Detection and Characterization of IS256, an Insertion Sequence in Staphylococcus aureus

By BRUCE R. LYON, MATTHEW T. GILLESPIE† AND RONALD A. SKURRAY*

Department of Microbiology, Monash University, Clayton, Victoria 3168, Australia

(Received 10 March 1987; revised 7 July 1987)

Resistance to the aminoglycosides gentamicin (Gm'), tobramycin (Tm') and kanamycin (Km') in strains of Staphylococcus aureus isolated in Australia is mediated by the transposon Tn4001. The 1.35 kb inverted repeat of this transposon exhibits many of the characteristics of an insertion sequence and has consequently been designated IS256. Tandem duplication of IS256 contiguous with Tn4001 results in an increase in the level of Gm'Tm'Km', thereby implying that the element possesses strong promoter sequences. Both contiguous and independent insertions of IS256 into the staphylococcal chromosome have been observed, the latter suggesting that the element may play a role in molecular rearrangements of the genome.

INTRODUCTION

Molecular analysis of strains of multiresistant Staphylococcus aureus isolated from clinical sources in Australia has revealed that resistance to the aminoglycosides gentamicin (Gm'), tobramycin (Tm') and kanamycin (Km') is commonly mediated by a transposable element designated Tn4001 (Lyon et al., 19843; Gillespie et al., 1987). This element is primarily detected on a family of structurally-related plasmids represented by the 28.4 kb prototype pSK1, but has also been demonstrated at various sites on the chromosomes of hospital staphylococci. Laboratory experiments have shown that Tn4001 exhibits a low specificity of insertion into the S. aureus plasmid pII147 and the chromosomes of both rec+ and rec strains (Lyon et al., 19846; B. Lyon, J. May & R. Skurray, unpublished data), and hence may constitute a valuable tool for transposon mutagenesis studies (G. Mahairas, B. Lyon, R. Skurray & P. Pattee, unpublished).

Electron microscopical analysis of heteroduplexed and self-annealed plasmid molecules has demonstrated that Tn4001 is approximately 4.7 kb in size and is composed of a unique 2.0 kb region, which encodes Gm'Tm'Km', flanked on either side by inverted repeat sequences of 1.35 kb (Lyon et al., 1984b, 1987). In this respect, Tn4001 resembles composite or class I transposons such as Tn5 and Tn10 which also possess an antibiotic resistance determinant(s) flanked by large inverted repeat sequences (Kleckner, 1981). Tn4001 shares substantial homology with the transposon-like determinants of Gm'Tm'Km' present on plasmids isolated in the USA, although the latter possess smaller inverted repeats of only 0.7 kb (Lyon et al., 1987). All attempts to achieve the transposition of the USA Gm'Tm'Km' determinant have been unsuccessful and the possibility exists that the reduced inverted repeats do not encode the necessary functions for transposition.

In the course of studying Tn4001 we have gathered evidence which implies that at least one of the inverted repeats of this transposon can act independently in the manner of an insertion sequence. The inverted repeat of Tn4001 has accordingly been designated IS256 and represents the first element of its type to be identified in the staphylococci.

† Present address: Research Centre for Cancer and Transplantation, Department of Pathology, University of Melbourne, Parkville, Victoria 3052, Australia.
Table 1. Characteristics of S. aureus strains

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Relevant phenotype*</th>
<th>Plasmid(s)†</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK430</td>
<td>Gm'Tm'Km'Ae'Eb'Qa'Tp'</td>
<td>pSK18</td>
<td>Tennent et al. (1985)</td>
</tr>
<tr>
<td>SK1589</td>
<td>Gm'Tm'Km'Ae'Eb'Qa'Tp'</td>
<td>pSK2</td>
<td>Gillespie et al. (1986)</td>
</tr>
<tr>
<td>SK982</td>
<td>Nv' Rf'</td>
<td>–</td>
<td>Lyon et al. (1984b)</td>
</tr>
<tr>
<td>SK1660</td>
<td>Gm'Tm'Km'Ae'Eb'Qa'Tp'</td>
<td>–</td>
<td>Gillespie et al. (1984, 1986)</td>
</tr>
<tr>
<td>SK1717</td>
<td>Gm'Tm'Km'Ae'Eb'Qa'Tp'</td>
<td>–</td>
<td>Gillespie et al. (1984, 1986)</td>
</tr>
<tr>
<td>SK1734</td>
<td>Gm'Tm'Km'Ae'Eb'Qa'Tp'</td>
<td>pSK3, pSK158</td>
<td>Gillespie et al. (1984, 1986)</td>
</tr>
<tr>
<td>SK1903</td>
<td>Gm'Tm'Km'Ae'Eb'Qa'Tp'</td>
<td>pSK52</td>
<td>Gillespie et al. (1984)</td>
</tr>
<tr>
<td>SK2201</td>
<td>Gm'Tm'Km'Ae'Eb'Qa'Tp'</td>
<td>pSK1α/β</td>
<td>This paper</td>
</tr>
<tr>
<td>SK2267</td>
<td>Gm'Tm'Km'Ae'Eb'Qa'Tp'</td>
<td>pSK1β</td>
<td>This paper</td>
</tr>
<tr>
<td>SK2268</td>
<td>Gm'Tm'Km'Ae'Eb'Qa'Tp'</td>
<td>pSK1β</td>
<td>This paper</td>
</tr>
</tbody>
</table>

* Abbreviations as in text with the addition of: Nv, novobiocin; Rf, rifampicin.
† Plasmids pSK3 (phenotypically cryptic), pSK52 (tetracycline resistance) and pSK158 (chloramphenicol resistance) are wild-type S. aureus plasmids with no homology to Tn4001.

Table 2. Characteristics of S. aureus and hybrid plasmids

<table>
<thead>
<tr>
<th>Plasmid no.</th>
<th>Phenotype*</th>
<th>Size (kb)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSK1α</td>
<td>Gm'Tm'Km'Ae'Eb'Qa'Tp'</td>
<td>28.4</td>
<td>Lyon et al. (1984a, 1986, 1987)</td>
</tr>
<tr>
<td>pSK1β</td>
<td>Gm'Tm'Km'Ae'Eb'Qa'Tp'</td>
<td>29.75</td>
<td>This paper</td>
</tr>
<tr>
<td>pSK18</td>
<td>Ac'Eb'Qa'Tp'</td>
<td>19.7</td>
<td>Lyon et al. (1984b, 1986)</td>
</tr>
<tr>
<td>pSK310</td>
<td>Gm'Tm'Km'Cm'</td>
<td>6.7</td>
<td>Lyon et al. (1987)</td>
</tr>
<tr>
<td>pSK449</td>
<td>Ac'Eb'Qa'Ap'</td>
<td>7.7</td>
<td>Tennent et al. (1985)</td>
</tr>
</tbody>
</table>

* Abbreviations as in text, with the addition of: Cm, chloramphenicol; Ap, ampicillin.

METHODS

Bacterial strains and plasmids. The relevant characteristics of the clinical and laboratory strains of S. aureus employed in this work are presented in Table 1. Properties of S. aureus plasmids and Escherichia coli hybrid plasmids are detailed in Table 2.

General methods. Standard culture media and the methods for the determination of antimicrobial susceptibilities and minimum inhibitory concentrations (MICs) of antibiotics were as previously described (Lyon et al., 1983; Gillespie et al., 1984).

DNA isolation, restriction analysis, DNA-DNA hybridization and electron microscopy. Purified plasmid DNA and whole-cell DNA was prepared as previously reported (Lyon et al., 1983, 1984). Digestion with the restriction endonucleases EcoRI, HaeIII, HincII, HindIII, HpaII, PvuII and TaqI (New England Biolabs), agarose gel electrophoresis, the estimation of DNA fragment sizes, the isolation of probe DNA, DNA-DNA hybridization and electron microscopy were performed as published (Lyon et al., 1983, 1987).

RESULTS

Duplication of the inverted repeat of Tn4001

The 4.7 kb Gm'Tm'Km' transposon Tn4001 has been detected on nine members of a family of structurally-related plasmids which, in addition to aminoglycoside resistance, variously encode resistance to acriflavine (Ac'), ethidium bromide (Eb') and quaternary ammonium compounds (Qa'), trimethoprim (Tp') and penicillin (Lyon et al., 1984b, 1987; Gillespie et al., 1987). A detailed restriction map of pSK1, the 28.4 kb prototype of this plasmid family, has been determined and the location of Tn4001 was established by comparison with the Gm'Tm'Km' plasmids pSK7 and pSK18 (Lyon et al., 1984b, 1987). A map of the Gm'Tm'Km' region of pSK1 (pSK1α) is presented in Fig. 1.

Analysis of plasmid DNA isolated from SK2201, a strain constructed by mixed culture transfer of pSK1 from a wild-type clinical strain to the plasmidless laboratory strain SK982 (Lyon et al., 1984b), has demonstrated the presence, within the pSK1 population, of a sub-
An insertion sequence in *S. aureus* Tn4001

Fig. 1. Restriction endonuclease map of the Gm'Tm'Km' region of pSK1α and the corresponding region of pSK1β. The inverted repeats of Tn4001 (IR_L and IR_R) are represented by the thick lines; the duplication in pSK1β is represented by the hatched area. Map coordinates are in kb and correlate with the published map of pSK1 (Lyon et al., 1987); restriction endonuclease sites are indicated by E (EcoRI), H (HindIII), Ha (HaeIII), Hc (HincII), Hp (HpaII), P (PvuII) and T (TagI); only relevant restriction endonuclease sites are shown. Also shown are the extents of the 2.5 kb HindIII and 1.3 kb TaqI-HincII hybridization probes derived from pSK310 and the extent of the 0.7 kb HaeIII-HindIII hybridization probe derived from pSK449, with the HindIII fragments of pSK1α and/or pSK1β hybridized by each of these probes shown under the relevant fragment.

population of larger plasmids. The two plasmid types (designated pSK1α and pSK1β, respectively) were segregated by screening single colonies of SK2201 to isolate strains SK2267 (carrying pSK1α) and SK2268 (carrying pSK1β). This purification step was facilitated by the finding that colonies carrying pSK1β were two to four times more resistant to gentamicin, tobramycin and kanamycin than colonies which carried pSK1α.

Restriction endonuclease analysis of pSK1β has revealed that this plasmid is identical to pSK1α except for the addition of 1.35 kb of DNA which maps near the Gm'Tm'Km' region. In HindIII digests of pSK1β this extra DNA appears as a single additional fragment of 1.35 kb (Fig. 2a, lanes C and E), while in HaeIII digests an additional fragment of 1.0 kb is observed (data not shown). These characteristic fragments would be expected if one (or both) of the inverted repeats of Tn4001 had undergone a tandem duplication as depicted for pSK1β in Fig. 1.

The additional DNA present in pSK1β was shown to possess homology with the inverted repeats of Tn4001 by hybridization with Tn4001-specific DNA fragments derived from the hybrid plasmids pSK310 and pSK449 (Fig. 1). When probed with the 2.5 kb HindIII fragment from pSK310 (Fig. 1), the 2.5 kb fragments in HindIII digests of pSK310 and pSK1α were hybridized as expected (Fig. 2b, lanes B and D), while both the 2.5 kb and 1.35 kb fragments of pSK1β were hybridized (Fig. 2b, lanes C and E). No hybridization was detected with pSK18 (Fig. 2b, lane F), a plasmid which is closely related to pSK1 but lacks Tn4001, and the Tp'-
encoding region (Lyon et al., 1986). When the 1·3 kb TaqI-HincII fragment of pSK310 (Fig. 1) was used as a probe, only the 2·5 kb HindIII fragments of pSK1α and pSK1β plasmids were hybridized (Fig. 2c, lanes C, D and E), thereby indicating that the additional DNA present in pSK1β does not share homology with the internal coding region of Tn4001. Proof that the additional DNA present in pSK1β is homologous with the inverted repeat of Tn4001 resulted from an experiment using the 0·7 kb HaeIII–HindIII fragment of pSK449 as a probe (Fig. 1). Fragments of 7·1 and 5·2 kb in HindIII digests of both pSK1α and pSK1β were hybridized by this probe, as they contain Tn4001 terminal sequences (Fig. 2d, lanes C, D and E), while hybridization of the 1·35 kb HindIII fragment of pSK1β indicated the presence of additional inverted repeat sequences (Fig. 2d, lanes C and E).

**Electron microscopic analysis of pSK1β**

Electron microscopic analysis of self-annealed plasmid DNA was employed to confirm the existence of the duplicated repeat and to locate its position relative to Tn4001. Two kinds of molecule with distinct secondary structures were detected in preparations of self-annealed pSK1β (Fig. 3). The first type of molecule exhibited the characteristic stem and loop structure of Tn4001 (Lyon et al., 1984b) with a double-stranded stem of approximately 1·4 kb and a single-stranded loop of 2·0–2·2 kb (Fig. 3a). The second type of molecule exhibited a larger single-stranded loop of 3·1–3·6 kb but possessed a single-stranded flanking segment (from the base of the double-stranded stem to end B) which was approximately 1·5 kb shorter than that seen in the former molecule (Fig. 3b).

The observed molecules can be explained if the smaller loop (Fig. 3a) was formed by annealing between the lone inverted repeat (e.g. IR₁ in Fig. 1) and the inner repeat (e.g. IRᵣ) of a tandem pair, in which case the outer repeat would form part of the single-stranded flanking segment. This segment would be shortened in the second molecule (Fig. 3b) if the outer repeat was annealed to the lone inverted repeat and the inner tandem repeat formed part of the larger...
An insertion sequence in *S. aureus*

Fig. 3. Electron micrographs of pSK1β DNA cleaved with *EcoRI*, denatured to single-stranded molecules and treated to enhance the formation of secondary structure. A tracing of each molecule is provided below for easier interpretation. The ends of each molecule are denoted by A and B (A marks the *EcoRI* site at 19.10 kb on the pSK1 physical map). The arrow marked C indicates a single-stranded loop of 2.0–2.2 kb; the arrow marked D indicates a single-stranded loop of 3.1–3.6 kb.

Fig. 3. Electron micrographs of pSK1β DNA cleaved with *EcoRI*, denatured to single-stranded molecules and treated to enhance the formation of secondary structure. A tracing of each molecule is provided below for easier interpretation. The ends of each molecule are denoted by A and B (A marks the *EcoRI* site at 19.10 kb on the pSK1 physical map). The arrow marked C indicates a single-stranded loop of 2.0–2.2 kb; the arrow marked D indicates a single-stranded loop of 3.1–3.6 kb.

single-stranded loop. Thus, these results confirm the presence of a tandemly duplicated repeat in pSK1 and indicate that the duplication has occurred to the right (Fig. 3, end B) of Tn4001 as depicted in Fig. 1.

**Detection of an insertion sequence on the *S. aureus* chromosome**

We have previously demonstrated the presence of Tn4001 on the chromosomes of a collection of Gmr Tm Kmr *S. aureus* isolated from clinical sources by probing HindIII digests of whole-cell DNA with the 1.3 kb *TaqI–HincII* fragment of pSK310 (Gillespie et al., 1987). Hybridization of such digests with the 2.5 kb HindIII fragment of pSK310 not only detected the 2.5 kb HindIII fragment of Tn4001 but also revealed the presence of one or more additional fragments which exhibit homology with the probe (Fig. 4a). These additional fragments did not result from tandem duplication of the inverted repeat of a chromosomal Tn4001, as no 1–35 kb HindIII fragments were evident, but must instead represent non-contiguous duplication or insertions of the repeat sequence. Moreover, in the case of the Gm Tm Km strain SK1589 (Fig. 4a, lane F) where, as expected, no 2.5 kb HindIII fragment was observed, five unique fragments exhibiting a degree of homology with the probe were detected. Homologous sequences were not detected for the Gm Tm Km strain SK1660, however (Fig. 4a, lane B).

The nature of the additional fragments observed in Fig. 4(a) was further investigated by probing an identical filter with the 0.7 kb *HaeIII–HindIII* fragment of pSK449 (Fig. 4b). The
Fig. 4. Hybridization analysis of whole-cell S. aureus DNA cleaved with HindIII, electrophoresed on a 0.8% (w/v) agarose gel and transferred to nitrocellulose paper. Lanes: A, pSK310 cleaved with HindIII; B, SK1660; C, SK1717, D, SK1734; E, SK1903; F, SK1589. Molecular size markers (HindIII-cleaved λ phage DNA) are shown at left and centre. (a) Autoradiograph following hybridization with the $^{32}$P-labelled 2.5 kb HindIII fragment of pSK310. The 5.6 kb fragment detected in lane C and the two larger fragments detected in lane F result from partial digestion of the DNA. (b) Autoradiograph following hybridization with the $^{32}$P-labelled 0.7 kb HaeIII–HindIII fragment of pSK449. The lane marked * contains HindIII-cleaved pSK449 DNA.

detection of additional fragments in Fig. 4(a) could be directly correlated with the presence of additional fragments possessing homology with the inverted repeat of Tn4001. Thus for example, for strain SK1734 (Fig. 4b, lane D), two of the fragments could be attributed to the presence of Tn4001, while the third resulted from the presence of an independent repeat sequence as suggested by Fig. 4(a) (lane D).

Confirmation that the homologous sequences detected in Fig. 4 were in fact closely related to the inverted repeat of Tn4001 was achieved by probing HaeIII–HindIII double digests of the DNA with the 0.7 kb HaeIII–HindIII fragment of pSK449 (data not shown). As expected, the only fragment found to hybridize in each instance was a 0.7 kb fragment equivalent to the probe.

**DISCUSSION**

The data presented in this paper provide strong evidence for the existence of an insertion sequence in multiresistant isolates of S. aureus. This 1.35 kb insertion sequence, which we have designated IS256, primarily acts as the terminal inverted repeats of the 4.7 kb Gm'Tm'Km' transposon Tn4001, but can also be detected as an autonomous insertion element.
An insertion sequence in S. aureus

IS256 was first observed in at least four members of the pSK1 plasmid family as a contiguous tandem duplication of one of the inverted repeats of Tn4001. Such duplication was shown to result in an increase in the level of Gm’Tm’Km’, but these plasmids possessing tandem repeats of IS256 were able to revert back to the a-type and confer low-level Gm’Tm’Km’, a finding which suggests that IS256 contains promoter sequences capable of acting in tandem to augment transcriptions of adjacent genes. In support of this notion, nucleotide sequencing of the resistance-encoding region of Tn4001 has revealed that the transcription start point of the Gm’Tm’Km’ gene occurs downstream from the IS256 duplication of pSK1β (Rouch et al., 1987). A similar phenomenon may have taken place amongst strains of S. aureus from the UK, where plasmids possessing an additional 0.75 kb of DNA as a result of transfer to a new host also mediated a fourfold increase in Gm’ (Townsend et al., 1984); some of these plasmids appear identical to those detected in Australian isolates. It is of interest that the tandem duplication of IS256 and subsequent increase in the level of Gm’ has been detected only after the transfer of these plasmids from their natural clinical host to the laboratory strain RN450, or a number of its derivatives; we have been unable to detect a β-form plasmid in any of 600 Australian clinical isolates or in clinical isolates that have been propagated in the presence of increased levels of gentamicin in an attempt to produce a β-form plasmid. This observation implies that the clinical host regulates the transpositional activity of IS256 on the pSK1 family of plasmids whereas RN450 does not; however, it should be noted that not all pSK1 family plasmids which carry Tn4001 are capable of undergoing induction or deregulation to produce a β-form plasmid. This latter point may reflect the innate transpositional activities of IS256 and Tn4001, which may be concordant. As such, the determination of the transpositional capabilities of IS256 in a recA background would be invaluable.

The presence of IS256 was also detected at multiple sites on the chromosomes of clinical isolates of S. aureus by DNA–DNA hybridization. In at least two of 28 strains examined, characteristic restriction fragment patterns suggested that a tandem duplication of IS256 adjacent to the chromosomal insertion of Tn4001 had occurred; however, no increase in the level of resistance to aminoglycosides was observed (data not shown). In other strains, the chromosomal insertions of IS256 were found to be independent of the transposon, thereby providing the first indication that insertion sequences may play a role in molecular rearrangements of the staphylococcal genome. The demonstration of an independent insertion of IS256 in a plasmid belonging to the pSK1 family (J. Tennent, J. May & R. Skurray, unpublished data), suggests that such activity may extend to extrachromosomal DNA.

Tn4001-like elements have been detected on self-transmissible plasmids isolated from S. aureus strains of USA origin (Lyon et al., 1987) and also from strains of Gm’ Streptococcus faecalis (Ferretti et al., 1986). In the former case, the inverted repeat sequences of the element were shown to be homologous with, though somewhat smaller than, IS256, while analysis of the nucleotide sequence of the S. faecalis element has revealed substantial identity with IS256 immediately adjacent to the region encoding aminoglycoside resistance (Rouch et al., 1987). In addition to acting as a potential focus for genetic rearrangement of chromosomal and extrachromosomal DNA, these data suggest that elements analogous with IS256 may play a crucial role in promoting the intraspecific together with the intergeneric spread of antimicrobial-resistance determinants.

We thank Linda Messerotti for skilful technical assistance. This work was supported by a Project Grant from the National Health and Medical Research Council (Australia).

REFERENCES


