Characterization of Mutations Contributing to Sulfathiazole Resistance in *Escherichia coli*

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A sulfathiazole-resistant dihydropteroate synthase (DHPS) present in two different laboratory strains of *Escherichia coli* repeatedly selected for sulfathiazole resistance was mapped to *folP* by P1 transduction. The *folP* mutation in each of the strains was shown to be identical by nucleotide sequence analysis. A single C→T transition resulted in a Pro→Ser substitution at amino acid position 64. Replacement of the mutant *folP* alleles with wild-type *folP* significantly reduced the level of resistance to sulfathiazole but did not abolish it, indicating the presence of an additional mutation(s) that contributes to sulfathiazole resistance in the two strains. Transfer of the mutant *folP* allele to a wild-type background resulted in a strain with only a low level of resistance to sulfathiazole, suggesting that the presence of the resistant DHPS was not in itself sufficient to account for the overall sulfathiazole resistance in these strains of *E. coli*. Additional characterization of an amplified secondary resistance determinant, *sur*, present in one of the strains, identified it as the previously identified bicyclomycin resistance determinant *ber*, a member of a family of membrane-bound multidrug resistance antiporters. An additional mutation contributing to sulfathiazole resistance, *sur*, has also been identified and has been shown to affect the histidine response to adenine sensitivity displayed by these purU strains.

Sulfonamide antimicrobial agents such as sulfanilamide, sulfathiazole, and sulfamethoxazole prevent the biosynthesis of reduced folate compounds by inhibiting the production of dihydropteroate from 6-hydroxymethyl-dihydropterin pyrophosphate and *p*-aminobenzoate (PABA) (7). Sulfonamide derivatives are structural analogs of PABA and compete with PABA for enzymatic condensation (catalyzed by dihydropteroate synthase or DHPS) with the dihydropterin substrate (7, 29). The dihydropterin-sulfonamide adducts are thought not to be further inhibitory to cellular function and to passively diffuse out of the cell (29). Since DHPS is inhibited from using PABA, the cell becomes depleted for the dihydropterin substrate necessary to produce dihydrofolate and tetrahydrofolate. Growth is thus thought to be inhibited by a lack of the vitamin cofactor necessary for the biosynthesis of macromolecular precursors (2, 13).

Pato and Brown (23) showed that a sulfonamide-resistant DHPS could be recovered from laboratory strains of *Escherichia coli* selected for resistance to sulfathiazole. Many transmissible R-determinant plasmids confer sulfonamide resistance on *E. coli* by expressing a sulfonamide-resistant form of DHPS (25, 32, 34), corroborating the role of a resistant DHPS in sulfonamide resistance. The acquisition of such plasmids is, in fact, the clinically significant mechanism by which *E. coli* becomes resistant to sulfonamide antimetabolites.

DHPS is encoded by *folP*, and the wild type as well as temperature-sensitive, sulfonamide-resistant variants have been cloned and sequenced (9, 10). Recently, the three-dimensional structure has been determined for the enzymes from *E. coli* (1) and *Staphylococcus aureus* (12). The mutant DHPS enzymes were each characterized to have elevated *Kₘ* for sulfathiazole (150- and 3,000-fold, respectively, and elevated *Kₘ* for PABA (10- and 300-fold, respectively). In addition, the activity of the mutant enzyme characterized by Dallas et al. (9) was only about 1/10 that of the wild-type enzyme, resulting in an auxotrophic requirement for the end products of folate metabolism. Wild-type and sulfanamide-resistant DHPS sequences have also been obtained from a variety of other microorganisms (6, 16, 26, 33, 35, 37).

Nichols and Guay (21) reported that one laboratory strain of *E. coli* selected for resistance to high levels of sulfathiazole contained a resistant DHPS, as well as a secondary resistance determinant, *sur*, present on a tandemly amplified segment of the chromosome. The level of sulfathiazole resistance correlated with the copy number of the *sur* amplification, but resistance was not abolished upon loss of the amplified DNA segment. *sur* was cloned from the resistant and wild-type strains and was shown to confer only a low level of resistance to sulfathiazole.

We have further characterized two laboratory strains of *E. coli* selected for resistance to high levels of sulfathiazole. Each contained a mutant *folP* allele in addition to at least one secondary mutation. We have characterized the contributions of the mutant *folP* and *sur* alleles and an additional mutation, *sur*, to sulfathiazole resistance in each strain and in wild-type backgrounds.

**MATERIALS AND METHODS**

**Microbiological methods.** The bacterial strains used in this study are listed in Table 1. Basal minimal medium for the growth of *E. coli* consisted of 0.4% (wt/vol) glucose and the inorganic salts recommended by Vogel and Bonner (36). Enriched medium was Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl [17]). Final antibiotic concentrations were as follows: ampicil-...
lin, 75 μg/ml; tetracycline, 10 μg/ml; kanamycin, 75 μg/ml; and nalidixic acid, 20 μg/ml. Amino acids were added at 20 μg/ml when necessary. Colorimetric screenings for pKAN1 and recombinants were facilitated by the addition of 40 μg of 3-bromo-4-chloro-3-indolyl-β-D-galactopyranoside per ml and 48 μg of iso-propyl-β-D-thiogalactopyranoside per ml to LB medium.

Selection of spontaneously arising sulfathiazole-resistant *E. coli* strains by the method of Siskind (32) was described previously (21). Because the growth of *E. coli* in the presence of sulfathiazole is dependent on the size of the inoculum and sulfathiazole is depleted from the medium over time, we established a standard method for determining the MIC of sulfathiazole for resistant strains. Cultures grown overnight were diluted 10⁻² in 0.9% saline, and 0.1 ml was spread onto plates containing minimal medium and different concentrations of sulfathiazole. Plates were incubated at 37°C and were scored for growth after 48 h, although plates containing minimal medium and different concentrations of sulfathiazole. Plates were incubated at 37°C and were scored for growth after 48 h, although colonies would continue to arise upon additional incubation of the plates. The lowest concentration at which no colonies were visible after 48 h was recorded as the MIC. The concentrations of sulfathiazole typically tested were 0, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, 14, and 16 μg/ml.

*E. coli* strains containing cotransductional markers for *folP* were constructed by transduction with bacteriophage P1 (18) by using as donors phage lysates derived from several strains containing Tn10 near 70 min (31). *E. coli* BN122 and BN123 were infected, and approximately 20 Tc⁺ recipients from each transduction were infected, and approximately 20 Tc⁺ recipients from each transduction experiment were selected with the aid of the program Primer, version 2 (Scientific and Educational Software). The primers used for amplification were 5'-TAG TCCAAGGGCTCTCCGTGTC-3' (for the 5' end of *folP*) and 5'-GATGGCCG ATCACACTGTGAT-3' (for the 3' end of *folP*). Amplification was carried out for 29 cycles with the following specific temperatures and times: denaturation at 94°C for 2.0 min, annealing at 61°C for 1.5 min, and extension at 72°C for 1.5 min. A 10-min extension at 72°C was carried out after 29 cycles of amplification. PCR products were purified with Geneclean (Bio 101) and were analyzed on a 0.8% agarose gel. A single PCR amplification product was obtained by using these conditions.

Direct PCR sequencing was performed according to the protocol supplied with the Sequenase PCR Sequencing kit purchased from United States Biochemicals.

**Enzyme assays.** DHPs assays were carried out as described by Richey and Brown (27). 6-Hydroxymethylpterin (Sigma Chemical Co.) was reduced by the method of Blijkens (5). 10°C-PABA was purchased from Amersham. 4-Amino-4-deoxychorismate synthase and 4-amino-4-deoxychorismate lyase activities were determined as described by Nichols et al. (22).

### RESULTS

Identification of *folP* mutations in two sulfathiazole-resistant *E. coli* strains. Spontaneously occurring sulfathiazole-resistant derivatives of *E. coli* BN102 were obtained by prolonged selection as described previously (21, 32), and two of the most resistant strains were chosen for analysis of the mutations contributing to resistance. The sulfathiazole MICs for *E. coli* BN122 (previously designated BN102sr-122 [21]) and BN123 were determined to be 10 and 14 μg/ml, respectively. The sulfathiazole MIC for the parent strain, *E. coli* BN102, was 0.25 μg/ml. Since each resistant strain had more than one mutation that contributed to sulfathiazole resistance (21) (see below), we first sought to study the role of *folP* mutations both in isolation from and in combination with the endogenous host backgrounds.

Each resistant strain was shown to express a sulfathiazole-resistance determinant (see below), and each had additional mutations that contributed to sulfathiazole resistance.

#### TABLE 1. *E. coli* strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference or derivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>BN102</td>
<td>trpA33 cysG (am) purU tyrT Tc⁺</td>
<td>BN112</td>
</tr>
<tr>
<td>BN122</td>
<td>folP122 ssu (brc) cysG (am) purU tyrT Tc⁺</td>
<td>BN112 × CAG12072</td>
</tr>
<tr>
<td>BN123</td>
<td>folP123 ssu trpA33 cysG (am) purU tyrT Tc⁺</td>
<td>BN122 × CAG12072</td>
</tr>
<tr>
<td>BN1038</td>
<td>folP122 zha-203::Tn10 ssu (brc) cysG (am) purU tyrT Tc⁺</td>
<td>BN123 × CAG12072</td>
</tr>
<tr>
<td>BN1039</td>
<td>folP122 zha-203::Tn10 ssu trpA33 cysG (am) purU tyrT Tc⁺</td>
<td>BN123 × CAG12072</td>
</tr>
<tr>
<td>BN1041</td>
<td>folP123 zha-203::Tn10 ssu trpA33 cysG (am) purU tyrT Tc⁺</td>
<td>BN123 × CAG12072</td>
</tr>
<tr>
<td>BN1042</td>
<td>folP122 zha-203::Tn10</td>
<td>MG1655 × BN1039</td>
</tr>
<tr>
<td>BN1057</td>
<td>folP123 zha-203::Tn10</td>
<td>BN123 × CAG12099</td>
</tr>
<tr>
<td>BN1066</td>
<td>folP123 zha-203::Tn10</td>
<td>BN123 × CAG12099</td>
</tr>
<tr>
<td>BN1079</td>
<td>folP122 zha-203::Tn10</td>
<td>MG1655 × BN1066</td>
</tr>
<tr>
<td>MG1655</td>
<td>Prototroph</td>
<td>31</td>
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<tr>
<td>CAG12072</td>
<td>zha-203::Tn10</td>
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</tr>
<tr>
<td>CAG12153</td>
<td>zhc-6::Tn10</td>
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<tr>
<td>CAG12099</td>
<td>zef-3129::Tn10</td>
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</tr>
<tr>
<td>CAG18451</td>
<td>zed-3069::Tn10</td>
<td>31</td>
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</table>
resistant DHPS (21) (Fig. 1). Derivatives of E. coli BN122 and BN123 that replaced the folP122 and folP123 alleles with wild-type folP (e.g., E. coli BN1038) were constructed by transduction with bacteriophage P1. In addition, wild-type backgrounds containing only the mutant folP alleles were constructed. As shown in Fig. 1, strains that received a wild-type folP allele produced a sulfathiazole-sensitive DHPS (50% inhibitory concentration, 2.2 μg/ml), and those that received a mutant allele produced a sulfathiazole-resistant DHPS (50% inhibitory concentration, 18 μg/ml). The phenotypic resistance to high levels of sulfathiazole correlated with the presence of a sulfathiazole-resistant DHPS in the E. coli BN122 and BN123 backgrounds (Table 2), but the presence of the folP122 or folP123 alleles in the E. coli MG1655 wild-type background resulted in only a low level of phenotypic resistance to sulfathiazole, even though a resistant DHPS was expressed.

**Nucleotide sequences of folP mutations.** A 1,065-bp DNA fragment containing the entire coding region of folP was amplified by PCR from strains containing wild-type and mutant folP alleles. The entire folP gene was sequenced directly from amplification present in the various databases by using the BLAST program (24) indicated a match with E. coli bcr, a gene isolated by its ability to confer resistance to bicyclomycin when it is overproduced (3). In the previously reported sequence, the predicted initiation site was equivalent to the Met at our position 22, and amino acid 99 was Val instead of our Asp (the previously reported nucleotide and amino acid sequences are available in database entries Gen-Bank U00008 and Swiss-Prot P28246, respectively). The product of sur (ber) is similar to that of the family of proton-motive force-dependent drug-H⁺ antagonists.

The reduction of amplification of sur in the E. coli BN122 background from four copies to one copy resulted in a decrease in the MIC from 10 to 4 μg/ml, consistent with the results reported previously (21). The presence of pBNSu'B in E. coli BN102 increased the MIC from 0.25 to 1 μg/ml. The presence of pBNSu'B in E. coli BN1042 (folP122) similarly altered the MIC for that strain. Thus, the increased gene dosage of sur (ber) either by chromosomal amplification or by the presence on a multicopy plasmid contributed a 2.5- to 4-fold increase in sulfathiazole resistance.

In order to test the function of the sur (ber) product, sur (ber) was interrupted with a Kn cassette and crossed into the chromosome of several strains. Disruption of sur (ber) in E. coli BN102 and BN123 had no effect on the phenotype of the strains, but disruption of sur (ber) in BN122 proved difficult, because recipients of the Kn cassette contained both the disrupted and complete copies of sur (ber) due to the amplification of the sur (ber) locus. Strains with a single copy of the interrupted sur (ber) locus were obtained after several generations of nonselective growth on rich medium containing kanamycin, and when transferred to minimal medium, these strains displayed a requirement for adenine. The adenine auxotrophy was mapped to 11 to 16 min by Hfr mating and to Kohara phage λE7(157) (14) by lytic complementation tests. Since this phage contained the purEK genes (30), we confirmed the complementation by subcloning a 3.0-kb BglII DNA fragment containing purEK (38) and demonstrated its ability to complement the adenine auxotrophy revealed by the sur (ber) interruption.

**Stability of pBNSu'B.** Like many plasmids that overproduce integral membrane proteins similar to proton motive force-dependent antiporters, pBNSu'B proved to be unstable in E. coli BN102 and its derivatives. The instability was observed as

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**TABLE 2. Phenotypic effects of folP alleles in various backgrounds**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Derivation</th>
<th>MIC (μg/ml)</th>
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</thead>
<tbody>
<tr>
<td>BN102</td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>MG1655</td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>CAG12072</td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>BN122</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>BN1039</td>
<td>BN122, zha-203::Tn10</td>
<td>12</td>
</tr>
<tr>
<td>BN1038</td>
<td>BN122, folP+/zha-203::Tn10</td>
<td>2</td>
</tr>
<tr>
<td>BN1042</td>
<td>MG1655, folP122</td>
<td>1</td>
</tr>
<tr>
<td>BN123</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>BN1041</td>
<td>BN123, zha-203::Tn10</td>
<td>14</td>
</tr>
<tr>
<td>BN1040</td>
<td>BN123, folP+/zha-203::Tn10</td>
<td>1</td>
</tr>
<tr>
<td>BN1083</td>
<td>MG1655, folP123</td>
<td>1</td>
</tr>
<tr>
<td>BN1057</td>
<td>BN123, sus/zef-3129::Tn10</td>
<td>4</td>
</tr>
<tr>
<td>BN1079</td>
<td>MG1655, sus123 zef/zef-3129::Tn10</td>
<td>1</td>
</tr>
</tbody>
</table>
a differential plating efficiency on LB-ampicillin and minimal-
aminopterin plates following transformation with pBNSu'B. Sup-
plementation of minimal medium plates revealed that either
PABA (1 μg/ml) or methionine (20 μg/ml) was capable of
increasing the plating efficiency of freshly transformed cells at
least 100-fold. Isolation of chromosomal mutations capable of
increasing E. coli BN102/pBNSu'B transformation efficiency
took advantage of the fact that E. coli BN102 transformed with
pBNSu'B and plated on LB-ampicillin produced two distinct
 colony sizes. When strains cured of plasmid were retrans-
mformed with pBNSu'B and plated on minimal-aminopterin and
minimal-aminopterin plates, strains derived from the smaller
colonies produced equal numbers of colonies on both LB-ampi-
cillin and minimal-aminopterin plates, while strains derived from
the larger colonies still showed differential plating efficiencies.
Six strains selected for increased plating efficiency were
tested for elevated levels of PABA or methionine synthesis.
One of the strains conferred resistance to 40 mM ethionine,
whereas the parental strain, E. coli BN102, was resistant to 20
mM ethionine, indicating elevated levels of synthesis of methi-
onine (11). Two of the strains were resistant to 70 μg of
sulfanilamide per ml, while the parent strain was resistant to 20
μg of sulfanilamide per ml, suggesting an increase in the level of
PABA biosynthesis. The levels of PABA biosynthetic en-
zymes in extracts of the sulfanilamide-resistant strains were
determined, and each sulfanilamide-resistant strain had a two-
fold increase in the relative specific activity of 4-amino-4-de-
oxychorismase synthase CoI (PabB) compared to that for the
parental strain. Three additional strains did not display elev-
ated levels of PABA or methionine, suggesting that pBNSu'B
stability could be affected by additional mutations.

An additional mutation contributing to sulfathiazole resis-
tance in E. coli BN123. A sulfathiazole resistance determinant
distinct from sur (bcr) was mapped to 44 min in E. coli BN123
by Hfr mating and cotransduction analysis. Replacement of the
new determinant, designated sur, with its wild-type allele re-
sulted in a 2.5-fold decrease in sulfathiazole resistance. In a
wild-type background, the sur-123 allele increased the level of
sulfathiazole resistance fourfold (Table 2). The effect of sur
appeared to be mediated through a balance of tetrahydrofolate and
its one-carbon derivatives, as determined by differences in
supplements required to relieve the adenine sensitivity con-
ferred upon these strains by their purU defect (19, 20). A
differential response to histidine was observed during adenine
inhibition in the otherwise isogenic sur-123 and sur strains (data
not shown). Because the sur mutation lay close to or in
his and it had affects on the histidine response to adenine
sensitivity consistent with alterations in histidine regulation, we
suggest that sur was likely to be a mutation that affected the
regulation of histidine biosynthesis.

**DISCUSSION**

We have selected E. coli strains for sulfathiazole resistance
and have shown that each contains a resistant DHPS. Two
independently isolated mutant folP alleles each contained the
same Pro64→Ser amino acid substitution. The site of the sub-
stitution lay within a highly conserved sequence block,
GGSTRPG-, which is identical among E. coli, Bacillus sub-
tilis, Neisseria meningitidis, and Streptococcus pneumoniae (9,
26, 33). The Pro64→Ser mutation is distinct from two other
mutations reported in E. coli folP, each of which was selected
for sulfathiazole resistance and simultaneous temperature sen-
sitivity. Both of the ts mutations lie at amino acid position
Phe28 but have different substitutions, Leu (9) and Ile (10).
While the determination of differences in the kinetic constants
of the Pro64→Ser enzyme is in process, we have not noted a
significant difference in the specific activity of the DHPS
present in crude extracts prepared from wild-type or mutant
strains (data not shown).

Pro64 is in a flexible loop of the protein that forms a portion
of the binding site for PABA and sulfanilamide, with the gua-
nidinium group of Arg63 forming a hydrogen bond with one of
the oxygens of sulfanilamide (1). Replacement of the chain-
bending proline with serine is likely to alter the structure of the
α-carbon chain in this region and thus alter the ability of the
Arg63 side chain to make stable contact with the inhibitor.

The conserved region from position 58 to 65 is also strongly
conserved in the DHPS domains of multifunctional enzymes
from Plasmodium falciparum (6, 35) and Pneumocystis carinii
(37), and amino acid substitutions at positions 62 and 63, as
well as other positions, have been found in sulfadoxine-resis-
tant strains of P. falciparum (6, 35). Interestingly, only G58 and
P64 are conserved in the highly resistant sulf- and sulfIII-en-
coded DHPS enzymes present on several R determinants.
While amino acid substitutions and duplications at other sites
within DHPS have been established as being responsible for
sulfonamide resistance in a variety of organisms, the combined
data also implicates the region between positions 58 and 65 as
an important site for substitutions that alter inhibitor or sub-
strate recognition.

Although the mutant chromosomal folP allele conferred a
fourfold increase in the MIC of sulfathiazole, the low level of
resistance is surprising in light of the fact that a resistant DHPS
has been characterized as the major mechanism of the acqui-
sition of resistance to sulfonamides in E. coli. High-level resis-
tance has been observed in E. coli by introduction of low-copy-
number R-determinant plasmids, indicating that resistance can
be attributed solely to a resistant DHPS. It is possible that
significant resistance can be achieved only if the regulation of
DHPS expression is uncoupled from its chromosomal context.

The prolonged selection procedure that we have used to
obtain *E. coli* strains resistant to high levels of sulfathiazole has resulted in several resistant strains with secondary mutations, that is, mutations outside of *folP*. One of the secondary mutations that we have characterized extensively is *sur* (*bcr*), originally detected as a determinant present on an amplified segment of the chromosome of *E. coli* BN122 (20). We have determined that the cloned copy of *sur* is identical to the wild-type gene and that it is identical to *bcr*, whose overexpression has also been correlated with bicyclocycin resistance (3). The *sur* (*bcr*) product is a member of the major facilitator family of membrane translocases (15). Several members of this family are proton motive force-dependent drug-**H** -antiporters, and it may be that *sur* (*bcr*) functions in an analogous manner, effluxing sulfathiazole from the cell. While a portion of the observed instability of pBNsuaB was very likely due to overexpression of an integral membrane protein, it is of interest that an end product (methionine) and an intermediate (PABA) of folate metabolism could stabilize the plasmid in strains grown on minimal medium. It is possible that PABA or another intermediate in folate biosynthesis, in addition to sulfathiazole, was also an efflux substrate for *sur* (*bcr*). It is not clear why the cloned *sur* (*bcr*), in conjunction with *folP122* in a wild-type background, did not elevate the level of resistance to that of *E. coli* BN122. It may be that the strains were compromised due to overexpression of the membrane protein or that additional mutations that are necessary for the manifestation of high levels of resistance to sulfathiazole in conjunction with elevated levels of *sur* (*bcr*) are present in *E. coli* BN122.

Another mutation, designated *sux*, has been detected in *E. coli* BN123. *sux* is distinct from *folP* and *sur* (*bcr*) and has been determined to play a large role in the resistance of *E. coli* BN123 to sulfathiazole. As with *folP* and *sur* (*bcr*), *sux* does not confer significant levels of resistance when taken out of the context of the *E. coli* BN123 background, suggesting that it, too, requires the context within which it arose to exert its maximum effect on sulfathiazole resistance.

The secondary mutations *sur* (*bcr*) and *sux* both affected sulfathiazole resistance primarily by influencing the metabolic pathways dependent upon one-carbon metabolism, especially the purine biosynthetic pathway. This result may in turn be influenced by the presence of the *purU* lesion in the parental strain. Further investigation of the role of secondary mutations in sulfathiazole resistance is under way with strains not initially compromised in their one-carbon metabolism.

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REFERENCES


27. Stock, J., D. P. Stahlby, C.-Y. Han, E. W. Six, and I. P. Crawford. 1990. An...


