Chromosome- and plasmid-mediated gentamicin resistance in *Staphylococcus aureus* encoded by Tn4001

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Summary. DNA sequences corresponding to the 4·7-kb gentamicin, tobramycin and kanamycin resistance (Gm^rTm^rKm^r) transposon Tn4001 have been detected on a series of nine structurally-related plasmids that mediate this phenotype in Australian isolates of *Staphylococcus aureus*. Tn4001 sequences have also been demonstrated on the chromosomes of Gm^rTm^rKm^r isolates that do not possess these plasmids, and the exhibited diversity of chromosomal sites occupied by this element implies that Tn4001 has transposed to the chromosome on numerous occasions *in vivo*. These results suggest that the rapid emergence of nosocomial Gm^rTm^rKm^r *S. aureus* in the early 1980s may have been the result of the transposition of Tn4001 from a chromosomal site to a readily disseminated plasmid.

Introduction

In nosocomial isolates of *Staphylococcus aureus*, resistance to gentamicin (Gm^r) and the related aminoglycosides tobramycin (Tm^r) and kanamycin (Km^r) has often been correlated with the presence of plasmids (Jaffe *et al.*, 1982; Gray *et al.*, 1983; Lyon *et al.*, 1983; Naidoo *et al.*, 1983; Townsend *et al.*, 1984b). These plasmids may encode additional functions including resistance to acriflavine (Ac^r), ethidium bromide (Eb^r), quaternary ammonium compounds (Qa^r), trimethoprim (Tp^r) and penicillin (Pc^r) and some are also capable of mediating their own transfer (McDonnell *et al.*, 1983; Goering *et al.*, 1985; Archer *et al.*, 1986; Gillespie and Skurray, 1986). In multiresistant strains of *S. aureus* from Australia, the 28·4-kilobase pair (kb) Gm^rTm^rKm^rAc^rEb^rQa^rTp^r plasmid pSK1 (Lyon *et al.*, 1984a, 1986) was shown to carry a 4·7-kb transposon, Tn4001, that encodes resistance to the aminoglycosides (Lyon *et al.*, 1984b). Analysis of plasmids in North American strains of *S. aureus* revealed that Gm^rTm^rKm^r was encoded by a transposon-like element that possessed substantial homology with Tn4001 (Lyon *et al.*, 1987), thereby suggesting

that Gm^rTm^rKm^r plasmids detected worldwide might carry elements analogous to Tn4001.

In several *S. aureus* isolates from Europe, Gm^rTm^rKm^r could not be linked to a plasmid locus (El Solh *et al.*, 1981; Kayser *et al.*, 1981; Witte and Dünhaupt, 1984; Coleman *et al.*, 1985), and in one instance the Gm^rTm^rKm^r determinant was cloned from a chromosomal site (El Solh *et al.*, 1986). Some Australian strains also exhibit chromosomal Gm^rTm^rKm^r and similarities amongst the aminoglycoside resistance levels of these strains and those bearing pSK1 (Lyon *et al.*, 1983; Gillespie *et al.*, 1984, 1985) suggest that the Gm^rTm^rKm^r determinants may be identical. Evidence that Tn4001 readily transposes from plasmids to the staphylococcal chromosome *in vitro* (Lyon *et al.*, 1984b; G. Mahairas, B. Lyon, R. Skurray and P. Pattee, unpublished observation) implies that this element may be similarly active *in vivo*. In this paper, we examine the location of transposon Tn4001 on plasmids and chromosomes of Gm^rTm^rKm^r strains of *S. aureus* from Australian hospitals.

Materials and methods

Bacterial strains and plasmids

Clinical isolates of *S. aureus* were obtained from Australian hospitals during the period 1976–1982 and many have undergone preliminary analysis (Lyon *et al.*, 1983, 1984a; Gillespie *et al.*, 1984, 1985, 1986). The size

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and resistance patterns of the plasmids detected in these strains are listed in table I. The relevant characteristics of *S. aureus* isolates employed in hybridisation studies are presented in table II.

General methods

Standard culture media and methods for the determination of antimicrobial susceptibilities and the transfer of plasmids by mixed culture have been described before (Lyon *et al.*, 1983, 1987; Gillespie *et al.*, 1985).

DNA isolation, restriction analysis and DNA-DNA hybridisation

Purified plasmid DNA was isolated as previously described (Lyon *et al.*, 1983) and whole-cell DNA was prepared by a rapid DNA-isolation technique (Lyon *et al.*, 1984a). Digestion with the restriction endonucleases *EcoRI*, *EcoRV*, *HaeIII*, *HincII*, *HindIII* and *TaqI* (New England Biolabs), agarose gel electrophoresis, the estimation of DNA-fragment sizes, the isolation of probe DNA and DNA-DNA hybridisation were performed as reported by Lyon *et al.* (1983, 1987).

Results

Detection of Tn4001 on Australian Gm^rTm^rKm^r plasmids

Plasmid-DNA analysis of 280 strains of multi-resistant *S. aureus* isolated from hospitals throughout Australia during 1980–1982 revealed that >50% of the organisms carried a member of a family of closely related plasmids of which pSK1 is the prototype (Lyon *et al.*, 1983, 1984a). All but one of the pSK1-related plasmids encode Ac^rEb^rQa^r and most mediate at least one other resistance phenotype which may include Gm^rTm^rKm^r, Tp^r or Pc^r (table I). Restriction endonuclease analysis of

these plasmids, such as the *EcoRI* and *HindIII* double digest presented in fig. 1A, demonstrated a high degree of similarity; the minor structural differences correlated with variations in plasmid phenotype.

Nine of the pSK1-related plasmids (pSK1, pSK4, pSK8, pSK9, pSK11, pSK14, pSK15, pSK16 and pSK17) mediate Gm^rTm^rKm^r, and all were found to possess a characteristic doublet of *HindIII* fragments equivalent to the *HindIII* fragments D and E (2.50 and 2.45 kb, respectively) of pSK1 (fig. 1A, lanes b, c, e, f, g, h, i, j and k). The two Gm^sTm^sKm^s plasmids pSK7 and pSK18 possessed the 2.45-kb *HindIII* fragment E but lacked the 2.5-kb *HindIII* fragment D (fig. 1A, lanes d and l). These findings correspond with previous evidence that the 2.5-kb *HindIII* fragment D of pSK1 maps symmetrically within the 4.7-kb Gm^rTm^rKm^r transposon Tn4001 and results from the presence of *HindIII* sites in the 1.35-kb inverted repeats of the element (Lyon *et al.*, 1984b).

The presence of Tn4001 sequences in all of the Gm^rTm^rKm^r plasmids was confirmed by hybridisation with a 2.5-kb *HindIII* fragment derived from the recombinant plasmid pSK310 which is equivalent to the *HindIII* fragment D of pSK1 (Lyon *et al.*, 1987). All nine plasmids were shown to possess a 2.5-kb fragment that hybridised to the probe (fig. 1B, lanes b, c, e, f, g, h, i, j and k). Moreover, these Gm^rTm^rKm^r plasmids produced *HaeIII* fragments of 3.9 kb that possessed sequences homologous to the 2.5-kb *HindIII* fragment of pSK310 (data not shown). This finding correlates with restriction-mapping data for Tn4001 indicating the presence of symmetrical *HaeIII* sites, 3.9 kb apart, within the transposon (Lyon *et al.*, 1987). However, the Gm^sTm^sKm^s plasmids, pSK7 and pSK18 failed to hybridise with the probe (fig. 1B, lanes d and l) or

Table I. *S. aureus* plasmids and their characteristics

Plasmid	Phenotype*	Size (kb)	Reference
pSK1	Gm ^r Tm ^r Km ^r Ac ^r Eb ^r Qa ^r Tp ^r	28.4	Lyon <i>et al.</i> (1984a, 1986, 1987)
pSK4	Gm ^r Tm ^r Km ^r Ac ^r Eb ^r Qa ^r Tp ^r Pc ^r	35.1	Lyon <i>et al.</i> (1984a)
pSK7	Ac ^r Eb ^r Qa ^r Tp ^r	23.7	Lyon <i>et al.</i> (1984a, 1987)
pSK8	Gm ^r Tm ^r Km ^r Ac ^r Eb ^r Qa ^r Tp ^r Pc ^r	35.1	Lyon <i>et al.</i> (1983)
pSK9	Gm ^r Tm ^r Km ^r Ac ^r Eb ^r Qa ^r	25.7	Lyon <i>et al.</i> (1984a)
pSK11	Gm ^r Tm ^r Km ^r Tp ^r Pc ^r	41.8	This paper
pSK14	Gm ^r Tm ^r Km ^r Ac ^r Eb ^r Qa ^r	24.4	Lyon <i>et al.</i> (1984a)
pSK15	Gm ^r Tm ^r Km ^r Ac ^r Eb ^r Qa ^r Pc ^r	31.1	Lyon <i>et al.</i> (1984a)
pSK16	Gm ^r Tm ^r Km ^r Ac ^r Eb ^r Qa ^r Tp ^r Pc ^r	35.1	Lyon <i>et al.</i> (1984a)
pSK17	Gm ^r Tm ^r Km ^r Ac ^r Eb ^r Qa ^r Tp ^r Pc ^r	36.4	This paper
pSK18	Ac ^r Eb ^r Qa ^r	19.7	Lyon <i>et al.</i> (1984b)

* Abbreviations as in text.

Fig. 1.A. Agarose gel (2.0% w/v) electrophoresis of purified *S. aureus* and *Escherichia coli* plasmid DNA cleaved with *Eco*RI and *Hind*III. The *Hind*III D and *Hind*III E fragments of pSK1 (lane b) are indicated by the letters D and E, respectively. The more intense bands in some lanes result from the presence of linear forms of plasmids pSK2 (4.4 kb) or pSK3 (1.6 kb) or both; these plasmids were often detected in clinical isolates (Lyon *et al.*, 1984a). Lane a, lambda virus DNA cleaved with *Eco*RI and *Hind*III, fragment sizes (in kb) are shown at left; b, pSK1; c, pSK4; d, pSK7 and pSK3; e, pSK8; f, pSK9; g, pSK11, pSK2 and pSK3; h, pSK14; i, pSK15, pSK2 and pSK3; j, pSK16 and pSK3; k, pSK17 and pSK3; l, pSK18; m, pSK310. (The lambda virus fragments were of less intensity than the plasmid fragments and do not reproduce well in the photograph).

B. DNA from gel A transferred to nitrocellulose and hybridised with a ³²P nick-translated probe consisting of the 2.5-kb *Hind*III fragment from pSK310.

produce a fragment of 3.9 kb when cleaved with *Hae*III, thereby confirming the absence of Tn4001 in these two plasmids.

Detection of Tn4001 sequences in strains exhibiting chromosomal Gm^rTm^rKm^r

Plasmid-DNA analysis of more than 200 Gm^r-Tm^rKm^r *S. aureus* isolates failed to detect the presence of plasmids responsible for this phenotype in c. 25% and prompted the conclusion that the resistance determinant was chromosomal in these strains (Lyon *et al.*, 1983, 1984a; Gillespie *et al.*, 1984, 1985). To test the hypothesis that this Gm^rTm^rKm^r determinant represented a chromosomal insertion of Tn4001, whole-cell DNA was isolated from selected clinical isolates of *S. aureus* (table II), cleaved with *Hind* III and hybridised with the 1.3-kb *Taq*I-*Hinc*II fragment of pSK310 which is specific for the central coding region of Tn4001; this probe was used in preference to the

2.5-kb *Hind*III fragment because it lacks homology with the inverted repeat sequences of Tn4001, which have been found to occur independently at numerous sites in the chromosome (B. Lyon, M. Gillespie and R. Skurray, unpublished observation). In digests of five of the Gm^rTm^rKm^r strains (SK1717, SK1734, SK1903, SK460 and SK457), a fragment equivalent to the 2.5-kb *Hind*III fragment from pSK310 (fig. 2A, lane a) was shown to be hybridised (fig. 2A, lanes d, e, f, g and h), whereas in the digest of the remaining Gm^rTm^rKm^r strain (SK1774), a fragment of 3.8 kb hybridised with the probe (fig. 2A, lane c). As expected, digests of the Gm^sTm^sKm^s strains SK1660 and SK1589 did not hybridise with the probe (fig. 2A, lanes b and i).

A similar hybridisation experiment employing *Hae*III-digested chromosomal DNA from these strains was conducted to provide more conclusive evidence for the presence of Tn4001 on the chromosome (data not shown). The 1.3-kb *Taq*I-*Hinc*II fragment from pSK310 hybridised with a

Table II. Selected *S. aureus* strains and their characteristics

Strain	Year of isolation	Phenotype*	Reference
SK1660	1976	Mc'Sm'Tc'Em'Cd'Hg'	Gillespie <i>et al.</i> (1984)
SK1774	1977	Mc'Sm'Tc'Gm'Tm'Km'Cd'Hg'	Gillespie <i>et al.</i> (1984)
SK1717	1977	Mc'Sm'Tc'Em'Gm'Tm'Km'Nm'Ak'Cd'Hg'	Gillespie <i>et al.</i> (1984)
SK1734	1978	Mc'Sm'Tc'Em'Gm'Tm'Km'Nm'Nm'Cm'Cd'Hg'	Gillespie <i>et al.</i> (1984)
SK1903	1978	Mc'Sm'Tc'Em'Gm'Tm'Km'Cd'Hg'	Gillespie <i>et al.</i> (1984)
SK460	1981	Mc'Sm'Tc'Em'Gm'Tm'Km'Nm'Ak'Cd'Hg'	Lyon <i>et al.</i> (1984a)
SK457	1981	Mc'Sm'Tc'Em'Gm'Tm'Km'Cm'Cd'Hg'	Gillespie <i>et al.</i> (1984)
SK1589	1981	Mc'Sm'Tc'Em'Cm'Cd'Hg'	Gillespie <i>et al.</i> (1986)

* Abbreviations as in text, with the addition of Mc, methicillin; Sm, streptomycin; Tc, tetracycline; Em, erythromycin; Nm, neomycin; Ak, amikacin; Cm, chloramphenicol; Cd, cadmium; Hg, mercury.

3.9-kb *Hae*III fragment in digests of strains SK1717, SK1734, SK1903, SK460 and SK457. This result is consistent with the carriage of *Tn4001* on the chromosome in these strains. When the DNA from strain SK1774 was digested with *Hae*III the probe hybridised with a fragment approximately 1.3 kb larger than the fragments detected in digests of the other Gm'Tm'Km' strains, a result which is

compatible with the *Hind*III digest hybridisation with this strain (fig. 2A, lane c).

Diversity of chromosomal sites occupied by *Tn4001*

To establish whether the transposon has inserted at identical or different sites on the chromosomes of these strains, chromosomal DNA from the

Fig. 2. Hybridisation analysis of clinical isolates of *S. aureus* demonstrating chromosomal Gm'Tm'Km'. DNA extracted from selected strains by the rapid method was cleaved with *Hind*III (A) or *Eco*RV (B), electrophoresed on an agarose gel 0.8% w/v, transferred to nitrocellulose, and hybridised with a 32 P nick-translated *Tn4001*-specific probe. Fragment sizes (in kb) of lambda virus DNA cleaved with *Hind*III are shown at left (A) and right (B). Lane a, pSK310 (in both A and B, pSK310 was digested with *Hind*III; the 2.5-kb fragment equivalent to the *Hind*III D fragment of pSK1 is indicated by the letter D); b, SK1660; c, SK1774; d, SK1717; e, SK1734; f, SK1903; g, SK460; h, SK457; i, SK1589.

S. aureus clinical isolates was digested with the endonucleases *EcoRI* and *EcoRV*, for which there are no sites within Tn4001. Hybridisation of the 1.3-kb *TaqI-HincII* probe with fragments of various sizes in *EcoRI* (data not shown) and *EcoRV* (fig. 2B) chromosomal digests indicated that Tn4001 had inserted at various chromosomal sites. There were only two strains, SK1717 and SK1734 (fig. 2B, lanes d and e), in which the transposon appear to be inserted in the same chromosomal fragment.

Discussion

Strains of Gm^rTm^rKm^r *S. aureus* were first isolated from hospital patients in Melbourne (Perceval *et al.*, 1976) and Sydney (Rountree, 1978) during the mid 1970s and, within 5 years, such organisms had reached epidemic proportions in many institutions (Pavillard *et al.*, 1982). The evidence presented in this and previous work (Lyon *et al.*, 1983, 1984a; Gillespie *et al.*, 1984, 1985) indicates that the gene(s) for Gm^rTm^rKm^r was (were) located exclusively on the chromosome in the earlier strains, whereas the majority of the later strains exhibited a plasmid-borne determinant. The demonstration that sequences specific to the 4.7-kb transposon Tn4001 are associated with both plasmid and chromosomal Gm^rTm^rKm^r suggests a mechanism whereby this situation could have arisen.

Analysis of Gm^rTm^rKm^r strains isolated in 1976 revealed that, like strain SK1774, these organisms possessed a chromosomal insertion some 1.3 kb larger than Tn4001, whereas subsequent isolates carried a smaller insert consistent with the presence of Tn4001. It is proposed that this change in size of the insert was responsible for producing a Gm^rTm^rKm^r element which is capable of transposing to diverse chromosomal sites and eventually to a plasmid vector, on which its rapid dissemination was assured. Although the nature of the additional 1.3-kb segment of DNA is uncertain, it does not appear to be equivalent to the 1.35-kb inverted repeat of Tn4001, which can act independently as an insertion sequence (B. Lyon, M. Gillespie and R. Skurray, unpublished observation). Furthermore, it is unlikely that the additional 1.3 kb of DNA possessed by Gm^r strains isolated in 1976

was responsible for the high-level aminoglycoside resistance displayed by these strains (Gillespie *et al.*, 1984) because strain SK1774, which also possessed this extra DNA, displayed low-level Gm^r.

As well as implying a low degree of insertional specificity, the diversity of chromosomal sites occupied by Tn4001 in clinical isolates of Gm^r-Tm^rKm^r *S. aureus* suggests that this transposon is highly mobile *in vivo*. It is surprising, therefore, that Tn4001 has probably only transposed to a plasmid *in vivo* on two occasions, once on to a heavy-metal-resistance plasmid to produce pSK23 (Gillespie and Skurray, 1986), and once on to a member of the pSK1 family. The latter is suggested by the finding that Tn4001 is inserted at an identical site in all nine Gm^rTm^rKm^r plasmids, hence we conclude that any structural differences between these plasmids must have evolved after this event. With respect to its cellular distribution, Tn4001 is unlike other transposons detected in clinical isolates of *S. aureus*, such as Tn554 and Tn4002, which display preference for a single site and orientation of insertion into the chromosome (Murphy *et al.*, 1985; Gillespie and Skurray, 1986; M. Gillespie, B. Lyon and R. Skurray, unpublished observation). It is most likely that Tn4001 inserted into a plasmid such as pSK7 from a chromosomal site and, indeed, every strain in which pSK7 has been detected also harbours a chromosomal Gm^rTm^rKm^r determinant (e.g., SK460). This transpositional event would have produced a plasmid equivalent to pSK1, the most common member of the family and a plasmid so evolutionally successful that it was found in 45% of multiresistant *S. aureus* strains isolated from Australian hospitals in the early 1980s (Lyon *et al.*, 1983, 1984a) and has also been detected elsewhere (Townsend *et al.*, 1984a). It will be interesting to follow the epidemiology of Gm^rTm^rKm^r in the future to determine whether Tn4001 dispenses with its plasmid vehicle and once again, like so many other staphylococcal resistance determinants (e.g., penicillin and tetracycline), becomes a stable component of the chromosome.

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