Protein Expression in *Escherichia coli* Minicells Containing Recombinant Plasmids Specifying Trimethoprim-Resistant Dihydrofolate Reductases

MARY E. FLING* AND LYNN P. ELWELL

Department of Microbiology, Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709

Deoxyribonucleic acid fragments containing the structural genes for several trimethoprim-resistant dihydrofolate reductases from naturally occurring plasmids were inserted into small cloning vehicles. The genetic expression of these hybrid plasmids was studied in purified *Escherichia coli* minicells. The type I dihydrofolate reductase, encoded by plasmid R483 and residing within transposon 7 (Tn7), had a subunit molecular weight of 18,000. The type II dihydrofolate reductase, specified by plasmid R67, had a subunit molecular weight of 9,000. These two enzymes were antigenically distinct in that anti-type II dihydrofolate reductase (R67) antibody did not cross-react with the type I (R483) protein. The trimethoprim-resistant reductase specified by plasmid R388 had a subunit molecular weight of about 10,500 and was immunologically related to the type II (R67) enzyme. A 9,000 subunit of the dihydrofolate encoded by the transposition element Tn402 was also antigenically related to the R67 reductase.

R-plasmid-mediated trimethoprim resistance was first described in clinical isolates of *Escherichia coli* and *Klebsiella aerogenes* (10). The mechanism of plasmid-associated trimethoprim resistance involves the synthesis of novel dihydrofolate reductases (DHFRs) which are highly resistant to trimethoprim; hence, the target site rather than the antibacterial agent is modified (2, 21). It has recently been demonstrated that R-plasmid-specified DHFRs can be separated into two broad classes based on different levels of sensitivity to trimethoprim and other related antifolate compounds (19). The so-called type I reductase (encoded by plasmid R483) has a 50% inhibitory concentration for trimethoprim several thousand-fold higher than that required to inhibit the *E. coli* chromosomal DHFR. The type II enzyme (encoded by plasmid R67) is for all practical purposes completely insensitive to trimethoprim, having 50% inhibitory concentrations several hundred-fold higher than those for the type I reductases.

Both types of plasmid-encoded enzymes show little difference from the chromosomal reductase in the binding of NADPH and dihydrofolate, and both resistant enzymes have molecular weights of about 35,000, approximately twice the size of the trimethoprim-sensitive, chromosomal reductases (19). Hence the chromosomal and plasmid-specified enzymes appear to be quite distinct from one another on the basis of molecular weight and enzyme characteristics. These observations lead to questions regarding the evolutionary origin(s) of the trimethoprim-resistant reductases as well as the relationships of the different plasmid-encoded enzymes to one another and to the trimethoprim-sensitive chromosomal enzyme.

Our approach to this question has been to isolate the DNA fragments containing the structural genes for both type I and II reductases, insert them into appropriate cloning vehicles, and then study the genetic expression of these genes in an *E. coli* minicell system.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** *E. coli* K-12 strain DS410 (*minA minB*), which produces minicells, has been described (5). To construct the hybrid plasmids shown in Fig. 1, we used conventional molecular cloning procedures (13) with some modifications (11). Mixtures of 1 to 2 µg of purified R plasmid DNA, the source of the DHFR DNA sequences, and 1 to 2 µg of cloning vector DNA, either pBR322 (5) or pSC101 (6), were digested to completion using the restriction endonucleases EcoRI or *Bam*HI (Miles Research Products).

The enzyme was inactivated by heating the DNA reaction mixture to 65°C for 5 min. The mixture was adjusted to ligation conditions (17) and ligated with T4 DNA ligase (Bethesda Research Laboratories, Inc.) for 36 to 42 h at 14°C. Competent *E. coli* HB101 cells transformed with DNA carrying the reductase gene were selected on Mueller-Hinton medium (Difco) supplemented with 50 µg of trimethoprim per ml and the antibiotic appropriate for the particular cloning vector.
Hybrid plasmid DNA was purified by cesium chloride-ethidium bromide density gradient centrifugation for characterization and transformation into the minicell-producing strain.

The transposition element Tn7, a 9-megadalton (Mdal) element determining trimethoprim and streptomycin resistance, was found originally in the R plasmid R483 which specifies a type I reductase (3). Plasmid ColEI::Tn7 was constructed by the transposition of Tn7 from plasmid R483 to ColEI (5).

Isolation and labeling of minicells. Minicells were isolated by the method of Kool et al. (16) as modified by Dougan and Sherratt (9). Minicells from 18-h cultures were purified by banding on 5 to 20% linear sucrose gradients and suspended in 1 ml of labeling medium in which methionine assay medium (Difco) was substituted for a synthetic amino acid mix. After incubation at 37°C for 20 min, lact[35S]methionine (about 800 Ci mmol⁻¹; New England Nuclear Corp.) was added (20 to 40 μCi ml⁻¹ of minicells), and incubation was continued for 45 min. Labeled minicells were pelleted and suspended in 50 μl of sample buffer (0.0625 M Tris, pH 6.8, 5% sodium dodecyl sulfate [SDS], 5% mercaptoethanol, 0.1% bromophenol blue, 10% glycerol), heated to 100°C for 5 min, and frozen at -70°C.

Electrophoresis and autoradiography. Proteins from disrupted minicells were fractionated on 15% SDS-polyacrylamide gels as described by Tyler and Sherratt (24). The gels were stained in 0.06% Coomassie blue G250 in 3.5% perchloric acid, destained in 7.5% acetic acid, dried, and autoradiographed for 5 to 10 days using Kodak X-Omat R film. Molecular weight standards employed were phosphorylase B, 94,000; bovine serum albumin, 68,000; ovalbumin, 43,000; carboxy anhydrase, 30,000; soybean trypsin inhibitor, 21,000; lysozyme, 14,300 (Biorad); and the cyanogen bromide cleavage products of horse heart myoglobin (range 2,512 to 16,949; BDH Chemicals, Ltd.).

Antibody preparation. Type II R67 DHFR was purified to homogeneity as reported by Smith et al. (32). New Zealand rabbits were immunized with injections of 502 μg of purified R67 DHFR in complete Freund adjuvant (Difco) intramuscularly. After 14 days, each rabbit was injected with 433 μg of purified enzyme in incomplete Freund adjuvant. Nine days later, a subcutaneous injection of 433 μg of DHFR in saline was administered. Rabbits were bled 4 weeks after the first injection. Whole sera were pooled and stored frozen.

Precipitation of antibody-antigen complexes with Staphylococcus aureus protein A. The Kessler procedure (15) as modified by Dougan et al. (7) was used to test anti-type II DHFR (R67) antiserum against labeled proteins synthesized in minicells harboring recombinant DNA templates. Supernatants of minicell extracts were reacted with 5 μl of specific antibody for 45 min, and then 38 μl of a 10% (wt/vol) solution of Formalin-fixed S. aureus cells (Calbiochem) was added. After 30 min at 24°C the S. aureus cells were pelleted through 1.0 M sucrose by low-speed centrifugation and washed. The pellet was suspended in 50 μl of sample buffer, denatured, and electrophoresed as described above. Controls to monitor the nonspecific binding of minicell-directed proteins to S. aureus protein A consisted of reaction mixtures in which anti-type II DHFR antiserum was omitted or in which normal rabbit serum was substituted for the antibody.

RESULTS

Construction of hybrid plasmids. In the present study, derivatives of ColEI::Tn7 were generated by digesting plasmid ColEI::Tn7 and pBR322 DNA with EcoRI and ligating this mixture of fragments. Two hybrid plasmid species were isolated and characterized. One type, for example, plasmid pFE504 (Fig. 1A), consisted of a 4.7-Mdal ColEI::Tn7 fragment inserted in the EcoRI site of plasmid pBR322. The other plasmids, from transformants selected on agar containing trimethoprim only, each had a single 4.7-Mdal fragment from plasmid ColEI::Tn7 (Fig. 1A, plasmid pFE506).

Plasmid R67, resistant to ampicillin, streptomycin, trimethoprim, chloramphenicol, sulfamethoxazole, and tetracycline (1), was the source of the type II DHFR DNA sequences. The R67 reductase gene located on a 6.5-Mdal EcoRI fragment, was inserted into the EcoRI site of pBR322 (Fig. 1B; plasmid pFE332). Specific DNA sequences were deleted from plasmid pFE332 by digesting the DNA with BamHI and ligating the cleavage products. Plasmid pFE364, isolated from a trimethoprim-resistant derivative, lacked two of the plasmid pFE332 BamHI fragments (Fig. 1B, pFE364).

An analogous series of hybrid plasmids was constructed using the same 6.5-Mdal EcoRI R67 fragment inserted in the plasmid pSC101 (Fig. 1; pFE413). Plasmids pFE425 and pFE420 were constructed by deleting BamHI fragments from pFE413 by the technique described for plasmid pFE364. Both plasmids lacked the largest BamHI fragment of R67. Plasmid pFE420 had a further deletion and was comprised of the 1.6-Mdal BamHI fragment of plasmid R67 and pSC101 DNA sequences (Fig. 1).

The trimethoprim-resistant DHFR of plasmid R388 resided in the hybrid plasmid pFE373 (Fig. 1C). It was constructed by inserting a 1.3-Mdal BamHI fragment from R388 into the cloning vector pBR322.

Synthesis of proteins in minicells. We studied the synthesis of a type II plasmid-encoded DHFR in minicells using the hybrid plasmids derived from R67 (Fig. 1B). The polypeptides synthesized in minicells containing these plasmids are illustrated in Fig. 2 and 3. The cloning vehicle pBR322 specified the synthesis of several proteins (Fig. 2D). The most prominent bands corresponded to β-lactamase proteins with molecular weights of 32,000, 30,000, and 27,000. Plasmid pFE364 specified, in addi-
Fig. 1. Restriction endonuclease maps of hybrid plasmids specifying trimethoprim-resistant DHFRs. The plasmids are represented as linear, opened at one of the junctions between the cloning vehicle and the inserted DNA. Symbols for restriction endonuclease sites are (I) EcoRI and (I) BamHI. Fragment masses are given as 10^6 daltons (Mdal). (A) Hybrid plasmids specifying type I (R483) reductases: ColEl::Tn7 and derivatives pFE504 and pFE506. ColEl DNA, open bar; Tn7 DNA, solid bar; cloning vehicle pBR322, hatched bar. (B) Hybrid plasmids specifying type II (R67) reductases. R67 DNA, solid bar. Cloning vehicles: pBR322, hatched bar; pSC101, stippled bar. (C) Hybrid plasmid specifying R388 reductase constructed by inserting a BamHI fragment from R388 (solid bar) into the cloning vehicle pBR322 (hatched bar).

Minicells containing plasmid pFE413 (Fig. 2B and 3B). The β-lactamase proteins were not synthesized by plasmid pFE420 (Fig. 2A and A'; Fig. 3C). The production of β-lactamase proteins by minicells containing plasmid pFE425 (Fig. 3A) and pFE413, but not pFE420, indicated that the R67 β-lactamase structural gene lies on the 1.3-Mdal BamHI fragment present in pFE425 but deleted from pFE413 in the construction of pFE420.

It is easier to visualize the novel proteins encoded by the R67 (pSC101) hybrids in Fig. 3. Minicells containing pSC101 synthesized at least six proteins, visible as faint bands in Fig. 3D. Plasmid pFE420 encoded most of the pSC101 proteins to its pBR322 component proteins, a novel polypeptide of about 9,000 (Figure 2C and C'). The prime designations for A and C (Fig. 2) indicate the presence of 5 μg of trimethoprim per ml during protein synthesis. The large protein of molecular weight 44,000 was presumably of bacterial origin, since it is synthesized in minicells containing no plasmid DNA.

The plasmids pFE413 and pFE420 contain the R67 reductase gene cloned into pSC101. Both of these hybrid plasmids specified a protein of molecular weight 9,000 (Fig. 2A, A', and B). Proteins that comigrated with the β-lactamase proteins were also synthesized by minicells harboring plasmid pFE413 (Fig. 2B and 3B). The β-lactamase proteins were not synthesized by plasmid pFE420 (Fig. 2A and A'; Fig. 3C). The production of β-lactamase proteins by minicells containing plasmid pFE425 (Fig. 3A) and pFE413, but not pFE420, indicated that the R67 β-lactamase structural gene lies on the 1.3-Mdal BamHI fragment present in pFE425 but deleted from pFE413 in the construction of pFE420.

It is easier to visualize the novel proteins encoded by the R67 (pSC101) hybrids in Fig. 3. Minicells containing pSC101 synthesized at least six proteins, visible as faint bands in Fig. 3D. Plasmid pFE420 encoded most of the pSC101 proteins to its pBR322 component proteins, a novel polypeptide of about 9,000 (Figure 2C and C'). The prime designations for A and C (Fig. 2) indicate the presence of 5 μg of trimethoprim per ml during protein synthesis. The large protein of molecular weight 44,000 was presumably of bacterial origin, since it is synthesized in minicells containing no plasmid DNA.

The plasmids pFE413 and pFE420 contain the R67 reductase gene cloned into pSC101. Both of these hybrid plasmids specified a protein of molecular weight 9,000 (Fig. 2A, A', and B). Proteins that comigrated with the β-lactamase proteins were also synthesized by minicells harboring plasmid pFE413 (Fig. 2B and 3B). The β-lactamase proteins were not synthesized by plasmid pFE420 (Fig. 2A and A'; Fig. 3C). The production of β-lactamase proteins by minicells containing plasmid pFE425 (Fig. 3A) and pFE413, but not pFE420, indicated that the R67 β-lactamase structural gene lies on the 1.3-Mdal BamHI fragment present in pFE425 but deleted from pFE413 in the construction of pFE420.

It is easier to visualize the novel proteins encoded by the R67 (pSC101) hybrids in Fig. 3. Minicells containing pSC101 synthesized at least six proteins, visible as faint bands in Fig. 3D. Plasmid pFE420 encoded most of the pSC101 proteins to its pBR322 component proteins, a novel polypeptide of about 9,000 (Figure 2C and C'). The prime designations for A and C (Fig. 2) indicate the presence of 5 μg of trimethoprim per ml during protein synthesis. The large protein of molecular weight 44,000 was presumably of bacterial origin, since it is synthesized in minicells containing no plasmid DNA.

The plasmids pFE413 and pFE420 contain the R67 reductase gene cloned into pSC101. Both of these hybrid plasmids specified a protein of molecular weight 9,000 (Fig. 2A, A', and B). Proteins that comigrated with the β-lactamase proteins were also synthesized by minicells harboring plasmid pFE413 (Fig. 2B and 3B). The β-lactamase proteins were not synthesized by plasmid pFE420 (Fig. 2A and A'; Fig. 3C). The production of β-lactamase proteins by minicells containing plasmid pFE425 (Fig. 3A) and pFE413, but not pFE420, indicated that the R67 β-lactamase structural gene lies on the 1.3-Mdal BamHI fragment present in pFE425 but deleted from pFE413 in the construction of pFE420.

It is easier to visualize the novel proteins encoded by the R67 (pSC101) hybrids in Fig. 3. Minicells containing pSC101 synthesized at least six proteins, visible as faint bands in Fig. 3D. Plasmid pFE420 encoded most of the pSC101 proteins to its pBR322 component proteins, a novel polypeptide of about 9,000 (Figure 2C and C'). The prime designations for A and C (Fig. 2) indicate the presence of 5 μg of trimethoprim per ml during protein synthesis. The large protein of molecular weight 44,000 was presumably of bacterial origin, since it is synthesized in minicells containing no plasmid DNA.
proteins (the 29,000 species appeared to be overproduced) and a rather large amount of the 9,000 protein (Fig. 3C). Plasmid pFE425 specified a very similar polypeptide pattern to those of pFE413 and pFE420 in which a 9,000 protein was a predominant, novel band (Fig. 3A). Therefore, to summarize our results relative to the type II plasmid reductase encoded by R67: (i) we have relocated the trimethoprim resistance DHFR gene from plasmid R67 into two separate cloning vehicles and have generated smaller derivatives; (ii) we have examined the polypeptides synthesized in E. coli minicells by four independent hybrid plasmids harboring the type II DHFR structural gene; and (iii) we find that in denaturing conditions (SDS-polyacrylamide gels) the putative type II reductase migrates as a polypeptide of molecular weight 9,000.

To analyze the type I plasmid-encoded DHFR in minicells, we used the ColE1::Tn7 derivative plasmids pFE504 and pFE506 (Fig. 1A). Further analysis of plasmid pFE506 showed that this 4.7-Mdal plasmid contained the replication origin of ColE1 and was able to replicate, even though more than half of the ColE1 sequences were deleted. In addition, 2.7 Mdal of Tn7 DNA sequences, including the trimethoprim and streptomycin resistance determinants, were present (Fling and Eure, unpublished data). The proteins synthesized in minicells harboring these plasmids are shown in Fig. 3F and G. Plasmid pBR322-encoded proteins are shown in well 3E. Plasmid pFE504 clearly directed the synthesis of β-lactamase as well as a polypeptide with a molecular weight of 18,000 (Fig. 3F). The cloning vehicle, pBR322, specified a polypeptide of approximately 18,900 (Fig. 2D and 3E), which was close to the molecular weight of the smaller polypeptide synthesized by pFE504. However, the pattern seen in Fig. 3G supports the argument that these are indeed separate and unrelated polypeptides. Two major polypeptides of molecular weight 30,000 (presumably the streptomycin-modifying enzyme [4]) and 18,000 were synthesized in minicells carrying pFE506 (Fig. 3G). As can be seen in Fig. 1A, plasmid pFE506 contains no pBR322 DNA sequences; however, pFE506 encodes as much of the 18,000-molecular-weight proteins as does pFE504. We conclude from the minicell data presented in Fig. 3 that the type I (R483) DHFR has a molecular weight of 18,000 in denaturing gels, whereas the type II (R67) DHFR has a molecular weight of 9,000.

**Proof of subunit structure using specific antibody.** To definitively show that the 9,000 polypeptide encoded by the R67 EcoRI fragment was related to the type II resistant reductase, the following experiment was performed. Antibody raised against purified type II enzyme in rabbits was added to the mixture of labeled polypeptides synthesized in minicells harboring the cloned type II DHFR structural gene. Enough formalized S. aureus cells were then added to bind all the immunoglobulin G present in the reaction mixture. S. aureus strains rich in protein A on their outer surfaces bind immunoglobulin G nonspecifically through the Fc fragment (12). The staphylococcal cells were pelleted, and the resultant protein complexes were denatured and electrophoresed. The polypeptides synthesized by plasmid pFE364, which contains a 1.6-Mdal fragment of R67 in addition to
pBR322, are shown in Fig. 4, lane B. Labeled minicell lysate plus antibody was electrophoresed, and the results are shown in Fig. 4, lane A. Clearly, the specific polypeptide recognized by the anti-DHFR type II antibody was the 9,000 species, the putative reductase subunit. An extremely small fraction of the β-lactamase proteins nonspecifically bound the staphylococcal protein A (Fig. 4, lane A; 7). An additional control was included in which radiolabeled minicell lysate was added to staphylococci in the absence of anti-DHFR type II antibody. Only a faint band corresponding to a small fraction of the β-lactamase protein could be observed when this sample was electrophoresed (data not shown).

We also analyzed the resistant DHFR specified by plasmid R388 (2). This particular reductase has a 50% inhibitory concentration for trimethoprim of at least 5,700 μM; however, its classification as either a type I or type II DHFR is uncertain (S. Smith, personal communication). The hybrid plasmid pFE373 contains the trimethoprim-resistant reductase originally harbored by R388 (Fig. 1). Labeled extracts derived from minicells containing pFE373 were reacted with anti-type II DHFR antibody. A polypeptide with a molecular weight of approximately 10,500 reacted specifically with the anti-type II DHFR (R67) antibody (results not shown). In addition, radiolabeled polypeptides, synthesized in minicells harboring the type I reductase derived from Tn7, and R34, a type I DHFR-specifying R plasmid (19), were reacted with the purified anti-type II DHFR antibody. There appeared to be no cross-reactivity between these two type I reductases and the anti-type II DHFR (R67) antibody.

Finally, minicells containing the cloned trimethoprim resistance DHFR gene of the transposition element Tn402 directed the synthesis of a 9,000 polypeptide that was specifically precipitated by anti-type II DHFR antibody (Fig. 4C). The hybrid plasmid pQRS272, containing the trimethoprim-resistant DHFR from transposon Tn402 of R751, was constructed by Roxanne Laux, Tom Petes, and James Shapiro from pBR322 and a HindIII fragment of λ imm434 cts1 5515 5519 ST::Tn402 (20). Hence, the reductase situated within Tn402 is related to the type II DHFR specified by naturally occurring R plasmid, R67.

**DISCUSSION**

The question of the origin(s) of these trimethoprim-resistant DHFRs is an intriguing and important one. Recently, Smith et al. have reported that the type II plasmid reductase is a tetramer consisting of four identical 8,500-molecular-weight subunits (22). The results presented in this investigation, studying the same plasmid DHFR but utilizing different techniques, support their conclusions. Furthermore, amino acid sequencing results have shown that no obvious homology exists between the type II (R67) DHFR subunit and any other sequenced bacterial or vertebrate DHFR (22, 22a).

A possible source of the plasmid-borne determinants is T4 bacteriophage-specified DHFR.
Although this phase enzyme resembles the plasmid reductases in certain respects, no immunodiffusion precipitin bands were observed when purified R483 reductase was allowed to react with antiserum raised against T4 DHFR (18). Furthermore, Mosher et al. report that the type I reductase is not enzymatically inactivated by anti-T4 DHFR antibody (18). It is, of course, possible that a bacteriophage(s) other than T4 is the ultimate source of the plasmid-borne reductase. An alternative explanation has been suggested (22), namely, that the plasmid DHFR is not a "classical" reductase at all but is, in fact, a meriacker oxidoreductase which has lost some earlier substrate specificity and has acquired the capacity to reduce dihydrofolate as a consequence. We have some data that support this possibility (Elwell, Walton, and Dornbusch, unpublished data). The transposon, Tn7, appears to share a significant amount of DNA sequence homology with the E. coli chromosome. The precise nature of these apparent Tn7-like nucleotides in the E. coli genome is unknown; however, it is a possibility that they represent an enzyme gene(s) universally found in the E. coli chromosome.

Two major conclusions can be drawn from the minicell data presented in this report. (i) The type I reductase encoded by R483 and the type II reductase specified by R67 are very dissimilar enzymes; they differ antigenically and in subunit structure. (ii) The type II, trimethoprim-resistant reductase can reside within a transposable DNA sequence. It remains to be seen how much additional diversity exists among R plasmid reductases. Tennhammar-Ekman and Skold (23) recently have compared four R plasmid-encoded reductases by using techniques including gel electrophoresis, analog inhibition profiles, heat sensitivity, and pH optimum curves. They conclude that the DHFRs specified by plasmids R483, R751, and R388 could be "clearly discerned as separate enzymes." These workers did not give subunit molecular weights for the enzymes they studied, and therefore direct comparisons with this study cannot be made. Zolg et al. have reported that the R388-encoded DHFR migrates in SDS-acrylamide gels as an 8,000 polypeptide (25). The difference between their reported value and our value of 10,500 may be attributable to the substantial error in determining the molecular weights of small polypeptides by this method. We interpret the small but consistent difference observed between the R388 and R67 proteins to mean that the R388 reductase differs from the R67-encoded DHFR in subtle biochemical respects, although antigenically the two enzymes are related.

Shapiro and Sporn (20) have shown that a trimethoprim resistance determinant can reside within a 5-Mda transposon, Tn402. E. coli minicells containing the Tn402 reductase sequences directed the synthesis of a 9,000 polypeptide that was specifically precipitated by anti-type II DHFR antibody. The fact that the type II reductase can reside within a transposon has important epidemiological implications in that this resistance determinant, as well as the type I DHFR, can be expected to disseminate by virtue of site-specific recombinational mechanisms. We intend to investigate the origins of the various R-plasmid reductases by heteroduplex analyses of the hybrid plasmids, against one another and against a cloned E. coli chromosomal reductase (Walton et al., unpublished data), and thus answer basic questions regarding the origin(s) of these most interesting enzymes.

ACKNOWLEDGMENTS
We thank Jerry Wolberg (Wellcome Research Laboratories) for providing us with anti-type II reductase antibody and Roxanne Laux, James Shapiro, and Tom Petes for pR85272. We are especially grateful to Gordon Dougan (Moynie Institute, Trinity College, Dublin) for teaching one of us (M.F.) the many fine points of the E. coli minicell technique.

LITERATURE CITED


