Gene Amplification Contributes to Sulfonamide Resistance in *Escherichia coli*

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A sulfathiazole-resistant strain of *Escherichia coli* was isolated and shown to contain a fourfold tandemly amplified segment of DNA 18 kilobase pairs in length in addition to a mutationally altered dihydropteroate synthase, the target enzyme for sulfonamide inhibition. The amplified DNA contained a gene designated *sur* that contributed to sulfathiazole resistance when present in greater amounts than those in the wild type. Sulfathiazole resistance was markedly decreased upon loss of the amplified DNA after nonselective growth. Plasmids that contained *sur* also conferred only weak sulfathiazole resistance on wild-type strains. Comparison of the restriction maps of the amplified DNA, wild-type DNA, and *sur*-containing plasmids showed that a DNA rearrangement occurred before or concomitant with the DNA amplification event. The DNA rearrangement resulted from an IS5 insertion, which, in conjunction with an IS5 element residing near *sur* in the wild-type strain, resulted in an -IS5-*sur*-IS5- configuration. Homologous recombination could account for duplication and subsequent amplification of the *sur* region. High-copy-number plasmids containing the *sur* locus did not express a sulfathiazole-resistant dihydropteroate synthase, nor did they overexpress wild-type dihydropteroate synthase. These data suggest that the high level of sulfathiazole resistance in this strain results from a synergistic effect of two different mutations.

Sulfonamide antibiotics prevent the biosynthesis of reduced folate compounds by inhibiting the production of 7,8-dihydropteroate (H_2 pteroate) from 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine (H_2 pterin) pyrophosphate and *p*-aminobenzoate (PABA) by H_2 pteroate synthase (3). Sulfathiazole and other sulfonamide analogs compete with PABA for enzymatic condensation with the H_2 pterin substrate (3, 23). The H_2 pterin-sulfonamide adducts are not in themselves inhibitory to cellular function and passively diffuse out of the cell (23). However, H_2 pteroate synthase is inhibited from using PABA, and the cell becomes depleted for both H_2 pterin and H_2 pteroate, the precursors of 7,8-dihydrofolate and 5,6,7,8-tetrahydrofolate. Cell growth is thus inhibited by lack of the vitamin cofactor necessary for the biosynthesis of purines, thymidine, several amino acids and vitamins, and *N*-formylmethionyl-tRNA (1, 6).

A common mode of resistance to sulfonamide antibiotics in *Escherichia coli* is via mutation of the structural gene encoding H_2 pteroate synthase, so that the altered enzyme product is sulfonamide resistant and discriminates more effectively between PABA and sulfonamides than does the wild-type enzyme (19, 32). For example, Swedberg et al. (32) isolated a sulfonamide-resistant strain of *E. coli* that was also temperature sensitive for growth. This strain produced a temperature-sensitive H_2 pteroate synthase that had a 10-fold higher K_m for PABA and a 150-fold higher K_m for sulfathiazole compared with those of the wild-type enzyme.

Sulfonamide resistance can also be conferred by several of the R-determinant plasmids found in *E. coli* strains (35). Sköld (29) showed that these plasmids complement the temperature-sensitive H_2 pteroate synthase described above at nonpermissive temperatures and express a sulfonamide resistance form of H_2 pteroate synthase. The sulfonamide resistant H_2 pteroate synthases encoded by plasmids R1 and R6-5 are about 10% smaller than the *E. coli* enzyme (M_r ,

45,000) and are resistant to 10^4 -fold higher concentrations of sulfathiazole. The K_m for PABA is approximately the same for the plasmid-encoded and chromosomal-encoded enzymes (33).

Apparently not all sulfonamide-resistant *E. coli* strains produce a resistant H_2 pteroate synthase. Pato and Brown (19), in one of the early studies on sulfonamide resistance in *E. coli*, found that one of their resistant strains did not contain a resistant enzyme. They hypothesized that sulfonamide resistance might also arise from alterations in cell permeability that reduce sulfonamide uptake. Subsequent experiments concerning permeability differences and sulfonamide resistance have yielded contradictory results (28). However, since both PABA and sulfonamides freely diffuse in and out of *E. coli* cells and do not seem to be actively transported (23), it seems unlikely that uptake or permeability mutations could contribute a major component to sulfonamide resistance.

We have isolated a sulfathiazole-resistant strain of *E. coli* that contains a DNA amplification in addition to a mutationally altered H_2 pteroate synthase gene. The amplified DNA enhances the level of resistance compared with that in strains that have lost the amplified DNA. Plasmid clones containing DNA from the amplification confer only weak sulfathiazole resistance on wild-type strains, suggesting either an interaction between the amplified gene product and an altered H_2 pteroate synthase or a synergistic effect of two types of resistance mechanisms. The association of IS5 with each endpoint of the amplified segment suggests a role for IS5 transposition and homologous recombination in the amplification mechanism.

MATERIALS AND METHODS

Bacterial strains. *E. coli* W3110 (*trpA33*) was made nalidixic acid resistant as described by Miller (18). The resulting strain, designated *E. coli* BN102, was used as the parental strain for selecting sulfonamide-resistant mutants. *E. coli*

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LE392 (*trpR55 metB1 hsdR514 galK2 galT22 lacY1 supE44 supF58*) was routinely used as the plasmid host. *E. coli* JM103 (Δ [*lac-pro*] *thi strA supE endA sbcB15 hsdR4/F' traD36 proAB lacI^q lacZM15*) was used as the M13 host strain (17).

Selection of sulfathiazole-resistant mutants. Spontaneous sulfathiazole-resistant mutants were isolated essentially as described by Sköld (29). VB minimal medium (100 ml) containing 40 μ g of tryptophan, 20 μ g of nalidixic acid, and 20 μ g of sulfathiazole per ml was inoculated with 10^7 to 10^8 cells of *E. coli* BN102 and incubated at 37°C with shaking until cultures had reached saturation. Most cultures required 10 to 16 days of incubation before growth was evident. Since only a few resistant cells in a population can deplete the sulfathiazole present in the medium, several further enrichment steps were required to obtain pure cultures. Cultures containing sulfathiazole-resistant mutants were diluted 1/500 into 5 ml of minimal tryptophan medium containing 5, 10, 15, or 20 μ g of sulfathiazole per ml and grown at 37°C with shaking. Cultures from the tube with the highest antibiotic concentration that had reached saturation after 2 days of growth were reinoculated into a similar series of tubes. After 2 days of growth, dilutions were made from the tube with the highest sulfathiazole concentration that contained a saturated culture and plated on solid media containing 2.5 or 5 μ g of sulfathiazole per ml. Single colonies that appeared after 2 to 3 days of incubation at 37°C were picked and streaked twice more on plates containing 2.5 μ g of sulfathiazole per ml. A single colony was chosen from the final plate, grown in 5 ml of minimal-tryptophan medium containing 5 μ g of sulfathiazole per ml, and stored in 50% glycerol at -20°C.

The level of resistance of each strain was estimated by the size of the zone of inhibition around a filter disk containing various amounts of sulfathiazole. Cultures were grown overnight in 5 ml of minimal tryptophan medium containing 5 μ g of sulfathiazole per ml. Cells were centrifuged and suspended in 5 ml of 0.85% saline, and 0.1 ml was added to molten soft agar and plated on minimal plates containing tryptophan. After the agar had cooled, a 13-mm filter disk was placed at the center of the plate, and 25 μ l of a solution containing 25, 50, or 100 μ g of sulfathiazole was applied to the filter disk. The plates were incubated at 37°C for 18 h, and the zone of inhibition was measured.

DNA manipulations. Restriction endonucleases were either obtained from commercial suppliers or prepared in this laboratory by published procedures. Restriction endonuclease digests were performed according to the recommendations of the supplier. Ligations, transformations, and plasmid preparations were carried out by standard methods (16).

DNA sequence analysis. DNA fragments were cloned into bacteriophage M13 mp8, mp9, mp18, or mp19 (17), and sequences were determined by the Sanger et al. dideoxy-chain termination method (25) as described previously (12).

Construction of *E. coli* libraries. DNA isolated from *E. coli* BN102*sur-122* was digested with *Hind*III or *Bam*HI and ligated to *Hind*III- or *Bam*HI-digested, dephosphorylated pBR322 DNA. After transformation into *E. coli* LE392, ampicillin-resistant colonies were pooled, plasmid DNA was prepared, and *E. coli* LE392 was transformed to sulfathiazole and ampicillin resistance. $\text{Su}^r \text{Ap}^r$ colonies were picked, plasmid DNA was prepared, and *E. coli* LE392 was again transformed to sulfathiazole resistance. Plasmids showing a high frequency of transformation to sulfathiazole resistance were saved for further analysis.

λ EMBL3 arms and packaging extracts were purchased from Stratagene. Libraries were constructed from DNA

prepared from *E. coli* BN102. The *E. coli* DNA was partially digested with *Sau*3AI and fractionated on sucrose gradients as described by Kaiser and Murray (11). DNA fragments between 15 and 22 kilobase pairs (kbp) were collected. Conditions for ligation and packaging were those recommended by Stratagene. Libraries were screened by hybridization with nick-translated plasmid probes (11).

Hybridization techniques. The *sur* gene dosage in *E. coli* BN102*sur-122* and several other mutant strains was quantitated by dot blot hybridization (10). DNA from each strain was sonicated for 15 s (producing fragments <4 kbp in length), and four dilutions of each sample were applied to charged nylon membranes in 0.4 N NaOH. Duplicate filters were hybridized with a 10-fold excess of ^{32}P -labeled probe DNA for 16 h at 65°C. A plasmid carrying the *pabA* gene, pBN*pabA* (13), was used as a single-copy reference sequence because *pabA* maps well away from the amplified region (data not shown). Filters were washed and exposed to X-ray film for several hours for a visual record. The dots were then cut from each filter, placed in scintillation fluid, and counted in a scintillation counter. Southern hybridizations were performed as described by Southern (31).

Enzyme assays. 6-Hydroxymethyl pterin (Sigma Chemical Co.) was reduced by the method of Blakely (2). ^{14}C -labeled PABA was obtained from Amersham Corp. H_2 pteroate synthase assays were performed as described by Richey and Brown (22).

RESULTS

Selection of sulfathiazole-resistant mutants. Ten sulfathiazole-resistant mutants were derived from *E. coli* BN102 (*trpA33 Nal^r*) as described by Sköld (29). The level of sulfathiazole resistance of each mutant strain was estimated by the size of the zone of inhibition around a 13-mm filter disk containing 25, 50, or 100 μ g of sulfathiazole and compared with that for *E. coli* BN102. The strain designated *E. coli* BN102*sur-122* showed the highest level of sulfathiazole resistance of the 10 mutant strains and was chosen for further characterization.

DNA was prepared from several of the sulfathiazole-resistant strains and digested with restriction endonuclease *Bam*HI. DNA from *E. coli* BN102*sur-122* showed three *Bam*HI fragments of unusually high intensity, with sizes of 12.0, 3.9, and 1.8 kbp (Fig. 1). The high intensity of a few specific fragments suggested that a portion of the genome had been amplified in this strain. Digestions with *Bam*HI, *Eco*RI, *Bgl*II, and *Cla*I all showed more than one fragment of high intensity. In each case the sum of the amplified fragment sizes was between 17.5 and 18 kbp. Digestion of DNA with *Hind*III showed a single intense band of approximately 18 kbp. Double digests of chromosomal DNA resulted in a restriction map that was circular in nature, suggesting that the amplified DNA either was organized as tandemly repeated units or existed as an extrachromosomal element. Attempts to isolate extrachromosomal DNA either by alkaline extraction or by ethidium bromide-CsCl density gradient centrifugation were unsuccessful. Additional data presented below confirmed the chromosomal location of tandemly amplified DNA.

Cloning a sulfathiazole resistance determinant. Plasmids containing portions of the amplified DNA were obtained by transforming pBR322 plasmid libraries constructed from *E. coli* BN102*sur-122* DNA into *E. coli* LE392 and selecting for growth in the presence of ampicillin and sulfathiazole. After several days of incubation, colonies were obtained from

FIG. 1. *Bam*HI digests of chromosomal DNA from wild-type *E. coli* and sulfathiazole-resistant mutants containing amplified DNA. Lanes: A, *Hind*III-digested λ DNA; B, *E. coli* BN102 (wild-type) DNA; C, unamplified BN102*sur*-122 DNA; D, BN102*sur*-122 DNA; E, F, and G, reamplified BN102*sur*-122 DNA. Arrows and associated numbers are used to denote sizes (kilobases) of lambda markers and amplified chromosomal DNA segments.

*Bam*HI and *Hind*III libraries. Six identical plasmids were obtained from the *Bam*HI library, and each contained two *Bam*HI fragments that corresponded to the 1.8- and 3.9-kbp fragments present in *Bam*HI digests of amplified chromosomal DNA (Fig. 2). One plasmid was retained and designated pBNSu'B. Attempts to subclone either of the *Bam*HI fragments independently did not yield plasmids conferring sulfathiazole resistance, suggesting that the gene conferring resistance was either interrupted or separated from its expression signals by *Bam*HI digestion. Plasmids isolated from the *Hind*III library contained either 13.5- or 18-kbp inserts. These two plasmids (pBNSu'H and pGG1, respectively) contained both *Bam*HI fragments that were present in

TABLE 1. Quantitation of *sur* dosage in sulfathiazole-resistant strains

Strain	cpm			Normalized copy no.
	pBN <i>pabA</i> (avg)	pBNSu'B (avg)	pBNSu'B/ pBN <i>pabA</i> ratio	
BN102 ^a	836	137	0.164	1.0
<i>sur</i> -122 ^b	460	324	0.704	4.3
<i>sur</i> -1 ^b	744	145	0.195	1.2
<i>sur</i> -2 ^b	614	117	0.191	1.2
<i>sur</i> -7 ^b	563	98	0.174	1.1
<i>sur</i> -8 ^b	927	145	0.156	1.0
<i>sur</i> -9 ^b	799	127	0.159	1.0

^a *E. coli* W3110 *trpA33* *Nal*^r.

^b Sulfathiazole-resistant derivative of *E. coli* BN102.

pBNSu'B, indicating that they were also derived from the amplified DNA.

Correlation of *sur* copy number with antibiotic resistance. The number of copies of the amplified DNA segment in *E. coli* BN102*sur*-122 was determined by dot blot DNA hybridization (10). Chromosomal DNA from the parental strain and several sulfathiazole-resistant mutants was probed with either ³²P-labeled pBNSu'B or pBN*pabA* (13). The normalized ratio of counts per minute of pBNSu'B hybridized to counts per minute of pBN*pabA* hybridized was used for comparisons of *sur* copy number, using the assumption that the *sur* and *pabA* genes were present at one copy each in the parental strain. Table 1 shows the results for several sulfathiazole-resistant strains. The hybridization data indicated that of several resistant strains, only *E. coli* BN102*sur*-122 had an amplification of the *sur* region, and that this strain contained approximately four copies of the *sur* gene.

Strains of *E. coli* BN102*sur*-122 containing various numbers of copies of the *sur* locus were isolated from a population that had been grown without sulfathiazole selection for 150 generations. Loss of copies during nonselective growth suggested that the amplified DNA was organized as tandem repeats. Colonies containing differing copies of *sur* were identified by Southern analysis of *Hind*III-digested chromosomal DNA probed with pBNSu'B. Strains carrying a single copy of *sur* were identified by the absence of hybridization to the 18-kbp DNA fragment characteristic of the tandem

FIG. 2. Restriction maps of chromosomal and cloned *sur* genes. Filled boxes represent IS5 elements. Restriction sites shown for the wild type are present also in each of the derivatives. (a) Restriction map of the wild-type chromosome deduced from double digests of chromosomal DNA and from Southern hybridization data. (b) Restriction map of the *Bam*HI insert in pBNSu'B. (c) Restriction map of the *Hind*III insert in pBNSu'H. The map is drawn to align with the wild-type map, and the position of IS5 is indicated by the triangle. (d) Restriction map of wild-type DNA present in λ EMBL3 *surC2*. (e) Structure of the tandem repeating unit in the amplified mutant, illustrating flanking IS5 elements.

FIG. 3. Correlation of loss of sulfathiazole resistance with loss of amplified DNA after growth in nonselective medium. The zone of inhibition is measured as the distance from the edge of a disk containing 50 μ g of sulfathiazole to the edge of the clear zone of growth inhibition. The point marked WT on the abscissa refers to the wild-type level of resistance measured for *E. coli* BN102.

amplification (data not shown) and the presence of a 13.5-kbp *Hind*III fragment representing a single *sur* copy. Strains carrying two copies of *sur* were identified by equal hybridization intensities of 18- and 13.5-kbp DNA fragments. Strains with three copies contained an 18-kbp fragment that hybridized with twice the intensity of the 13.5-kbp fragment.

Determination of the resistance of the strains containing different *sur* copy number by zone of inhibition (Fig. 3) indicated that resistance decreased as the number of *sur* copies decreased and that maximum resistance was obtained with three to four copies of *sur* present. However, the sulfathiazole susceptibility of strains containing a single copy of *sur* did not reach the wild-type level of *E. coli* BN102, suggesting that additional factors besides *sur* amplification contributed to sulfathiazole resistance in *E. coli* BN102*sur*-122.

***sur* does not encode H₂pteroate synthase.** To determine whether the amplified segment of DNA encoded H₂pteroate synthase, crude cell extracts were prepared from *E. coli* BN102, BN102*sur*-122, and BN102 containing pBNSu'B or pBNSu'H, and H₂pteroate synthase activity was measured (22) in the presence of increasing concentrations of sulfathiazole. The parent strain, *E. coli* BN102, as well as the parent containing plasmids pBNSu'B or pBNSu'H expressed a sulfathiazole-sensitive H₂pteroate synthase (Fig. 4). In contrast, *E. coli* BN102*sur*-122 expressed a sulfathiazole-resistant H₂pteroate synthase, and the expression of the resistant enzyme was not affected by loss of the amplified DNA (data not shown). Furthermore, the specific activity of H₂pteroate synthase was similar in each crude extract tested. These data suggested that *sur* did not encode H₂pteroate synthase, nor did it affect the level of H₂pteroate synthase expression. Therefore, *sur* amplification seems likely to have enhanced sulfathiazole resistance by a mechanism unrelated to over-expression of a wild-type or mutationally altered H₂pteroate synthase.

IS5 insertion sequences are present at the junctions of the amplified DNA. The identical DNA amplification that was present in *E. coli* BN102*sur*-122 could be reestablished in strains that had lost the amplification by selecting for increased resistance to sulfathiazole. *E. coli* BN102*sur*-122 derivatives containing a single copy of the *sur* locus were subjected to the same procedure used to isolate the original

FIG. 4. Sulfathiazole sensitivity of H₂pteroate synthases from *E. coli* BN102 (●), BN102*sur*-122 (○), BN102(pBNSurB) (X), and BN102(pBNSurH) (*).

mutants. Resistant strains were obtained after only 2 days of growth in media containing 10 μ g of sulfathiazole per ml. DNA was prepared from representative mutants, and the presence and extent of amplification in each were determined by agarose gel electrophoresis after digestion with *Bam*HI. Three newly isolated mutants each contained amplified DNA, and the extent of the amplification was similar to that in *E. coli* BN102*sur*-122 (Fig. 1). Quantitative dot blot determinations indicated that the level of amplification (four or five copies) in each new mutant strain was also similar to that found in *E. coli* BN102*sur*-122 (Table 2). Attempts to isolate additional amplification mutants directly from *E. coli* BN102 were unsuccessful, even when a kanamycin cassette was located near *sur* and selection was for increased kanamycin resistance. These data suggested that *E. coli* BN102*sur*-122 had undergone an unusual or rare event that allowed reisolation of the same DNA amplification after loss of the original copies.

To investigate the nature of the novel junctions of the amplified DNA, restriction maps of wild-type, amplified, and cloned DNA were compared. Wild-type restriction maps were constructed from λ EMBL3 clones isolated by hybridization to pBNSu'B and from chromosomal digests probed with pBNSu'B. The restriction map of a 20-kbp segment of

TABLE 2. Quantitation of *sur* dosage in unamplified and reamplified derivatives of BN102*sur*-122

Strain	cpm			Normalized copy no.
	pBNpabA (avg)	pBNSu'B (avg)	pBNSu'B/pBNpabA ratio	
BN102	89	150	1.7	1.0
<i>sur</i> -122(1u) ^a	93	149	1.6	1.1
<i>sur</i> -122(1r) ^b	91	573	6.3	3.9
<i>sur</i> -122(2r) ^b	88	698	7.9	4.9
<i>sur</i> -122(3r) ^b	93	741	8.0	5.0

^a Unamplified derivative of BN102*sur*-122.

^b Reamplified derivative of BN102*sur*-122.

FIG. 5. Autoradiogram of a Southern hybridization experiment with the ^{32}P -labeled 6.6-kbp *Bgl*II fragment as a probe hybridized to *Hind*III digests of *E. coli* BN102 genomic DNA (a), *E. coli* BN102*sur*-122 genomic DNA (b), and pBNSu'H plasmid DNA (c).

the wild-type chromosome corresponding to the amplification is illustrated in Fig. 2a. The restriction map of the wild-type chromosome was colinear with the map of amplified DNA and pBNSu'B. However, portions of the pBNSu'H map were not colinear with either the wild-type chromosome or amplified DNA, suggesting that pBNSu'H contained a novel junction between amplified and single-copy DNA or had undergone a rearrangement during the cloning procedures.

The DNA insert of pBNSu'H was identical to a chromosomal *Hind*III fragment of *E. coli* BN102*sur*-122 containing the novel junction between the amplified DNA and single-copy sequences of the chromosome. Figure 5 shows an autoradiogram of a Southern hybridization experiment with the ^{32}P -labeled 6.6-kbp *Bgl*II fragment derived from pBNSu'H hybridized to a *Hind*III digest of *E. coli* BN102 (parental), *E. coli* BN102*sur*-122 (amplified mutant), and pBNSu'H DNA. In the parental strain, a single *Hind*III fragment of approximately 12 kbp hybridized to the probe. In the amplified mutant, the 18-kbp DNA fragment representing multiple copies of the amplified segment hybridized very strongly to the probe, whereas the single-copy fragment containing one of the amplification junctions hybridized less strongly. The single-copy junction fragment was 13.5 kbp in length and aligned precisely with the insert present in pBNSu'H. The cloned fragment present in pBNSu'H therefore represented the junction fragment between the amplified and single-copy sequences, and this junction fragment contained a DNA insertion relative to the homologous fragment present in the unamplified parental chromosome.

When ^{32}P -labeled pBNSu'H was used as a hybridization probe to chromosomal DNA digests, the probe hybridized to multiple DNA fragments in each restriction endonuclease digest, suggesting the presence of a repetitive DNA sequence, most likely an insertion element. The position of the repetitive element in pBNSu'H was determined by using various fragments as hybridization probes to digests of chromosomal DNA. The results (not shown) indicated that the repetitive element was primarily contained within the 950-bp *Eco*RI-*Bgl*II fragment indicated in Fig. 2.

The repetitive element was identified as IS5 by DNA sequence analysis. The 550-, 950-, and 1,150-bp *Eco*RI-*Bgl*II DNA fragments from pBNSu'H were cloned into *Eco*RI- and *Bam*HI-digested M13 mp8 and M13 mp9 (17), and oppositely oriented fragments were identified by hybridization of several M13 mp8 and M13 mp9 clones. DNA sequences were determined by the Sanger et al. method (25) for about 250 bp from each site. Comparison of these sequences with the *E. coli* DNA sequences contained in GenBank (version R35.0, March 1986) identified several of the sequences as portions

FIG. 6. Southern hybridization of the ^{32}P -labeled 950-bp *Eco*RI-*Bgl*II IS5 fragment of λ EMBL*sur*C2 to digests of λ EMBL*sur*C2 and pBNSu'H. Lanes: a and b, ethidium bromide-stained agarose gel of *Eco*RI- and *Bgl*II-digested λ EMBL*sur*C2 and pBNSu'H, respectively; c and d, autoradiogram of IS5 fragment hybridized to *Eco*RI- and *Bgl*II-digested λ EMBL*sur*C2 and pBNSu'H, respectively.

of the insertion sequence IS5 (5, 14, 26). DNA sequences from the *Eco*RI site of the 550-bp fragment and from the *Bgl*II site of the 1,150-bp *Eco*RI-*Bgl*II fragment identified the 16-bp inverted repeat sequences and the four-base target duplication (CTAA) characteristic of IS5 insertion (5).

The *Eco*RI and *Bgl*II sites present within IS5 also aligned with *Eco*RI and *Bgl*II sites in the wild-type chromosome at the "left" side of the amplified sequences. Restriction sites for *Eco*RI, *Bgl*II, and *Cla*I at the left junction were completely colinear in the wild type and the amplification mutant and suggested that the wild-type strain contained an IS5 element at this position. This conclusion was verified by using the 950-bp *Eco*RI-*Bgl*II fragment from λ EMBL3 *sur*C2 as a hybridization probe for sequences in pBNSu'H. The probe hybridized to IS5 sequences in pBNSu'H (Fig. 6) and established the presence of IS5 at both boundaries of the amplified DNA.

DISCUSSION

Sulfonamide resistance in *E. coli* has been previously characterized as the acquisition of a gene encoding a sulfonamide-resistant H_2 pteroate synthase either by mutation of the chromosomal H_2 pteroate synthase gene (19, 33) or by acquisition of an extrachromosomal element containing a resistant H_2 pteroate synthase gene (29). The latter mechanism is so prevalent that two different H_2 pteroate synthase genes have been identified on broad-host-range plasmids, suggesting that plasmids carrying sulfonamide resistance determinants have evolved at least twice (34). We isolated an *E. coli* strain with two independent mutations, an alteration of the H_2 pteroate synthase gene, and an amplification of a gene we designated *sur*. That some of the sulfathiazole-resistant strains we isolated contain two mutations is not surprising, given the protracted selection procedures necessary to obtain and isolate resistant strains.

Although the amplified *sur* gene contributes a major portion of the sulfathiazole resistance in *E. coli* BN102*sur*-122, the presence of *sur* on high-copy-number plasmids does not by itself confer a high degree of resistance on susceptible strains of *E. coli*. This suggests that the amplified product of *sur* and the mutationally altered H₂pteroate synthase in the same strain cooperate synergistically to confer higher levels of resistance than either mutation alone. A similar phenomenon has been reported in *Streptococcus pneumoniae*. A sulfonamide-resistant *S. pneumoniae* strain containing three different mutations, including an altered H₂pteroate synthase, was isolated (8). However, cloned DNA containing the altered H₂pteroate synthase gene by itself did not confer sulfonamide resistance (15). It may be that at least two different mutations, and possibly more, are required for sulfonamide resistance to occur in *E. coli* as well *S. pneumoniae*. If this is true, then it may be that other resistant strains in our collection contain multiple mutations, although only *E. coli* BN102*sur*-122 appears to have a DNA amplification.

Since resistance to other antifolates such as methotrexate or trimethoprim is frequently associated with the amplification or overproduction of the target enzyme dihydrofolate reductase (27, 30), we tested the possibility that the amplified *sur* locus resulted in increased expression of H₂pteroate synthase. We found that reducing the copy number of *sur* in *E. coli* BN102*sur*-122 did not affect the expression of the mutationally altered H₂pteroate synthase and that plasmids containing *sur* did not specify an altered H₂pteroate synthase or affect the expression of H₂pteroate synthase in wild-type strains. The mechanism of the contribution to sulfathiazole resistance by *sur* is so far undetermined.

The DNA amplification responsible for increased *sur* dosage has been characterized as a tandem repeat 18 kbp in length, with IS5 sequences at both ends and at the novel junction. One IS5 sequence was found to reside in the wild-type chromosome near *sur*, and a second copy found in the amplified strain has been newly transposed to this site. A logical mechanism for the DNA amplification event would involve (i) transposition of a new IS5 element to the site on the opposite side of *sur* from the resident element, (ii) homologous recombination between the two directly oriented IS5 sequences to establish a duplication, and (iii) homologous recombination between any two portions of the duplication to establish higher orders of amplification. This model is supported by experiments that show identical reamplification after loss of the original amplified DNA.

The *sur* amplification differs from the type of illegitimate amplifications characterized in *E. coli* for *ampC* (4) and for *lacZ* (36). In these cases, the boundaries of the amplified DNA are characterized by short (6- to 12-bp) homologous sequences. The DNA amplification we have observed is more similar to that observed in r-determinant amplification (transition) involving recombination between insertion sequences (7, 20, 21, 24, 35). The role of transposon-mediated DNA rearrangements associated with DNA amplification has also been observed in P1 plasmids carrying Tn2653 (9).

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