

N-terminal amino-acid sequence and subunit structure of the type IV trimethoprim-resistant plasmid-encoded dihydrofolate reductase

C. J. THOMSON, HILARY-KAY YOUNG* and S. G. B. AMYEST†

Department of Bacteriology, The Medical School, University of Edinburgh, Teviot Place, Edinburgh EH8 9AG and *Department of Biological Sciences, University of Dundee, Dundee DD1 4HN

Summary. The type IV plasmid-mediated dihydrofolate reductase (DHFR), from a clinical strain of *Escherichia coli* isolated in South India, was prepared from a transconjugant containing the original clinical plasmid, *E. coli* J62-2 (pUK1123), and from *E. coli* C600 (pUK1150) containing a 2.6-kb *Hind*III fragment of pUK1123 cloned into plasmid pBR322. Both preparations were purified by methotrexate affinity chromatography. Automatic amino-acid sequencing of the N-terminal of the purified type IV enzyme from both sources gave an identical sequence which was clearly distinct from other plasmid-mediated trimethoprim-resistant DHFRs. The type IV DHFR showed most homology with the endogenous, chromosomally-encoded *E. coli* enzyme. Amino-acid sequence analysis also showed that the type IV enzyme preparation from *E. coli* J62-2 harbouring the original clinical plasmid, pUK1123, also contained the *E. coli* DNA-binding protein NS1. Analysis by polyacrylamide gel electrophoresis suggested that the type IV enzyme, in its native form, consists of a DHFR of M_r 33 000 coupled to a DNA-binding protein.

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) (DHFR).¹ Shortly after its introduction in 1968, plasmids which encoded resistance to trimethoprim (Tp) were identified in gram-negative bacteria.² The mechanism of resistance was subsequently identified as the production of an additional trimethoprim-resistant DHFR which enabled the blocked endogenous chromosomally-encoded enzyme to be bypassed.³

The DHFRs encoded by resistance plasmids (R-plasmids) in gram-negative bacteria have been divided into seven major types based on their biochemical properties.⁴ Some of the genes responsible have also been sequenced.^{5–10} Initially, plasmid-mediated trimethoprim resistance was dominated by the spread of two genes encoding DHFR types I and II. However, since 1982, a

number of new enzymes have been identified, the most unusual of these being the type IV DHFR.

Bacteria harbouring plasmids encoding the type IV plasmid-encoded DHFR were found in South India in 1984.¹¹ This enzyme confers low-level resistance on the host bacterium so may be a potential intermediate between a sensitive enzyme and the highly-resistant plasmid-mediated DHFR. The enzyme differs from the six other types identified in several ways: (1) it is an inducible enzyme, which is rare for a plasmid-encoded resistance enzyme in gram-negative bacteria; (2) it has a large M_r (46 700); and (3) it is only partially resistant to trimethoprim (ID_{50} Tp = 0.2 μ M).¹¹ Examination of the induction mechanism of the type IV enzyme and its response to challenge with trimethoprim has suggested that this resistance mechanism is more similar to a resistance mechanism sometimes employed by the bacterial chromosome, namely the production of a mutant enzyme which is moderately resistant to trimethoprim and is hyperproduced, thus swamping the available drug, with sufficient activity remaining to continue the bacterial reduction of dihydrofolate to the active tetrahydrofolate form.^{12,13}

It is important to establish how the type IV

Received 27 Oct. 1989; accepted 5 Dec. 1989.

†Correspondence should be sent to Dr S. G. B. Amyes.

enzyme relates to the other plasmid-mediated DHFRs. We report the use of automatic amino-acid sequencing to give a rapid partial sequence sufficient to determine evolutionary relationships among trimethoprim-resistant DHFRs.

Materials and methods

Bacterial strains and plasmids

The type IV DHFR was prepared for sequence analysis from two bacterial strains: (1) *Escherichia coli* J62-2 (pUK1123), an *E. coli* transconjugant containing the original clinical trimethoprim R-plasmid from a clinical strain of *E. coli* isolated at the Christian Medical College Hospital, Vellore, South India in 1984;¹¹ (2) *E. coli* C600 (pUK1150), an *E. coli* clone containing plasmid pBR322 with a 2.6-kb *Hind*III fragment of pUK1123 encoding the type IV DHFR gene.¹⁴

Dihydrofolate reductase preparation

DHFR was prepared from 10-L overnight cultures in Iso-Sensitest Broth (Oxoid) grown at 37°C and shaken vigorously.¹¹ Bacteria were harvested by centrifugation at 6000 *g* for 15 min 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 (2 × 30 s, 8 µm; MSE Soniprep) and the lysate was cleared by centrifugation at 40 000 *g* for 1 h at 4°C. DHFR activity was assayed at 37°C in 40 mM sodium phosphate buffer, pH 6.0.³

Enzyme purification and sequence analysis

DHFR was precipitated from the crude preparation by the addition of ammonium sulphate to 50–80% saturation. The enzyme was resuspended in buffer A and eluted from a Sephadex G-75 gel filtration column (2 cm² × 90 cm). The fractions showing peak activity were pooled and then applied to a column (0.375 cm² × 20 cm) containing methotrexate-agarose. This column was washed with buffer A until the absorbance at 280 nm was less than 0.01 OD units and all the unbound protein had been removed. The DHFR was then eluted with 2 ml of 0.5 M K₂HPO₄, containing 4 µmol dihydrofolate, followed by 75 ml of 0.1 M K₂HPO₄ containing 1 µM dihydrofolate;¹⁵ 5-ml fractions were collected. The three eluted fractions showing peak activity were pooled and concentrated, with Amicon Centiprep and Centricon concentrators, to 60 µl. Prior to sequence analysis, each preparation was checked for purity by reverse-phase HPLC analysis. The single peak of protein, determined on the reverse-phase HPLC, was analysed on an Applied Biosystems 477A protein sequencer.¹⁶

Molecular mass (M_r) determination

Molecular mass determination by Sephadex gel filtration was performed as described earlier.¹¹ SDS and

native polyacrylamide gel electrophoresis (PAGE) were performed on a Pharmacia Phast system according to the manufacturer's instructions. For SDS gels, protein samples were boiled for 3 min in a mixture of SDS and bromophenol blue. For native gels, samples were simply dissolved in a solution of bromophenol blue. Protein bands were visualised by staining with Coomassie Blue. For SDS gels, a Sigma molecular size marker kit (14 000–70 000) was used, and for native gels bovine serum albumin (M_r 66 000), ovalbumin (M_r 45 500) and trypsin inhibitor (M_r 20 100) were employed as standards.

Results

Molecular mass of the type IV DHFR

The type IV DHFR was purified by methotrexate affinity chromatography from *E. coli* J62-2 (pUK1123) and *E. coli* C600 (pUK1150). In both cases, the purified protein was analysed by HPLC and a single protein peak was observed; this was identified as DHFR.

We have shown previously that a partially-purified preparation of the type IV DHFR, encoded by plasmid pUK1123, has a M_r of 46 700 when measured by Sephadex G-75 gel filtration.¹¹ We now examined the methotrexate-agarose purified enzyme, encoded by plasmid pUK1123, on SDS-free PAGE. On a homogeneous 20% acrylamide gel, the enzyme from plasmid pUK1123 comigrated with ovalbumin, corresponding to a M_r of 45 500. Staining of the gel with the non-specific protein stain Coomassie Blue (fig. 1) confirmed the high purity obtained by methotrexate-agarose affinity chromatography.

The M_r of the ammonium sulphate purified enzyme from the cloned DHFR gene in plasmid pUK1150 was determined in a calibrated Sephadex G-75 column and found to be 33 000 (fig. 2). This lower M_r could have arisen from two causes; (1) the cloning of the type IV gene into plasmid pBR322 may have removed some of the sequence of the gene or it may have put a new stop codon into the sequence allowing the translation of a protein of smaller size; or (2) the protein from the original plasmid pUK1123 may have been composed of two non-identical subunits and only one of the genes had been cloned. If (2) was the case, the original protein would show different M_r values when measured by PAGE with SDS.

SDS-PAGE for M_r determination

The type IV DHFR from the cloned gene in plasmid pUK1150 was treated with SDS and then applied to a 10–15% gradient SDS-containing

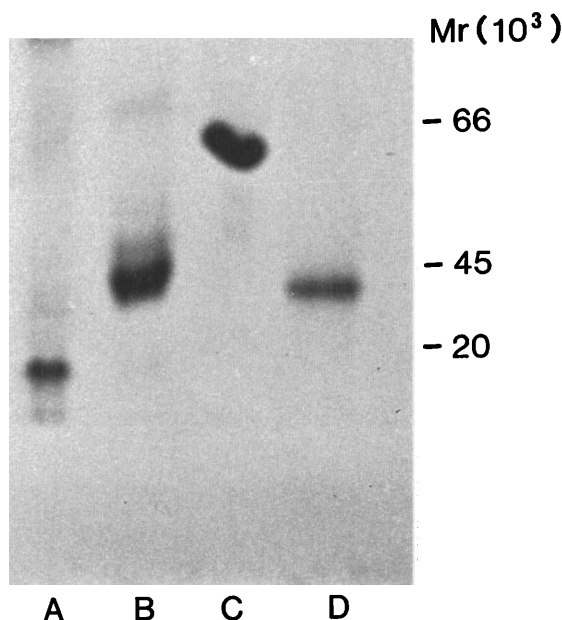


Fig. 1. SDS-free polyacrylamide gel electrophoresis of the purified type IV DHFR from *E. coli* J62-2 (pUK1123). Bands were visualised after staining with Coomassie Blue. A, trypsin inhibitor; B, ovalbumin; C, bovine serum albumin; D, type IV DHFR.

polyacrylamide gel. When it was electrophoresed until the dye front reached the end of the gel, it migrated as a single protein band of M_r *c.* 30 000. This result suggests that the enzyme from the cloned gene was a monomeric protein. On the other hand, when the purified DHFR encoded by the clinical plasmid pUK1123 was treated in the same manner, it migrated as one major band and a series of small bands (fig. 3). The major band had a M_r of 33 000. The smallest of the minor bands corresponded to a M_r of *c.* 10 000. The other minor bands were of similar intensity and appeared to have M_r values that were multiples of the smallest band, suggesting that they might be dimeric and trimeric forms of the M_r 10 000 protein. If this was the case, it indicated that the type IV enzyme of M_r *c.* 46 000, found on SDS-free PAGE and gel filtration was, in fact, made up of more than one subunit.

N-terminal amino-acid sequence analysis

HPLC was used to establish that the two enzyme preparations derived from plasmids pUK1123 and pUK1150 were pure proteins. The proteins from the respective HPLC peaks were applied to an Applied Biosystems 477A protein sequencer and a clear result was obtained for the first 50 amino acids of both enzymes (fig. 4). As expected, the DHFR

sequences obtained from *E. coli* J62-2 (pUK1123) and *E. coli* C600 (pUK1150) were identical and quite distinct from those trimethoprim-resistant plasmid-mediated DHFRs that have already been sequenced.⁵⁻¹⁰ There was, however, one distinct difference between the enzyme preparation derived from the clinical plasmid (pUK1123) and that from the clone. In the former, the presence of a second protein was apparent in the sequence analysis confirming that the native type IV DHFR was composed of non-identical subunits. The first 19 amino acids were distinguishable and comparison with known sequences has identified it as an exact match with the NS1 protein, a DNA-binding protein in *E. coli*.¹⁷ The presence of this second protein in the pUK1123-derived preparation was particularly interesting because this DHFR preparation had appeared as a single protein on HPLC and on SDS-free PAGE. In the preparation derived from the cloned plasmid pUK1150, there was no evidence at all of any second protein. Furthermore, the NS1-binding protein has a very distinctive pattern when measured on SDS-PAGE. It migrates as not only a monomer of M_r 9250, but also as a dimer, trimer and tetramer.¹⁸ This is the pattern that was found with the minor bands of the second protein on SDS-PAGE (fig. 3).

Discussion

Since the introduction of trimethoprim in 1968, several plasmid-mediated DHFRs have been identified that confer resistance to trimethoprim. These enzymes have, in the past, been largely distinguished by their biochemical properties⁴ although the amino-acid sequence of many of them is now known.⁵⁻¹⁰ Some of the more recently identified enzymes are clearly related to earlier plasmid-mediated DHFRs. For example, amino-acid sequence analysis has revealed that the types Ia and V are closely related.¹⁰ The unique nature of the type IV enzyme, however, suggests that it may not be derived from any of the other plasmid enzymes. Indeed its sensitivity to trimethoprim and the low level resistance it confers would suggest that it is a precursor to the other plasmid-mediated enzymes.

In this investigation, the N-terminal sequence of the type IV enzyme was determined with the use of the Applied Biosystems 477A automatic amino-acid sequencer which allowed rapid sequence analysis. Two properties of the type IV DHFR make it particularly suitable for sequencing by this method; (1) the majority of the active site of all DHFRs is situated at the N-terminal of the protein;^{19,20} and (2) purification of the native

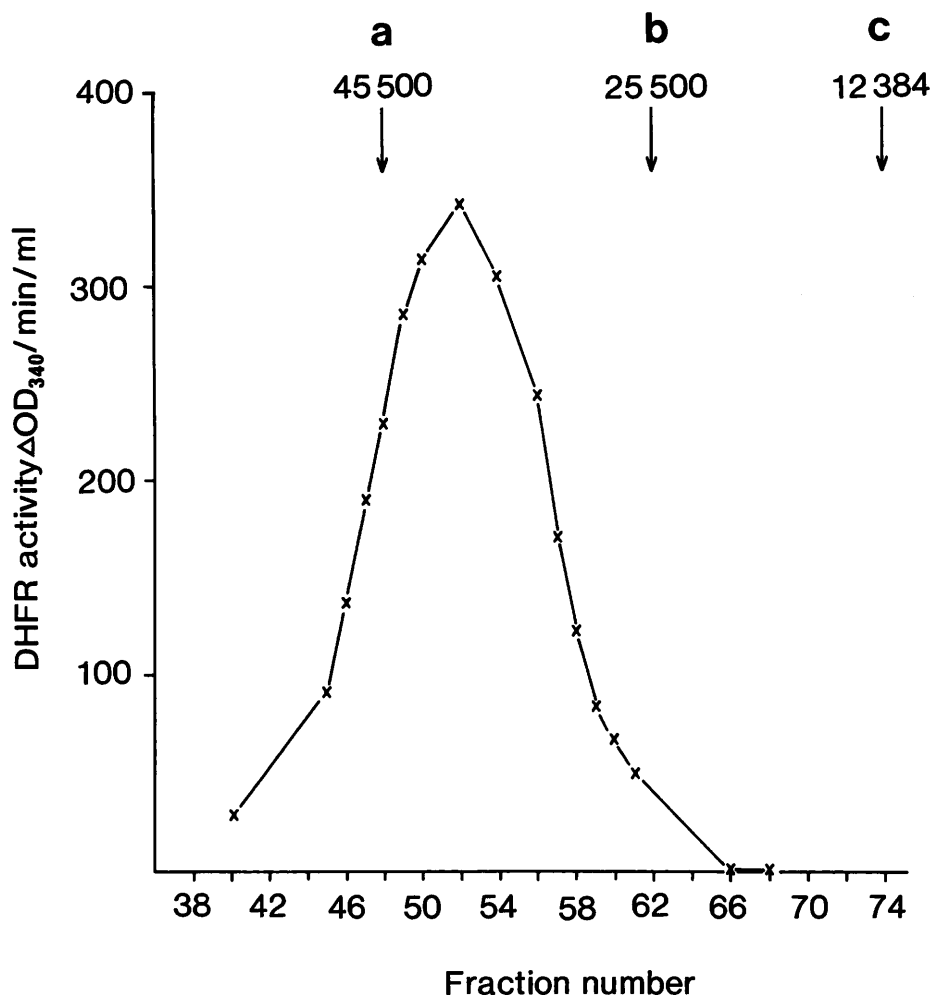


Fig. 2. DHFR activity of an ammonium sulphate (50–80% saturation) precipitate from *E. coli* C600 (pUK1150) analysed by Sephadex G-75 gel filtration. Elution of marker proteins is also shown—a, ovalbumin; b, chymotrypsinogen; c, cytochrome C.

protein is relatively easy with the use of methotrexate-agarose affinity chromatography, a result of the methotrexate sensitivity of the type IV enzyme, a relatively rare property amongst plasmid-encoded trimethoprim-resistant DHFRs.

Comparison of the first 50 amino acids of the type IV sequence with those for the other plasmid-mediated DHFRs sequenced so far (types I–V)^{5–10} showed the type IV enzyme to be clearly distinct. However, significant homology occurred with the *E. coli* K12 chromosomally-encoded enzyme. Twenty direct matches were found between the two enzymes and nine further amino acids were homologous with the amino acids at the same position in the chromosomal enzyme.²¹ This high degree of similarity between the type IV DHFR and the *E. coli* chromosomally-encoded enzyme is, perhaps, not surprising because the type IV enzyme, like the

chromosomally-encoded enzyme, confers only partial resistance to trimethoprim. Furthermore, studies on the induction mechanism of the type IV enzyme have indicated that its mechanism of resistance is more similar to the hyper-production mechanism of resistance mediated by deregulation of the *E. coli* chromosomally-encoded enzyme rather than the classical by-pass mechanism mediated by trimethoprim R-plasmids.²²

One surprising finding was the presence of the DNA-binding protein NS1 in the purified enzyme preparation from the original clinical plasmid (pUK1123). Analysis of this preparation by HPLC gave a single peak. As a further check on purity the preparation was run on native PAGE and stained with Coomassie Blue, and only a single band was obtained at M_r 45 500 confirming its purity. This corresponds to the M_r of the type IV enzyme when

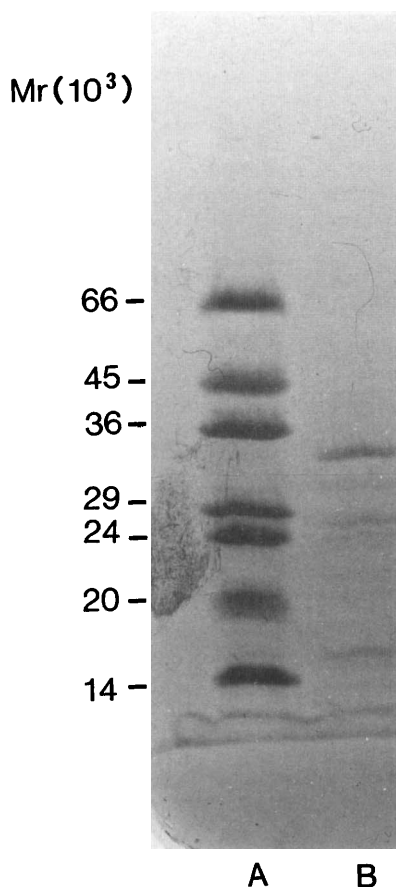


Fig. 3. SDS-PAGE of the purified type IV DHFR from *E. coli* J62-2 (pUK1123). Bands were visualised after staining with Coomassie Blue. A, Sigma mol. wt markers (M_r 14 000–70 000); B, type IV DHFR.

measured by gel filtration. The results indicate that the native type IV enzyme is a complex of the two proteins. SDS-PAGE analysis of this preparation confirmed the existence of subunits of differing size. Purified enzyme from the cloned type IV DHFR from pUK1150 contained no DNA-binding protein. The cloned enzyme has a lower M_r and is monomeric when examined on SDS-PAGE. The most likely explanation for the lower M_r is the absence of the complexed DNA-binding protein.

The reason why the type IV enzyme is complexed with a DNA binding protein is not clear but it may be related to the induction mechanism, since proteins which bind to DNA have been implicated in the control of gene expression.^{23,24} The absence of the binding protein in enzyme encoded by the cloned gene probably results from the lack of the NS1 gene in the restriction fragment cloned into pBR322. As pBR322 is a multi-copy vector, the necessity for induction of the type IV enzyme to produce trimethoprim resistance has been removed in the clone,¹⁴ thus the selection pressure for the NS1 protein gene was not present in the cloning process.

The amino-acid sequence reported in this paper has been registered in the protein database of the National Biomedical Research Foundation (NBRF), Washington, DC, USA, and has been assigned the accession number A33004.

We thank the Wellcome Trust for grant number 16376/1.5 which supported this work. We thank the WELMET Protein Characterisation Facility, University of Edinburgh for the HPLC analysis and sequencing. WELMET is supported by the Wellcome Trust, Edinburgh and Heriot-Watt Universities and the Salvesens Trust.

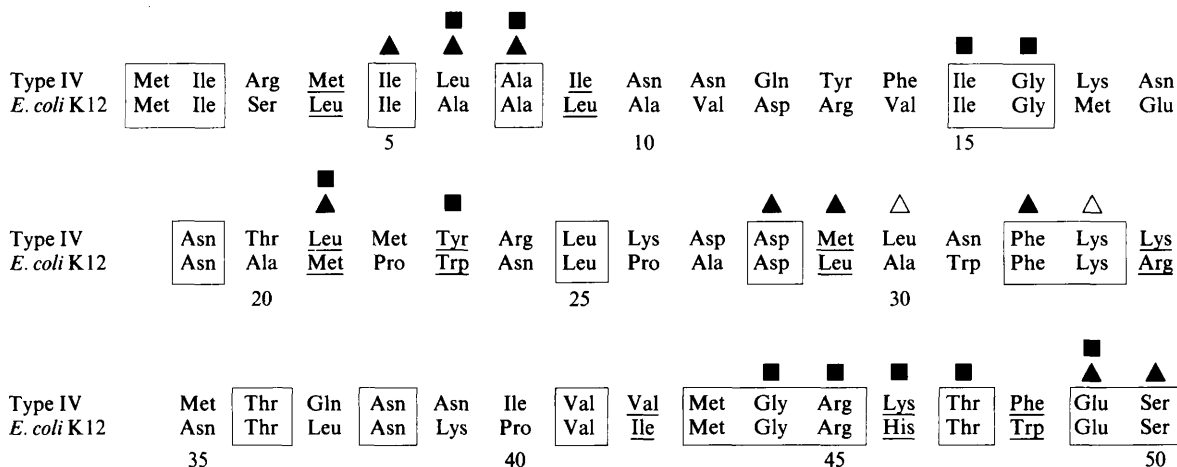


Fig. 4. N-terminal amino-acid sequences of the type IV plasmid-encoded DHFR and the *E. coli* K12 chromosomally-encoded enzyme. The amino acids are identified by standard three-letter abbreviations and are numbered according to the convention of Rouch *et al.*¹⁹ Identical amino acids are shown within the line boxes. Homologous amino acids are underlined. The amino acids involved in the active site are shown by the symbols at the top of the diagram: ▲ indicates the binding positions of trimethoprim; △ indicates the additional binding positions of methotrexate; ■ indicates the binding positions of NADPH. Positions taken from Rouch *et al.*¹⁹

REFERENCES

- Burchall J J, Hitchings G H. Inhibitor binding analysis of dihydrofolate reductases from various species. *Mol Pharmacol* 1965; **1**: 126–136.
- Fleming M P, Datta N, Grüneberg R N. Trimethoprim resistance determined by R factors. *Br Med J* 1972; **1**: 726–728.
- Amyes S G B, Smith J T. R-factor trimethoprim resistance mechanism: an insusceptible target site. *Biochem Biophys Res Commun* 1974; **58**: 412–418.
- Amyes S G B. The success of plasmid-encoded drug resistance genes in clinical bacteria: an examination of plasmid-mediated ampicillin and trimethoprim resistance genes and their resistance mechanisms. *J Med Microbiol* 1989; **28**: 73–83.
- Stone D, Smith S L. The amino acid sequence of the trimethoprim resistant dihydrofolate reductase specified in *Escherichia coli* by R-plasmid R67. *J Biol Chem* 1979; **254**: 10857–10861.
- Zolg J W, Hänggi U J. Characterization of R plasmid-associated, trimethoprim-resistant dihydrofolate reductase and determination of the nucleotide sequence of the resistance gene. *Nucleic Acids Res* 1981; **9**: 697–710.
- Fling M E, Richards C. Nucleotide sequence of the trimethoprim resistant dihydrofolate reductase gene harboured by Tn7. *Nucleic Acids Res* 1983; **11**: 5147–5158.
- Flensburg J, Steen R. Nucleotide sequence analysis of the trimethoprim resistant dihydrofolate reductase encoded by R-plasmid R751. *Nucleic Acids Res* 1986; **14**: 5933.
- Fling M E, Kope J, Richards C. Characterization of plasmid pAZ1 and the type III dihydrofolate reductase gene. *Plasmid* 1988; **19**: 30–38.
- Sundstrom L, Radstrom P, Swedberg G, Sköld O. Site-specific recombination promotes linkage between trimethoprim- and sulfonamide-resistance genes. Sequence characterization of dhfrV and sulI and a recombination active locus of Tn21. *Mol Gen Genet* 1988; **213**: 191–201.
- Young H-K, Amyes S G B. A new mechanism of plasmid trimethoprim resistance: characterization of an inducible dihydrofolate reductase. *J Biol Chem* 1986; **261**: 2503–2505.
- Sheldon R, Brenner S. Regulatory mutants of dihydrofolate reductase in *Escherichia coli* K12. *Mol Gen Genet* 1976; **147**: 91–97.
- Tennhammar-Ekman B, Sundstrom L, Sköld O. New observations regarding evolution of trimethoprim resistance. *J Antimicrob Chemother* 1986; **18** Suppl C: 67–76.
- Towner K J, Young H-K, Amyes S G B. Biotinylated DNA probes for trimethoprim-resistant dihydrofolate reductases types IV and V. *J. Antimicrob. Chemother* 1988; **22**: 285–291.
- Kaufman B T. Methotrexate-agarose in the purification of dihydrofolate reductase. *Methods Enzymol* 1974; **34**: 272–281.
- Hayes J D, Kerr L A, Cronshaw A D. Evidence that glutathione S-transferases B1-B1 and B2-B2 are the products of separate genes and that their expression in human liver is subject to inter-individual variation. Molecular relationships between the B1 and B2 subunits and other alpha class GST. *Biochem J* 1989; (in press).
- Mende L, Timm B, Subramanian R. Primary structure of two homologous ribosome-associated DNA-binding proteins of *Escherichia coli*. *FEBS Lett* 1978; **96**: 395–398.
- Rouviere-Yaniv J, Kjeldgaard N O. Native *Escherichia coli* Hu protein as a heterotypic dimer. *FEBS Lett* 1979; **106**: 297–300.
- Rouch D A, Messerotti L J, Loo L S L, Jackson C A, Skurray R A. Trimethoprim resistance transposon Tn4003 from *Staphylococcus aureus* encodes genes for a dihydrofolate reductase and thymidylate synthetase flanked by three copies of IS257. *Mol Microbiol* 1989; **3**: 161–175.
- Novak P, Stone D, Burchall J J. R plasmid dihydrofolate reductase with a dimeric subunit structure. *J Biol Chem* 1983; **258**: 10956–10959.
- Smith D R, Calvo J M. Nucleotide sequence of the *E. coli* gene coding for dihydrofolate reductase. *Nucleic Acids Res* 1980; **8**: 2255–2274.
- Thomson C J, Amyes S G B. Induction mechanism of the type IV dihydrofolate reductase. *Abstracts of the 4th European Congress of Clinical Microbiology* 1989; 407.
- Ptashne M. How eukaryotic transcriptional activators work. *Nature* 1988; **335**: 683–689.
- Magasanik B. Reversible phosphorylation of an enhancer binding protein regulates the transcription of bacterial nitrogen utilization genes. *Trends Biochem Sci* 1988; **13**: 475–479.