Volume 266, number 1,2, 159-162 FEBS 08524 June 1990

Identical genes for trimethoprim-resistant dihydrofolate reductase from *Staphylococcus aureus* in Australia and Central Europe

Alexander Burdeska, Michael Ott, Willi Bannwarth and Rudolf L. Then

Pharmaceutical Research Department, F. Hoffmann-La Roche Ltd., CH-4002 Basel, Switzerland

Received 14 March 1990

The nucleotide sequence of a 1.25 kb *Bgl*II/*Eco*RI fragment from the 34 kb trimethoprim (Tp)-resistant plasmid pABU1 of *Staphylococcus aureus* 157/4696, isolated in Zürich, was determined. It contained the entire Tp-resistant dihydrofolate reductase gene, 197 bp of the thymidylate synthetase, 395 bp of a truncated gene and 111 bp of IS257R1. With the exception of one single base pair at position 862 the sequence of the whole fragment was identical to nucleotides 1633 to 2885 of the Tp-resistant transposon Tn*4003* in plasmid pSK1 from an Australian *S. aureus* isolate. This suggests the worldwide dissemination of Tn*4003* in multiresistant *Staphylococci*.

Trimethoprim resistance; Dihydrofolate reductase; *Staphylococcus aureus*; Transposon Tn*4003*

1. INTRODUCTION

Multiresistant staphylococci, which in addition to their methicillin resistance carry resistances to other antibiotics such as gentamicin, erythromycin or tetracycline, cause serious therapeutic problems [1]. Cotrimoxazole is an effective alternative in the treatment of infections by multiresistant staphylococci, but resistance to Tp, one component of cotrimoxazole, has been reported since the early 1980s [2]. The first plasmid-encoded Tp-resistant DHFR has recently been described by Young et al. [3]. This DHFR was isolated from a multiresistant *Staphylococcus aureus* from Australia. We recently found that a similar or identical enzyme was present in a multiresistant *Staphylococcus aureus* from Central Europe [4]. Tp-resistance in this strain was transferable and resided on a 28 kb plasmid [1]. In this study, we cloned the gene into pUC18, expressed it in *Escherichia coli* and determined its nucleotide sequence.

Correspondence address: A. Burdeska, F. Hoffmann-La Roche Ltd., Pharmaceutical Research, Building 70.211, CH-4002 Basel, Switzerland

Abbreviations: Pc, penicillin G; Cd, cadmium; Cm, chloramphenicol; Ap, ampicillin; MTX, methotrexate; DHFR, dihydrofolate reductase; Tp, trimethoprim; TS, thymidylate synthetase; ORF, open reading frame

The nucleotide sequence(s) presented here has (have) been submitted to the EMBL/GenBank database under the accession number no. Y07536

2. EXPERIMENTAL

2.1. *Bacterial strains and plasmids*

S.aureus 157/4696 possessing plasmid pABU1 (34 kb, Tp^r, Pc^r, Cd^r) and *S.aureus* ATCC 25923 were recently described [1,4]. The host strains employed were *S.aureus* 113 (NCTC 8325 r-m+) [5] and *E.coli* M15 (C600-r-Δ*lac*M15) [6]. Plasmid pABU1a (3.5 kb, Cm^r) also resides in *S.aureus* 157/4696. Plasmid pABU1/3 (Ap^r,Tp^r) is pUC18, containing a 1.4 kb-*Eco*RI/*Hin*dIII fragment of pABU1 and plasmid pABU1/1 (Ap^r, Tp^r) is pUC18, containing a 2.1 kb *Eco*RI-fragment of pABU1.

2.2. *Cloning vectors and restriction enzymes*

The cloning vectors pUC18, pUC19 as well as bacteriophage M13mp18RF, M13mp19RF [7] and all restriction enzymes were supplied by Boehringer Mannheim, Switzerland.

2.3. *General methods*

Isolation of plasmids from *S.aureus* 157/4696 was carried out by the alkaline lysis method of Bennett et al. [8] by disrupting the cell wall with Lysostaphin (Sigma) and Lysozyme (Pharmacia). Protoplast transformation of *S.aureus* 113 was performed by the method of Götz et al. [9]. Cloning procedures and the use of restriction enzymes followed published procedures [10].

The DNA nucleotide sequence was determined with the aid of bacteriophage M13 by the chain termination method of Sanger et al. [11]. Fragments were sequenced in two ways: (i) with ^{35}S-dATP and the USB Sequenase Kit Version 2.0 and (ii) with the automated sequencer 370A and Taq polymerase (both Applied Biosystems Inc.). Purification of DHFR, determination of activity, and kinetic parameters were performed as previously described [4]. One unit of DHFR activity was taken as the conversion of 1 μmol of dihydrofolate per min.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

Table I
Sensitivity of different DHFRs towards Tp and MTX

DHFR source	IC_{50} Tp (μM)	IC_{50} MTX (nM)
S.aureus 157/4696 (pABU 1)[a]	14	3.0
S.aureus 113 (recipient, chromosomal)[a]	0.08	1.4
S.aureus 113 (pABU 1), transformant[a]	12	3.0
S.aureus 157/4696 (chromosomal)[b]	0.04	1.1
E.coli M15 (recipient, chromosomal)[a]	0.01	4.2
E.coli M15 (pABU1/3)[a/b]	15/19	−/1.7

[a] Determined in crude extract
[b] Partially purified enzyme

3. RESULTS

3.1. *Plasmid isolation and transfer*

S.aureus 157/4696 possesses two plasmids, the 28 kb pABU1 (Tp^r,Pc^r,Cd^r) and the 3.5 kb (pABU1a (Cm^r). Both plasmids could be transferred to *S.aureus* 113 via protoplast transformation and selection on DM3 agar containing TP (10 mg/l) and Cm (16 mg/l), respectively. The original strain *S.aureus* 157/4696 and all transformants exhibited high level resistance to Tp (minimal inhibitory concentration >1024 mg/l).

3.2. *Cloning and expression of the DHFR from* S. aureus *157/4696 in* E.coli *M15*

A 2.1 kb *Eco*RI- and a 1.4 kb *Eco*RI/*Hin*dIII fragment of pABU1 with the intact DHFR-gene (see Fig. 1) were cloned into the cloning vector pUC18 used to transform *E.coli* and the Tp-resistant clones were selected on Mueller-Hinton agar (Difco) containing Tp (10 mg/l) + Ap (50 mg/l). Specific activity of DHFR, determined in crude extracts (corrected for NADPH oxidase activity) were 194 mU/mg protein for *S.aureus* 157/4696, 50 mU/mg protein and 49 mU/mg protein for the Tp-sensitive strains *S.aureus* 113 and *S.aureus* ATCC 25923 respectively, 206 mU/mg protein for *S.aureus* 113 (pABU1), 3.6 mU/mg protein for *E.coli* M15 and 872 mU/mg protein for *E.coli* M15 (pABU1/3). Concentrations of Tp and MTX which inhibited the enzyme activity by 50% (IC_{50}), were compared for several bacterial strains producing these different DHFRs and are shown in Table I.

The sensitivity towards Tp of the plasmid-coded DHFR is in the μM-range, as previously described [4], although this enzyme is highly sensitive to MTX (IC_{50} = 3 nM).

The 2.1 kb *Eco*RI fragment was characterized more precisely by the use of additional restriction endonucleases and the location of the gene for Tp-resistance was defined by subcloning analysis (Fig. 1).

3.3. *Nucleotide sequence of the Tp^r-gene*

Two portions of the *Eco*RI-fragment from pABU1 were selected for determination of the nucleotide sequence; the 0.8 kb *Bgl*II/*Hin*dIII-fragment and the 1.25 kb *Bgl*II/*Eco*RI-fragment shown in Fig. 1. Both fragments were first subcloned into pUC18 and in a second step into M13mp18RF/M13mp19RF. The latter contains the entire gene of the Tp-resistant DHFR while the former contains only a part of it and did not produce Tp^r *E.coli* clones. The sequence of the whole 1.25 kb *Bgl*II/*Eco*RI fragment is shown in Fig. 2. Two open reading frames (ORF) were recognized on the same strand. The first ORF which starts at nucleotide 243 and has its stop codon at position 726 could be identified as the ORF for the DHFR gene due to the internal *Hin*dIII site. That this *Hin*dIII site interrupts the DHFR gene is known from the subcloning analysis. The second ORF starts at nucleotide 738 and has its stop codon at position 1158. A third putative ORF lies upstream of these two and has its stop codon at position 196. The start codon of this putative ORF must lie upstream of the *Eco*RI site which limits the *Bgl*II/EcoRI fragment.

The first 35 N-terminal amino acid residues of the chromosomal DHFR from *S.aureus* 157/4696 have recently been determined [4]. They were compared to the sequence of the DHFR from plasmid pABU1 as well

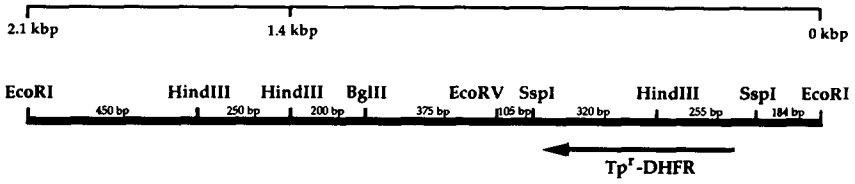

Fig. 1. Restriction map of the 2.1 kb *Eco*RI-fragment containing the Tp^r-determinant.

```
      ---► TS
   1  GAATTCATTC ATACATTTGG AGATGCACAT ATTTATTCCA ATCATATGGA
  51  TGCAATACAT ACACAATTAT CAAGAGATAG TTATCTACCA CCTCAATTAA
 101  AAATTAATAC AGATAAATCA ATTTTTGATA TTAATTATGA AGATTTAGAA
                                                 TS xxx
 151  TTGATAAATT ATGAATCACA CCCTGCTATT AAAGCACCTA TTGCGGTATA
                                                 O►DHFR
 201  AAAAGATGCA TTAATATTTC GCATACAGAA AGGAGGTATA CCATGACATT
 251  ATCAATAATT GTCGCTCACG ATAAACAAAG AGTCATTGGG TACCAAAATC
 301  AATTACCTTG GCACTTACCA AATGATTTAA AGCATATTAA ACAACTGACC
 351  ACTGGGAATA CACTTGTAAT GGCACGGAAA ACTTTTAATT CTATAGGGAA
 401  GCCATTGCCA AATAGACGTA ACGTCGTACT CACTAACCAA GCTTCATTTC
 451  ACCATGAAGG GGTAGATGTT ATAAACTCTC TTGATGAAAT TAAAGAGTTA
 501  TCTGGTCATG TTTTTATATT TGGAGGACAA ACGTTATACG AAGCAATGAT
 551  TGACCAGGTA GATGATATGT ATATCACAGT AATAGATGGA AAGTTTCAAG
 601  GAGACACATT CTTTCCACCA TACACATTCG AAAACTGGGA AGTCGAATCT
 651  TCAGTAGAAG GTCAACTAGA TGAAAAAAAT ACTATACCGC ATACATTCTT
                          DHFR xxx          O►ORF140
 701  ACATTTAGTG CGTAGAAAAG GGAAATAGGA GGCAATTATG GCTAAACAAA
 751  TTATCGTCAC GGATTCAACC TCTGATTTAT CACATGAATA TTTAAAACAA
 801  CATAACATTC ATGTTATACC ATTAAGCCTG ACAATCGACG GGAAATCTTA
 851  CACTGATCAA GTTGATATCT CTTCAAGTGA GTATATCGAT CATATTGAAA
 901  ATGATGCAGA CGTCAAAACA AGTCAACCAC CTATTGGTCG ATTTATTGAA
 951  ACATATGAGC AATTAGCTCA AGATGACGTT GAAATTATAA GTATTCATCT
1001  TTCGTCAGGC TTAAGTGGTA CTTATAATAC TGCTGTTCAA GCGAGCCATA
1051  TGGTAGATGG TAATATTACA GTGATTGATT CTAAATCTAT TTCGTTTGGG
                                        O►IS257RI
1101  TTAGGTTATC AAATTAAGCA AATTGTTGAA TTGGTTCTGT TGCAAAGTAA
       ORF140xxx
1151  AAAAATATAG CTAACCACTA ATTTATCATG TCAGTGTTCG CTTAACTTGC
1201  TAGCATGATG CTAATTTCGT GGCATGGCGA AAATCCGTAG ATC
```

Fig. 2. Nucleotide sequence of the 1.25 *Bgl*II/*Eco*RI fragment from pABU1. Arrows with circle indicate start, and xxx stop of ORF.

as to several other bacterial DHFRs, in order to evaluate homologies in the N-terminal region (Fig. 3). The highest degree of homology was observed between the *S.aureus* chromosomal DHFR and the DHFR from pABU1.

4. DISCUSSION

A computer search for homology [17] of the 1.25 kb nucleotide sequence with known DNA nucleotide sequences revealed a surprisingly high similarity with part of the Tp-resistance transposon Tn*4003* from the staphylococcal multiresistance plasmid pSK1, which has recently been published [12]. The 1.25 kb *Bgl*II/*Eco*RI fragment spans from nucleotide 1633 to 2885 of Tn*4003*. Upstream of the Tp-resistance gene it contains 197 basepairs of the 3′-end of the TS gene and downstream 395 basepairs of the 5′-end of the ORF140 as well as 111 basepairs of IS257R1. Only one single base pair exchange (transition of C into T at position 862 in the 1.25 kb fragment, corresponding to position 2494 of Tn*4003*) within the truncated ORF140 was found. Thus the genes for Tp-resistant DHFR from a *Staphylococcus* from Australia and another one from Central Europe (strain 157/4696 was isolated in Zürich, Switzerland) are identical, as well as the adjacent sequences of the gene for TS and IS257RI as far as determined. The previously discussed differences in some enzymatic parameters between the SI and the enzyme from *S.aureus* 157/4696 are therefore due to experimental variation and not to differences in the protein [4]. This leads to the assumption that transposon Tn*4003* has spread worldwide, since a DNA probe of the S1 DHFR from plasmid pSK1 also hybridized to DNA from strains from North America (S.G.B. Amyes, and S. Tait, personal communication). This highly conserved sequence of the Tp-resistant staphylococcal DHFR is surprising when compared to the multitude of Tp-resistant DHFRs found in Gram-negative bacteria [18], where even among the same type sequence differences are found. The type II enzymes from plasmids R388 and R67 e.g. differ in 17 out of 78 amino acids [19].

	10 20 30	Homology (%)
S.aureus 157/4696 (pABU1)	MTLSIIVAHDKQRVIGYQNQLPWHLPNDLKHIKQLT	-
S.aureus (pSK1)	MTLSIIVAHDKQRVIGYQNQLPWHLPNDLKHIKQLT	100
S.aureus (chromosomal)	TLSILVAHDLQRVIGFENQLPWHLPNDLKHVKKLS	80
E.coli K12 (chromosomal)	MISLIAALAVDRVIGMENAMPWNLPADLAWFKRNT	46
E.coli (Tn7)	VKLSLMVAISKNGVIGNGPDIPWSAKGEQLLFKAIT	34
L.casei	TAFLWAQNRDGLIGKDGHLPWHLPDDLHYFRAQT	37
S.faecium	MFISMWAQDKNGLIGKDGLLPWRLPNDMRFFREHT	37

Fig. 3. Similarities in the amino acid sequence at the N-terminus (first 35 residues) of several bacterial DHFRs. Sequence references are: *S.aureus* (pSK1) [12], *S.aureus* 157/4696 chromosomal DHFR [4], *E.coli* K12 chromosomal DHFR [13], *E.coli* type I DHFR (Tn7) [14], *L.casei* DHFR [15], *S.faecium* [16].

The question of the origin of the Tp-resistant staphylococcal DHFR is highly interesting. The comparison carried out by Rouch et al. [12] led them to suggest a chromosomal gene of a Gram-positive bacterium such as *Bacillus* as ancestor. The comparison of the staphylococcal S1 enzyme with other DHFRs gave 33% overall amino acid homology with the enzymes from *B.subtilis*, *L.casei* and *S.faecium* and slightly less for *E.coli*. However, as shown in Fig. 3, 80% homology is found between the chromosomal *S.aureus* DHFR and the plasmid derived enzyme from Tn*4003* or from plasmid pABU1 although only 35 amino acids of the chromosomal staphylococcal enzyme have as yet been determined [4]. Six of the seven amino acids which differ between this enzyme and the Tn*4003* coded DHFR can be explained by a single base pair exchange. Unless the full sequence of the chromosomal *S.aureus* DHFR proves to possess a much lesser degree of overall homology than the 35 residues at the amino terminus, we assume that this enzyme derives from the Tp-sensitive chromosomal *S.aureus* DHFR. Further support for this assumption is the possibility of selecting Tp-resistant *S.aureus* by serial passages over increasing concentrations of Tp. The mutant strains obtained harboured DHFRs with reduced sensitivity towards Tp, but nearly unchanged sensitivity towards MTX, showing behaviour very similar to that of the S1 enzyme (unpublished results). Once such a mutation has occurred under selection pressure of Tp, the mutated gene could have transposed from the chromosome to a plasmid with the aid of IS257 elements. To resolve this question, the full sequence of the chromosomal DHFR gene from *S.aureus* will have to be determined. The failure to cross-hybridize type S1 DHFR DNA with *S.aureus* chromosomal DNA [20] may be due to a lack of specificity of the probe used. We are presently employing a 0.58 kb *Ssp*I fragment, which includes the whole gene for the DHFR, as a probe, in order to collect more information on the dissemination of the Tp-resistance gene among staphylococci and preliminary experiments indicate hybridization of this probe with the chromosomal DNA of *S.aureus* ATCC 25923.

Acknowledgements: We wish to thank D. Doran, H. Gmünder, A. Dorn and J. Meyer for stimulating discussions, F. Hermann, P. Caspers, U. Certa for experimental help and P. Hartman and J. Nielsen for help during preparation of the manuscript.

REFERENCES

[1] Burdeska, A. and Then, R.L. (1990) J. Med. Microbiol. 31, 11-14.
[2] Lyon, B.R., May, J.W. and Skurray, R.A. (1983) Antimicrob. Agents Chemother. 23, 817-826.
[3] Young, H.K., Skurray, R.A. and Amyes, S.G.B. (1987) Biochem. J. 243, 309-312.
[4] Hartman, P.G., Stähli, M., Kocher, H.P. and Then, R.L. (1988) FEBS Lett. 242, 157-160.
[5] Iordanescu, S. and Surdeanu, M. (1976) J. Gen. Microbiol. 96, 277-281.
[6] Stüber, D., Ibrahimi, I., Cutler, C., Dobberstein, B. and Bujard, H. (1984) EMBO J. 3, 3143-3148.
[7] Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Gene 33, 109-119.
[8] Bennett, P.M., Heritage, J. and Hawkey, P.M. (1986) J. Antimicrob. Chemother. 18, 421-424.
[9] Götz, G., Kreutz, B. and Schleifer, K.A. (1983) Mol. Gen. Genet. 189, 340-342.
[10] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[11] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467.
[12] Rouch, D.A., Messerotti, L.J., Loo, L.S.L., Jackson, C.A. and Skurray, R.A. (1989) Mol. Microbiol. 3, 161-175.
[13] Smith, D.R. and Calvo, J.M. (1980) Nucleic Acids Res. 8, 2255-2274.
[14] Fling, M.E. and Richards, C. (1983) Nucleic Acids Res. 11, 5147-5158.
[15] Freisheim, J.H., Bitar, K.G., Reddy, A.V. and Blankenship, D. (1978) J. Biol. Chem. 253, 6437-6444.
[16] Gleisner, J.M., Petersen, D.L. and Blakley, R.L. (1974) Proc. Natl. Acad. Sci. USA 71, 3001-3005.
[17] Devereux, J., Haeberli, P. and Smithies, O. (1984) Nucleic Acids Res. 12, 387-395.
[18] Huovinen, P. (1987) Antimicrob. Agents Chemother. 31, 1451-1456.
[19] Freisheim, J.H., and Matthews, D.A. (1984) in: Folate Antagonists as Therapeutic Agents, Vol. 1 (Sirotnak, F.M., Burchall, J.J., Ensminger, W.B. and Montgomery, J.A., eds). Academic Press, pp. 83, Academic Press, New York.
[20] Gillespie, M.T., Lyon, B.R., Loo, L.S.L., Matthews, P.R., Stewart, P.R. and Skurray, R.A. (1987) FEMS Microbiol. Lett. 43, 165-171.