Trimethoprim resistance determinants encoding a dihydrofolate reductase in clinical isolates of *Staphylococcus aureus* and coagulase-negative staphylococci

JAN M. TENNENT*, HILARY-KAY YOUNG††, B. R. LYON, S. G. B. AMYE‡ and R. A. SKURRAY

Department of Microbiology, Monash University, Clayton, Victoria 3168, Australia and †Department of Bacteriology, Medical School, University of Edinburgh, Edinburgh EH8 9AG

Summary. The molecular and biochemical basis of resistance to high concentrations (MIC ≥ 1000 mg/L) of trimethoprim (Tpf(H)) was examined in Australian isolates of *Staphylococcus aureus* and coagulase-negative staphylococci. The Tpf(H) determinant (dfrA) was located within a 2.75-Kb BglII fragment on the *S. aureus* aminoglycoside-resistance plasmids pSK1 and pSK16 as judged by comparative restriction mapping with two naturally-occurring variants, pSK9 and pSK14, which did not encode trimethoprim resistance. This was confirmed in DNA-DNA hybridisation experiments in which a 0.9-Kb sequence of pSK1 DNA was used as a specific probe for the Tpf(H) gene. Plasmid DNA from three strains of coagulase-negative staphylococci, and the chromosomal material of one other isolate, were found to share homology with the probe DNA. Dihydrofolate reductases produced by these strains were virtually identical to the type S1 enzyme encoded by the *S. aureus* plasmid pSK1. Interspecies transfer may have been responsible for the conservation of Tpf(H) gene sequences among staphylococci.

Introduction

Trimethoprim inhibits chromosomally-encoded dihydrofolate reductase (DHFR) required for bacterial metabolism of folic acid (Smith and Amyes, 1984). High-level plasmid-mediated resistance to trimethoprim (Tpf(H)) in *Escherichia coli* involves the production of an insusceptible target enzyme which allows the inhibited metabolic step to be bypassed (Amyes and Smith, 1974). Resistance to trimethoprim has also been encountered in multi-resistant strains of *Staphylococcus aureus* (MRSA) and coagulase-negative staphylococci isolated in Australia and elsewhere (Hamilton-Miller et al., 1981; Lyon et al., 1983; Richardson, 1983; Townsend et al., 1984; Archer et al., 1986). Resistance to high concentrations of trimethoprim (≥ 1000 mg/L) in c. 50% of Australian isolates of MRSA is mediated by the 28.4-Kb plasmid pSK1 which also encodes resistance to acriflavine, quaternary ammonium compounds and the aminoglycosides gentamicin, tobramycin and kanamycin (Gillespie et al., 1986; Lyon et al., 1987). The trimethoprim-resistance gene from pSK1, designated *dfrA* (Lyon and Skurray, 1987), encodes the production of a trimethoprim-resistant dihydrofolate reductase (*Young et al.*, 1987). This plasmid-encoded enzyme, the first to be characterised in staphylococci, differs from all previously characterised plasmid-encoded dihydrofolate reductases and we have designated it type S1 (*Young et al.*, 1987).

The spread of resistance to antimicrobial agents in nature may be accomplished by the transfer of resistance determinants between *S. aureus* and coagulase-negative staphylococci (Lacey, 1975). In continuance of our investigations into the relevance of this hypothesis to the emergence and spread of MRSA in Australian hospitals (*Tennent et al.*, 1984, 1986), we have compared the genetic and...
molecular characteristics of the trimethoprim resistance exhibited by epidemiologically-related isolates of MRSA and coagulase-negative staphylococci and have characterised the trimethoprim-resistant dihydrofolate reductases produced by these strains.

Materials and methods

Bacterial strains and plasmids

Clinical isolates of S. aureus and coagulase-negative staphylococci were selected from a collection of multiresistant strains obtained from Australian hospitals between 1979 and 1982. The coagulase-negative staphylococci were identified as either S. epidermidis (114 strains) or as 'other coagulase-negative staphylococci' (S. haemolyticus, S. saprophyticus, S. simulans, S. warneri, S. hominis; 50 strains) on the basis of phosphatase activity and aerobic production of acid from maltose, mannitol, sucrose, or trehalose (Kloos and Schleifer, 1975). Two strains of 'other coagulase-negative staphylococci' that were highly resistant to trimethoprim were identified as S. hominis type I by the API Staph system.

S. aureus strains carrying plasmids for use as size standards, and plasmid-free strains of S. aureus (SA113, SK982) used as recipients in DNA transfer experiments, have been described previously (Lyon et al., 1983; Tennent et al., 1986). A description of the S. aureus plasmids and strains examined in this study is presented in table I; plasmid pSK1 is representative of a family of S. aureus multiresistance plasmids which range in size from 20 to 42 Kb and principally encode resistance to antiseptics or disinfectants and to aminoglycoside antibiotics (Gillespie et al., 1987b). Methods for the curing of plasmids and for the construction of the S. aureus-E. coli hybrid plasmid pSK407 from pSK1 have been described elsewhere (Tennent et al., 1983 and 1986).

General procedures

Trimethoprim sensitivity and minimum inhibitory concentrations (MICs) were determined on Diagnostic Sensitivity Test Agar (Oxoid) supplemented with 5% defibrinated horse blood lysed with saponin. The methods used for testing sensitivity to other antimicrobial agents, for transformation of plasmid DNA, and for plasmid elimination, were as described previously (Lyon et al., 1983; Gillespie et al., 1984). The transfer of plasmid DNA in mixed culture was performed as described by McDonnell et al. (1983).

DNA manipulation procedures

Isolation of plasmid DNA, digestion with the restriction enzymes BglII, EcoRI, EcoRV, HindIII, and HpaII (New England Biolabs, Inc., Beverley, MA, USA), and agarose gel electrophoresis, were performed as previously described (Tennent et al., 1984). HindIII digests of lambda DNA were used as standards; fragment sizes in kilobase pairs (Kb) were as published (Daniels et al., 1983). DNA was transferred to nitrocellulose for hybridisation analysis by the bidirectional procedure of Smith and Summers (1980).

A 0.9-Kb EcoRI-EcoRV DNA fragment, which spans the dfrA structural gene, was prepared by electrophoration from pSK407, nick-translated with (α-32P)dATP (1800 Ci/mmol) and hybridised under conditions of high stringency with nitrocellulose filters carrying target DNA, as described by Maniatis et al. (1982). Washed and dried filters were exposed to Fuji RX film at -70°C for sufficient time to produce an acceptable autoradiograph.

Dihydrofolate reductase

DHFR was prepared from 3-L stationary-phase cultures grown in Brain Heart Infusion Broth (Oxoid) by the method of Young et al. (1987). Enzyme activity was assayed by the method of Amyes and Smith (1974) and characterised as described previously (Young et al., 1987).

Results

Trimethoprim resistance in S. aureus

Susceptibility tests of clinical strains of S. aureus revealed that plasmids in the same family as pSK1, such as pSK16, also conferred high-level resistance to trimethoprim (MIC > 1000 mg/L) on their hosts (table I). Derivatives cured of pSK1 (strain SK553) or pSK16 (strain SK296) were no longer highly resistant to trimethoprim but were more resistant than S. aureus NCTC 6571. The level of trimethoprim resistance in SK553 was similar to that found with the clinical isolates of S. aureus that carried plasmids pSK9 and pSK14, and S. aureus strain SK434 that lacked a plasmid belonging to the pSK1 family (table I).

Plasmid location of the trimethoprim-resistance gene in S. aureus

The structural gene for Tp’(H) which has been localised to a 0.75-Kb sequence of DNA contained within the 2.75-Kb BglII fragment of pSK1 (Lyon et al., 1986) is shown in fig. 1. The S. aureus aminoglycoside-resistance plasmids pSK9 and pSK14 did not encode the Tp’(H) phenotype although their resistance profiles were similar to those of pSK1 and pSK16 (table I). Digestion of the Tp’(H) plasmid pSK16 with BglII showed that, like pSK1, it possessed a 2.75-Kb BglII fragment (fig. 2A, lanes b and e) whereas plasmids pSK9 and pSK14 (fig. 2A, lanes c and d) did not. Further restriction endonuclease analyses showed that
Table I. Characteristics of *S. aureus* strains and plasmids

<table>
<thead>
<tr>
<th><em>S. aureus</em> strain</th>
<th>MIC of Tp (mg/L)</th>
<th>Plasmid*</th>
<th>Plasmid size (Kb)</th>
<th>Plasmid resistance pattern</th>
</tr>
</thead>
</table>
| SK429              | >1000            | pSK1     | 28.4              | Gm Tm Km Ac Eb Qa Tp
| SK529              | 1000             | pSK1     | 28.4              | Gm Tm Km Ac Eb Qa Tp
| SK271              | >1000            | pSK16    | 35.1              | Gm Tm Km Ac Eb Qa Tp
| SK257              | 20               | pSK9     | 25.7              | Gm Tm Km Ac Eb Qa
| SK74               | 20               | pSK14    | 24.4              | Gm Tm Km Ac Eb Qa
| SK553t             | 20               | ...      | ...               | ...
| SK296t             | 20               | ...      | ...               | ...
| SK434§             | 20               | ...      | ...               | ...
| NCTC 6571          | 1.25             | ...      | ...               | ... \\

Gm; gentamicin; Tm, tobramycin; Km, kanamycin; Ac, acriflavine; Eb, ethidium bromide; Qa, quaternary ammonium compounds; Tp, trimethoprim (MIC > 1000 mg/L); Pc, penicillin

* Some strains carry more than one plasmid; only the pSK1-family plasmid is described.
† SK553 is a pSK1-cured derivative of SK529.
§ SK296 is a pSK16-cured derivative of SK271.
§ SK434 is a clinical isolate which lacks a pSK1-family plasmid.

Plasmids pSK9 and pSK14 were identical to pSK1 except that deletions of 2.7 Kb and 4.0 Kb, respectively, had occurred in the region of these plasmids corresponding to the position of the *dfrA* gene (fig. 1). Both deletions were found to overlap the *BglI* site at 12-05 on the pSK1 map and thus accounted for the distinct *BglI* profiles of pSK9 and pSK14.

A 0.9-Kb EcoRI-EcoRV fragment of plasmid pSK407 (Lyon et al., 1986), which maps between co-ordinates 13.55 and 14.45 on the pSK1 map (fig. 1) and consists almost entirely of the *dfrA* determinant, was used to probe the *BglI* digests of the *S. aureus* plasmids. Strong hybridisation signals were obtained between the probe and the 2.75-Kb *BglI* fragments of plasmids pSK1 and pSK16 (fig. 2B, lanes b and e). Similarly-sized *BglI* restriction fragments from three other plasmids, pSK4, pSK8 and pSK17 found in clinical isolates of *S. aureus* that were highly resistant to trimethoprim (Gillespie et al., 1987b), also demonstrated homology with the probe DNA (data not shown). In contrast, the pSK407-derived probe did not hybridise with any fragments of pSK9 or pSK14 (fig. 2B, lanes c and d), thus confirming the absence of *dfrA* sequences on these plasmids as suggested by the restriction mapping data. On the basis of these results, the 0.9-
Hybridisation analysis of trimethoprim-resistant coagulase-negative staphylococci

Rapidly-isolated DNA from four of the Tpₜ(H) strains of coagulase-negative staphylococci, and from one representative S. aureus strain harbouring pSK1 (SK429), was analysed by agarose gel electrophoresis (fig. 3A), transferred to nitrocellulose and then hybridised with the radiolabelled 0.9-Kb EcoRI-EcoRV fragment from plasmid pSK407 (fig. 3B). Three bands of DNA in strain SK47 (fig. 3B) were considered suitable for use as a dfrA-specific probe.

**Trimethoprim resistance in coagulase-negative staphylococci**

Screening of 164 clinical isolates of multiresistant coagulase-negative staphylococci showed that 71 strains (43%) were resistant to trimethoprim 50 mg/L. For 63 (89%) of these 71 resistant strains, the MIC of trimethoprim was 100 mg/L, whereas the MIC for the other eight (11%) isolates was >1000 mg/L. Plasmid elimination and transfer studies failed to demonstrate a plasmid locus for the Tpₜ(H) determinant in these strains, of which six were identified as S. epidermidis and two as S. hominis type I.
TRIMETHOPRIM RESISTANCE IN STAPHYLOCOCCI

3B, lane b), which were presumed to have corresponded to the covalently closed circular (ccc), open circular (oc) and linear forms of a 7-9-kb plasmid present in this isolate, hybridised with the probe DNA. Plasmids of different sizes in strains SK76 (15.5 kb) and SK400 (17.5 kb), and the presumptive chromosomal material of SK683, also demonstrated homology with the dfrA-specific probe (fig. 3B, lanes c, d, and e).

Dihydrofolate reductases encoded by trimethoprim-resistance genes

The plasmid-encoded dihydrofolate reductases from strains SK76, SK47 and SK400 were very similar to one another and to the chromosomally-encoded enzyme from strain SK683 (table II). However, the specific activity of the chromosomal dihydrofolate reductase from SK683 was much lower than that from the other three strains. All four enzymes had properties similar to those of the type S1 dihydrofolate reductase encoded by S. aureus plasmid pSK1, including a remarkably high affinity for methotrexate. The small variations in ID50 values for trimethoprim are unlikely to be significant, especially when they are considered in conjunction with the Ki results for trimethoprim. The type S1 dihydrofolate reductase differed from the chromosomal enzyme in S. aureus NCTC 6571 and was also distinct from the enzyme in the trimethoprim-sensitive S. epidermidis strain SK360 (table II).

Discussion

Over half the strains of MRSA isolated in Australia harbour a 20-42-kb plasmid that belongs to a family of structurally-related plasmids which includes pSK1, pSK4, pSK8, pSK9, pSK14, pSK16 and pSK17 (Gillespie et al., 1987b; Lyon et al., 1984a; Lyon and Skurray, unpublished data). In this study, a 0-9-kb fragment located within the 2.75-Kb BgII fragment of pSK1 was used as a dfrA-specific probe. Homologous fragments of 2.75 kb were also present in the BgII digestion profiles of other S. aureus plasmids to which the TpR(H) phenotype had previously been assigned by plasmid elimination and transfer experiments. As expected, those S. aureus plasmids that did not mediate TpR(H) failed to hybridise with the probe DNA. On this basis, isolates of TpR(H) coagulase-negative staphylococci were examined for the presence of DNA sequences homologous to the S. aureus TpR(H) determinant. Three isolates of coagulase-negative staphylococci carried plasmid DNA which hybridised with the probe DNA and, in a fourth strain, the probe bound to DNA which formed a band at the position appropriate to chromosomal DNA. The relatively low DHFR activity associated with this fourth strain suggests that the gene was located on a low copy-number replicon as would be expected for the chromosome. Together, these results suggest that an identical TpR(H) determinant was present among some Australian isolates of S. aureus and coagulase-negative staphylococci and we believe this to be the first indication that the S. aureus dfrA determinant may be present at both plasmid and chromosomal loci in coagulase-negative staphylococci.

The interspecies dissemination of such a TpR(H) determinant may have occurred by plasmid transfer. Archer et al. (1986) showed that conjugative

Table II. Dihydrofolate reductases encoded by plasmids and the chromosome in S. aureus and coagulase-negative staphylococci

<table>
<thead>
<tr>
<th>Source of enzyme</th>
<th>Specific activity*</th>
<th>Mₜ</th>
<th>Tp ID5₀</th>
<th>Mtx ID5₀</th>
<th>TD5₀</th>
<th>DHF Km</th>
<th>Tp Ki</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tp-sensitive strains (chromosomal)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. aureus NCTC 6571</td>
<td>14-8</td>
<td>21 600</td>
<td>0-04</td>
<td>0-0025</td>
<td>&gt;12.0</td>
<td>80-0</td>
<td>0-0065</td>
</tr>
<tr>
<td>S. epidermidis SK360</td>
<td>8-2</td>
<td>22 600</td>
<td>0-065</td>
<td>0-00023</td>
<td>&gt;12.0</td>
<td>4-7</td>
<td>0-0033</td>
</tr>
<tr>
<td>Tp-resistant strains (plasmid)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. aureus pSK1 (type S1)†</td>
<td>39-3</td>
<td>19 700</td>
<td>50-0</td>
<td>0-002</td>
<td>&gt;12.0</td>
<td>10-8</td>
<td>11-6</td>
</tr>
<tr>
<td>S. epidermidis SK76†</td>
<td>27-1</td>
<td>19 300</td>
<td>15-0</td>
<td>0-004</td>
<td>&gt;12.0</td>
<td>6-2</td>
<td>6-3</td>
</tr>
<tr>
<td>S. epidermidis SK47†</td>
<td>51-2</td>
<td>20 200</td>
<td>56-0</td>
<td>0-006</td>
<td>&gt;12.0</td>
<td>4-2</td>
<td>4-5</td>
</tr>
<tr>
<td>S. hominis I SK400†</td>
<td>101-0</td>
<td>19 900</td>
<td>14-8</td>
<td>0-0023</td>
<td>&gt;12.0</td>
<td>12-0</td>
<td>3-8</td>
</tr>
<tr>
<td>S. epidermidis SK683</td>
<td>7-7</td>
<td>19 500</td>
<td>60-0</td>
<td>0-004</td>
<td>&gt;12.0</td>
<td>8-1</td>
<td>9-0</td>
</tr>
</tbody>
</table>

* = nmoles dihydrofolate reduced/min/mg of protein; † = plasmid-mediated enzymes; Mtx = Methotrexate, Tp = Trimethoprim; DHF = Dihydrofolate; TD50 = Time taken for the enzyme to lose half its activity at 45°C; ID50 = Concentration inhibiting 50% of enzyme activity.
plasmids bearing homologous trimethoprim resistance genes could be transferred between *S. aureus* and *S. epidermidis*. In contrast, none of the Australian isolates of Tp'H coagulase-negative staphylococci were able to transfer plasmid-borne Tp'H to *S. aureus* in mixed cultures or by transformation. Furthermore, the pSK1-family of *S. aureus* Tp'H plasmids were all larger than the genotypically-similar plasmids detected in coagulase-negative staphylococci; the structural relationships between the Tp'H plasmids from the different staphylococcal species are currently being investigated. Alternatively, the spread of a single Tp'H determinant among the staphylococci may have occurred by recombination between largely dissimilar plasmids or between plasmid and chromosomal DNA. High-level resistance to trimethoprim has hitherto been described as a resistance determinant (dfrA) encoded for only by plasmids of the pSK1 family in Australian isolates of *S. aureus* (Tennent et al., 1985; Lyon et al., 1986). However, the same determinant appears to be present on plasmids unrelated to the pSK1 family in *S. aureus* isolates from North America (Coughter et al., 1987) and, in this report, we have shown that dfrA may be present on different replicons, including the chromosome, in coagulase-negative staphylococci. These results suggest that this staphylococcal gene can be translocated. Indeed, preliminary data have shown the Tp'H region of pSK1 to be flanked by directly repeated copies of the insertion sequence IS257 (Gillespie et al., 1987a; Lyon and Skurray, 1987).

We have designated the putative trimethoprim resistance transposon Tn4003 (Lyon and Skurray, 1987) and are presently screening DNA from the Tp'H coagulase-negative staphylococci for the presence of IS257 sequences.

The trimethoprim-resistance gene of pSK1 encodes the production of the type S1 dihydrofolate reductase which is quite unlike the plasmid enzymes of gram-negative bacteria (Young and Amyes, 1986; Young et al., 1987). However, the degree of insusceptibility to trimethoprim of the type S1 DHFR is very similar to the type 1 enzyme. The type 1 enzyme accounts for the majority of plasmid-borne trimethoprim resistance in gram-negative organisms. The gene for this enzyme resides on a transposon (Barth et al., 1976) but the level of resistance to trimethoprim may also have some selective advantage. If this is so, the gene encoding the S1 enzyme may be equally successful in gram-positive bacteria.

The presence of common, or closely related, trimethoprim-resistance determinants in *S. aureus* and coagulase-negative staphylococci strongly suggests that interspecies exchange of genetic material has occurred in vivo among Australian isolates of staphylococci. How such transfer occurs, and which species acts as the source of resistance determinants, are unknown. The transfer of antibiotic resistance from coagulase-negative staphylococci to *S. aureus* on skin in vivo (Jaffe et al., 1980; Naidoo and Noble, 1981) suggests that transfer may have occurred in the natural habitat of these organisms under selective pressure of antimicrobial use.

We thank M. Gillespie for useful discussions, L. Messerotti for technical assistance and S. Ferguson for the identification of some of the strains. This work was supported by a Project Grant from the National Health and Medical Research Council of Australia and Wellcome Trust Grant no. 16376/1.5.

**REFERENCES**


TRIMETHOPRIM RESISTANCE IN STAPHYLOCOCCI


