ANTIBIOTIC RESISTANCE

A retrospective molecular analysis of gentamicin resistance in *Staphylococcus aureus* strains from UK hospitals

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The composite transposon Tn4001, and a related chromosomal Tn4001-like element, encode resistance to the aminoglycosides gentamicin, tobramycin and kanamycin (GmTmKm^r) in Australian strains of *Staphylococcus aureus*. Southern hybridisation analysis of GmTmKm^r *S. aureus* strains isolated from various hospitals in the UK between 1975 and 1985 indicated that they predominantly encoded chromosomal copies of Tn4001 or a Tn4001-like element. However, a strain isolated in 1985 was found to carry Tn4001 on a plasmid related to pSK1, the prototypical multiresistance plasmid commonly detected in *S. aureus* strains from Australian hospitals.

Introduction

Since the mid-1970s, nosocomial strains of *Staphylococcus aureus* resistant to gentamicin and the related aminoglycosides, tobramycin and kanamycin (GmTmKm^r) have been isolated in many parts of the world, including Europe [1–4], North America [5, 6] and Australia [7, 8]. In *S. aureus*, GmTmKm^r has generally been found to be mediated by a bifunctional enzyme that specifies 6'-acetyltransferase [AAC(6')] and 2"-phosphotransferase [APH(2'')] aminoglycoside modifying activities [9, 10].

In strains of *S. aureus* from Australian hospitals, GmTmKm^r has most often been reported to be encoded on the 4.5-kb transposon Tn4001 that is composed of a 1.9-kb central region flanked by inverted copies of the 1.3-kb insertion element IS256 [11–14]. The central region of Tn4001 carries an *aacA-aphD* gene which mediates GmTmKm^r via production of the AAC(6')-APH(2'') bifunctional enzyme [15].

Gentamicin resistance in Australian *S. aureus* isolates

was first reported in 1976 [7]. In GmTmKm^r strains isolated between 1976 and 1980, Tn4001 or a related Tn4001-like element were detected at various chromosomal sites [8, 16, 17]. This Tn4001-like element is composed of an entire Tn4001 with an additional 1.7 kb segment of DNA located in the central region [16 and C.L. Wright, A. Hettiaratchi, N. Firth and R. A. Skurray, unpublished observations].

In most methicillin- and gentamicin-resistant *S. aureus* strains isolated in Australia since 1980—the so-called 'classic' or 'epidemic' Australian MRSA—Tn4001 has been located on a group of structurally related, multiresistance plasmids, designated the pSK1 family [12, 16, 18, 19]. Plasmids of this family typically carry the *qacA* multidrug resistance gene which encodes resistance to various antiseptics and disinfectants [20, 21]. In addition, members of the pSK1 family of plasmids may confer resistance to trimethoprim encoded on the transposon-like structure Tn4003 [22, 23] and penicillin via the transposon Tn4002 [24].

Some epidemic methicillin- and gentamicin-resistant *S. aureus* strains isolated in UK hospitals carry plasmids that appear to be members of the pSK1 family and encode GmTmKm^r on Tn4001 [25, 26], whereas others possess chromosomal copies of Tn4001 [27]. A retrospective analysis of *S. aureus* strains isolated from hospitals in the UK between 1966 and 1986 was carried out to ascertain the involvement of Tn4001 and Tn4001-like elements in the emergence of GmTmKm^r.

Received 11 April 1997; accepted 16 June 1997.

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Materials and methods

Bacterial strains and plasmids

The *S. aureus* strains were isolated from various UK hospitals between 1966 and 1986 and held in a collection at the Laboratory of Hospital Infection, Central Public Health Laboratory, Colindale, London. The gentamicin-resistant strains and their relevant characteristics are listed in Table 1.

S. aureus strains used as controls included the rifampicin- and novobiocin-resistant laboratory strain SK982 [11], and the Australian GmTmKm^r clinical strains SK529, SK1717 and SK1774. *S. aureus* SK529 carries the plasmids pSK1, pSK2 and pSK3 [17]; strains SK1717 and SK1774 carry chromosomal copies of Tn4001 and a Tn4001-like element, respectively [16].

The *Escherichia coli* plasmid pSK310, consisting of the 2.5-kb *Hind*III fragment of Tn4001 from pSK1 cloned into the *Hind*III site of pACYC184 [12], was used to obtain the 1.3-kb *Hind*III-*Taq*I Tn4001-specific probe.

General methods

Standard culture media, methods for determining antimicrobial susceptibilities and minimum inhibitory concentrations (MICs) of antibiotics, mixed culture transfer and plasmid elimination procedures were as described previously [8, 17].

Molecular analysis

Isolation of whole-cell DNA from *S. aureus*, digestion with restriction endonucleases, agarose gel electrophoresis and Southern hybridisation were performed

as described by Lyon *et al.* [12, 17]. *E. coli* plasmid DNA was isolated by standard methods [28]. DNA for use as a radiolabelled probe was electro-eluted from an agarose gel and nick translated with [α -³²P] dATP [28].

Results

Characterisation of UK GmTmKm^r *S. aureus* strains

Of 74 *S. aureus* strains isolated from various hospitals in the UK between 1966 and 1986, inclusive, 19, all isolated in the period from 1975 to 1985, were found to encode GmTmKm^r; many of these strains, particularly those isolated after 1978, showed resistance to other antimicrobial agents (Table 1).

Agarose gel electrophoresis of whole-cell DNA isolated from 10 strains representative of the 19 GmTmKm^r *S. aureus* isolates is shown in Fig. 1a (lanes e–n). Whole-cell DNA from the Australian clinical GmTmKm^r isolate SK529 is also shown (Fig. 1a, lane b). This strain carries the multiresistance plasmid pSK1 (28.4 kb), the chloramphenicol resistance plasmid pSK2 (4.5 kb) and the cryptic plasmid pSK3 (1.5 kb) [17]. Fig. 2 shows a physical and genetic map of pSK1 with an expanded map of Tn4001 [29].

Each of the 19 GmTmKm^r UK strains, except for *S. aureus* SK3738 (Fig. 1a, lane m), carried up to three plasmids of various sizes. Nine strains including *S. aureus* SK3715, SK3718, SK3727, SK3735 and SK3763 (Fig. 1a, lanes e, f, i, k and n, respectively) and *S. aureus* SK3730, SK3739, SK3747, and SK3749 (data not shown) were found to carry plasmids > 20 kb in size.

Table 1. Characteristics of GmTmKm^r *S. aureus* strains

Strain no.	Year isolated	Hospital	Additional resistance to
SK3715*	1975	Westminster, London	Pc Ak Cd
SK3718*	1976	St George's, London	Pc Ak Cd
SK3720	1976	St George's, London	Pc Tc Cd
SK3721*	1976	Royal United, Bath	Ak
SK3722	1977	Royal United, Bath	Ak
SK3723	1977	School of Medicine, Leeds	Pc
SK3724*	1977	Royal Infirmary, Glasgow	Pc Eb Fa
SK3727*	1977	Edgware General, London	Pc Tc Em Cd Hg
SK3730	1978	Withington, Manchester	Tc Em Nm Ak Tp
SK3731*	1978	Royal Berkshire, Reading	Em
SK3734	1979	Withington, Manchester	Tc Em Nm Ak Tp Eb
SK3735*	1979	Hillingdon, London	Mc Pc Tc Em Cl Cm Eb Cd Hg
SK3736	1979	Southport General Infirmary	Tc Em Nm Ak Tp Eb
SK3738*	1980	Southport General Infirmary	Pc Tc Em Nm Ak Tp
SK3739	1980	University College, London	Mc Pc Tc Em Cm Cd Hg
SK3747	1982	Southend, Essex	Mc Pc Tc Em Nm Ak Cl Tp
SK3749	1982	Whittington, London	Mc Pc Tc Ak Eb Nv
SK3754*	1984	Southend, Essex	Mc Pc Tc Em Cl Tp
SK3763*	1985	Leicester Royal Infirmary	Mc Pc Tc Em Cl Tp Cd Hg

Ak, amikacin; Cd, cadmium; Cl, clindamycin; Cm, chloramphenicol; Eb, ethidium bromide; Em, erythromycin; Fa, fusidic acid; Hg, mercury; Mc, methicillin; Nm, neomycin; Nv, novobiocin; Pc, penicillin; Tc, tetracycline; Tp, trimethoprim.

*Strains used in experiments described in Figs. 1, 3 and 4.

Fig. 1. (A) Agarose (1.0% w/v) gel electrophoresis of whole-cell *S. aureus* DNA: lane a, SK982, a gentamicin-sensitive control strain; b, SK529; c, SK1717; d, SK1774; e, SK3715; f, SK3718; g, SK3721; h, SK3724; i, SK3727; j, SK3731; k, SK3735; l, SK3738; m, SK3754; n, SK3763. The positions of the three plasmids pSK1, pSK2 and pSK3 found in the Australian *S. aureus* clinical strain, SK529 (lane b), are labelled; the open circular (oc) form of pSK2 is also indicated. The chromosomal band is labelled (Chr). (B) Autoradiograph of DNA from the above gel transferred to nitrocellulose and hybridised with an α - ^{32} P nick-translated probe consisting of the 1.3-kb *Hind*II-*Taq*I fragment of Tn4001.

Detection of Tn4001 sequences in UK GmTmKm^r *S. aureus* strains

Whole-cell DNA from all 19 strains was initially hybridised with a 1.3-kb *Hind*II-*Taq*I fragment from Tn4001, which is specific for the central region of Tn4001 that encodes the *aacA-aphD* gene (Fig. 2) [15, 16]. Fig. 1b shows hybridisation of this probe to undigested whole-cell DNA from the gel in Fig. 1a. The probe did not hybridise to DNA of the gentamicin-sensitive control, *S. aureus* SK982 (Fig. 1b, lane a), but did hybridise to DNA from the three Australian GmTmKm^r strains: *S. aureus* SK529 (Fig. 1b, lane b), which carries Tn4001 on pSK1 [12]; *S. aureus* SK1717 (Fig. 1b, lane c), which carries a chromosomal copy of Tn4001; and *S. aureus* SK1774 (Fig. 1b, lane d), which carries a chromosomal copy of the Tn4001-like element [16].

Of the UK strains examined, only *S. aureus* SK3763 possessed a plasmid, designated pSK575, which hybridised to the *Hind*II-*Taq*I probe (Fig. 1b, lane n). With whole-cell DNA isolated from this strain and from *S. aureus* SK529 (Fig. 1b, lane b) there was also some hybridisation of the probe to DNA migrating at a position equivalent to that of the chromosome. *S. aureus* SK529 cured of pSK1 (SK553) and *S. aureus*

SK3763 cured of pSK575 (SK4009) were both GmTmKm^s, and whole-cell DNA of these two cured derivatives did not hybridise with the 1.3-kb *Hind*II-*Taq*I fragment of Tn4001. Thus, for *S. aureus* SK529 and SK3763, hybridisation of Tn4001 probe to DNA migrating at the level of the chromosome was probably due to the presence of contaminating plasmid DNA co-migrating with the chromosome.

In the other 18 GmTmKm^r strains the probe hybridised to a single band migrating at a position equivalent to that of the chromosome, as shown by the examples in Fig. 1b, lanes e–m.

To further compare the GmTmKm^r determinants in the UK strains with Tn4001, whole-cell DNA from the 10 strains shown in Fig. 1 was digested with *Hind*III and probed with the 1.3-kb *Hind*II-*Taq*I fragment of Tn4001. Tn4001 possesses a 2.5-kb *Hind*III fragment which encompasses the *aacA-aphD*-encoding central region and 298 bp of each flanking IS256 element (Fig. 2) [14, 15]. As expected, the probe hybridised with a 2.5-kb *Hind*III fragment from the Tn4001-carrying Australian *S. aureus* strains SK529 and SK1717 (Fig. 3, lanes b and c). The probe also hybridised to an equivalently sized band in seven of the UK strains –

Fig. 2. Physical and genetic maps of plasmids pSK1 and pSK4 from GmTmKm^r Australian strains of *S. aureus* [19] and pSK575 from the GmTmKm^r UK *S. aureus* strain SK3763. The sizes of the plasmids are indicated on the right. The three plasmids are aligned relative to the *aacA-aphD* gene of Tn4001 which confers GmTmKm^r. An expanded map of Tn4001 shows relevant restriction sites and the extent of the 1.3-kb *Hind*II-*Taq*I fragment from the *aacA-aphD*-encoding central region which was used as a probe. The locations of the other determinants encoded by these plasmids are also shown. The *qacA* gene encodes multi-drug resistance to antiseptics and disinfectants; *dfrA* encodes trimethoprim resistance and is located on the transposon-like structure Tn4003; and *blaZ* mediates penicillin resistance and is encoded on the transposon Tn4002. A dotted line indicates the approximate position of a 2.5-kb segment absent from pSK575 in comparison to pSK4. Inverted copies of IS256 on Tn4001 and directly repeated copies of IS257 associated with Tn4003 are represented by □ and ■, respectively. Restriction sites are indicated by B, *Bgl*II; E, *Eco*RI; Ha, *Hae*III; Hd, *Hind*II; H, *Hind*III; S, *Sal*I; T, *Taq*I; for clarity, only selected sites are shown.

Fig. 3. Autoradiograph of whole-cell *S. aureus* DNA cleaved with *Hind*III and hybridised with α -³²P nick-translated probe consisting of the 1.3-kb *Hind*II-*Taq*I fragment of Tn4001. Fragments were separated by electrophoresis on an agarose 1.0% w/v gel then transferred to nitrocellulose for hybridisation. Sizes (in kb) of the two fragments found to hybridise with the probe are shown on the left. Lanes a–n contain DNA from the same strains as those shown in Fig. 1.

S. aureus strains SK3721, SK3724, SK3727, SK3731, SK3735, SK3754 and SK3763 (Fig. 3, lanes g–k, m, n) – indicating the presence of Tn4001 on the plasmid pSK575 in *S. aureus* SK3763 and on the chromosome in the other six strains. In experiments in which whole-cell DNA was cleaved with *Hae*III and probed with the 1.3-kb *Hind*II-*Taq*I fragment of Tn4001, fragments equivalent to the 3.9-kb *Hae*III fragment of Tn4001 (Fig. 2) hybridised with the probe in the seven UK strains described above (data not shown). Therefore, it is likely that these strains each carry an entire copy of Tn4001.

In contrast to the 2.5-kb Tn4001 *Hind*III fragment seen in the seven UK isolates described above, *S. aureus* strains SK3715, SK3718 and SK3738 were found to possess a 4.2-kb hybridising fragment (Fig. 3, lanes e, f and l) which corresponded to that of the larger Tn4001-like element carried in the chromosome of the Australian strain SK1774 (Fig. 3, lane d). As with *S. aureus* SK1774, *Hae*III digests of DNA from all three UK strains showed a fragment of 5.6 kb which hybridised to the Tn4001-specific probe, suggesting that these strains also carry the larger Tn4001-like element (data not shown).

Diversity of chromosomal sites occupied by *Tn4001* and the *Tn4001*-like element

Whole-cell DNA from the nine UK *S. aureus* strains described above and the two Australian strains SK1717 and SK1774, was digested with *Eco*RI, for which there are no sites in either *Tn4001* or the *Tn4001*-like element [16]. These digests were probed with the 1.3-kb *Hind*III-*Taq*I fragment of *Tn4001* (Fig. 4).

With each strain, the *aacA-aphD* specific probe hybridised to only one chromosomal *Eco*RI fragment which ranged in size from 8 to > 20 kb (Fig. 4, lanes c–m), suggesting that each strain carries a single copy of either *Tn4001* or the *Tn4001*-like element, located at a variety of chromosomal sites.

Characterisation of pSK1-family plasmid pSK575

The *Tn4001*-specific probe also hybridised to 9.5-kb and 18.5-kb fragments of plasmids pSK1 (Fig. 4, lane b) and pSK575 (Fig. 4, lane n), respectively. Transfer of plasmid pSK575 from *S. aureus* SK3763 to the laboratory strain SK982 by mixed culture transfer demonstrated that this plasmid encoded resistance to penicillin and trimethoprim as well as GmTmKm^r. Plasmid pSK575 was found to possess a restriction endonuclease digestion profile very similar to that of the pSK1 family plasmid, pSK4 (Fig. 2) [19, 24], which mediates resistance to penicillin via a β -lactamase gene, *blaZ*, on *Tn4002* [24] and resistance to trimethoprim encoded by a dihydrofolate reductase (DHFR) gene, *dfrA* on the transposon-like structure, *Tn4003* [22, 23]. However, plasmid pSK575 appeared

to lack a 2.5-kb segment corresponding to a region that normally encodes the *qacA* antiseptic and disinfectant resistance gene on pSK1 family plasmids [20, 30].

Discussion

Gentamicin-resistant *S. aureus* strains were first detected in Australian hospitals in the mid-1970s [7] and in all isolates examined from that time until 1980, the determinant for GmTmKm^r was shown to be encoded by either the composite transposon *Tn4001* or a *Tn4001*-like element, both located exclusively on the chromosome [16]. However, in most epidemic GmTmKm^r strains of *S. aureus* isolated in Australia during and after 1980, GmTmKm^r was encoded by *Tn4001* on members of the pSK1 family of plasmids [8, 11, 17]. It has been suggested that *Tn4001* transposed from the chromosome to a GmTmKm^s member of the pSK1 family of plasmids, such as pSK7, producing a plasmid equivalent to pSK1 [8, 16, 30]. As *Tn4001* is inserted at an identical site in all members of the pSK1 family examined, transposition of *Tn4001* to a family member probably occurred only once with structural differences between the various plasmids evolving later [16, 30].

Results of the present study similarly show that GmTmKm^r *S. aureus* strains isolated in UK hospitals between 1975 and 1980 carry *Tn4001* or the *Tn4001*-like element exclusively on the chromosome. One of the strains included in this study, *S. aureus* SK3715, was one of the first reported GmTmKm^r *S. aureus* strains isolated in the UK [2]. This strain, and two other UK strains, SK3718 and SK3738 isolated in 1976 and 1980, respectively, appear to carry the same chromosomal *Tn4001*-like element as that seen in some of the first GmTmKm^r strains isolated in Australia [16]. The copies of *Tn4001* and the *Tn4001*-like elements carried by these UK strains were located at various chromosomal sites, suggesting that they have arisen by independent insertion events and supporting the notion that, like *Tn4001* [11], the *Tn4001*-like element is transposable.

Previous reports have suggested that some epidemic GmTmKm^r strains present in UK hospitals in the 1980s could carry *Tn4001* on a pSK1-like plasmid [25–27]. In one of these studies [25], most GmTmKm^r *S. aureus* strains isolated from the London Hospital and the Royal Free Hospital, London, during 1983 and 1984, carried plasmids which, from restriction analysis, appear to belong to the pSK1 family. The resistance properties and plasmid profiles of these UK epidemic strains indicated that they are probably identical to, and hence share a clonal origin with, epidemic strains from Australian hospitals [25–27]. In contrast to these epidemic strains, five of six strains isolated between 1980 and 1985 from various UK hospitals and examined in the present study encoded

Fig. 4. Autoradiograph of whole-cell *S. aureus* DNA cleaved with *Eco*RI and hybridised with an α -³²P nick-translated probe consisting of the 1.3-kb *Hind*III-*Taq*I fragment of *Tn4001*. Fragments were separated by electrophoresis on an agarose 1.0% w/v gel and then transferred to nitrocellulose for hybridisation. Lanes a–n contain DNA from the same strains as those shown in Fig. 1. Fragment sizes (in kb) of co-electrophoresed *Hind*III-cleaved λ virus DNA are shown on the left.

the Tn4001 GmTmKm^r determinant on the chromosome. Only *S. aureus* SK3763, isolated in 1985, carried Tn4001 located on a pSK1-like plasmid, pSK575. This plasmid was very similar to the pSK1-family plasmid, pSK4, isolated from Australian strains of *S. aureus* (Fig. 2).

The retrospective molecular analysis of UK hospital strains indicates that the pattern of emergence of GmTmKm^r in Australian and UK *S. aureus* strains is very similar. All strains examined from both countries from the mid-1970s until 1980 carry Tn4001 or the Tn4001-like element at various chromosomal sites. It is possible that chromosomal integration of these elements provided an opportunity for stabilisation of GmTmKm^r in *S. aureus* populations following transfer of a Tn4001-carrying plasmid from an unidentified donor species. Furthermore, it seems likely that Tn4001 and its derivatives have been exclusively responsible for the spread of gentamicin resistance among *S. aureus* strains in the UK and Australian hospitals.

We thank Dr R. R. Marples for generously providing the collection of UK *S. aureus* strains during a period when one of us (R.A.S.) was working in the laboratory of Professor K. G. H. Dyke, Microbiology Unit, Department of Biochemistry, University of Oxford. This work was supported in part by Project Grants from the National Health and Medical Research Council (Australia).

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