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

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Summary. The molecular and biochemical basis of resistance to high concentrations ($\text{MIC} \geq 1000$ mg/L) of trimethoprim ($\text{Tp}^r_{(\text{H})}$) was examined in Australian isolates of *Staphylococcus aureus* and coagulase-negative staphylococci. The $\text{Tp}^r_{(\text{H})}$ determinant (*dfrA*) was located within a 2.75-Kb *Bgl*II 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 $\text{Tp}^r_{(\text{H})}$ 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 $\text{Tp}^r_{(\text{H})}$ 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 ($\text{Tp}^r_{(\text{H})}$) 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; Town-

send *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

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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.*, 1984b; 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 (Lyon *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 *Bgl*II, *Eco*RI, *Eco*RV, *Hind*III, and *Hpa*II (New England Biolabs, Inc., Beverly, MA, USA), and agarose gel electrophoresis, were performed as previously described (Tennent *et al.*, 1984). *Hind*III 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 *Eco*RI-*Eco*RV DNA fragment, which spans the *dfrA* structural gene, was prepared by electroelution 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 $Tp^{r(H)}$ which has been localised to a 0.75-Kb sequence of DNA contained within the 2.75-Kb *Bgl*II 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 $Tp^{r(H)}$ phenotype although their resistance profiles were similar to those of pSK1 and pSK16 (table I). Digestion of the $Tp^{r(H)}$ plasmid pSK16 with *Bgl*II showed that, like pSK1, it possessed a 2.75-Kb *Bgl*II 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

<i>S. aureus</i> strain	MIC of Tp (mg/L)	Plasmid*	Plasmid size (Kb)	Plasmid resistance pattern
SK 429	> 1000	pSK 1	28.4	Gm Tm Km Ac Eb Qa Tp _(H)
SK 529	1000	pSK 1	28.4	Gm Tm Km Ac Eb Qa Tp _(H)
SK 271	> 1000	pSK 16	35.1	Gm Tm Km Ac Eb Qa Tp _(H) Pc
SK 257	20	pSK 9	25.7	Gm Tm Km Ac Eb Qa
SK 74	20	pSK 14	24.4	Gm Tm Km Ac Eb Qa
SK 553†	20	...	...	...
SK 296‡	20	...	...	...
SK 434§	20	...	...	...
NCTC 6571	1.25	...	...	...

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

* Some strains carry more than one plasmid; only the pSK 1-family plasmid is described.

† SK 553 is a pSK 1-cured derivative of SK 529.

‡ SK 296 is a pSK 16-cured derivative of SK 271.

§ SK 434 is a clinical isolate which lacks a pSK 1-family plasmid.

plasmids pSK 9 and pSK 14 were identical to pSK 1 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 *Bg*II site at 12.05 on the pSK 1 map and thus accounted for the distinct *Bg*II profiles of pSK 9 and pSK 14.

A 0.9-Kb *Eco*RI-*Eco*RV fragment of plasmid pSK 407 (Lyon *et al.*, 1986), which maps between co-ordinates 13.55 and 14.45 on the pSK 1 map (fig. 1) and consists almost entirely of the *dfrA* determinant, was used to probe the *Bg*II digests of the *S. aureus* plasmids. Strong hybridisation signals were

obtained between the probe and the 2.75-Kb *Bg*II fragments of plasmids pSK 1 and pSK 16 (fig. 2B, lanes b and e). Similarly-sized *Bg*II restriction fragments from three other plasmids, pSK 4, pSK 8 and pSK 17 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 pSK 407-derived probe did not hybridise with any fragments of pSK 9 or pSK 14 (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-

Fig. 1. Restriction endonuclease map of the Tp^r_(H) region of pSK 1, together with the corresponding regions of pSK 9 and pSK 14. Also shown is the location of the 5.1-Kb *Eco*RI fragment of pSK 1 cloned in the formation of pSK 407. Areas of pSK 1 absent from pSK 9 and pSK 14 are indicated by dotted lines; the hatched region represents the extent of the 0.9-Kb *Eco*RI-*Eco*RV hybridisation probe derived from pSK 407 (see text). Map co-ordinates are in Kb; restriction endonuclease sites are indicated by Bg (*Bg*II), E (*Eco*RI), Hp (*Hpa*II) and V (*Eco*RV). A complete map of pSK 1 has appeared elsewhere (Lyon *et al.*, 1986).

Fig. 2. A. Agarose gel (1.4% w/v) electrophoresis of purified staphylococcal DNA cleaved with the restriction endonuclease *Bgl*II: Lane (a) lambda phage DNA cleaved with *Hind*III as standards with fragment sizes in Kb shown on the left; (b) pSK1; (c) pSK9; (d) pSK14; (e) pSK16. pSK1 and pSK16 were prepared from laboratory-derived transferrants, whereas pSK9 and pSK14 were isolated from their clinical host strains (table I) which each carry two plasmids. In both cases, the additional plasmid was identified as pSK3 (which does not possess a *Bgl*II site); the ccc and oc forms of pSK3 appear in lanes c and d as intense bands of DNA. B. Autoradiograph of the gel shown in (A) after hybridisation with the 0.9-Kb *Eco*RI-*Eco*RV fragment of pSK407. No homologous sequences were detected in lanes c and d (see text).

Kb *Eco*RI-*Eco*RV DNA fragment of pSK407 was 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 $Tp^{r(H)}$ determinant in these strains, of which six were identified as *S. epidermidis* and two as *S. hominis* type I.

Hybridisation analysis of trimethoprim-resistant coagulase-negative staphylococci

Rapidly-isolated DNA from four of the $Tp^{r(H)}$ 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 *Eco*RI-*Eco*RV fragment from plasmid pSK407 (fig. 3B). Three bands of DNA in strain SK47 (fig.

Fig. 3. A. Agarose gel (1% w/v) electrophoresis of rapidly-isolated DNA from strains of coagulase-negative staphylococci resistant to high levels of trimethoprim (MIC > 1000 mg/L): Lane (a) plasmid standards with sizes in Kb shown on the left; (b) SK47; (c) SK76; (d) SK400; (e) SK683; (f) SK429-*S. aureus* (Lyon *et al.*, 1983). B. Autoradiograph of the gel in A after hybridisation with the $Tp^{r(H)}$ -specific probe.

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 very 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 *Bgl*II fragment of pSK1 was used as a *dfrA*-specific probe. Homologous fragments of 2.75 Kb were also present in the *Bgl*II digestion profiles of other *S. aureus* plasmids to which the $Tp^{(H)}$ phenotype had previously been assigned by plasmid elimination and transfer experiments. As expected, those *S. aureus* plasmids that did not mediate $Tp^{(H)}$ failed to hybridise with the probe DNA. On this basis, isolates of $Tp^{(H)}$ coagulase-negative staphylococci were examined for the presence of DNA sequences homologous to the *S. aureus* $Tp^{(H)}$ 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 $Tp^{(H)}$ 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 $Tp^{(H)}$ 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

Source of enzyme	Specific activity*	M _r	Tp ID50 μM	MtxID50 μM	TD50 min	DHF Km μM	Tp Ki μM
Tp-sensitive strains (chromosomal)							
<i>S. aureus</i> NCTC 6571	14.8	21 600	0.04	0.0025	> 12.0	80.0	0.0065
<i>S. epidermidis</i> SK360	8.2	22 600	0.065	0.00023	> 12.0	4.7	0.0033
Tp-resistant strains (plasmid)							
<i>S. aureus</i> pSK1 (type S1)†	39.3	19 700	50.0	0.002	> 12.0	10.8	11.6
<i>S. epidermidis</i> SK76†	27.1	19 300	15.0	0.004	> 12.0	6.2	6.3
SK47†	51.2	20 200	56.0	0.006	> 12.0	4.2	4.5
<i>S. hominis</i> I SK400†	101.0	19 900	14.8	0.0023	> 12.0	12.0	3.8
(Chromosomal)							
<i>S. epidermidis</i> SK683	7.7	19 500	60.0	0.004	> 12.0	8.1	9.0

* = 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 I 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.

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