pRJ5: a naturally occurring *Staphylococcus aureus* plasmid expressing constitutive macrolide–lincosamide–streptogramin B resistance contains a tandem duplication in the leader region of the *ermC* gene

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The 2.55 kb *Staphylococcus aureus* plasmid, pRJ5, confers constitutive resistance to macrolide–lincosamide–streptogramin B (MLS) antibiotics. pRJ5 is nearly identical to the inducible MLS resistance plasmid pT48, and has homology with the *S. aureus* plasmids pE194 and pSN2. The *Hind*III–C and/or *Hind*–B fragments were required for stable maintenance of the plasmid and probably carry *palA*. Plasmids pRJ5 and pT48 were shown to belong to the same incompatibility group, Inc12 (L). DNA sequencing showed that pRJ5 contains a 28 bp direct tandem duplication in the leader/attenuator region of *ermC*. This is likely to change the secondary structure of the methylase mRNA, allowing constitutive expression of *ermC*. The type of mutation found on plasmid pRJ5 is different from those observed in similar 2.5 kb constitutive MLS-resistance plasmids isolated from other Gram-positive bacteria, including staphylococci.

Introduction

Resistance to macrolide–lincosamide–streptogramin B (MLS) antibiotics was encountered almost as soon as these drugs were introduced into clinical practice. The most common form of MLS resistance is caused by N⁶,N⁶-dimethylation of an adenine residue in the 23S ribosomal RNA, which reduces the affinity between the antibiotic and the ribosome (Dubnau, 1984). In some isolates, production of the methylase enzyme is inducible by sub-inhibitory concentrations of erythromycin (Em) or the related antibiotic oleandomycin (Dubnau, 1984) by a mechanism that involves translational attenuation. However, in many strains the expression of the methylase is constitutive (Novick *et al.*, 1979a; Bastos *et al.*, 1980; Lampson & Parisi, 1986; Monod *et al.*, 1986; Catchpole & Dyke, 1990).

In *Staphylococcus aureus*, MLS resistance is commonly mediated by small (2–4 kb) class I multicopy plasmids (Lyon & Skurray, 1987; Novick, 1989) of either the pE194 family or the pSN2 family. Class I plasmids replicate by a rolling circle mechanism (RCR) (Te Riele

et al., 1986; Gruss & Ehrlich, 1989; Novick, 1989). Three plasmid-encoded elements are used in RCR: a plus origin, a replication protein (Rep) and a minus origin (*palA*).

Plasmid pE194 encodes the *ermC* gene, which confers inducible MLS resistance, and it is to date the only member of its family. pE194 is 3.7 kb in size, and has been extensively studied in both *S. aureus* (Weisblum *et al.*, 1979; Iordanescu & Surdeanu, 1980; Byeon & Weisblum, 1990; Sozhamannan *et al.*, 1990) and *Bacillus subtilis* (Gryczan *et al.*, 1980; Shivakumar *et al.*, 1980; Horinouchi & Weisblum, 1982).

pSN2 is a 1288 bp cryptic plasmid. Its only significant ORF encodes a protein required for initiation of plasmid replication (Khan & Novick, 1982). The pSN2 family includes a series of closely related plasmids that contain an MLS resistance determinant linked to the basic pSN2 replicon. Those plasmids possess MLS resistance determinants which are homologous with *ermC*, although their replication regions do not share homology with that of pE194 (Projan *et al.*, 1987; Catchpole *et al.*, 1988; Catchpole & Dyke, 1990, 1991).

In this study we present a molecular characterization of pRJ5, a plasmid from *S. aureus* that encodes MLS resistance (Bastos *et al.*, 1980), including the DNA sequence of the *ermC* leader region. The sequence relations between pRJ5 and other MLS resistance

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The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number L04687.

Table 1. *Bacterial strains and plasmids*

Strain	Plasmid	Size (kb)	Phenotype associated with plasmid*	Source or reference
MB3	pRJ5	2.55	MLS ^r I ⁻	Bastos <i>et al.</i> (1980); this study
RN1801	pT127	4.4	Tc ^r , Inc3 (C)	Iordanescu (1976)
RN2442	pE194	3.72	MLS ^r I ⁺ Inc11 (K)	Iordanescu (1976)
SA1105	pE2222	2.5	MLS ^r I ⁻ Inc12 (L)	Iordanescu & Surdeanu (1980)
SA1117	pSA6522	7.0	Tc ^r , Inc3 Inc12	Iordanescu & Surdeanu (1980)
T48	pT48	2.47	MLS ^r I ⁺	Catchpole <i>et al.</i> (1988)
Sk31	pSN2	1.28	—	Khan & Novick (1982)
RN451	—	—	—	Novick (1967)

* I⁺, inducible phenotype; I⁻, constitutive phenotype.

plasmids were also investigated by comparing restriction endonuclease maps and functional organization and by hybridization.

Methods

Bacterial strains, growth conditions and plasmids. *S. aureus* strains and plasmids are listed in Table 1. The strains were grown in CY medium (Novick, 1967) and stored at -70 °C until needed. Subcultures were made in either trypticase soy broth (TSB, Difco) or trypticase soy agar (TSA, Difco). When necessary, media were supplemented with tetracycline (Tc) and/or erythromycin (Em) at 5 µg ml⁻¹. The cultures were incubated at 32 °C.

For the analysis of plasmid stability, and for segregation analysis, exponential-phase cultures were diluted 1000-fold and grown in TSB, in the absence of selection, until the culture had regained its original density. This was repeated 10 times. The proportion of plasmid-free cells was determined by serial dilution and plating on drug-free agar followed by replica-plating on drug-containing agar.

Plasmid transfer. Transductions with phage ϕ 11 were done as described previously (Bastos *et al.*, 1980). Transformation was performed as described by Murphy *et al.* (1981).

Plasmid DNA isolation. Whole cell lysates were prepared as described by Giambiagi-Marval *et al.* (1990). Plasmid DNA was prepared by caesium chloride/ethidium bromide density-gradient centrifugation of cleared lysates, prepared as described by Novick *et al.* (1979b).

Restriction endonuclease analysis. Restriction endonucleases were purchased from New England Biolabs and used as specified by the manufacturer.

Gel electrophoresis. Restriction endonuclease digests of plasmid DNA and uncut plasmids were analysed by electrophoresis in 0.7–1.5% (w/v) agarose horizontal slab gels and/or in 5.0% (w/v) polyacrylamide vertical slab gels (Sambrook *et al.*, 1989). In both cases, both gel buffer and running buffer were 89 mM-Tris, 89 mM-boric acid and 2.5 mM-EDTA. Size standards for linear DNA fragments were bacteriophage λ DNA cleaved with *Bst*EII or with *Hind*III and *Eco*RI and bacteriophage ϕ X174 DNA cleaved with *Hae*III (Ausubel *et al.*, 1990).

DNA hybridization. Southern blot hybridization was done as described previously (Bastos & Murphy, 1988). Probe DNA was labelled by nick-translation as described by Sambrook *et al.* (1989).

PCR cloning and sequencing. Primers corresponding to nucleotides 1616–1634 and 1889–1917 of pT48 were used to amplify a fragment from plasmid pRJ5, equivalent to a 301 bp fragment of pT48, that included the region encoding the leader peptide and the 5' part of *ermC*. To prepare template DNA, cells from strain MB3 were scraped with a toothpick from a TSA plate incubated for 18 h, washed twice in 10 mM-Tris, 0.1 mM-EDTA, pH 7.8, resuspended in 200 µl of the same buffer, and lysed by the addition of 100 µg lysostaphin ml⁻¹. After incubation for 30 min, lysis was completed by the addition of SDS to a final concentration of 0.2%. Two microlitres of this preparation was used for PCR.

PCR reactions used Vent polymerase (New England Biolabs) in a reaction mixture containing each primer at 100 µM and other components as specified by the manufacturer. Reactions were cycled at 94 °C for 1 min, 45 °C for 45 s and 72 °C for 45 s, for 25 cycles. A fragment of approximately 325 bp was purified by electrophoresis on a 3% (w/v) NuSieve gel (FMC Bio Products). The excised band was melted at 65 °C and incubated without further purification with T4 DNA-ligase and *Sma*I-cleaved M13mp11 RF1 DNA. The ligation mixture was used to transform *Escherichia coli* JM109. Six clones, all with inserts in the same orientation, were sequenced by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977), using Sequenase (US Biochemicals) and [α -³⁵S]dATP (New England Nuclear).

Computer analysis. Possible RNA secondary structures were predicted by the computer program of Zuker & Stiegler (1981).

Results and Discussion

Structural organization of pRJ5 and its relationship to other S. aureus plasmids

A restriction map of pRJ5 is presented in Fig. 1. The homology between pRJ5 DNA and pE194 or pSN2 was investigated by Southern hybridization, using pE194 and pSN2 as probes (Fig. 1). The size of pRJ5 was estimated as 2.55 kb. pRJ5 contains a 1.35 kb region homologous to pE194 located between positions 910 and 2260. A comparison of the restriction maps of pRJ5 and pE194 (Fig. 1) indicates that some of the restriction sites in the region of homology between these plasmids are conserved. This region includes the *ermC* gene, encoding a 23S rRNA methylase, and the leader region that controls

Fig. 1. Comparative analysis of the restriction maps and the functional organization of the plasmids pE194 (Horinouchi & Weisblum, 1982; Byeon & Weisblum, 1990), pRJ5, pT48 (Catchpole *et al.*, 1988) and pSN2 (Khan & Novick, 1982). The plasmids are linearized to emphasize the alignment of homologous regions. Relevant restriction sites are indicated as follows: A, *AccI*; B, *BclI*; C, *Clal*; H, *HpaI*; Hp, *HpaII*; Ha, *HaeIII*; Hh, *HhaI*; Hd, *HindIII*; Hf, *HinfI*; P, *PstI*; S, *SacI*; T, *TaqI*; X, *XbaI*. For plasmids pE194, pT48 and pSN2, the nucleotide positions are from the DNA sequence. Numbering starts from a *MboI* site for pE194, from a *HindIII* site for pRJ5 and pT48 and from a *HpaII* site for pSN2. ORFs are designated by arrows. *pre*, plasmid recombination protein; *cop*, copy number control; *rep*, replication protein; *erm*, methylase; *palA*, minus-strand conversion signal; *RS_A*, recombination site A. The presumed locations of *ermC*, *rep* and *palA* are shown for pRJ5. The solid bar indicates the pSN2 homologous region and the cross-hatched bar indicates the pE194 homologous region in pRJ5.

its expression. We conclude that the MLS resistance determinant of pRJ5 is very similar to *ermC*, and that the remainder of pRJ5 bears no detectable homology with pE194. Therefore, pRJ5 cannot be placed in the pE194 family of class I plasmids.

pRJ5 also showed homology to plasmid pSN2, between positions 150 and 900. This homology corresponds, in pSN2, to the region encoding RepL, the protein involved in initiation of plasmid replication (Khan & Novick, 1982; Novick, 1989). Based on these results, pRJ5 can be placed in the pSN2 family of class I plasmids.

The restriction map of pRJ5 is nearly identical to that of pT48 (Fig. 1), which also belongs to the pSN2 family (Catchpole *et al.*, 1988). The *HindIII*-B fragment of pT48 encloses most of *palA*, an imperfect palindrome of about 156 bp (Catchpole *et al.*, 1988). If the *HindIII*-B

fragment of pRJ5 also contains *palA* sequences, the removal of this fragment would generate plasmid instability. *palA* mutants of *S. aureus* plasmids have a marked decrease in copy number and are highly unstable (Gruss *et al.*, 1987). To delete the small *HindIII* fragments B and C, pRJ5 DNA was completely digested with *HindIII*, religated, and used to transform protoplasts of strain RN451 with selection for Em^R. The transformants were analysed for the presence of plasmid DNA, which was barely visible in agarose gels (data not shown), indicating that the copy number was low. The deletion derivatives were also highly unstable, being lost from 94% of cells after approximately 50 generations in the absence of selection. Plasmid DNA from some deletion derivatives was transferred to nitrocellulose and hybridized to ³²P-labelled pRJ5 DNA. Multimeric forms of pRJ5, mainly dimers, were detected (data not

Table 2. Incompatibility between *pRJ5*, *pT48* and *pSA6522*

The phenotype associated with each plasmid is shown in Table 1. At least 2 transductants from each cross were subjected to segregation analysis by growth at 32 °C for about 100 generations under nonselective conditions. Strain RN451 was used as the host strain. Each plasmid was shown to be stable in RN451.

Donor plasmid	Resident plasmid	Colonies tested	Colonies resistant to	
			Em	Tc
pSA6522	pRJ5	1118	18 (1.61%)	1113 (99.55%)
pSA6522	pT48	174	0	174 (100%)
pSA6522	pE2222	358	69 (19.27%)	358 (100%)
pT127	pRJ5	1977	1947 (98.48%)	1975 (99.9%)

shown). These data confirm our assumption that *pRJ5* is similar to *pT48*, and that the *HindIII*-C and/or *HindIII*-B fragments of *pRJ5* encompass at least part of a *pala*-like sequence.

Incompatibility relationship between plasmids *pRJ5* and *pT48*

At least 14 distinct incompatibility (Inc) groups have been defined for plasmids of *S. aureus* (Novick, 1989). We tested the relationships of *pRJ5* and *pT48* with other *S. aureus* plasmids.

So far, plasmids *pE12* and *pIM13* were the only members of the *pSN2* family whose Inc group has been determined. They were assigned to the Inc12 (1) group (Novick, 1989); this group was therefore tested first. Plasmid *pE2222* is a representative member of this group and it also encodes *Em^R* (Iordanescu & Surdeanu, 1980). However, the family of this plasmid had not yet been determined. Since *pE2222*, *pRJ5* and *pT48* carry

the same resistance marker (*Em^R*), the Inc relationships between them were studied using a recombinant plasmid (*pSA6522*) formed between *pE2222* (with a mutation in the *erm* gene) and *pSA0301* (a *Tc^R* plasmid that belongs to group Inc 3).

Heteroplasmid strains carrying plasmid *pT127* and *pRJ5* were stably inherited which demonstrated that *pRJ5* is compatible with plasmids from the Inc3 (C) group (Table 2). Heteroplasmid clones carrying plasmids *pSA6522* and *pE2222* were used to confirm that the Inc12 system was still functional in the recombinant plasmid *pSA6522*.

Heteroplasmid clones were constructed by transduction of *pSA6522* into the host strains carrying either *pRJ5* or *pT48*, with selection for resistance to *Tc* and *Em*. In each cross, at least two transductants were tested for plasmid segregation. Plasmid *pSA6522* could efficiently displace both *pRJ5* and *pT48* (Table 2). These results were confirmed by agarose gel electrophoresis of plasmid DNA isolated from 20 colonies resistant to either *Tc* or *Em* (data not shown). It can be concluded that *pE2222*, *pRJ5* and *pT48* are mutually incompatible and therefore belong to Inc12. This also confirms the close relationship between *pRJ5* and *pT48* and suggests that *pRJ5* and *pE2222* belong to the *pSN2* family.

Incompatibility relationship between *pRJ5* and *pSN2*

Plasmid *pRJ5* was transduced into strain SK31, which carries the cryptic plasmid *pSN2*, with selection for *Em^R*. Two transductants were subjected to segregation analysis as described above. After about 100 generations of growth, each of the 673 colonies tested after plating on drug-free agar were *Em^R*. Forty colonies were screened for plasmids by agarose gel electrophoresis and all of them contained both plasmids *pRJ5* and *pSN2* (data not

Fig. 2. Nucleotide sequence of the *ermC* control region from *pRJ5*. The 283 bp sequence from a *MboI* site of *pRJ5* shows the control region for *ermC* and the 5' end of the methylase gene. Structures labelled A, A', C1, C2, C2', C3 and C4 represent repeat sequences. SD indicates a Shine–Dalgarno sequence. The termination codon for the putative leader peptide is indicated by double bars. The arrows indicate the beginning and the end of a 28 bp tandem duplication found in *pRJ5*. Positions are numbered with respect to *pT48* sequence.

Fig. 3. (a) A predicted inactive conformation of the *ermC* leader region in pT48 (Catchpole *et al.*, 1988). (b) A possible change in the conformation of the *ermC* regulatory region due to a 28 bp direct tandem duplication in pRJ5. A, A', C1, C2, C2', C3 and C4 are repeat sequences. SD-1 and SD-2 are the Shine-Dalgarno sequences for the leader peptide and the methylase, respectively. Heavy lines above the sequence indicate the tandem duplication found in pRJ5. Numbers are nucleotide coordinates according to the published sequence for pT48 (Catchpole *et al.*, 1988).

shown). Thus pRJ5 coexists stably with pSN2 without selection, and therefore these plasmids belong to distinct Inc groups. According to Dr K. G. H. Dyke (personal communication), pT48 is also compatible with pSN2.

Molecular basis for the constitutive expression of the ermC gene from plasmid pRJ5

The restriction map of pRJ5 is nearly identical to that of pT48. However, pRJ5 confers constitutive MLS resistance, whereas pT48 confers inducible resistance. To determine the molecular basis for this difference, a small

fragment of pRJ5, including the leader peptide and the 5'-terminus of *ermC*, was cloned into M13mp11 and its DNA sequence was determined (Fig. 2). A comparison with the equivalent region of plasmid pT48 (Catchpole *et al.*, 1988) showed that a 28 bp direct tandem duplication occurred in plasmid pRJ5, beginning at nucleotide 1775 with respect to pT48 sequence.

A model for the inducible expression of MLS resistance was proposed by Gryczan *et al.* (1980) and Horinouchi & Weisblum (1980), based on studies performed with the *ermC* gene from plasmid pE194. The *ermC* gene from pT48 is almost identical to *ermC* from

pE194 (99 % base identities) (Catchpole *et al.*, 1988). The model proposes that a 141 bp leader region at the 5' end of the *ermC* mRNA is capable of folding to form a secondary hairpin structure. This structure forms a translational attenuator by sequestering the ribosome binding site (SD-2) and the initiation codon (AUG) for the methylase, thus preventing full expression of MLS resistance. In addition, the 5' controlling region contains a small ORF capable of encoding a 19 amino acid peptide. It is believed that induction occurs when Em-sensitive ribosomes, in the process of translating the small leader peptide, become stalled due to the inhibiting effect of Em. This precipitates a change in the secondary structure of the mRNA to a conformation in which the initiation signals SD-2 and AUG become unpaired. The mRNA is now accessible to ribosomes, which initiate translation of the methylase gene.

The model described above is supported by the properties of both regulatory mutations and naturally occurring plasmids which confer constitutive MLS resistance. Several changes which result in constitutive expression have been identified among mutants of pE194. A point mutation in the repeat sequence C3 disrupted its ability to form a stem-loop structure with repeat sequence C4, thus freeing SD-2 (Gryczan *et al.*, 1980; Horinouchi & Weisblum, 1980). Duplication of repeat segments C3 and C4 ensures that one set of initiation signals is always unpaired (Gryczan *et al.*, 1980). A third type of constitutive mutant involves deletions of the leader peptide region (Hahn *et al.*, 1982). However, in naturally occurring plasmids conferring constitutive MLS resistance, only deletions affecting the leader peptide gene repeats C1 and C2 have been reported.

Analysis of the DNA sequence of the constitutive MLS resistance determinants from plasmids pNE131 (Lampson & Parisi, 1986), pIM13 (Monod *et al.*, 1986), pJ74 (Catchpole *et al.*, 1988) and pA22 (Catchpole & Dyke, 1990) revealed deletions of 107 bp, 107 bp, 71 bp and 58 bp, respectively, in the leader/attenuator region. Since the only difference found between pRJ5 and pT48 in this region is a 28 bp direct tandem duplication, we assume that this is the cause of the constitutive phenotype conferred by pRJ5.

According to the translational attenuation model for regulation of MLS resistance, the tandem duplication in pRJ5 should result in an alternate conformation of the mRNA, allowing constitutive expression of the methylase. The predicted folding of the *ermC* leader region for plasmid pT48 in the uninduced state is shown in Fig. 3(a) (Catchpole *et al.*, 1988). Only SD-1 is accessible to ribosomes, so only the leader peptide is translated. SD-2 is sequestered within an energetically favourable base-paired structure, and methylase is not expressed. In pRJ5, the tandem duplication should

permit the formation of a structure similar to that in Fig. 3(a), but in which SD-2 and the associated AUG codon are exposed. Fig. 3(b) shows one such structure. The direct tandem duplication found in pRJ5 provides an extra sequence, designated C2', which if paired to C1, would form a hairpin with a very low stability ($\Delta G = -7.5$ kcal) compared to the hairpin formed by C1 and C2 in pT48 ($\Delta G = -13.9$ kcal). This structure is probably not formed *in vivo*. Thus the primary effect of the duplication would be to destabilize pairing of C1 and C2. Consequently, C2 would pair with C3, forming a stable hairpin ($\Delta G = -18.5$ kcal) which would release C4 and make the expression of MLS resistance constitutive. This hairpin has a higher stability than that formed by C3 and C4 in pT48 ($\Delta G = -16.9$ kcal).

The stable C2-C3 pairing proposed for plasmid pRJ5, including only the second hairpin, is identical to structures proposed for plasmids pJ74, pNE131, pIM13 and pA22 (Catchpole & Dyke, 1990) and is in agreement with Gryczan *et al.* (1980), Horinouchi & Weisblum (1980) and Mayford & Weisblum (1989), who postulated that disruption of the integrity of C3 plus C4 to free sequence SD-2 constitutes the key event in induction of the *ermC* transcript.

In conclusion, it is suggested that the tandem duplication is responsible for the constitutive MLS resistance specified by pRJ5. This report shows that pRJ5 differs from all other constitutive MLS resistance plasmids, and shows that several different mutations have occurred in natural isolates.

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