 1000 nM (black), 100 nM (dark grey), buffer only control (light grey).

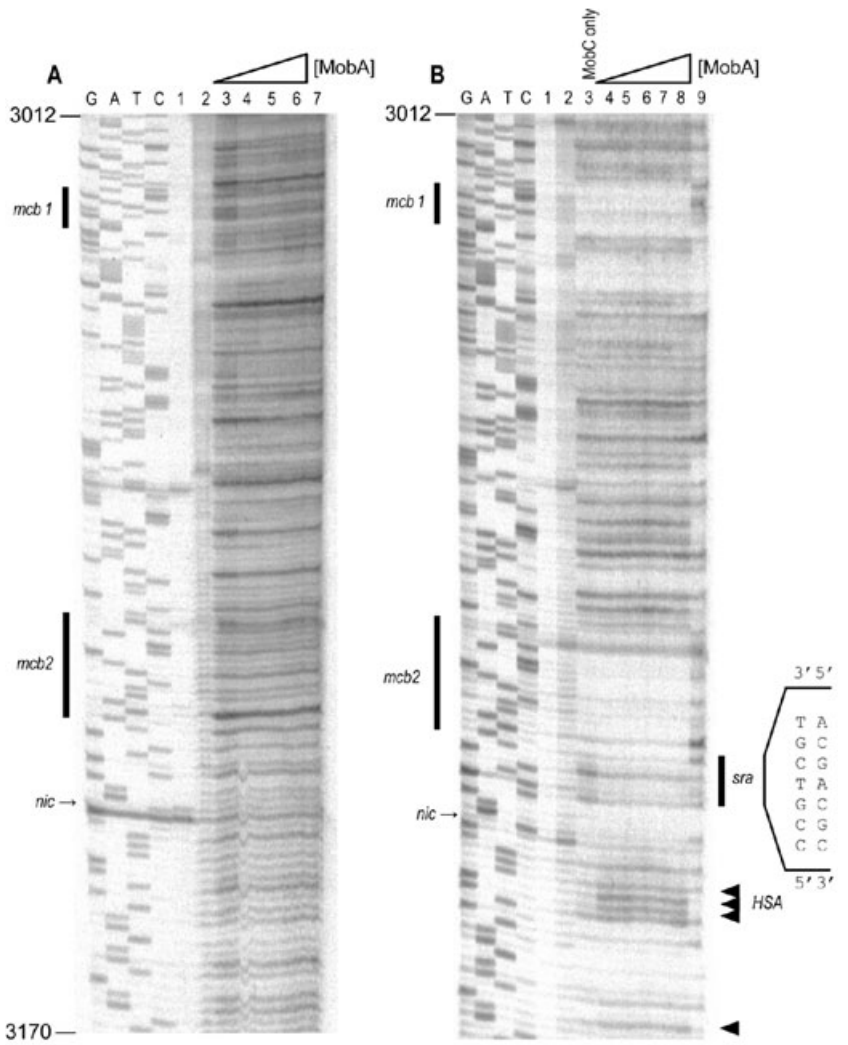

Fig. 4. Analysis of MobA binding at *oriT* by DNase I footprinting. DNase I footprinting was performed on the upper strand of *oriT* in the absence (A) and presence (B) of pre-bound GSH–MobC. A supercoiled pC221*cop903* template was used and fragments probed by primer extension; data obtained using primer NIC+98 is shown. Lane identity of (A): lane 1, DNA only/no DNase I; lane 2, $MobAH_6$ only/no DNase I; lanes 3–6, $MobAH_6$ (nM): 50, 100, 250, 450; lane 7, DNase I only. Lane identity of (B): lanes 1 and 2, as (A); lane 3, GSH–$MobC^{221}$ only: 120 nM; lanes 4–8, GSH–MobC (120 nM) + $MobAH_6$ (nM): 50, 100, 250, 450, 450; lane 9, DNase I only control. Sequencing ladder standards G, A, T, C are as indicated. Solid bars indicate protection, designated *mcb1*, *mcb2* and *sra*. MobA-induced DNase I hypersensitivity (*HSA*) is indicated by black arrowheads. The position of *nic* is indicated with an arrow.

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 $oriT_{nic}$. This $oriT_{nic}$ was cloned into pCER19, as pCER21*wt* (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*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 pCER21*wt* substrate at the 5′ nucleotide of the *nic* dinucleotide from 5′-GC-3′ to 5′-TC-3′ to create the substrate pCER*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 pCΔ-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 pCΔ, and both novobiocin

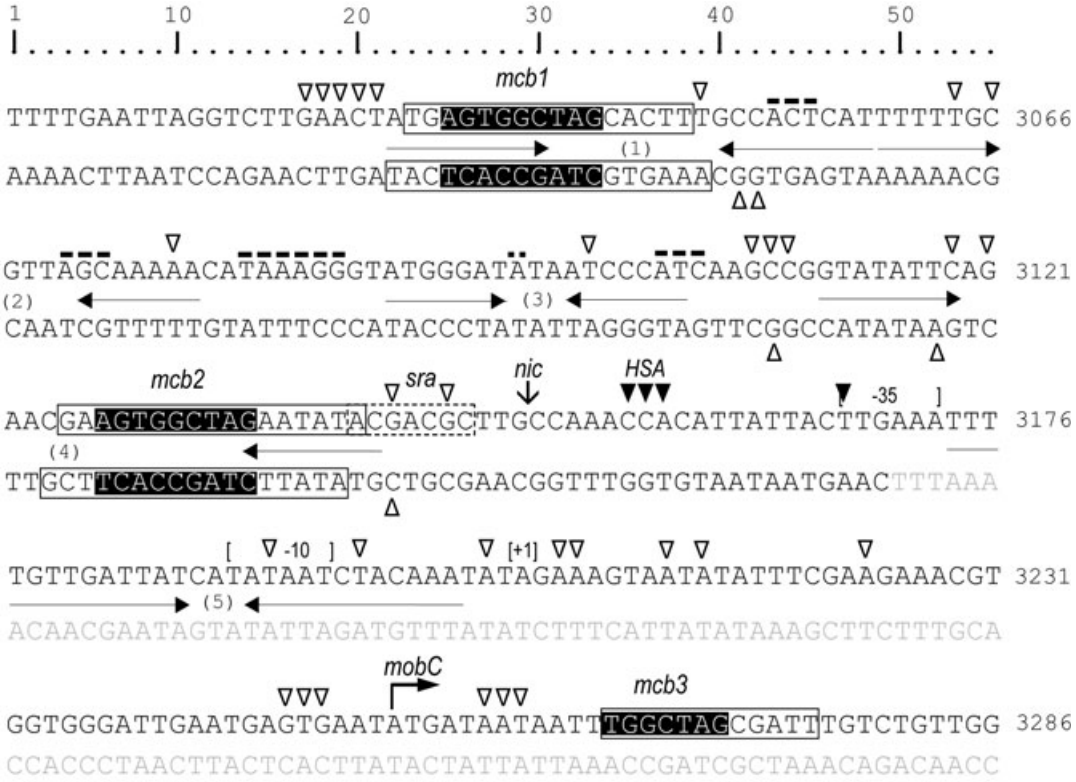

Fig. 5. Summary of footprinting data for the binding of MobC and MobA. Double-stranded DNA encompassing bases 3012–3285 (5′–3′) of pC221 (GenBank Accession No. X02166 as modified by Smith and Thomas, 2004). Sequences in black type correspond to the regions footprinted in Figs 1 and 4. Principle regions protected by MobC are indicated by solid boxes and labelled. The conserved sequence is highlighted in black. Those protected by MobA are indicated by dashed boxes. *Nic* is indicated by an arrow. Regions of diminished DNase I hypersensitivity upon binding of MobC are indicated by dashed bars. The predicted promoter for *mobC* is labelled (–35, –10) and the start codon indicated by a bent arrow. The positions of inverted repeats, based on Smith and Thomas (2004), are indicated.

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 pCER*77mcb* (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 $oriT_{nic}$, which could bear functional significance (Fig. 7A).

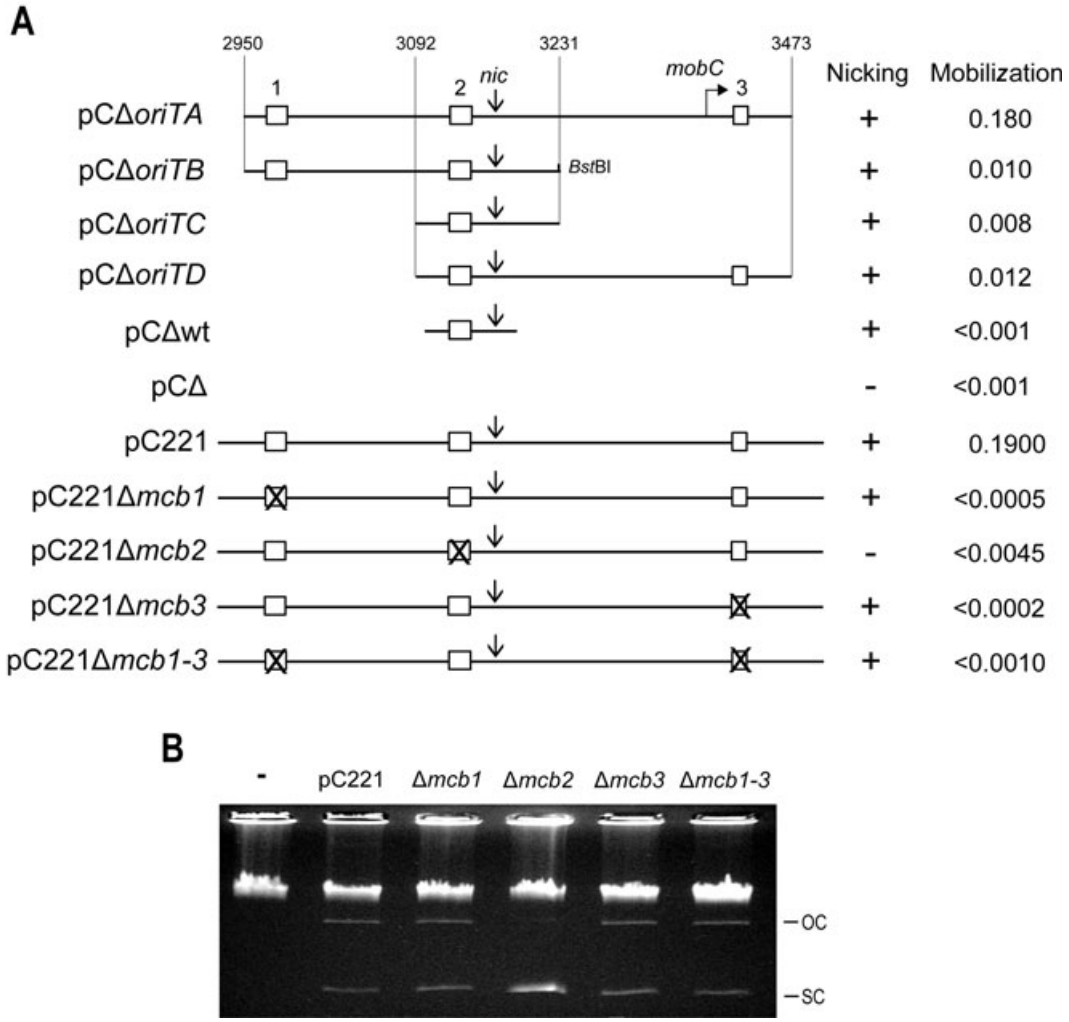

Fig. 6. A. Schematic diagram detailing the regions of *oriT* cloned and assessed for nicking and mobilization. The co-ordinates given are those of pC221 (GenBank Accession No. X02166 as modified by Smith and Thomas, 2004). The MobC binding sites (*mcb*) are indicated by numbered boxes. *Nic* is indicated by a small arrow and *mobC* by a bent arrow. *oriTA* (514 bp), *oriTB* (256 bp), *oriTC* (128 bp), *oriTD* (386 bp). Substrate nicking *in vitro* is indicated (+). The efficiency of mobilization of each construct is shown, and represents the number of target *oriT* molecules transferred for every transfer of pGO1 (calculated as described in the *Experimental procedures*). In the pC221Δ*mcb* mutants 'X' signifies mutation at that position.
B. Agarose gel electrophoresis of whole cell lysates showing *in vivo* nicking phenotype of pC221*mcb* mutants present in RN4220 donor cells also carrying pGO1. A control lane (–) indicates lysate of a pGO1-only donor.

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 pCER*77sra* (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

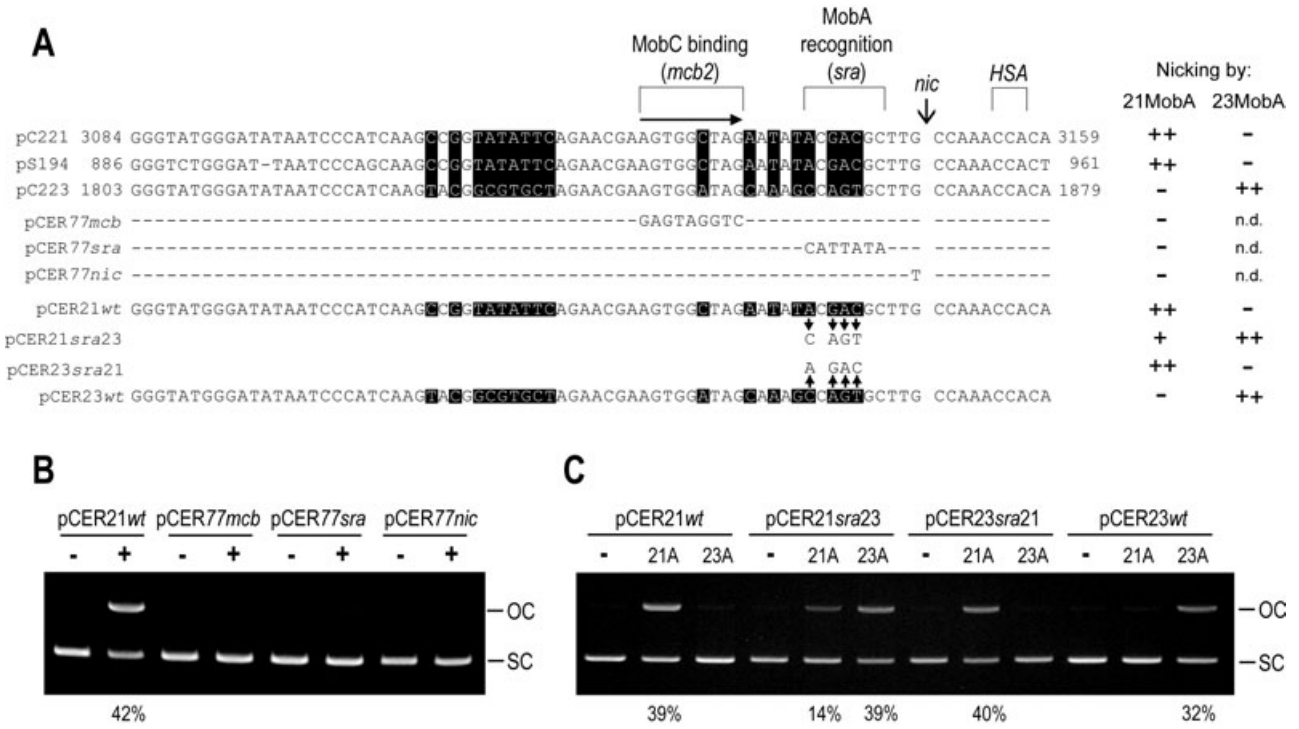

Fig. 7. Effect on nicking activity of mutation within $oriT_{nic}$.
A. Alignment of pC221 (GenBank Accession No. X02166), pS194 (GenBank Accession No. X06627) and pC223 (GenBank Accession No. NC_005243) *oriT* regions. Bases that are not conserved between pC221/pS194 and pC223 are highlighted black. The positions of MobC binding (*mcb2*), MobA recognition (*sra*) and hypersensitivity in the presence of MobA (*HSA*) are labelled. *Nic* is indicated by an arrow. The susceptibility to nicking in the presence of either pC221 MobA (21MobA) or pC223 MobA (23MobA) is listed for each. The $oriT_{nic}$ substrates that were synthesized with incorporated mutations made at *mcb2* (pCER*77mcb*), *sra* (pCER*77sra*) and *nic* (pCER*77mcb*) were based on the pC221 sequence of the alignment. The $oriT_{nic}$ substrates pCER21*wt* and pCER23*wt* correspond to the wild-type pC221 and pC223 sequences, and were used as controls for the *sra*-swap experiment. The four bases swapped between these sequences to generate the *sra*-swap substrates pCER21*sra*23 and pCER23*sra*21 are indicated.
B and C. Nicking assays performed on supercoiled cloned mutant and *sra*-swap $oriT_{nic}$ substrates. The nicking reactions were prepared as described (Caryl *et al*., 2004). DNA only controls for each substrate are indicated (–). The position of nicked, open-circular DNA (OC) and supercoiled DNA (SC) is given. (B) Nicking of pC221 sequence based *mcb*, *sra* and *nic* mutants was performed using pC221 $MobAH_6$ and GSH–MobC, with pCER21*wt* as a positive control. (C) Nicking of *sra*-swap $oriT_{nic}$ substrates was in the presence of pC221 $MobAH_6$ (21A) or pC223 $MobAH_6$ (23A), and pC221 GSH–MobC. Percentages indicate percentage of total plasmid DNA in the nicked open-circular DNA form.

wild-type pC221 (pCER21*wt*) and pC223 (pCER23*wt*) sequences; and two *sra*-swap substrates, pCER21*sra*23 and pCER23*sra*21. *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 pCER21*sra*23 by 21MobA (14%), compared with 23MobA (39%); and no detectable nicking of pCER23*sra*21 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, *srj*, 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 on the outside of bent DNA and restricted 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 $oriT_{nic}$ 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 $oriT_{nic}$ (Fig. 7A), which, despite their similarity, can only be nicked by their cognate relaxases.

Second, comparison of the pC221 $oriT_{nic}$ 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 $oriT_{nic}$ 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 high-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, TraI36, of the unrelated F-plasmid (Stern and Schildbach, 2001) and TrwC of R388 (Guasch *et al.*, 2003).

We have not yet been able to demonstrate a direct protein–protein interaction between MobC and MobA, nor is there any unequivocal data for such interactions between TraJ–TraI of RP4 (Ziegelin *et al.*, 1989) or NikA–NikB (Furuya and Komano, 1995). While not conclusive, a protein–protein interaction between the homologous *Agrobacterium tumefaciens* pTi plasmid VirD1–VirD2 proteins has been suggested on the basis of nuclear localization and co-immunoprecipitation in mammalian cells (Relic *et al.*, 1998).

In summary, we have located three sequences within *oriT* required for interaction with the accessory protein MobC, and a further site for the subsequent interaction with the relaxase MobA. Nicking by the latter is dependent on the presence of the plasmid-specific sequence adjacent to *nic*, in concert with a single *mcb* site. However, all three *mcb* sites are required for formation of a productive complex capable of mobilization. Finally, it is worth noting the presence of the third MobC binding site within the *mobC* gene itself. Such a location presents the possibility of an autoregulatory role for MobC; a role that would link the establishment of the relaxation complex with the expression of these proteins required for its formation.

Experimental procedures

Bacterial strains, growth conditions and manipulation of DNA

Bacterial strains, plasmids and primers are listed in Table 1. *E. coli* strain DH5α 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 DH5α 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^{-1}; Chloramphenicol (Cm) 10 μg ml^{-1}; Erythromycin (Em) 5 μg ml^{-1}; Gentamicin (Gm) 5 μg ml^{-1}; Novobiocin (Nv) 5 μg ml^{-1}; Rifampicin (Rf) 5 μg ml^{-1}.

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* DH5α. The shuttle vector pCΔ 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 pCΔ 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 pCΔ. 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
Strain		
E. coli DH5α	F$^-$, ϕ80d*lacZ* ΔM15, *endA*1, *recA*1, *HsdR*17 (r_k^-, m_k^+), *supE*44, *thi-1*, *GyrA*96, *relA*1, Δ(*lacZYA-argF*)*U169*, λ$^-$	BRL
S. aureus RN4220	Attenuated, *mec*A, TSSE$^-$, *rsbU*$^-$, *agr*$^-$, restriction deficient, modification proficient derivative of strain + 8325–4.	Kreiswirth *et al.* (1983)
S. aureus RN2677	8325 derived ϕ11, ϕ12, ϕ13 lysogen, *agr*$^+$, *sak*$^+$, *rsbU*$^+$, *hib*$^-$, restriction deficient, spontaneous Nv^r and Rf^r mutant, recipient strain for conjugation.	Kornblum *et al.* (1986) T.J. Foster[a]
Plasmids		
pC221	4555 bp: Cm^r, *inc4*, *repD*, *mobCAB*	Novick and Bouanchaud (1971)
pC221*cop903*	4168 bp: Cm^r, *inc4*, *repD*, *mobCAB* (Δ806-1185: copy no. mutation)	Projan *et al.* (1985)
pCER19	3063 bp: pUC19: Ap^r, *ori* (pMB1), *lacZ*, *cer* (dimer resolution)	Caryl *et al.* (2004)
pCER21T	3575 bp: pCER19 containing pC221*cop903 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
pCER21*wt*	3127 bp: wild type 77 bp pC221 $oriT_{nic}$	This work
pCER*77mcb*	3127 bp: 77 bp pC221 $oriT_{nic}$ mutated at *mcb*	This work
pCER*77sra*	3127 bp: 77 bp pC221 $oriT_{nic}$ mutated at *sra*	This work
pCER*77nic*	3127 bp: 77 bp pC221 $oriT_{nic}$ mutated at *nic*	This work
pCER21*sra*23	3127 bp: 77 bp pC221 $oriT_{nic}$ with pC223 *sra* swap	This work
pCER23*sra*21	3127 bp: 77 bp pC223 $oriT_{nic}$ with pC221 *sra* swap	This work
pCER23*wt*	3127 bp: wild type 77 bp pC223 $oriT_{nic}$	This work
pCΔ	5625 bp: pE194 : pUC19 based shuttle vector.	This work
pCΔ21wt	5689 bp: Shuttle vector containing wt 77 bp pC221 $oriT_{nic}$	This work
pCΔ*oriTA*	6128 bp: Shuttle vector containing 514 bp *oriTA*	This work
pCΔ*oriTB*	5880 bp: Shuttle vector containing 256 bp *oriTB*	This work
pCΔ*oriTC*	5753 bp: Shuttle vector containing 139 bp *oriTC*	This work
pCΔ*oriTD*	6000 bp: Shuttle vector containing 386 bp *oriTD*	This work
pC221Δ*mcb1*	4555 bp: pC221 with a 9 bp mutation of *mcb1*	This work
pC221Δ*mcb2*	4555 bp: pC221 with a 9 bp mutation of *mcb2*	This work
pC221Δ*mcb3*	4555 bp: pC221 with a 9 bp mutation of *mcb3*	This work
pC221Δ*mcb1–3*	4555 bp: pC221 with a 9 bp mutations of *mcb1* and *mcb3*	This work
pGO1	52 314 bp: Gm^r, Tp^r, Qam^r, *tra*$^+$	T.J. Foster[a]

Oligonucleotides	Sequence (5′–3′)[b]	Description
PforORITA	AACTGCAGAAATTTATGAACGTATAGC	PstI forward *oriTA*
BrevORITA	CGGGATCCTTGGCAATCGATTGTCGC	BamHI reverse *oriTA*
PforORITC	AACTGCAGGATATAATCCCATCAAGCCG	PstI forward *oriT*C
PrevORITC	CGGGATCCACGTTTCTTCGAAATATATTAC	BamHI reverse *oriTC*
NIC+203	CATATTCGGATTCGCTC	Primer extension + strand
NIC+98	CACTCATTCAATCCCACC	Primer extension + strand
NIC–178	GAACGTATAGCAACCAC	Primer extension – strand
HPA23–	AAGTTTATAGCAACCAC	Primer extension + strand
21P+	AACGAAGTGGCTAGAATATA	*mcb* oligoduplex + strand
21P–	TATATTCTAGCCACTTCGTT	*mcb* oligoduplex – strand
21Pmut+	AACGA**GAGTAGGTC**AATATA	mutagenic *mcb* + strand
21Pmut–	TATATT**GACCTACTC**TCGTT	mutagenic *mcb* – strand
ORIT–MCB+	GGGTATGGGATATAATCCCATCAAGCCGGTATATTCAGAACGA **GAGTAGGTC**AATATACGACGCTTGCCAAACCACAG	pC221 $oriT_{nic}$ with mutated *mcb* + strand
ORIT–MCB–	GATCCTGTGGTTTGGCAAGCGTCGTATATT**GACCTACTC**TCGT TCTGAATATACCGGCTTGATGGGATTATATCCCATACCCTGCA	pC221 $oriT_{nic}$ with mutated *mcb* – strand
ORIT–SRA+	GGGTATGGGATATAATCCCATCAAGCCGGTATATTCAGAACGA AGTGGCTAGAATAT**CATTATA**TTGCCAAACCACAG	pC221 $oriT_{nic}$ with mutated *sra* + strand
ORIT–SRA–	GATCCTGTGGTTTGGCAA**TATAAGT**ATATTCTAGCCACTTCGT TCTGAATATACCGGCTTGATGGGATTATATCCCATACCCTGCA	pC221 $oriT_{nic}$ with mutated *sra* – strand
ORIT–NIC+	GGGTATGGGATATAATCCCATCAAGCCGGTATATTCAGAACGA AGTGGCTAGAATATACGACGCTT**T**CCAAACCACAG	pC221 $oriT_{nic}$ with mutated *nic* + strand
ORIT–NIC–	GATCCTGTGGTTTGG**A**AAGCGTCGTATATTCTAGCCACTTCGT TCTGAATATACCGGCTTGATGGGATTATATCCCATACCCTGCA	pC221 $oriT_{nic}$ with mutated *nic* – strand
21WT+	GGGTATGGGATATAATCCCATCAAGCCGGTATATTCAGAACGA AGTGGCTAGAATATACGACGCTTGCCAAACCACAG	pC221 $oriT_{nic}$ + strand
21WT–	GATCCTGTGGTTTGGCAAGCGTCGTATATTCTAGCCACTTCGT TCTGAATATACCGGCTTGATGGGATTATATCCCATACCCTGCA	pC221 $oriT_{nic}$ – strand
21SRA23+	GGGTATGGGATATAATCCCATCAAGCCGGTATATTCAGAACGA AGTGGCTAGAATAT**CCAGT**GCTTGCCAAACCACAG	pC221 $oriT_{nic}$ with pC223 *sra* + strand
21SRA23–	GATCCTGTGGTTTGGCAAGC**ACTGG**ATATTCTAGCCACTTCGT TCTGAATATACCGGCTTGATGGGATTATATCCCATACCCTGCA	pC221 $oriT_{nic}$ with pC223 *sra* – strand

Table 1. *cont.*

Oligonucleotides	Sequence (5′–3′)[b]	Description
23SRA21+	GGGTATGGGATATAATCCCATCAAGTACGGCGTGCTAGAACGA AGTGGATAGCAAAG**ACGAC**GCTTGCCAAACCACAG	pC223 *oriT*$_{nic}$ with pC221 *sra* + strand
23SRA21–	GATCCTGTGGTTTGGCAAGC**GTCGT**CTTTGCTATCCACTTCGT TCTAGCACGCCGTACTTGATGGGATTATATCCCATACCCTGCA	pC223 *oriT*$_{nic}$ with pC221 *sra* – strand
23WT+	GGGTATGGGATATAATCCCATCAAGTACGGCGTGCTAGAACGA AGTGGATAGCAAAGCCAGTGCTTGCCAAACCACAG	pC223 *oriT*$_{nic}$ + strand
23WT–	GATCCTGTGGTTTGGCAAGCACTGGCTTTGCTATCCACTTCGT TCTAGCACGCCGTACTTGATGGGATTATATCCCATACCCTGCA	pC223 *oriT*$_{nic}$ – strand
PST–BSTXI+	AGGCTGCAGTAGCAACCACATTTTTGG	PstI and BstXI forward
BAM–CLAI–	CGGGATCCAATCGATTGTCGCGTTTC	BamHI and ClaI reverse
MCB1A+	AGCTTCATCATGCTGTATG	Forward *mcb1*
MCB1A–	AAAGTG**GACCTACTC**CATAGTTCAAGACCTAATTC	Reverse *mcb1* mutation
MCB1B+	ACTATG**GAGTAGGTC**CACTTTGCCACTCATTTTTTGCG	Forward *mcb1* mutation
MCB2A–	TATATT**GACCTACTC**TCGTTCTGAATATACCGG	Reverse *mcb2* mutation
MCB2B+	GAACGA**GAGTAGGTC**AATATACAACGCTTGCC	Forward *mcb2* mutation
MCB3A–	CAAATC**TGATGCAAG**ATTATTATCATATTCACTC	Reverse *mcb3* mutation
MCB3B+	AATAAT**CTTGCATCA**GATTTGTCTGTTGGGGAAAAC	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.

duced using primers PforORITA/BrevORITA. *oriTB* was prepared by digestion of *oriTA* with PstI/BstBI and cloning into pCΔ digested with PstI/AccI. *oriTC* was prepared using PforORITC and BrevORITC; and *oriTD* using PforORITC and BrevORITA. Ligated recombinants were transformed into *E. coli* DH5α (Dagert and Ehrlich, 1979) under Ap selection. Recombinant shuttle vectors prepared from *E. coli* were screened by restriction analysis and confirmed by DNA sequence analysis. They were then introduced into the mating donor strain (*S. aureus* RN4220, pGO1, pC221) after first passaging through plasmid-free *S. aureus* RN4220 (Table 1) by electroporation as described (Schenk and Laddaga, 1992) using 0.2 cm gap cuvette, 2.3 kV pulse voltage, 2.5 ms.

Site-directed mutations of *mcb* sites in the context of pC221 *oriT* were prepared by overlap extension PCR using pC221 as a template. Each mutant *oriT* was prepared in two separate reactions (A and B) with primers for the two halves overlapping at the site of the desired mutation. The primers used for mutation at *mcb1* were MCB1A+ and MCB1A– (reaction A), and MCB1B+ and BAM–CLAI– (reaction B). Primers for *mcb2* were PST–BSTXI+ and MCB2A– (reaction A), and MCB2B+ and BAM–CLAI– (reaction B). Primers for *mcb3* were PST–BSTXI+ and MCB3A– (reaction A), and MCB3B+ and BAM–CLAI– (reaction B). The products of reactions A and B were gel purified and subjected to a final PCR amplification using primers (PST–BSTXI+ and BAM–CLAI–) in order to amplify the final product from the overlap-extended A+B template, and incorporate appropriate restriction site tags. The mutagenized *oriT* DNA (511 bp) products were digested with BamHI and PstI and cloned into similarly digested pCER19 and transformed into *E. coli* DH5α. Recombinant DNA was selected by α-complementation using a complete LB/S-Gal/IPTG medium (Sigma) and screened by restriction analysis. Once isolated, recombinant DNA for each mutant was digested with BstXI and ClaI, releasing the mutagenized *oriT* DNA. This fragment was ligated with a similarly digested reciprocal part of the pC221 and transformed by electroporation as described above. Mutant plasmids were confirmed by sequencing.

For *oriT*$_{nic}$ constructs based on pCER19, complementary strands corresponding to either wild-type or specifically mutated 77 bp *oriT*$_{nic}$ were synthesized incorporating PstI 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 200 mM KCl, 25 mM Tris.HCl (pH 8.0), 2 mM $CaCl_2$ and incubated at 21°C for 2 h. Following cleavage, the mixture was adjusted to 0.5 M KCl and passed through a benzamidine column (Pharmacia; 1 ml) at 0.5 ml min^{-1} to remove thrombin. Protein solutions were dialysed and concentrated as previously reported (Caryl *et al*., 2004).

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

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

Primers 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-[^{33}P]-ATP (3000 Ci mmol^{-1}) (MP Biomedicals, Irvine, CA).

Primer extension reactions contained 0.07 pmol of DNase I-digested plasmid DNA, 1.2 pmol 5′-end-labelled oligonucleotide primer, 0.25 units of AmpliTaq 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 $MgCl_2$, 0.025% v/v Tween 20. Dideoxy nucleotide sequencing ladders were prepared according to the manufacturer's instructions using 0.02 pmol supercoiled pC221*cop903* 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 nM 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 pre-rinsed 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 $NiSO_4$ (500 μM) at 5 μl min^{-1}. H_6MobC^{221} 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 Ni^{2+} 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 pCΔ 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 OD_{600} equivalent of 0.1 and incubated at 30°C until mid-log phase.

Volumes of donor and recipient cultures equivalent to ~3×10^8 cells (1 ml culture at OD_{600} 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.

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