Plasmid-mediated trimethoprim-resistance in *Staphylococcus aureus*

Characterization of the first Gram-positive plasmid dihydrofolate reductase (type S1)

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The trimethoprim-resistance gene located on plasmid pSK1, originally identified in a multi-resistant *Staphylococcus aureus* from Australia, encodes the production of a dihydrofolate reductase (type S1), which confers a high degree of resistance to its host and is quite unlike any plasmid-encoded dihydrofolate reductase hitherto described. It has a low M_r (19700) and has a higher specific activity than the constitutive Gram-negative plasmid dihydrofolate reductases. The type S1 enzyme is heat-stable and has a relatively low affinity for the substrate, dihydrofolate (K_m 10.8 μM). It is moderately resistant to trimethoprim, and is competitively inhibited by this drug with an inhibitor-binding constant of 11.6 μM . This is the first identification and characterization of a plasmid-encoded trimethoprim-resistant dihydrofolate reductase derived from a Gram-positive species.

INTRODUCTION

Trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine] prevents the bacterial NADPH-dependent reduction of dihydrofolate to tetrahydrofolate by competitively inhibiting the enzyme dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP⁺ oxidoreductase, EC 1.5.1.3) (Burchall & Hitchings, 1965). Three years after the introduction of trimethoprim, bacterial plasmids, which were able to resist the action of the drug, emerged in Gram-negative bacteria (Fleming *et al.*, 1972). The resistance mechanism, encoded by these plasmids, is the synthesis of an additional trimethoprim-resistant dihydrofolate reductase, which enables the inhibited target of the host bacterium to be by-passed (Amyes & Smith, 1974a).

The dihydrofolate reductases encoded by resistance plasmids (R-plasmids) in Gram-negative bacteria have been subdivided into four major classes. The type I enzymes (M_r 35000) are the most common and consist of two identical subunits (M_r 18000). They are heat-labile and have K_i values for trimethoprim of about 20 μM (Pattishall *et al.*, 1977; Amyes & Smith, 1978; Tennhammar-Ekman & Sköld, 1979). The type II enzymes consist of four subunits, each with an M_r of about 9000 (Smith *et al.*, 1979; Fling & Elwell, 1980). These enzymes are heat-stable and have K_i values for trimethoprim of about 0.15 mM (Tennhammar-Ekman & Sköld, 1979; Amyes & Smith, 1976). The third type of enzyme is monomeric (M_r 16900) and is much more sensitive to trimethoprim (K_i 19 nM) (Fling *et al.*, 1982; Joyner *et al.*, 1984). The type IV enzyme has recently been identified and differs from the other three because it is inducible (Young & Amyes, 1986). It also has a larger M_r (46700) but a low K_i for trimethoprim (63 nM).

Until 1983, high-degree plasmid-mediated trimethoprim-resistance had only been found in Gram-negative

bacteria. Recently, however, some multi-resistant strains of *Staphylococcus aureus*, which have been responsible for an increasing number of hospital infections in Australia, were also found to be trimethoprim-resistant (Lyon *et al.*, 1983, 1984). Some of these trimethoprim-resistant strains possessed a 28.4 kb plasmid encoding resistance to trimethoprim in addition to acriflavine and quaternary ammonium compounds and the aminoglycosides gentamicin, tobramycin and kamamycin (Townsend *et al.*, 1984; Tennent *et al.*, 1985; Lyon *et al.*, 1986; Gillespie *et al.*, 1986). Plasmid pSK1, the prototype of these plasmids, has been found in *Staphylococcus aureus* strains isolated throughout Australia (Lyon *et al.*, 1984). This plasmid was shown to carry a trimethoprim-resistance gene by mixed-culture transfer and curing studies (Tennent *et al.*, 1985; Lyon *et al.*, 1986). Like the type I and type II trimethoprim R-plasmids from Gram-negative bacteria, plasmid pSK1 conferred a high degree of resistance, enabling the host strain to grow in solution containing 800 mg of trimethoprim/l. The trimethoprim-resistance gene of plasmid pSK1, which lies within a 51 kb fragment of the plasmid flanked by two *EcoR*I restriction sites, has been cloned into the plasmid vector pACYC184 to produce the hybrid plasmid pSK407 (Lyon *et al.*, 1986).

In the present study we have characterized the dihydrofolate reductase that was specified by the clinical plasmid pSK1 by examining the enzyme encoded by the hybrid plasmid pSK407. The enzyme, designated type S1, differs both physically and biochemically from all previous plasmid dihydrofolate reductases.

MATERIALS AND METHODS

Bacterial strains and plasmids

Staphylococcus aureus SK2267 (pSK1) is a laboratory-derived strain into which the 28.4 kb *IncI* clinical

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Table 1. Specific activity of dihydrofolate reductase

Bacterial strain	Specific activity of dihydrofolate reductase (nmol of dihydrofolate reduced/min per mg of protein)	
	Grown in the absence of trimethoprim	Grown in the presence of trimethoprim (10 mg/l)
<i>E. coli</i> BHB2600 (pACYC184)	1.7	
<i>E. coli</i> BHB2600 (pSK407)	39.4	33.4
<i>S. aureus</i> N.C.T.C. 6571	14.8	
<i>S. aureus</i> SK2267 (pSK1)	129.6	91.2

plasmid pSK1 (Tennent *et al.*, 1985) was introduced by mixed-culture transfer. The trimethoprim-sensitive *S. aureus* N.C.T.C. 6571 (Amyes & Smith, 1974b) was donated by Pamela M. Waterworth. *Escherichia coli* K-12 strain BHB2600 (F-803 *supE supF hsdR met*) (Hohn, 1979) containing the 4.3 kb tetracycline- and chloramphenicol-resistant cloning vector pACYC184 (Chang & Cohen, 1978) was used as a trimethoprim-sensitive control. Plasmid pSK407 was a hybrid plasmid of pACYC184 containing the 5.1 kb *Eco*R1 restriction fragment of plasmid pSK1 that possessed the trimethoprim-resistance structural gene (Lyon *et al.*, 1986).

Enzyme preparation and assay

For the determinations of specific activity, dihydrofolate reductase was prepared from 1-litre overnight cultures of bacteria grown in Oxoid Isosensitest broth with vigorous agitation at 37 °C as detailed by Young & Amyes (1986). The cells were harvested by centrifugation (6000 *g* for 10 min at 15 °C) and resuspended in buffer A (50 mM-sodium phosphate buffer, pH 7.4, containing 10 mM-2-mercaptoethanol and 1 mM-EDTA). The bacteria were disrupted by sonication, and the cell debris was removed by centrifugation (40000 *g* for 1 h at 4 °C). Dihydrofolate reductase activity was assayed at 30 °C in 40 mM-sodium phosphate buffer, pH 6, as described previously (Amyes & Smith, 1974a). One unit of dihydrofolate reductase activity is defined as the amount of enzyme required to reduce 1 nmol of dihydrofolate/min. Protein concentrations were determined by the method of Waddell (1956). For enzyme purification, 5-litre cultures were prepared as above. (NH₄)₂SO₄ was added to 50% saturation. After centrifugation (14000 *g* for 30 min at 4 °C), further (NH₄)₂SO₄ was added to the supernatant to give 80% saturation, and, after further centrifugation, the pellet was dialysed against buffer A. It was applied to a Sephadex G-75 column (2 cm² × 90 cm) previously equilibrated with buffer A and eluted with the same buffer (Young & Amyes, 1986). The *M_r* was determined by Sephadex exclusion chromatography as described previously (Amyes & Smith, 1974a). Heat-sensitivity, enzyme kinetics and inhibitor studies were performed as previously described (Amyes & Smith, 1976).

RESULTS

S. aureus SK2267 (pSK1) from *E. coli* BHB2600 (pSK407) were grown both in the presence and in the absence of trimethoprim (10 mg/l). *S. aureus* N.C.T.C. 6571 and *E. coli* BHB2600 (pACYC184) were cultured solely in the absence of the drug. The specific activities

of dihydrofolate reductase in the cleared lysates are shown in Table 1. The control *E. coli* strain BHB2600 (pACYC184), which lacked any trimethoprim-resistance gene, had a dihydrofolate reductase specific activity of 1.7 nmol of dihydrofolate reduced/min per mg of protein. On the other hand, the specific activity of the control trimethoprim-sensitive *S. aureus* strain was about 7 times greater. The presence of the trimethoprim-resistance gene in *S. aureus* considerably increased the specific activity of dihydrofolate reductase. A similar increase was found in the *E. coli* strain harbouring the hybrid trimethoprim-resistance plasmid pSK407. In all cases, the production of dihydrofolate reductase was constitutive, and no significant increase in activity was observed when the strains were grown in the presence of trimethoprim. Since the chromosomal dihydrofolate reductase activity of *S. aureus* was high and could affect the purification of the plasmid enzyme, the dihydrofolate reductase of the hybrid plasmid, pSK407, was purified and its properties were compared with those of the

Fig. 1. Sephadex G-75 gel filtration of *E. coli* dihydrofolate reductase

●, Dihydrofolate reductase activity obtained after Sephadex exclusion chromatography of a 50–80%-satn.-(NH₄)₂SO₄ precipitate from *E. coli* BHB2600 (pSK407). ○, Elution of standard marker proteins (*A₂₈₀*): A, ovalbumin (*M_r* 45 500); B, chymotrypsinogen (*M_r* 25 000); C, cytochrome *c* (*M_r* 12 384).

chromosomal enzyme derived from *S. aureus* N.C.T.C. 6571.

Dihydrofolate reductase activity was purified from *E. coli* BHB2600 (pSK407) grown in the presence of 10 mg of trimethoprim/l and from *S. aureus* N.C.T.C. 6571 grown in the absence of the drug. Both preparations gave a single peak of dihydrofolate reductase activity after Sephadex gel filtration. The plasmid enzyme peak is shown in Fig. 1. In an attempt to distinguish the plasmid enzyme from the *E. coli* host dihydrofolate reductase, the assays were repeated in the presence of 4 μ M-trimethoprim, a concentration sufficient to eliminate chromosomal dihydrofolate reductase activity. The peak was decreased slightly, but the maximum enzyme activity was still found at the same position (results not shown). When the peak from *S. aureus* N.C.T.C. 6571 was assayed in the same concentration of trimethoprim, no enzyme activity could be seen. When the gel filtrations were repeated in the presence of standard proteins, the peak of activity for the plasmid dihydrofolate reductase corresponded to an M_r of 19700 (Fig. 1), whereas the enzyme peak for *S. aureus* N.C.T.C. 6571 corresponded to 21600. The failure to detect a second peak of activity identifiable as the host enzyme in the *E. coli* BHB2600 (pSK407) preparation was probably due to the large amount of plasmid-determined enzyme present and its similar size to the *E. coli* enzyme (Amyes & Smith, 1974a).

The peak fraction of the plasmid enzyme (59) had a specific activity of 815.0, which represented a greater than 20-fold purification of the crude extract, and the peak fraction of the *S. aureus* chromosomal enzyme (57) had a specific activity of 484.7, which represented a greater than 30-fold purification. These two fractions were used in the further characterization of the enzymes. The plasmid enzyme was heat-stable, retaining more than 70% of its activity after maintenance for 12 min at 45 °C, as was the chromosomal enzyme, which retained 100% of its activity under the same conditions (Table 2).

The activities of both enzymes were assayed in the presence of increasing concentrations of trimethoprim and methotrexate in order to determine the concentration of each antifolate required to give 50% inhibition (ID_{50}). The ID_{50} of trimethoprim for the plasmid enzyme was 50 μ M and that of methotrexate was 2 nM. In both cases the enzyme activity was inversely proportional to the logarithm of the inhibitor concentration. The corresponding values for the *S. aureus* chromosomal enzyme were 40 nM for trimethoprim and 2.5 nM for methotrexate (Table 2).

The activity of the peak fractions was investigated under conditions of partial saturation with dihydrofolate

Fig. 2. Lineweaver-Burk plots of the partially purified plasmid dihydrofolate reductase

The reciprocal of the dihydrofolate concentration ($1/[S]$) is plotted against the reciprocal of the rate of dihydrofolate reductase activity ($1/v$) in the presence and in the absence of trimethoprim. $\times$, No trimethoprim; O , 20 μ M-trimethoprim; $\bullet$, 50 μ M-trimethoprim.

and full saturation with NADPH (50 mM). In control studies we have found that concentrations of NADPH above 10 mM do not limit the rate of either of these enzymes. The results, analysed by the method of Lineweaver & Burk, show that the K_m value for dihydrofolate of the plasmid enzyme (Fig. 2) was 10.8 μ M. This is similar to the type I, type II and type IV Gram-negative plasmid dihydrofolate reductases but is about 25-fold greater than the value obtained with the type III enzyme (Amyes, 1986). The *S. aureus* chromosomal enzyme had a higher K_m for dihydrofolate than those of the new plasmid enzyme and the *E. coli* chromosomal dihydrofolate reductase.

When the assays were repeated in the presence of trimethoprim it was found that the maximum velocity of the plasmid dihydrofolate reductase remained the same (Fig. 2), indicating that the drug causes competitive inhibition of the enzyme. The K_i for trimethoprim was 11.6 μ M, similar to that reported for the type I enzyme (Pattishall *et al.*, 1977). Trimethoprim also competitively inhibited the *S. aureus* chromosomal dihydrofolate reductase, with a K_i of 6.5 nM, similar to that of the *E. coli* host enzyme (Amyes & Smith, 1976).

Table 2. Comparison of dihydrofolate reductases originally encoded by plasmid pSK1 and the chromosome of *S. aureus* N.C.T.C. 6571

Abbreviations: Mtx, methotrexate; Tp, trimethoprim; DHF, dihydrofolate; TD_{50} , time taken for the enzyme to lose half its activity at 45 °C.

Enzyme (original source)	M_r	Tp ID_{50} (μ M)	Mtx ID_{50} (μ M)	TD_{50} (min)	DHF K_m (μ M)	Tp K_i (μ M)
Type S1 (pSK1)	19700	50.0	0.002	> 12.0	10.8	11.6
<i>S. aureus</i> (N.C.T.C. 6571)	21600	0.04	0.0025	> 12.0	80.0	0.0065

DISCUSSION

The properties of the *S. aureus* plasmid dihydrofolate reductase characterized here show marked differences from the four types of Gram-negative plasmid-determined enzymes (for review see Amyes, 1986). This is the first plasmid dihydrofolate reductase from staphylococci to be identified and characterized, and we therefore describe it as the type S1.

The M_r of the type S1 enzyme is similar to that of the *S. aureus* chromosomal enzyme. However, the other properties of the S1 dihydrofolate reductase suggest that it is not related to the chromosomal enzyme. In addition, genetic probing experiments with the trimethoprim-resistance gene from pSK1 failed to show homology with the bacterial chromosome of sensitive staphylococci (L. Loo & R. A. Skurray, unpublished work).

The structural gene encoding the type S1 dihydrofolate reductase has been shown, by transposon mutagenesis of the cloned gene in the hybrid plasmid pSK407, to be a maximum of 0.75 kb and a minimum of 0.55 kb. This gene could be predicted to encode a protein with an M_r between about 20000 and 27000 (Lyon *et al.*, 1986). The actual size of the plasmid enzyme is very close to the minimum predicted from the size of the structural gene, and this fact suggests that the whole of the dihydrofolate reductase is encoded by the gene and that it is not made up of identical subunits, as are the type I and type II Gram-negative plasmid enzymes. In this one respect, the S1 dihydrofolate reductase is like the type III plasmid enzyme.

In its physical properties, the S1 enzyme is most similar to the type I Gram-negative plasmid dihydrofolate reductase and, more especially, the smaller type Ib variant (Young & Amyes, 1985). It has an equivalent K_m value for dihydrofolate and K_i for trimethoprim. It also has approximately the same ID_{50} value for trimethoprim, but surprisingly has the same ID_{50} value for methotrexate as the *S. aureus* chromosomal enzyme. The type I enzyme is produced constitutively in amounts up to 10 times that of the *E. coli* chromosomal enzyme. Similarly, the S1 enzyme is produced in the same order of magnitude relative to the *S. aureus* chromosomal enzyme. However, the enzymes are probably not directly related to each other. They have very different responses to heat treatment, and, unlike the type I enzyme, the S1 dihydrofolate reductase does not seem to be made up of identical subunits. In addition, an *S. aureus* plasmid-encoded trimethoprim-resistance gene, which hybridized with the Australian trimethoprim-resistance gene, showed no homology with the type I plasmid dihydrofolate reductase structural gene (Archer *et al.*, 1986).

The type I plasmid gene is by far the most widespread plasmid dihydrofolate reductase structural gene in Gram-negative bacteria. In many respects, the biochemical properties of the S1 gene product are similar to the type I gene product, and, although they are not directly

related to one another, the two genes do seem to have evolved convergently to produce dihydrofolate reductases with similar trimethoprim-resistance characteristics. Future studies will show if the type S1 dihydrofolate reductase gene shares the same promiscuity as the type I Gram-negative structural gene.

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