The Nucleotide Sequence of *Staphylococcus aureus* Plasmid pT48 Conferring Inducible Macrolide–Lincosamide–Streptogramin B Resistance and Comparison with Similar Plasmids Expressing Constitutive Resistance

By I. CATCHPOLE, C. THOMAS,† A. DAVIES AND K. G. H. DYKE*

Microbiology Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK

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The complete nucleotide sequence of a naturally occurring *Staphylococcus aureus* plasmid, pT48 (from *S. aureus* strain T48), has been determined. The 2475 bp plasmid confers inducible resistance to macrolide–lincosamide–streptogramin B (MLS) type antibiotics. It is similar to the constitutive MLS resistance plasmid, pNE131, from *Staphylococcus epidermidis* and shows homology with *S. aureus* plasmids pSN2 and pE194. It contains a *palA* structure homologous to that on *S. aureus* plasmid pT181. The open reading frame, ORF B, within the pSN2 homologous region has a frameshifted C-terminus, relative to pNE131, resulting in a smaller, 158 amino acid putative polypeptide. The pE194 homologous region has the *ermC* resistance determinant and retains the leader region, deleted in pNE131, required for inducible expression of an adenine methylase.

Another naturally occurring *S. aureus* strain, J74, shows constitutive resistance to erythromycin and contains a small plasmid, pJ74, which is similar to pNE131 but with a different deletion in the leader sequence. The results are consistent with the translational attenuation model for *ermC* expression.

INTRODUCTION

Staphylococcus aureus strains that are resistant to erythromycin are also often resistant to other macrolides, lincosamides and streptogramin B (MLS) antibiotics (Dubnau, 1984; Weisblum, 1984). Such naturally occurring MLS resistance is also seen in a variety of other organisms: *Bacillus* spp. (Docherty *et al.*, 1981; Gryczan *et al.*, 1984; Mahler & Halvorson, 1980), *Streptococcus* spp. (Horinouchi *et al.*, 1983; Clewell, 1981) and *Streptomyces* spp. (Fujisawa & Weisblum, 1981), including *Streptomyces erythraeus*, the producer of erythromycin. In these species, MLS resistance is mediated by specific *N*⁶,*N*⁶-dimethylation of adenine in 23S rRNA. This results in reduced ribosomal affinity for MLS antibiotics (Dubnau, 1984; Weisblum, 1984; Lai *et al.*, 1973; Lai & Weisblum, 1971; Skinner *et al.*, 1983). Production of the methylase enzyme involved is often inducible by sub-inhibitory concentrations of erythromycin or the related antibiotic oleandomycin (Dubnau, 1984; Weisblum, 1984; Murphy, 1985; Horinouchi *et al.*, 1983; Gryczan *et al.*, 1984).

Three different determinants involved in MLS resistance have been described in *Staphylococcus aureus*: *ermA* (Murphy, 1985; Murphy *et al.*, 1985), *ermB* (Novick *et al.*, 1979; Khan & Novick, 1980) and *ermC* (Horinouchi & Weisblum, 1982). The former two genes are located on the transposons Tn554 (Murphy, 1985; Murphy *et al.*, 1985) and Tn551 (Novick *et al.*, 1979; Khan & Novick, 1980) respectively, whereas *ermC* was first described on the 3.7 kb

Abbreviations: MLS, macrolide–lincosamide–streptogramin B; ORF, open reading frame.

† Present address: Department of Biochemistry, University of Leicester, Leicester LE1 7RU, UK.

plasmid pE194 (Horinouchi & Weisblum, 1982). Expression of *ermA* and *ermC* determinants is inducible by erythromycin. Nucleotide sequence analysis and molecular studies of *ermC* have led to the postulation of a model for this regulation (Dubnau, 1984; Weisblum, 1984). The mechanism involved is post-transcriptional (Gryczan *et al.*, 1980; Horinouchi & Weisblum, 1980) and, by analogy to the transcriptional attenuation seen in amino acid biosynthesis genes (Kolter & Yanofsky, 1982), is described as translational attenuation.

There have been a number of reports in the literature of small, 2.0–2.5 kb, MLS-resistance-conferring plasmids, both in *S. aureus* and in *Staphylococcus epidermidis* (Bastos *et al.*, 1980; Iordanescu & Surdeanu, 1980; Parisi *et al.*, 1981; Keller *et al.*, 1983). Such plasmid-borne MLS resistance is generally constitutive, but the sequence of the plasmid pNE131 from *S. epidermidis* shows strong homology with *ermC* (Lampson & Parisi, 1986*a, b*). pNE131 has a 1074 bp region of homology with pE194 which contains the determinant *ermM*, almost identical to *ermC* apart from a 107 bp piece, absent in the former. This results in the loss of some of the complementary base-pairing regions within the 5' leader of the *ermM* mRNA which are critical for inducible expression of *ermC* (Lampson & Parisi, 1986*a*; Dubnau, 1984; Weisblum, 1984). An almost identical plasmid to pNE131, found naturally in *Bacillus subtilis*, pIM13, has also recently been sequenced (Monod *et al.*, 1986).

In this paper we present the complete nucleotide sequence (2475 bp) of a naturally occurring *S. aureus* plasmid, pT48. Although similar to pNE131, it retains all the complementary repeats required for the inducibility of *ermC* expression, some of which are deleted in the *S. epidermidis* plasmid. A further *S. aureus* plasmid, pJ74, is described. This bears close homology to pNE131, but has a different mRNA leader deletion, resulting in constitutive MLS resistance. A region of pT48 homologous to the small cryptic *S. aureus* plasmid pSN2 (Khan & Novick, 1982) is also discussed, together with features of the remainder of pT48, in relation to the replication of this and other small *S. aureus* plasmids (Gruss *et al.*, 1987; Novick *et al.*, 1987).

METHODS

Organisms, growth conditions and plasmids. *Escherichia coli* strains JM103 and JM107 were obtained from Bethesda Research Laboratories and were grown in 2 × TY medium (Yanisch-Perron *et al.*, 1985).

S. aureus strains were grown in CY medium as described by Dyke & Noble (1984). Strains T48 and J74 were originally isolated from infected patients in Miami, Florida, USA, and London, UK, respectively. pT48 was the only plasmid present in strain T48, whereas J74 contained, in addition to the 2.5 kb erythromycin-resistance-conferring plasmid, pJ74, a 3.2 kb plasmid, thought to be involved in conferring resistance to cadmium ions (unpublished observations). Strain RN4220, a restriction-minus derivative of the 8325-N strain, which contains neither antibiotic resistance markers nor plasmid (Fairweather *et al.*, 1983), was used as a host for transfer of plasmids by the polyethylene glycol protoplast fusion technique (Götz *et al.*, 1981), to obtain strains with single plasmids in the same background.

Tests for inducible or constitutive nature of MLS resistance. The inducible or constitutive nature of MLS resistance was determined on solid medium using erythromycin (10 µg) and tylosin tartrate (500 µg) discs, as described by Weisblum *et al.* (1979).

Plasmid preparation and restriction endonuclease analysis. Plasmid DNA was prepared by a cleared lysate procedure and purified by caesium chloride/ethidium bromide density-gradient centrifugation as described by Novick *et al.* (1979). Plasmid pJ74 (2.5 kb) was separated from the 3.2 kb J74 plasmid by low-melting-point agarose gel electrophoresis and isolated by standard DNA fragment purification procedures (Maniatis *et al.*, 1982). Restriction endonuclease maps were determined for pT48 and pJ74 using single and double enzyme digestions and any ambiguities were resolved using the nucleotide sequence data. Restriction enzymes were purchased from a variety of sources: Amersham, Boehringer Mannheim, New England Biolabs and Bethesda Research Laboratories; they were used according to the manufacturers' instructions.

Nucleotide sequence determination. Restriction enzyme fragments from pT48 and pJ74 were cloned into the M13 filamentous phage vectors mp10, mp11, mp18 and mp19 (Norrander *et al.*, 1983). DNA sequence was determined by dideoxynucleotide chain termination in the presence of [α^{35} S]dATP (Sanger *et al.*, 1977). Labelled fragments were separated on buffer-gradient polyacrylamide/urea gels (Biggin *et al.*, 1983). Klenow fragment and T4 DNA ligase were supplied by Boehringer Mannheim. Dideoxynucleotides, dideoxynucleotides and [α^{35} S]dATP were purchased from Amersham. All were used in accordance with the manufacturer's instructions.

Fig. 1. Partial restriction endonuclease map, and sequencing strategy, for pT48. A dashed arrow shows also the sequencing strategy for a region of pJ74. Restriction enzyme site key: *, *HaeIII*; ∇ , *HgiAI*; $\bullet$, *HindIII*; $\times$, *HinfI*; $\blacklozenge$, *HpaI*; $\square$, *MboI*; $\blacksquare$, *TaqI*.

RESULTS AND DISCUSSION

Nucleotide sequence of pT48

A restriction enzyme map of pT48 was obtained using standard techniques and this is shown partially in Fig. 1. This also demonstrates the sequencing strategy used to determine the complete nucleotide sequence of pT48, shown in Fig. 2. In both figures the same *HindIII* site is used as the reference point for base-pair numbering. Overlapping sequences were obtained for all the sites used for cloning into M13 except for the unique *BclI* and *SstI* sites, and a single *HindIII* site. The sequence was determined on both strands for about 65% of pT48. The plasmid contains 2475 bp.

Nucleotide sequence of part of pJ74

The size and restriction enzyme map of pJ74 is indistinguishable from that of pT48 except for the loss of a unique *HaeIII* site and a deletion of about 70 bp between the unique *SstI* and *BclI* sites of the latter plasmid. pJ74 confers constitutive MLS resistance whereas pT48 confers inducible resistance. To determine the molecular basis for this difference the small *SstI/BclI* fragment of pJ74 was cloned into M13mp19 and the sequence determined in one direction from the *SstI* site (see Fig. 1). This sequence is shown in Fig. 3; the 235 nucleotides obtained encompass the region of the deletion and the absent *HaeIII* site with respect to pT48.

Comparative analysis of plasmid sequence data

The nucleotide sequence of the *S. aureus* plasmid pT48 is similar to those of *S. epidermidis* plasmid pNE131 (Lampson & Parisi, 1986b) and *B. subtilis* plasmid pIM13 (Monod *et al.*, 1986). It also contains regions homologous to the *S. aureus* plasmids pE194, conferring MLS resistance (Horinouchi & Weisblum, 1982), and pSN2, cryptic (Khan & Novick, 1982). A comparison of these homologies and the structural features of the homologous plasmids are shown, together with plasmids pJ74, in Fig. 4. The differences between the six plasmids are discussed in the following sections.

(a) *Region of homology to pE194.* pT48 contains a 1182 bp region of homology to pE194, beginning at nucleotide 911 (Fig. 2), which includes the *ermC* gene conferring a 23S rRNA methylase and the leader region controlling its expression (Horinouchi & Weisblum, 1982). This region is basically the same as in plasmid pNE131, the major difference being a 107 bp deletion in the leader region of the latter plasmid. Exactly the same deletion is found in the *B. subtilis* constitutive MLS resistance plasmid pIM13 (Monod *et al.*, 1986). The regions specifying the methylase and the leader are discussed separately. The only differences between pT48 and pNE131 within the pE194 homologous region which is not involved in MLS resistance are (see Fig. 2): within pT48 with respect to pNE131 there is an extra G at nucleotide 962 (also present in pIM13), a G at nucleotide 2019 instead of a C, and a GT at 2060-2061 instead of a TG; an A is absent between nucleotides 2061 and 2062; and there is an insertion of the dinucleotide TT as bases 2079 and 2080. The differences are all as in pT48 in the sequence of pE194, except for the latter TT insertion which is absent (Horinouchi & Weisblum, 1982).

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HindIII-MboI-----IR-----{-----pSN2 homology 70
 AAGCTTTTGA'TCTACATTAA'GCGTGCAGGT'TTTTGTCTA'TTGTAGAGC'ATTTTATGTT'ATACTAGTCC
 TTCGAAAACT'AGATGTAATT'CGCACGTCCA'AAAAACAGAT'AAACATCTGC'TAAAAACAA'TATGATCAGG
 EndAspL
 140T
 TTGAAAGAAT'AATAATCAGA'TAATGCATTT'TCTTGTTTTT'CATTTCCTC'TTGCTCAAAG'TTCCCAAATT
 AACTTTCTTA'TTATTAGTCT'ATTACGTAAA'AGAACAAAA'GTAAACGGAG'AACGAGTTTC'AAGGGTTTAA
 ysPheSerTy rTyrAspSer LeuAlaAsnG luGlnLysG1 uAsnAlaGlu GlnGluPheA snGlyPheG1
 aqI 210
 CGAGTAAGAG'GTATTTTGT'TTTTGGTCGT'CGCCTCTCAT'TAGTAGTTCA'GGGTTTAAAC'TTAATACTCC
 GCTCATTCTC'CATAAAACA'AAACCAGCA'GCGGAGAGTA'ATCATCAAGT'CCCAAATTGT'AATTATGAGG
 uLeuLeuLeu TyrLysGlnL ysGlnAspAs pGlyArgMet LeuLeuGluP roAsnLeuMe tLeuValGly
 DdeI 280
 AGTTTTTCTT'TTTATAATAT'TTCCTTCTTC'TAAGATTTTA'AGTGTGTGTA'TTACTGTTTG'TAGACTTGT
 TCAAAAAGAA'AAATATTATA'AAGGAAGAAG'ATTCTAAAA'TCACACAAT'AATGACAAAC'ATCTGAAACA
 ThrLysArgL ysIleIleAs nGlyGluGlu LeuIleLysL euThrThrI1 eValThrGln LeuSerThrG
 AluI AluI 350
 CCTGTAGCTT'TTGCTATTTCT'TCTTGTGTGA'GCTATCATTG'TATTGTACT'TAAGTGGACA'TTATCTAGGA
 GGACATCGAA'AACGATAAAG'AGAACAACAT'CGATAGTAAC'ATAACAATGA'ATTCACCTGT'AATAGATCCT
 lyThrAlaLy sAlaIleGlu ArgThrThrA lalleMetTh rAsnAsnSer LeuHisValA snAspLeuI1
 HpaI 420
 TATAGTTAAC'GATTTTAAGT'TTTTTCCGC'CAATCATATC'TAACATACCT'ATTAATTGCA'CTATATATGC
 ATATCAATTG'CTAAAATTCA'AAAAAGGCG'GTTAGTATAG'ATTGTATGAA'TAATTAAAGT'GATATATACG
 eTyrAsnVal IleLysLeuL ysLysGlyG1 yIleMetAsp LeuMetSerI leLeuGlnVa lIleTyrAla
 490
 CTTTACGAAG'TTACCAGACG'TTTGTTTACG'GTATAACTTG'TCTACCTCTA'TGACTTCTCC'ACTTTCTTCG
 GAAATGCTTC'AATGGTCTCG'AAACAAATGC'CATTATGAAC'AGATGGAGAT'ACTGAAGAGG'TGAAGAAAGC
 LysValPheA snGlySerTh rGlnLysArg TyrLeuLysA spValGluI1 eValGluGly SerGluGluA
 DdeI 560
 TCTATGAGCC'TCTGAGAGCC'TTTATAGACT'GTTCATATC'TTTCTTTCAT'CTTTTCTCA'CTCCTTATTT
 AGATACTCGG'AGACTCTCGG'AAATATCTGA'CAAGGTATAG'AAAGAAAGTA'GAAAAGAGT'GAGGAATAAA
 spIleLeuAr gGlnSerGly LysTyrValT hrGlyTyrAr gGluLysStart-ORF B SD
 630
 TAAACTATTC'TAACTATATC'ATAACTGTT'TAAAAAAGAA'AGAACATTTG'TTAAAGAAA'TTGAACAAAA
 ATTTGATAAG'ATTGATATAG'TATTGACAAG'ATTTTTTTTT'TCTTGTAAAC'AATTTTCTTT'ATCTTGTTT
 -10 -35
 700
 ATGAGTGAAG'AATTAGAACA'AACAAATTC'TTATAACCT'TATCATCTCA'ACCTATATTA'AGATTTTACC
 TACTCAGTTT'TTAATCTTGT'TGTTTTAAGG'AATATTGGA'ATAGTAGAGT'TGGATATAAT'TCTAAATAGG
 HinfI 770
 TAGTTGAATC'TTCTTTTCTA'TATAAAGCGT'CGGAGCATAT'CAGGGGGTTA'TCTAACGTAA'ATGCTACCCCT
 ATCAACTTAG'AAGAAAAGAT'ATATTTCGCA'GCCTCGTATA'GTCCCCCAAT'AGATTGCATT'TACGATGGGA
 AatII 840
 TCGGCTCGCT'TTCGCTCGGC'ATTGACGTCA'GATACTGCAC'CCCTGAACC'CCCATGCTCC'AACAGCAAAA
 AGCCGAGCGA'AAGCGAGCCG'TAAGTGCAGT'CTATGACGTG'GGGACTTGG'GGGTACGAGG'TTGTGCTTTT
 Fnu4HI pSN2 homology 910H
 AGGAAACTTT'TTGTCTCTTT'TCCGACGCTT'ATTCGCTTCG'CTCATATTTA'TATAGAAAAG'AAGCGAATGC
 TCCTTTGAAA'AACGACGAAA'AGGCTGCGAA'TAAGCGAAGC'GAGTATAAAT'ATATCTTTTC'TTCGCTTACG
 {haI-----ClaI/HinfI-----RsaI-----pE194 homology 980
 GCAAAGACA'TAATCGATTC'ACAAAAAATA'GGTACACGAA'AAACAAGTTA'AGGGATGCAG'TTTATGCATC
 CGTTTTCTGT'ATTAGCTAAG'TGTTTTTAT'CCATGTGCTT'TTGTTCAT'TCCCTACGTC'AAATACGTAG
 AluI AluI 1050
 CCTTAACCTA'CTTATTAAT'AATTTATAGC'TATTGAAAAG'AGATAAGAAT'TGTTCAAAGC'TAATATTGTT
 GGAATTGAAT'GAATAATTTA'TTAAATATCG'ATACTTTTC'TCTATTCTTA'ACAAGTTTCG'ATTATAACAA
 End LysAsnPheL euLysTyrSe rAsnPheLeu SerLeuPheG lnGluPheSe rIleAsnAsn
 1120
 TAAATCGTCA'ATTCCTGCAT'GTTTTAAGGA'ATTGTTAAAT'TGATTTTTTG'TAAATATTTT'CTTGATTTCT
 ATTTAGCAGT'TAAGGACGTA'CAAAATTCCT'TAACAAATTA'ACTAAAAAAC'ATTTATAAAA'GAACATAAGA
 LeuAspAspI leGlyAlaHi sLysLeuSer AsnAsnPheG lnAsnLysTh rPheIleLys LysTyrGluL
 HpaI 1190
 TTGTTAACCC'ATTTCTAAC'GAAATAATTA'TACTTCTGTT'TATCTTTGTG'TGATATTCTT'GATTTTCTTC
 AACCAATTGGG'TAAAGTATTG'CTTTATTAAT'ATGAAGACAA'ATAGAAACAC'ACTATAAGAA'CTAAAAAAG
 ysAsnValTr pLysMetVal PheTyrAsnI yrLysGlnLy sAspLysHis SerIleArgS erLysLysAr
 AluI 1260
 TATTTAATCT'GATAAGTGAG'CTATTCACCT'TAGGTTTAGG'ATGAAAATAT'TCTCTTGGA'CCATACCTTAA
 ATAAATTAGA'CTATTCACCT'GATAAGTGAA'ATCCAAATCC'TACTTTTATA'AGAGAACCTT'GGTATGAATT
 gAsnLeuArg IleLeuSerS erAsnValLy sProLysPro HisPheTyrG luArgProVa lMetSerLeu
 1330
 TATAGAAATA'TCAACTCTGT'CCATTAAAG'TAATGCCAAT'GAGCGTTTTG'TATTTAATAA'TCTTTTAGCA
 ATATCTTTAT'AGTTGAAGAC'GGTAATTTTC'ATTACGGTTA'CTCGCAAAAC'ATAAATATT'AGAAAACTCG
 IleSerIleA spValGluAl aMetLeuLeu LeuAlaLeuS erArgLysTh rAsnLeuLeu ArgLysAlaP

AACCCGTATT'CCACGATTAA'ATAAATCTCA'TCAGCTATAC'TATCAAAAAC'AATTTTGGCT'ATTATATCTG
 TTGGGCATAA'GGTGCTAATT'TATTAGAGT'AGTCGATATG'ATAGTTTTTG'TTAAACGCA'TAATATAGAC
 heGlyTyrGl uValIleLeu TyrIleGluA spAlaIleSe rAspPheVal IleLysArgI leIleAspTh
 1400R
 AluI
 sal 1470
 TACTTATGTT'ATAAGGTATA'TTACCAAATA'TTTTATAGGA'TTGGTTTTTA'GGAAATTTAA'ACTGCAATAT
 ATGAATACAA'TATTCCATAT'AATGGTTTAT'AAAATATCCT'AACCAAAAAT'CCTTTAAATT'TGACGTTATA
 rSerIleAsn TyrProIleA snGlyPheIl eLysTyrSer GlnAsnLysP roPheLysPh eGlnLeuIle
 1540
 BclI
 ATCCTTGT'TT'AAAACCTGGA'AATTATCGTG'ATCAACAAGT'TTATTTCTG'TAGTTTTGCA'TAATTTATGG
 TAGGAACAAA'TTTTGAACCT'TTAATAGCAC'TAGTTGTTCA'AATAAAGAC'ATCAAAACGT'ATTAATATCC
 AspLysAsnL euValGlnPh eAsnAspHis AspValLeuL ysAsnGluTh rThrLysCys LeuLysHisA
 1680
 RsaI HaeIII1610DdeI
 TCTATTTCAA'TGGCAGTTAC'GAAATTACAC'CTCTGTACTA'ATTCAAGGGT'AAAATGGCCT'TTCTCTGAGC
 AGATAAAGTT'ACCGTCAATG'CTTTAATGTG'GAGACATGAT'TAAGTTCCCA'TTTTACCGGA'AAAGGACTCG
 spIleGluIl eAlaThrVal PheAsnCysA rgGlnValLe uGluLeuThr PheHisGlyL ysGlySerGl
 1750
 A'-----C4-----C3-----
 AGTAATAAAG'TTTTGACTGT'GTTTTATATT'TTCTCGTTC'ATTATAACCC'TCTTTAATTT'GGTTATAATG
 TCATTATTTT'AAAACCTGACA'CAAATATAA'AAAGAGCAAG'TAATATTGGG'AGAAATTTAA'CCAATATTAC
 ThrIlePheA snGlnSerHi sLysIleAsn LysGluAsnStart ermC SD-22
 1820
 HinfI-----C2-----C1-----
 AATTTTGCTT'ATTAACGATT'CATTATAACC'ACTTATTTTT'TGTTGGTTG'ATAATGAACT'GTGCTGATTA
 TTAACACGAA'TAATTGCTAA'GTAATATTGG'TGAATAAAAA'ACAAACCAAC'TATTACTTGA'CAAGCTAAT
 EndLysLy sAsnProGln TyrHisValT hrSerIleVa
 1890
 A-----SstI
 CAAAAAATCT'AAAAATGCC'ATATTTTTTC'CTCCTTATAA'AATTAGTATA'ATTATAGCAC'GAGCTCTGAT
 GTTTTTATGA'TTTTACGGG'TATAAAAAAG'GAGGAATATT'TAATCATAT'TAATATCGTG'CTCGAGACTA
 lPheIleSer PheIleGlyStart leader SD-1 -10
 1960
 MboI
 AAATATGAAC'ATGATGAGTG'ATCGTTAAAT'TTATACTGCA'ATCTGATGCG'ATTATTGAAT'AAAAGATATG
 TTTTACTTGT'TACTACTCAC'TAGCAATTTA'AATATGACGT'TAGACTACGC'TAATAACTTA'TTTCTATAC
 -2030
 AGAGATTAT'CTAGTTTCTT'TTTTACAAGA'AAAAAGAAA'GTTCTTAAAG'GTTTATAGT'TTGGTTCGTA
 TCTCTAAATA'GATCAAGAA'AAAAATGTCT'TTTTCTTT'CAAGAATTC'CAAAATATCA'AAACGAGCAT
 2100
 RS-A-----pE194 homology-----
 GAGCACACGG'TTTAACGACT'TAATTACGAAG'TAAATAAGT'CTAGTGTTG'TTAGACTTTA'ATGTTTTTTA
 CTCGTGTGCC'AAATGCTGA'ATTAATGCTTC'ATTATTC'AATCATACAA'AACTGGAAT'TACAAAAAAT
 2170
 HgiAI-----RS-B-----palA-----
 AAGGCATTAG'TGCACTTAAG'CGTCAGAGCAT'GGCTTTATG'CCGAGAAAAC'TATTGGTTGG'AATGGCGTGT
 TTCCGTAATC'ACGTGAATTC'GCAGTCTCGTA'CCGAAATAC'GGCTCTTTTG'ATAACCAACC'TTACCGCACA
 2240
 HindIII-----RsaI-----
 GTGTTAGCCA'AAGCTTTTGG'GAGTTGGTTGG'GGGTTTCAT'GGGATTAATC'CCATGAAAGT'ACCAACTCAA
 CACAATCGGT'TTCGAAACCG'CTCAACCAACC'CCCAAAGTA'CCCTAATTAG'GGTACTTTCA'TGGTTGAGTT
 2310
 pal A-----
 CAACACACTA'ACGCCGTGTT'GTTCCAAACCA'TAGGAAAT'GGAATAAGCA'ATTAGTATA'TGAGAGTATA
 GTTGTTGAT'TGCGGACAAC'CAAGGTTGGTT'ATCCTTAA'CCTTATTCGT'TAATCATATT'ACTCTCATAT
 2380
 RsaI-----
 ATGTTGGTAT'AACGTTAGTA'TAATGATGCTT'TTTTTCATT'ATATTTTTTA'TGTACTTTAA'ACCTGCACGC
 TACAAACATA'TTGCAATCAT'ATTATACGAA'AAAAAGTAA'TATAAAAAAT'ACATGAAAT'TGACGCTGCG
 2450
 HindIII-----IR-----
 TTAGCGAAT'TAGAAAAAGC'TTAATCGCATT'TCATAGATT'GACCTCCCAA'TAATACGCTG'GTGTTATGG
 AATACGCTTA'ATCTTTTTCG'AATTAGCGTAA'AGTATCTAA'CTGGAGGGTT'ATTGATGCAC'CAACAATAAC
 IR
 GAGGTCAATC'TATTTTCATTT'GCGTT
 CTCCAGTTAG'ATAAGTAAA'CGCAA

Fig. 2. Complete nucleotide sequence of pT48. Numbering starts from the *Hind*III site shown as the zero co-ordinate in Fig. 1. The amino acid sequence of the putative polypeptides described by open reading frame (ORF) B, *ermC* and the *ermC* leader peptide are shown. Structures labelled A, A', C1, C2, C3 and C4 represent six complementary base-pairing sequences. SD indicates a Shine-Dalgarno sequence. The ends of homology of pSN2 and pE194 are marked, as are the two recombination sequences: RS-A and RS-B (see text). Two large palindromic structures are shown: palA (see Fig. 6) and IR (inverted repeat) (see Fig. 7).

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-10          SD-1
SstI      *1          *2          HindIII          *3
GAGCTCGCTA' TAATTATACT' AATTTTATAA' GGTGGTTATA' ATGAATCGTT' AATAAGCAAA' ATTCACTATA
-----
                                C2                                C3

SD-2 ermC          MetAsnGlu LysAsnIleL ysHisSerG1 nAsnPheIle ThrSerLysH
ACCAAATTAA' AGAGGGTTAT' AATGAACGAG' AAAAATATAA' AACACAGTCA' AAACTTTATT' ACTTCAAAAC
-----
                                C4                                A'

isAsnIleAs pLysIleMet ThrAsnIleA rgLeuAsnG1 uHisAspAsn IlePheGluI leGlySerG1
ATAATATAGA' TAAATAATG' ACAAATATAA' GATTAAATGA' ACATGATAAT' ATCTTTGAAA' TCGGCTCAGG
                                DdeI

yLysGlyHis PheThrLeuG luLeu
*4
AAAAGGGCAT' TTTACCCTTG' AATTA

```

Fig. 3. Nucleotide sequence of the *ermC* control region from pJ74. The sequence of 235 bp from the unique *SsrI* site of pJ74 shows the control region for *ermC* and the N-terminal portion of the methylase. Features are as in Fig. 2 excepting: *1, loss of GT (nucleotides 1880 and 1879) from comparable region of pT48; *2, ends of 71 bp deletion with respect to pT48 sequence (nucleotides 1853 and 1781); *3, nucleotide C is a T at position 1749 in pT48; *4, nucleotide G is a C at position 1579 in pT48.

Fig. 4. Functional map of pT48 and comparison to other homologous plasmids. The plasmids are shown linearized to maximize the overlap of homologous regions. ORFs are designated by large open boxes, as is the control region required for inducible expression of *ermC*. A small arrow denotes the position of the leader peptide within *ermC*. Gaps represent deletions with respect to the sequence of plasmid pT48 within the *ermC* leader region. A further deletion within pIM13 with respect to pNE131 is encircled and marked with an asterisk. RS-A (dotted line), and RS-B (part of *palA*: open arrow heads) represent two distinct recombination sequences. The position of a large inverted repeat (IR) is indicated in thick black. Filled-in arrow heads show the ends of homologous regions. , pSE194 homologous regions.

Table 1. Codon differences within *ermC* methylases

The comparison of nucleotide sequences for the *ermC* methylase codons uses capital letters to represent conserved bases and lower case letters where differences occur. Only codons which show a base sequence difference between the *ermC* regions of the four plasmids (pT48, pNE131, pIM13 and pE194) are shown. Novel amino acids resulting from nucleotide base changes are italicized.

Plasmid	Codon no.									
	42	50	99	108	121	134	142	145	177	189
pT48	GGc	cAG	TtT	ACa	gAT	AGa	TTg	cTT	AaT	CAg
	Gly	Gln	Phe	Thr	Asp	Arg	Leu	Leu	Asn	Gln
pNE131	GGg	cAG	TtT	ACg	gAT	AGg	TTc	tTT	AaT	CAg
	Gly	Gln	Phe	Thr	Asp	Arg	<i>Phe</i>	<i>Phe</i>	Asn	Gln
pIM13	GGg	cAG	TtT	ACg	gAT	AGa	TTg	tTT	AaT	CAg
	Gly	Gln	Phe	Thr	Asp	Arg	Leu	<i>Phe</i>	Asn	Gln
pE194	GGc	aAg	TaT	ACg	aAT	AGa	TTg	cTT	AgT	CAa
	Gly	<i>Lys</i>	<i>Tyr</i>	Thr	<i>Asn</i>	Arg	Leu	Leu	<i>Ser</i>	Gln

(i) *Region specifying the methylase (ermC)*. A 244 amino acid open reading frame (ORF) of pT48 bearing >99% homology to *ermC* of pE194 is assumed to encode the methylase enzyme (Horinouchi & Weisblum, 1982). The codon usage of these two *ermC* genes is compared with that of the *ermM* methylase (from pNE131) and the similar gene from pIM13 in Table 1. All four methylases are 244 amino acids long. The methylases conferred by pT48 and pNE131 share identical amino acids apart from positions 142 and 145, where in both cases Leu in pT48 has changed to Phe in pNE131. The pIM13 methylase is identical to that of pNE131 apart from codons 134 and 142, which are as in pT48 for the *B. subtilis* plasmid. The methylase specified by pE194 shows four further changes from that of pT48 and, therefore, six in all from that of pNE131. Most of the changes significantly alter the type of amino acid found in that position and therefore must represent amino acids that have no significant role in methylase function. The differences represent point mutations distributed throughout the gene. These changes, taken together with those in the rest of the pE194 homologous region, imply that, within the process of genetic drift, pT48 is more closely related to pE194 than is pNE131 or pIM13, for this region. It is interesting that for pJ74, where only the first 48 codons of the methylase are known (Fig. 3), codon 42 is as in pNE131. This represents a change of C to G from pT48 to pNE131 and pJ74. In all cases it results in the loss of the unique *Hae*III site found in pT48.

(ii) *Leader region controlling methylase expression*. For pE194 a translational attenuation model has been proposed to explain the inducibility of its *ermC* gene by sub-inhibitory concentrations of erythromycin (Dubnau, 1984; Weisblum, 1984). The molecular basis of this model relies upon the translation of a 19 amino acid leader peptide (Dubnau, 1985) and the presence of six complementary base-pairing regions 5' to the methylase-coding region (Horinouchi & Weisblum, 1982). This is known as the *ermC* leader region. An identical region, apart from an extra A at nucleotide 1748 (Fig. 2), can be found in plasmid pT48, which also shows inducible *ermC* expression. The other plasmids for which this region has been sequenced, pJ74 (Fig. 3), pNE131 (Lampson & Parisi, 1986a) and pIM13 (Monod *et al.*, 1986) all confer constitutive MLS resistance. The leader regions of these plasmids show large deletions with respect to pT48 (see Fig. 4): 71 bp for pJ74 (1783–1852 bp) and 107 bp for pNE131 and pIM13 (1737–1842 bp). These data are shown in Fig. 5 together with a schematic representation of the control of *ermC* expression in pT48. The Pribnow box (–10), transcription start site, leader peptide and Shine–Dalgarno sequences (1 and 2) are so designated from their identity to such features in pE194 (Horinouchi & Weisblum, 1982). The region shown in Fig. 5 is identical to that in plasmids pJ74, pNE131 and pIM13 apart from the differences shown.

Fig. 5 shows the simplest interpretation of the translational attenuation model (Dubnau, 1984; Weisblum, 1984) as applied to pT48. The methylase encoded by *ermC* mediates *N*⁶,*N*⁶-dimethylation of adenine in 23S rRNA and this results in reduced affinity of the 50S ribosomal

Fig. 5. Control region for inducible *ermC* expression of pT48, showing regions absent in pJ74, pNE131 and pIM13. The DNA sequence is shown for pT48 of the *ermC* mRNA leader region as inactive (a) and active (b) conformations, with reference to expression of the *ermC* methylase. A, A', C1, C2, C3 and C4 are complementary base-pairing regions, and SD-1 and SD-2 are the Shine-Dalgarno sequences for the leader peptide and the methylase, respectively. The regions absent in pJ74 and pNE131 (pIM13) are shown in (b). * = C for pJ74 and absent in pE194.

sub-unit for MLS antibiotics. MLS resistance is conferred since the antibiotics can no longer inhibit protein synthesis by stalling the ribosomal peptidyl transferase enzyme. Induction relies upon the use of this inhibition. Two possible conformations of the *ermC* leader region are shown as DNA sequence in Fig. 5, but the mechanism works at the mRNA level. In (a), the uninduced state, only SD-1 is accessible to ribosomes, so only the leader peptide is translated. SD-2 is sequestered within the energetically favourable complementary base-paired structures shown and methylase is not expressed. In the presence of erythromycin, ribosomes can stall whilst translating the leader peptide and can stabilize the active conformation shown in Fig. 5(b). In this instance, the region C1 is prevented from binding its complementary repeat C2, and now the more energetically favourable situation is for C2 to bind C3, thus freeing region C4. This unmasks SD-2, allowing the methylase enzyme to be translated (Dubnau, 1984; Weisblum, 1984). In the case of pJ74, both the leader peptide and region C1 are deleted, leaving its *ermC* mRNA leader effectively stuck with C2 and C3 paired as in conformation (b). This can explain why MLS resistance is constitutively expressed in pJ74. A similar phenotype is shown by pNE131, but as can be seen in Fig. 5(b), both the deletion and the explanation for constitutivity are slightly different. In pNE131, the leader peptide, C1, C2 and C3 are deleted, so that no complementary base-pairing regions can form. However, the leader region is again fixed in a conformation in which SD-2 is permanently exposed, and so methylase expression is constitutive. Both of these naturally occurring deletions are similar to those obtained *in vitro* with pE194, either by growth of bacterial strains on tylosin plates (Horinouchi & Weisblum, 1981), or generated by Bal31 exonuclease digestion (Hahn *et al.*, 1982), to produce constitutive mutants. Indeed, the 3' end-point of the deletion in pJ74 (at nucleotide 1783 in pT48) exactly matches the 3' end of one such mutant selected on tylosin (Horinouchi & Weisblum, 1981). The role of antibiotic usage in the clinical environment in producing such constitutive mutants has been discussed (Lampson & Parisi, 1986a).

It is interesting that the extra A-T base-pair found at nucleotide 1748 in pT48, but absent in the equivalent region of pE194, results in greater stability of both the C2:C3 and C3:C4 complementary base-paired structures (Horinouchi & Weisblum, 1982). In both cases this is by virtue of an extra A:T base pairing. The equivalent region in pJ74 contains a G-C base-pair which lowers the energy of the C2:C3 structure by creating an interior loop, instead of a bulge loop (as in pE194), in the base-paired secondary structure (Tinoco *et al.*, 1973).

(b) *Region of homology to pSN2.* pT48 has 87% homology to an 878 bp region of the cryptic *S. aureus* plasmid pSN2 (Khan & Novick, 1982), from nucleotides 33 to 910 (Fig. 4). This includes a 158 amino acid ORF designated ORF B by its similarity to the equivalent ORF in pNE131 (Lampson & Parisi 1986b). These two ORF Bs have identical DNA sequences apart from the deletion of two bases (AG) between nucleotides 93-94 in pT48, which results in a C-terminal frameshift. As a consequence, ORF B in pT48 is four amino acids shorter than in pNE131, and its final eight amino acids are different. The C-terminus of the pT48 ORF B is the same as that of the pSN2 ORF apart from two changes of Asp to Glu at amino acids 152 and 158 (Khan & Novick, 1982). The equivalent ORF in pIM13, ORF 3, is truncated with respect to that of pT48 due to a deletion from nucleotide 2464-109 in the former plasmid compared to the pT48 sequence. This results in the removal of the final 14 amino acids of the pT48 ORF and their replacement by only two: Ile and Asp (Monod *et al.*, 1986). The '-10' and '-35' sequences, and the Shine-Dalgarno 'ribosome-binding site' are assumed to be as in pNE131 (Lampson & Parisi, 1986b).

pSN2 has been shown to direct the synthesis of 20 kDa and 12 kDa polypeptides in coupled transcription-translation systems, based on cell extracts, *in vitro*. This is presumably due to its ORF (Khan & Novick, 1982). Although the function of the encoded polypeptide/s is unknown, a role has been suggested in plasmid replication and maintenance for the pIM13 ORF 3 which appears to encode only a 16 kDa polypeptide (Monod *et al.*, 1986).

The only other differences between pT48 and pNE131 within this region are: a T insertion at nucleotide 52, the sequence TCGCT (bases 873-877) is reduced to CTG in pNE131 (the pT48 sequence is also seen in pSN2 and pIM13), and nucleotide 904 is a C instead of a T, again as in pSN2 (See Fig. 2).

(ii) *Inverted repeat (IR)*. An imperfect inverted repeat of approximately 70 bp is shown as its lowest free energy structure, a palindrome of ΔG about $-45.5 \text{ kcal mol}^{-1}$ ($-190.4 \text{ kJ mol}^{-1}$) in Fig. 7 (Tinoco *et al.*, 1973). The palindrome encompasses nucleotides 2369-32 in pT48, and similar structures have not been seen in other *S. aureus* plasmids. The deletion in pIM13 from nucleotide 2464-109 in the pT48 sequence also results in the loss of most of this palindrome, although inverted repeats of about 21 bp are still present (Monod *et al.*, 1986). It is as yet unclear if this structure has a defined function but it may be postulated to act as a transcriptional terminator for the mRNA encoding the ORF B polypeptide, although it is a little large for such a structure. It may also have a functional role in the resolution of pT48 multimeric replication complexes to supercoiled plasmid monomers (unpublished data, this laboratory). Plasmid pIM13 is naturally highly multimeric (Monod *et al.*, 1986). It is interesting that one edge of the palindrome starts exactly as one end of the pSN2 homologous region finishes, i.e. at nucleotide 32 (see Fig. 7). There are three changes within this region in pT48 compared to pNE131: the C present at nucleotide 2386 is a T in pNE131, a C is inserted at base pair 2423 in the former plasmid and there is the deletion of an A between nucleotides 2435-2436 in pT48. The latter two differences are as in pT48 for pIM13 and lead to increased stability of the structure shown in Fig. 7 for pT48.

It is also probable that mutations changing inducible MLS resistance to the constitutive phenotype can occur independently in different clinical environments. Transfer of such a mutated plasmid from *S. aureus* to *S. epidermidis*, rather than in the opposite direction, is implicated on this occasion (Lampson & Parisi, 1986*b*; Cohen *et al.*, 1982). Transfer to *B. subtilis* followed by further mutations also seems likely in the case of pIM13 (Monod *et al.*, 1986).

Note added in proof. Whilst this paper was in press, a report of the nucleotide sequence of another *S. aureus* plasmid, pE5, conferring inducible MLS resistance was published (Projan *et al.*, 1987). The sequence of pE5 is nearly identical to that of pT48.

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