

GENETIC CHARACTERIZATION OF PLASMID pRJ5 OF *Staphylococcus aureus* COMPARED TO PLASMID pE194

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ABSTRACT

pRJ5, a naturally occurring constitutive macrolide, lincosamide and streptogramin B (MLS) resistance plasmid of *Staphylococcus aureus*, was compared to pE194, a plasmid that confers the inducible phenotype.

pRJ5 was stable in all strains of *S. aureus* tested, even under growth at 43°C, which distinguished it from pE194 which was shown to be thermo-sensitive for replication. pRJ5, like pE194, was highly unstable in *Bacillus subtilis* when the cells were grown in nonselective conditions. Multimeric forms of pRJ5 DNA were detected in the few cells of *B. subtilis* that retained this plasmid.

pE194 was transduced by phages phi11 and phi443 at frequencies 400 and 20- fold higher, respectively, than pRJ5. Both plasmids were co-transduced with the plasmid pRJ4.

pRJ5 was shown to be compatible with pE194. Therefore they belong to distinct Inc groups.

Hybridization studies revealed that pRJ5 shares a 1.35 kb region of homology to pE194, which is limited to the *erm* gene, conferring MLS resistance.

INTRODUCTION

Staphylococcal resistance to the macrolide antibiotic erythromycin (Em) usually involves co-resistance to other macrolides, such as oleandomycin, and also to other chemically distinct groups of antibiotics, the lincosamides and streptogramin B (Weisblum, 1985). The MLS antibiotics inhibit protein synthesis in prokaryotes by binding to the 50S ribosomal subunit. Resistance is conferred by N⁶-N⁶ dimethylation

of an adenine residue of 23S rRNA. This causes reduced binding of all three classes of antibiotics, giving rise to the MLS^r phenotype (Dubnau, 1984).

This study explores the relationship between two naturally occurring MLS^r plasmids of *Staphylococcus aureus*. Plasmid pE194, reported by Iordanescu (1976), specifies Em-induced resistance and it was the first MLS^r plasmid described in *S. aureus*. pE194 is 3,728 bp in length and it has been extensively studied in both its natural host (Weisblum *et al.*, 1979; Iordanescu and Surdeanu, 1980; Byeon and Weisblum, 1990; Sozhamannan *et al.*, 1990) and *Bacillus subtilis*, where it was found to replicate and express resistance (Gryczan *et al.*, 1980; Shivakumar *et al.*, 1980; Horinouchi and Weisblum, 1982). pRJ5 is a 2.55 kb plasmid isolated in our lab and it was the first naturally occurring plasmid coding for constitutive MLS^r described in *S. aureus* (Bastos *et al.*, 1980).

In this communication, several properties of both plasmids were compared, including spontaneous loss at 32°C and 43°C, stability in different bacterial hosts and transduction frequency by different transducing phages. The incompatibility relationship and the presence of homology between them were also investigated.

MATERIAL AND METHODS

Bacterial strains and plasmids

S. aureus strains and plasmids used in this study are listed in Table I. Strains MB3, RN2442, RN3775, RN1801, RN451 and RN450 are derivatives of strain NCTC8325 (Novick, 1967). RN451 is lysogenic for phage phi11. To obtain strain MB19, strain A443 was cured of plasmid pRJ5 and transformed with plasmid pE194.

Growth conditions and genetic crosses

Trypticase soy broth (TSB, Difco) was used for liquid cultures of *S. aureus* and LB broth (Schleif and Wensink, 1981) was used for liquid cultures of *B. subtilis*. Solid medium was either trypticase soy agar (TSA, Difco; for *S. aureus*) or LA (for *B. subtilis*). When necessary, the media were supplemented with tetracycline (Tc) or erythromycin (Em) at 5 µg/ml. Cultures were grown at 32°C, unless otherwise indicated.

Plasmid stability and segregation analysis were studied by plating after either 10 or 100 generations of growth in nonselective broth and scoring for retention of plasmid markers.

Transductions with either phage phi11 or phi443 were done as described previously (Bastos *et al.*, 1980). Transformation of *S. aureus* and *B. subtilis* protoplasts

Table I - *S. aureus* strains and plasmids.

Strain	Plasmid(s)	Size (kb)	Phenotype associated with plasmid	Source or reference
A443	pRJ5	2.55	MLS ^r	Bastos <i>et al.</i> , 1980
	pRJ4	4.4	Tc ^r	
E129	pRJ5	2.55	MLS ^r	Bastos <i>et al.</i> , 1980
MB3	pRJ5	2.55	MLS ^r	This study
RN2442	pE194	3.72	MLS ^r Inc11 (K)	Iordanescu, 1976
MB19	pE194	3.72	MLS ^r Inc 11 (K)	This study
	pRJ4	4.4	Tc ^r	
RN3775	pSA4502	8.1	Tc ^r Inc3 (C) Inc11 (K)	Iordanescu and Surdeanu, 1980
RN1801	pT127	4.4	Tc ^r Inc3 (C)	Iordanescu, 1976
SCC-8	-	-	-	Bastos <i>et al.</i> , 1980
RN451	-	-	-	Novick, 1967
RN450	-	-	-	Novick, 1967

MLS, macrolide - lincosamide - streptogramin B; Tc, tetracycline; Inc, incompatibility.

was performed as described by Murphy *et al.* (1981) and Chang and Cohen (1979), respectively.

Curing experiments

Growth of broth cultures at high temperature (43°C) was used to cure staphylococci of drug resistance as described previously (Bastos and Penido, 1981).

Isolation and in vitro manipulation of plasmid DNA

Whole cell lysates were prepared as described by Giambiagi-Marval *et al.* (1990). Plasmid DNA for restriction endonuclease analysis and for nick-translation reactions was prepared by CsCl-ethidium bromide density-gradients centrifugation of cleared lysates prepared as described by Novick *et al.* (1979).

Restriction mapping and agarose gel electrophoresis were performed essentially as described by Sambrook *et al.* (1989). Southern blots and hybridizations were performed as described by Bastos and Murphy (1988).

Restriction enzymes and DNA polymerase I were obtained from New England Biolabs and were used as specified by the manufacturer.

RESULTS AND DISCUSSION

Analyses of the stability of plasmids pRJ5 and pE194 in S. aureus and B. subtilis

To compare the stability of pRJ5 relative to pE194, in different bacterial hosts, these plasmids were transferred to strains of *S. aureus* and *B. subtilis*, either by transduction or by protoplast transformation. Strains containing either pRJ5 or pE194 were subjected to approximately 10 generations of nonselective growth at 32°C and analysed for the maintenance of Em^R. As shown in Table II, pRJ5 and pE194 were quite stable in strains of *S. aureus*, irrespective of their phage groups. On the other hand, both plasmids were lost at high frequencies from the *B. subtilis* strains. These results showed that plasmids pRJ5 and pE194, although able to replicate and express resistance in *B. subtilis*, are both highly unstable in this microorganism, unless grown in the presence of Em.

Table II - Stability of plasmids pRJ5 and pE194 in strains of *S. aureus* and *B. subtilis* grown at 32°C.

Bacterial species	Genetic background (phage group)	Plasmid	Colonies tested	Percentage of Em ^r colonies	
<i>S. aureus</i>	A443 (II)	pRJ5	1,856	99.95	
		pE194	1,840	99.89	
	E129 (II)	pRJ5	2,588	99.42	
		RN451 (I/III)	pRJ5	1,812	98.84
			pE194	2,085	100
<i>B. subtilis</i>	BD170	pRJ5	1,100	66.36	
		pE194	1,094	35.28	
	IS75	pRJ5	1,395	6.74	

Em resistance was scored after 10 generations of growth in nonselective conditions.

Whole cell lysates analysed in one of the agarose gels, run to confirm the elimination of pRJ5 from the *B. subtilis* cells, were transferred to nitrocellulose and hybridized to ³²P-labelled pRJ5 DNA. No DNA bands homologous to pRJ5 were detected in those colonies that had lost Em^r (Figure 1,B, lanes *e* to *j*). In those colonies that retained Em^r, pRJ5 hybridized not only to the CCC and OC forms of the plasmid, but also to additional DNA bands migrating above them (Figure 1,B, lanes *a* to *d*). These additional bands probably correspond to multimeric forms of pRJ5, mainly dimers, present in *B. subtilis* cells. pRJ5 multimerization was not observed in *S. aureus* (lane *k*).

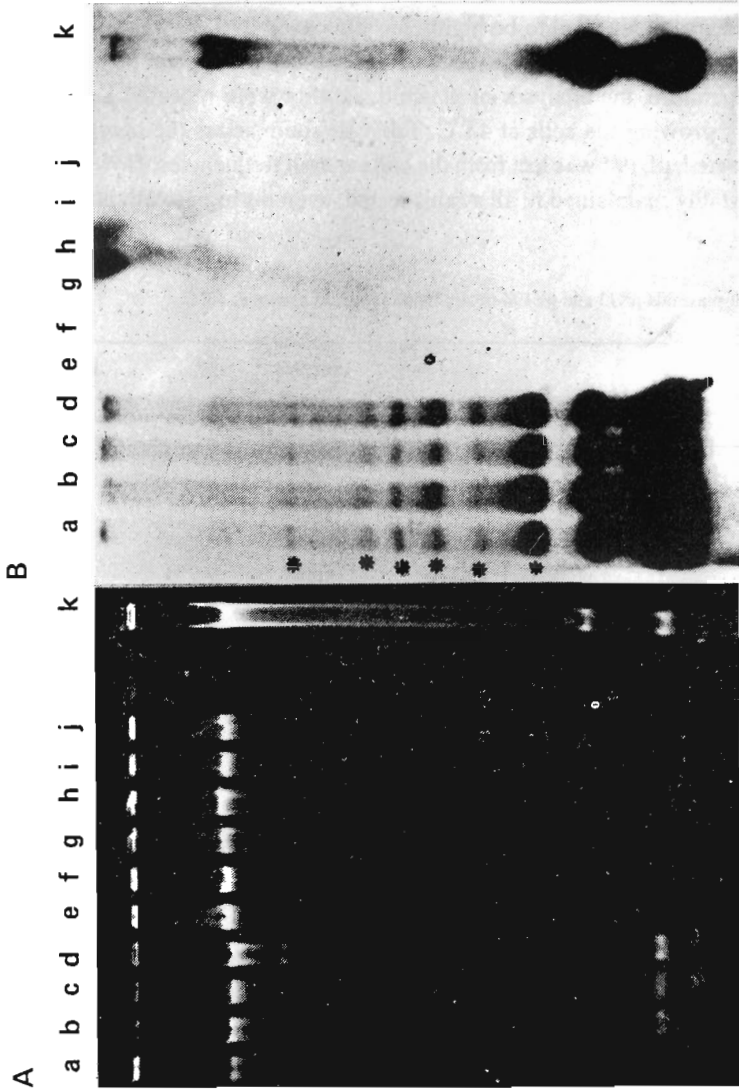


Figure 1 - DNA-DNA hybridization analysis. Whole cell lysates were prepared from either Em^r or Em^s colonies derived from *B. subtilis* BD170 carrying plasmid pRJ5, separated on 0.8% (w/v) agarose gel, transferred to a nitrocellulose membrane and probed with pRJ5 DNA (A). Photograph of ethidium bromide stained agarose gel. Lanes a-d, Em^r colonies; lanes e-j, colonies cured of Em^r; lane k, *S. aureus* strain MB3. OC, relaxed plasmid DNA; CCC, supercoiled plasmid DNA. Asternisks indicate multimeric forms of plasmid DNA.

Plasmid multimerization has been shown to cause plasmid instability by lowering the number of segregating units during cell division (Summers and Sherratt, 1984). Therefore multimerization may contribute to pRJ5 instability in *B. subtilis*.

pE194 has been reported to be highly unstable even in *S. aureus* when its host strains are grown at 43°C (Iordanescu and Surdeanu, 1980). To test the stability of plasmid pRJ5 at this temperature, the analyses of plasmid stability were repeated as described before, except for growing the cells at 43°C. Table III summarizes the results of these analyses. As expected, pE194 was lost from the cells at high frequencies. However, pRJ5 was found to be stably maintained in all strains tested, even during growth at 43°C.

Table III - Stability of plasmids pRJ5 and pE194 in strains of *S. aureus* grown at 43°C.

Genetic background (phage group)	Plasmid	Colonies tested	Percentage of Em ^r colonies
A443 (II)	pRJ5	1,803	99.78
	pE194	1,624	2.46
E129 (II)	pRJ5	1,831	99.40
RN451 (I/III)	pRJ5	1,793	95.98
	pE194	2,151	0.88

Em-resistance was scored after 10 generations of growth in nonselective conditions.

Transduction frequencies of plasmids pRJ5 and pE194

To measure the transduction frequencies of plasmids pRJ5 and pE194, different strains were used as donors of these plasmids in transductions to appropriate plasmid-negative recipients, with selection for Em^r.

Small *S. aureus* plasmids are typically transduced by phages as phi 11 or 80 £ at frequencies between 10⁻⁷ and 10⁻⁶ per plaque-forming units (pfu) (Iordanescu *et al.*, 1978). As shown in Table IV, the transduction frequencies for plasmid pE194 ranged from 4.3x10⁻⁶ to 2.5x10⁻⁵, whereas those for pRJ5 ranged from 1.1x10⁻⁸ to 1.3x10⁻⁶. Plasmid pE194 was always transduced at higher frequencies than pRJ5.

The usual mechanism of generalized transduction in *S. aureus* has been extensively studied using phi 11 as the transducing phage. This phage seems to infect only strains that belong to phage groups I and/or III. It has been predicted (Iordanescu, 1976) and demonstrated (Novick *et al.*, 1986) that transducing particles contain linear

Table IV - Plasmid transduction frequency.

Donor strain (plasmid)	Transducing phage	Recipient strain	Transduction frequency
MB3 (pRJ5)	011	RN450	1.1×10^{-8}
RN2442 (pE194)	011	RN450	4.3×10^{-6}
A443 (pRJ5)	0443	SCC-8	1.3×10^{-6}
MB19 (pE194)	0443	SCC-8	2.5×10^{-5}

Lysates were prepared by U.V. induction of the lysogenic donor strains and used to infect the recipient strains at a multiplicity of infection of 0.1. Transduction frequencies represent a mean of at least three independent experiments and are given as the number of transductants per pfu.

head-to-tail concatemers of small plasmids the same size as their own genome. Based on these studies it was proposed that the frequency of plasmid transduction would then depend on the copy number of the plasmid in the donor strain, its ability to form concatemers and the ability of the phage to encapsulate them.

Plasmid multimerization has been shown to occur for plasmid pRJ5, at least under certain conditions (Figure 1). Therefore, with respect to phage ϕ i11, the hypotheses to explain the lowest transduction frequencies observed for plasmid pRJ5 could be: its apparently lower copy number in strain MB3 compared to strain A443 (data not shown) and the inability of phage 011 to encapsulate concatemeric pRJ5 DNA. Since the encapsulation of concatemeric plasmid DNA depends on the presence of DNA sequences that mimic the phage *pac* site (site used to initiate sequential encapsulation of phage DNA), it is possible that pRJ5 lacks DNA sequences homologous to the *pac* site of phage 011 and that these sequences are present on plasmid pE194.

The highest transduction frequencies for plasmid pRJ5 were observed when ϕ i443 was used as the transducing phage. These results suggest that plasmid pRJ5 may have characteristics that favor its transduction by phage ϕ i443.

ϕ i443 is a temperate phage that was found lysogenizing strain A443, in which pRJ5 was first detected. Differently from ϕ i11, phage ϕ i443 seems to infect only strains that belong to phage group II, such as SCC-8. However, to date, nothing is known about the biology of this phage. But since pRJ5 seems to be maintained in the background of strain A443 with a higher copy number than in the background of strain RN451, the lysogenic strain for phage ϕ i11 (data not shown), the increased copy number of pRJ5 in A443 could explain the higher transduction frequency of this plasmid by phage ϕ i443. Alternatively, pRJ5 might carry DNA sequences homologous to the *pac* site of ϕ i443, which would favor the encapsulation of concatemers of this plasmid.

Both strains A443 and MB19 carry a second plasmid, pRJ4, coding for Tc^r. Therefore the co-transduction of either pRJ5 or pE194 and pRJ4 by phage phi443 was also investigated by scoring the number of Em^r transductants that became simultaneously Tc^r. These analyses showed 10% of co-transduction of plasmids pRJ5 and pRJ4 and 6% of co-transduction of plasmids pE194 and pRJ4. The two co-transduced plasmids were found to be physically independent on examination of the co-transductant clones by agarose gel electrophoresis (data not shown).

It was demonstrated that most of the known *S. aureus* plasmids contain one or two specific recombination sites, RS_A and RS_B, that are involved in plasmid cointegration and co-transduction (Novick *et al.*, 1984; Gennaro *et al.*, 1987). RS_B cointegrates are formed by a phage-determined recombination system (Novick *et al.*, 1984) and recombination at RS_A is mediated by a plasmid-encoded trans-acting protein, Pre (plasmid recombination) (Gennaro *et al.*, 1987). Both sites and the *pre* gene was found in plasmids pE194 and pT181. pRJ4 is a plasmid almost identical to pT181 (unpublished results) and therefore should carry both recombination sites and the *pre* gene. pRJ5 seems to carry only a complete RS_A site but lacks a complete RS_B site and the *pre* gene (to be published elsewhere). Therefore, the presence of RS_B and/or RS_A on pE194, pRJ4 and pRJ5 and the presence of the *pre* gene on pE194 and pRJ4 could explain the co-transduction observed with these plasmids.

Incompatibility relationship between plasmids pRJ5 and pE194

Incompatibility appears to be the most reliable genetical criterion for plasmid classification. Applied to *S. aureus* plasmids, this criterion has already led to delimitation of at least 14 Inc groups (Novick, 1989). pE194 is the prototype plasmid of group Inc11 (K).

To test the Inc relationship, heteroplasmid strains were constructed by transductions, with selection for a suitable antibiotic resistance marker. Two transductants from each cross were analysed for plasmid segregation after approximately 100 generations of nonselective growth. The presence or the absence of the plasmid was always confirmed by agarose gel electrophoresis. Spontaneous loss of each plasmid used to construct the heteroplasmid clones was assayed by the same procedure. The results of these experiments are summarized in Table V. All plasmids studied were found to be stably maintained in their respective host strains, being lost from the cells spontaneously at very low frequencies (from 0 to 2.98%, depending on the plasmid).

Since pRJ5 and pE194 carry the same single resistance marker, the Inc relationship between them was studied making use of a recombinant plasmid between pE194 and pSA0301 (a Tc^r plasmid that belongs to group Inc3). The recombinant

Table V - Incompatibility relationship between pRJ5 and pE194.

Donor plasmid	Resident plasmid	Colonies tested	Percentage of Colonies	
			Em ^r	Tc ^r
pSA4502	pRJ5	4,254	99.65	99.58
pSA4502	pE194	797	10.01	95.11
pT127	pRJ5	1,977	98.48	99.90

The donor plasmid was transduced into the host strain carrying the resident plasmid with selection for Tc^r and Em^r. Two transductants from each cross were subjected to segregation analysis, during growth at 32°C, for about 100 generations in nonselective conditions.

Strain RN451, which contains neither antibiotic resistance marker nor plasmids, was used as host strain for all plasmids in order to obtain plasmids in the same background.

plasmid, pSA4502, carries a mutation in the pE194 gene coding for MLS^r (Iordanescu and Surdeanu, 1980).

Heteroplasmid clones carrying plasmids pT127 and pRJ5 were used as a control of compatibility between pRJ5 and plasmids from the Inc3(C) group. This compatibility was then confirmed (Table V). Heteroplasmid strains carrying both pSA4502 and pE194 were tested to confirm that the Inc11 system was still functional in pSA4502. As expected, pSA4502 could displace efficiently pE194 from the heteroplasmid clones. On the other hand, plasmid pRJ5 was shown to coexist stably with pSA4502 without selection of either of them. It can be concluded that pRJ5 and pE194 are compatible and therefore they belong to distinct Inc groups.

The presence of two Inc functions has been demonstrated, namely the copy-control inhibitor (*cop*) and the replication origin (*ori*), in small *S. aureus* plasmids coding for drug resistance (Novick, 1987 and 1989). On the basis of the data presented above, we can assume that plasmids pRJ5 and pE194 do not share homologous *cop* and *ori* regions.

Homology between plasmids pRJ5 and pE194

A map of restriction sites for plasmid pRJ5 (Figure 2) was constructed by standard techniques of single and double restriction endonuclease digestions. In addition, restriction digests from pRJ5 were transferred to a nitrocellulose membrane and hybridized to ³²P-labelled pE194 DNA. The region of homology found is also depicted

in Figure 2. pRJ5 contains a 1.35 kb region of homology to pE194 located between positions 910 and 2260. A gross comparison of restriction maps between pRJ5 and pE194 (Horinouchi and Weisblum, 1982) revealed a conservation of some restriction sites between pRJ5 and pE194, which is limited to the region of homology found between these plasmids. In pE194, the conserved region encompasses the *ermC* gene which codes for the 23S rRNA methylase responsible for MLS^r . This homology was already expected since there seems to be a high degree of sequence conservation among the 23S rRNA methylase genes found on different MLS^r plasmids (Novick and Murphy, 1985).

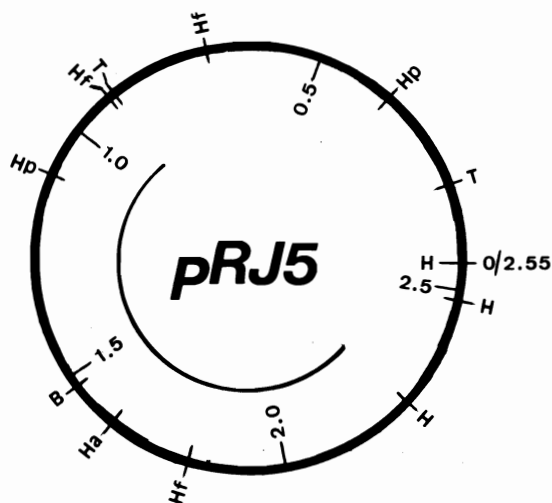


Figure 2 - Restriction map of plasmid pRJ5. Only relevant restriction sites are indicated as follows: *Hind*III (H); *Taq*I (T); *Hpa*I (Hp); *Hin*fI (Hf); *Bcl*I (B) and *Hae*III (Ha). The reference point of the map is one of the *Hind*III sites. The distances are given in kb. The region of homology to pE194 is indicated with a light line.

The remainder of pRJ5 bears no detectable homology with pE194. The restriction maps of both plasmids also differ markedly outside the homologous region. These data are in agreement with the compatibility observed between pRJ5 and pE194. If these plasmids had similar nucleotide sequences for all regions involved in replication and maintenance, we would expect them to be incompatible.

Although the *erm* genes of plasmids pRJ5 and pE194 seem to be highly homologous, pRJ5 confers constitutive MLS resistance whereas pE194 confers inducible resistance. Whether *ermC* is the ancestral gene of the methylase gene found in pRJ5 is currently being investigated. By comparing the complete nucleotide sequences of both plasmids, we may be able to discern more clearly the ancestral relationship between these two MLS^r genes.

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RESUMO

Este trabalho compara o plasmídeo pRJ5 de *Staphylococcus aureus*, o qual confere resistência constitutiva aos antibióticos do grupo MLS (macrolídeos, lincosamidas e estreptogramina B), com o plasmídeo pE194, que confere resistência induzida.

pRJ5 mostrou-se estável em todas as estirpes de *S. aureus* analisadas, nas condições de cultivo a 32°C ou a 43°C, o que o diferenciou de pE194, que comportou-se como termo-sensível para duplicação. Ambos os plasmídios mostraram-se altamente instáveis em *Bacillus subtilis*, quando as células foram cultivadas sob condições não seletivas. Nas poucas células de *B. subtilis* que mantiveram o pRJ5, foram detectadas formas multiméricas deste plasmídeo.

O pE194 foi transduzido pelos fagos phi11 e phi443 em frequências, respectivamente, 400 e 20 vezes maiores que o pRJ5. Ambos os plasmídios foram co-transduzidos com o plasmídeo pRJ4.

O pRJ5 mostrou-se compatível com o pE194. Portanto, eles pertencem a grupos Inc diferentes.

Existe uma região de homologia entre os plasmídios pRJ5 e pE194, com cerca de 1,35 kb de extensão. Esta homologia está restrita ao gene *erm* que confere a resistência a drogas.

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