JOURNAL OF BACTERIOLOGY, Sept. 1987, p. 4320–4326
0021-9193/87/094320-07$02.00/0

Vol. 169, No. 9

Sulfonamide Resistance in *Streptococcus pneumoniae*: DNA Sequence of the Gene Encoding Dihydropteroate Synthase and Characterization of the Enzyme

PALOMA LOPEZ,[1,2] MANUEL ESPINOSA,[2] BILL GREENBERG,[1] AND SANFORD A. LACKS[1]*

Biology Department, Brookhaven National Laboratory, Upton, New York 11973,[1] and Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, 28006 Madrid, Spain[2]

Received 27 February 1987/Accepted 11 June 1987

A chromosomal gene of *Streptococcus pneumoniae* carrying a spontaneous mutation to sulfonamide resistance was identified. Comparison of its DNA sequence with the wild-type sequence showed that the mutation, *sul*-d, consisted of an insert of 6 base pairs, a repeat of an adjacent 6-base-pair segment. The gene encoded a 34-kilodalton polypeptide, SulA, which as a dimer or trimer constituted the enzyme dihydropteroate synthase. This was shown by enzyme activity measurements, expression in minicells of *Bacillus subtilis*, and the amino-terminal sequence of the polypeptide product. Subcloning of the gene in an *Escherichia coli* expression vector allowed purification of the enzyme to 80% homogeneity in a single step and at high yield. Although a deleted plasmid, pLS83, produced the mutant dihydropteroate synthase, it did not confer sulfonamide resistance in vivo. It is suggested that the SulA polypeptide is also a component of an enzyme that acts in another step of folate biosynthesis and that this step is inhibited in vivo by either free or conjugated sulfonamides.

One of the earliest examples (37) of inhibition of cell growth by an analog of a cellular metabolite was the antibacterial action of sulfanilamide and its reversal by *p*-aminobenzoate (PAB). The latter was shown to be a component of folate, an essential vitamin (1). Analysis of folate biosynthesis in several bacterial species (27, 31), including *Streptococcus pneumoniae* (24), revealed the following pathway:

$$6\text{-}CH_2OH\text{-}7,8\text{-}H_2\text{pterin} \xrightarrow{+ATP} 6\text{-}CH_2OH\text{-}7,8\text{-}H_2\text{pterin pyrophosphate} \xrightarrow{+PAB} 7,8\text{-dihydropteroate} \xrightarrow{+\text{glutamate, }+ATP} \text{folate}$$

The enzymatic step considered most likely to be affected by sulfonamides is the reaction catalyzed by dihydropteroate synthase (Fig. 1). Synthesis of dihydropteroate was inhibited by sulfonamides in extracts of *Escherichia coli* (3) and *S. pneumoniae* (36). However, sulfonamides are also incorporated in place of PAB by the enzymatic reaction (3, 28), and the resultant analogs of dihydropteroate could inhibit another step in the pathway. A mutation to sulfanilamide resistance, called *sul*-d, was obtained in *S. pneumoniae* (13), and this mutation was shown to affect the enzymatic properties of dihydropteroate synthase in bacterial extracts by increasing the K_m for PAB and the K_i for sulfanilamide (25).

A 10-kilobase (kb) chromosomal fragment of *S. pneumoniae* containing the *sul*-d mutation was cloned in the recombinant plasmid pLS80 (33). The wild-type allele was transferred to this plasmid by chromosomal facilitation of plasmid establishment (19) to give pLS80s. After transfer of pLS80 to *Bacillus subtilis*, a deleted derivative, pLS83, was obtained (19). Despite deletions of 4.5 and 2.5 kb on either side of the *sul*-d-containing remnant, the gene containing that mutation remained intact in pLS83. This gene, *sulA*, which encodes dihydropteroate synthase, is the subject of the present report.

The DNA sequences of the sulfonamide-resistant (*sul*-d) and wild-type (*sul*-s) alleles of the *sulA* gene were determined. An open reading frame was identified and shown to encode a protein product with dihydropteroate synthase activity. This protein was produced in large amounts, purified, and partially characterized. Contrary to expectation, the *sul*-d mutant dihydropteroate synthase produced by pLS83 in *S. pneumoniae* did not by itself confer sulfonamide resistance.

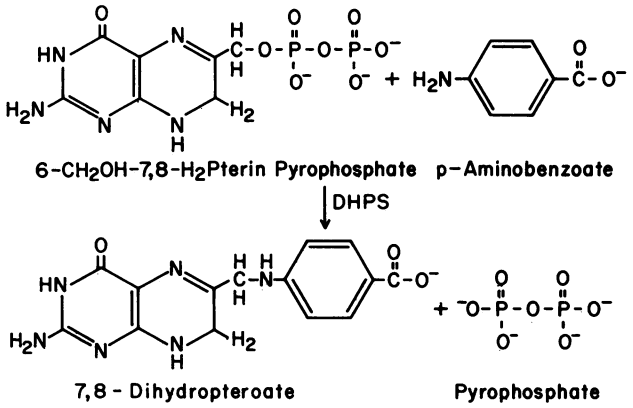

FIG. 1. Reaction catalyzed by dihydropteroate synthase (DHPS).

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used are listed in Table 1.

Growth and transformation of bacteria. Cultures of *S. pneumoniae* and *Bacillus subtilis* were grown and transformed as described previously (19). *E. coli* was grown in L broth and transformed by the method of Kushner (15). Cultures were treated with plasmid DNA at 1.0 μg/ml. Transformants of *S. pneumoniae* and *B. subtilis* were selected in agar medium containing tetracycline at 1.0 and 50 μg/ml, respectively; transformants of *E. coli* were selected with ampicillin at 50 μg/ml.

Preparation and manipulation of plasmid DNA. Purified plasmids were prepared by the method of Currier and Nester (7). Crude plasmid preparations were made from *E. coli* by the method of Birnboim and Doly (2) and from *S. pneumoniae* by a modification of that method (33). Cleared lysates of *B. subtilis* were prepared as described (9).

Procedures for DNA manipulation were similar to those described by Maniatis et al. (21). To construct pLS26, the pAR2192 vector was cleaved with *Eco*RI and treated with alkaline phosphatase; it was then ligated to the fragment with *Eco*RI termini remaining after treatment of pLS83 with *Bgl*I and *Eco*RI. Plasmids and restriction fragments were analyzed by gel electrophoresis in 1% agarose or 5% polyacrylamide and staining with ethidium bromide.

DNA sequence determination. Trace amounts of RNA were removed from plasmid samples by treatment with pancreatic RNase and gel filtration. After cleavage with restriction enzymes, the DNA fragments were treated with alkaline phosphatase and labeled at their 5′ ends with [γ-^{32}P]ATP and T4 polynucleotide kinase. Nucleotide sequences were determined by the chemical method of Maxam and Gilbert (23).

Analysis of plasmid-encoded proteins. Minicells of *B. subtilis* were prepared and labeled with [^{35}S]methionine as described previously (8). Details of the procedures used for labeling and analyzing proteins produced in the *E. coli* BL21(DE3) expression system have been presented earlier (18).

Preparation of cell extracts. Cultures of *S. pneumoniae* were grown at 37°C to an OD_{650} of 0.8 in a casein hydrolysate-based medium (16) supplemented with 0.2% sucrose. *E. coli* BL21(DE3) containing plasmids was grown with shaking at 37°C in M9 medium with ampicillin at 0.2 mg/ml until it reached an OD_{600} of 0.5, at which time isopropylthio-galactoside (IPTG) was added to 0.5 mM, and the culture was incubated for 2 h more. In each case cells from 1 liter were centrifuged, washed, and suspended in 10 ml of gel filtration buffer containing 10 mM Tris hydrochloride (pH 7.6), 0.5 M NaCl, and 3 mM β-mercaptoethanol. The cells were disrupted by passage through a French pressure cell at 20,000 lb/in^2, and the crude extracts were clarified by centrifugation for 20 min at 20,000 × *g*. Protein was determined by the method of Lowry et al. (20).

Assay of dihydropteroate synthase. One substrate for the reaction, 6-hydroxymethyldihydropterin pyrophosphate (Fig. 1), was prepared by reacting 6-hydroxymethylpterin (Sigma Chemical Co.) with pyrophosphoric acid and purifying the product by chromatography on DEAE-dellulose by the method of Shiota et al. (32). A yield of 3 mg was obtained from 25 mg of starting material. The 6-hydroxymethylpterin pyrophosphate was reduced to the dihydro compound with dithionite, as described by Futterman (10), shortly before use.

Each assay mixture contained, in a volume of 0.2 ml, 0.1 M Tris hydrochloride (pH 8.1), 10 mM $MgCl_2$, 0.1 M β-mercaptoethanol, 25 μM 6-hydroxymethyldihydropterin pyrophosphate, 50 μM [^{14}C]PAB (10 mCi/mmol), and the enzyme sample. After incubation for 1 h at 37°C, the reaction was stopped by heating for 1 min at 100°C. Samples (0.1 ml) were applied to Whatman 3MM paper, and descending chromatograms were developed with 0.1 M potassium phosphate buffer (pH 7.0) until the front moved 30 cm from the origin. Under these conditions the reaction product, dihydropteroate, remains at the origin, whereas unreacted PAB migrates with an R_f of 0.78. Areas (2 cm^2) containing the origin were excised from the chromatograms, placed in a vial with scintillation fluid, and counted for radioactivity. One unit of enzyme activity was defined as the production of 1 nmol of dihydropteroate per h at 37°C.

Determination of amino-terminal polypeptide sequence. Protein purified by gel filtration was subjected to preparative electrophoresis in the presence of sodium dodecyl sulfate on a 10 to 20% polyacrylamide gradient gel prepared from "ultrapure" acrylamide (International Biotechnologies). The main polypeptide band was visualized by placing the gel in 4 M sodium acetate at 4°C for 30 min (12) and excised. The polypeptide was electroeluted with an ISCO sample concentrator with a chamber solution of 50 mM NH_4HCO_3 and 0.1% sodium dodecyl sulfate. It was lyophilized and dissolved in 0.4 ml of water four times to remove NH_4HCO_3. Approximately 1 nmol of polypeptide was subjected to

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Derivation	Relevant features	Reference
S. pneumoniae			
708	Multiple mutant of R6	Wild-type *sul* (*sul*-s)	19
772	Transformant of 708	Sulfonamide resistant (*sul*-d)	19
B. subtilis CU403*divIVB1*	Mutant of CU403	Minicell producer	26
E. coli BL21(DE3)	Defective lambda lysogen	Inducible T7 RNA polymerase	34
Plasmids			
pLS1	Deletion of pMV158	Vector for *sul* and *polA*	33
pLS80	Cloned 10-kb *sul* insert	Contains *sul*-d mutation	19
pLS80s	*sul*-s recombinant of pLS80	Contains *sul*-s allele	19
pLS83	*B. subtilis* pLS80 transformant	Deleted on both sides of *sul*-d	19
pLS830	Recombinant of pLS83	Deleted upstream of *sul*-d	19
pSM23	Cloned pneumococcal *polA*	No *sulA* gene present	22
pAR2192	Constructed from pBR322	Has T7 RNA polymerase promoter	Rosenberg et al.[a]
pLS26	pLS83 *sul* fragment in pAR2192	Hyperexpression of SulA (d)	This work

[a] A. H. Rosenberg, B. N. Lade, C. Chui, S.-W. Lin, J. J. Dunn, and F. W. Studier, Gene, in press. The authors redesignated pAR2192 vector pET-5.

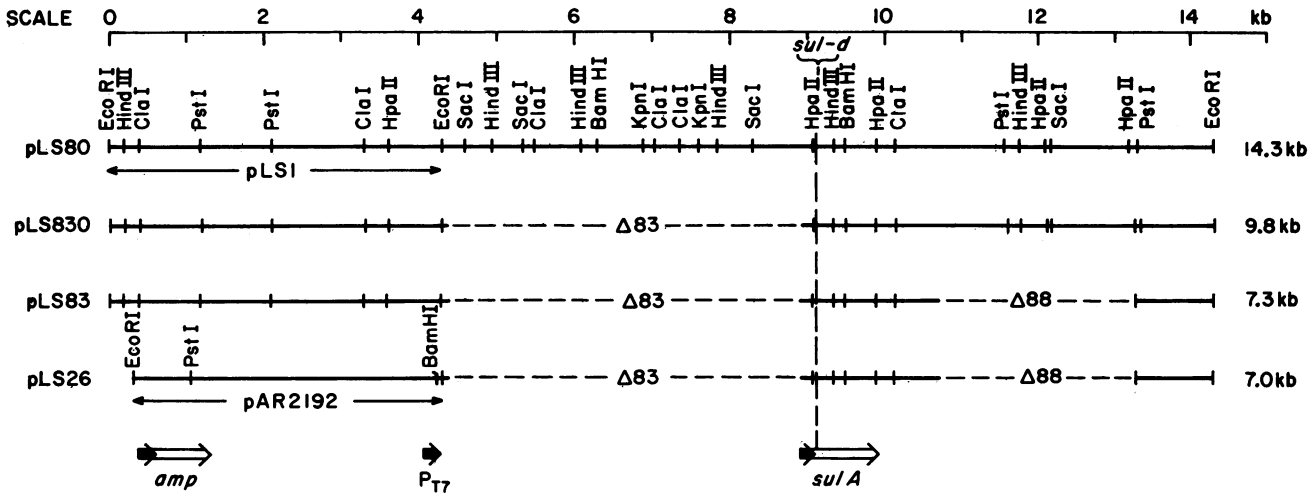

FIG. 2. Maps of plasmids containing the *sulA* gene encoding dihydropteroate synthase. Restriction maps are shown linearized at a common *Eco*RI site; dashes indicate deleted segments. Promoters (solid arrows) and translated products (open arrows) are shown for pLS26.

amino-terminal sequence determination in a gas-phase sequenator (Applied Biosystems).

RESULTS

DNA sequence of the *sulA* gene. The *sul*-d mutation had been localized to a particular *Hpa*II fragment of pLS80 (19) (Fig. 2). The DNA sequence in the vicinity of the mutation was determined for both the mutant (*sul*-d) DNA in pLS80 and the corresponding wild-type (*sul*-s) DNA in pLS80s. A restriction map of this segment and the strategy used to determine its sequence are shown in Fig. 3. Analysis of open reading frames in the segment revealed a single large open frame reading from left to right in phase 2 (Fig. 3). This gene was called *sulA*.

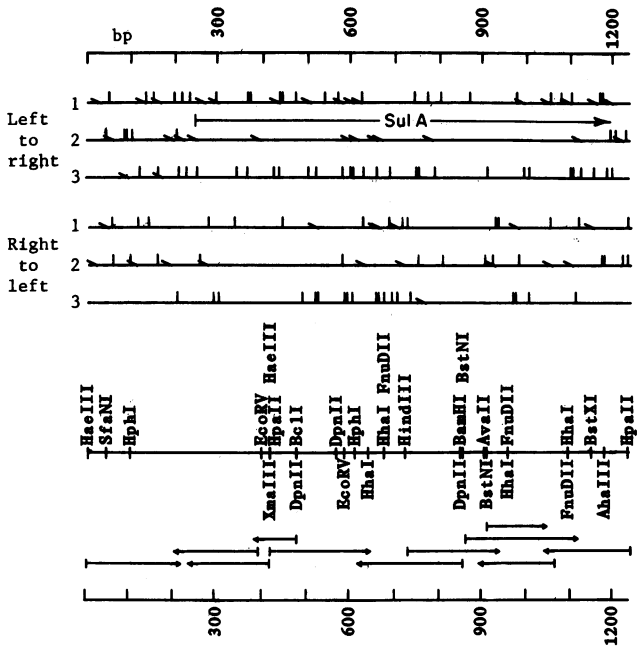

FIG. 3. Sequencing strategy and open reading frames for DNA in the vicinity of the *sul*-d mutation. Arrows at bottom show segments sequenced: vertical marks indicate sites of end labeling; arrowheads indicate extent of sequence determined. Reading frames in all three phases are depicted above for both directions: vertical marks indicate terminator codons; oblique marks indicate potential ATG start codons. The region encoding SulA is shown.

Figure 4 presents the DNA sequence of *sulA* and adjacent portions of the cloned chromosomal DNA. The amino acid sequence of the polypeptide beginning at the first ATG codon in the open reading frame is also shown. Upstream from the start site was a putative promoter for transcription of *sulA*. Its sequence, TgtcCA[17 nucleotides]TAaAAT, corresponds to the consensus sequence for procaryotic promoters (29), as indicated by the upper-case letters and the spacing between the −35 and −10 portions of the sequence. The putative ribosome-binding site for translation of the *sulA* product contained a Shine-Dalgarno sequence, AGGga-GTGA (base symbols in upper-case letters are complementary to 16S rRNA) (30). The *sulA* gene would encode a polypeptide of 34 kilodaltons (kDa). Restriction mapping and sequencing of pLS83 showed that this plasmid contained the entire *sulA* gene together with the signals for transcription and translation.

Expression of the *sulA* product. Plasmid pLS83 was transferred into a minicell-producing strain of *B. subtilis* so that polypeptides encoded by it could be examined. Extracts of minicells labeled with [^{35}S]methionine were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, and an autoradiogram of labeled polypeptides is shown in Fig. 5. Minicells containing the pLS1 vector alone (lane 1) gave a diffuse band at 38 kDa, corresponding to the Tet protein (18), and several weaker bands, which were observed previously (8). With pLS83 (lane 2), an additional strong band corresponding to a polypeptide of approximately 34 kDa was present. This is apparently the *sulA* product, SulA.

To increase its expression and further characterize the SulA protein, the 3.0-kb *Eco*RI fragment of pLS83 containing the chromosomal insert was inserted into the *Eco*RI site of pAR2192, which contains a phage T7 RNA polymerase promoter, to give pLS26 (Fig. 2). This plasmid was introduced into *E. coli* BL21(DE3), which contains the phage T7 RNA polymerase gene under control of the *lac* repressor (34). In this strain the polymerase is inducible by IPTG.

```
5'-GGCCTTTTTCCATTTCGTCGAAAATGGCAACAGCATCTTCTACTGATTGTTAATGGGATTGCTTTCTGTTTGTTGGCTCTTGTGGTGATTATGAGTCGGA  100
   CATTAGGAATTTCTGTGTAAAAGTTTTTATGTAGGAACCGACCTCTTTCTACTAGGGAAAGATGAATGCAATCGTGTCCATCTTTTTCTTTTTATGGTAA  200
                                                                                  -35
   AATAGAAAAATAATATGATGAAAATCCTTGAGGGAGTGACCGATATGTCAAGTAAAGCCAATCATGCAAAGACAGTTATTTGCGGAATTATCAATGTAAC  300
   -10                                S.D.      fMetSerSerLysAlaAsnHisAlaLysThrValIleCysGlyIleIleAsnValThr
                                                 *  *  *  *  *  *  *  *  *  *

   CCCAGACTCCTTTTCGGACGGTGGTCAATTTTTTGCTCTTGAGCAGGCACTCCAGCAGGCTCGTAAATTGATAGCAGAAGGAGCCAGTATGCTCGATATC  400
    ProAspSerPheSerAspGlyGlyGlnPhePheAlaLeuGluGlnAlaLeuGlnGlnAlaArgLysLeuIleAlaGluGlyAlaSerMetLeuAspIle

   GGCGGAGAATCGACTCGGCCGGGCAGTAGCTATGTTGAGATAGAAATAGAAGAGGAAATCCAGCGTGTTGTTCCAGTGATCAAAGCGATTCGCAAGGAAA  500
   GlyGlyGluSerThrArgProGlySerSerTyrValGluIleGluIleGluGluIleGlnArgValValProValIleLysAlaIleArgLysGlu

   GTGATGTCCTCATCTCTATTGATACTTGGAAGAGCCAAGTAGCAGAGGCTGCTTTGGCTGCTGGTGCCGATCTAGTCAATGATATCACTGGTCTTATGGG  600
SerAspValLeuIleSerIleAspThrTrpLysSerGlnValAlaGluAlaAlaLeuAlaAlaGlyAlaAspLeuValAsnAspIleThrGlyLeuMetGly

   TGATGAGAAAATGCCTCATGTGGTAGCTGAAGCGAGAGCGCAAGTGGTCATCATGTTTAATCCAGTTATGGCGCGACCTCAGCACCCTAGCTCGCTCATC  700
    AspGluLysMetProHisValValAlaGluAlaArgAlaGlnValValIleMetPheAsnProValMetAlaArgProGlnHisProSerSerLeuIle

   TTCCCTCATTTTGGTTTTGGTCAAGCTTTTACAGAGGAAGAGTTAGCTGACTTTGAAACATTGCCAATCGAAGAATTGATGGAGGCTTTCTTTGAACGAG  800
   PheProHisPheGlyPheGlyGlnAlaPheThrGluGluGluLeuAlaAspPheGluThrLeuProIleGluGluLeuMetGluAlaPhePheGluArg

   CACTAGCGAGAGCGGCAGAAGCTGGTATTGCACCAGAAAATATCCTGTTGGATCCAGGAATTGGCTTTGGTCTGACCAAGAAAGAAAATCTGCTTCTTTT  900
AlaLeuAlaArgAlaAlaGluAlaGlyIleAlaProGluAsnIleLeuLeuAspProGlyIleGlyPheGlyLeuThrLysLysGluAsnLeuLeuLeuLeu

   ACGGGACCTGGATAAACTACATCAGAAGGGCTATCCAATCTTTCTCGGAGTGTCGCGCAAGCGATTTGTCATCAATATCCTAGAGGAGAATGGTTTTGAA 1000
    ArgAspLeuAspLysLeuHisGlnLysGlyTyrProIlePheLeuGlyValSerArgLysArgPheValIleAsnIleLeuGluGluAsnGlyPheGlu

   GTCAATCCTGAGACAGAGCTTGGTTTCCGCAATCGGGACACGGCTTCGGCTCATGTAACCAGTATCGCTGCAAGACAGGGTGTAGAAGTGGTGCGCGTGC 1100
ValAsnProGluThrGluLeuGlyPheArgAsnArgAspThrAlaSerAlaHisValThrSerIleAlaAlaArgGlnGlyValGluValValArgVal

   ATGACGTAGCTAGTCACAGGATGGCAGTTGAAATTGCCTCTGCCATTCGTCTGGCTGATGAAGCGGAAAATTTAGATTTAAAACAATATAAATAAGATGA 1200
HisAspValAlaSerHisArgMetAlaValGluIleAlaSerAlaIleArgLeuAlaAspGluAlaGluAsnLeuAspLeuLysGlnTyrLys

   AAGAAATTGAAAACAATCAATGGATTGCTAACTACCGG-3'
```

FIG. 4. Nucleotide sequence of the *sulA* gene and its vicinity. The sequence of one DNA strand is shown beginning at an *Hae*III site and ending at an *Hpa*II site. The predicted amino acid sequence of the SulA polypeptide is shown together with a putative promoter and mRNA ribosome-binding site. Underlined and labeled are the −35 and −10 portions of the promoter and the Shine-Dalgarno sequence. Determination of the amino-terminal sequence of the purified dihydropteroate synthase polypeptide revealed the residues marked by asterisks. The 6-base segment underlined at positions 446 to 451 marks the *sul*-d mutation; this segment is absent in the wild type.

A comparison of dihydropteroate synthase activity in strains of *S. pneumoniae* and *E. coli* carrying various plasmids indicated that this enzyme corresponded to the SulA protein encoded by pLS83 and pLS26. Strains of *S. pneumoniae* containing either the *sul*-s or *sul*-d allele of *sulA* produced approximately the same amount of dihydropteroate synthase, and the presence of the multicopy plasmid pLS83 increased this amount 10-fold (Table 2). The level of dihydropteroate synthase in *E. coli* BL21(DE3) containing a control plasmid, pSM23, after induction of the T7 RNA polymerase with IPTG and incubation for 2 h was not very different from the level attributable to the chromosomal gene in *S. pneumoniae*. Induction of the strain containing pLS26, however, gave a level of dihydropteroate synthase 1,000-fold higher.

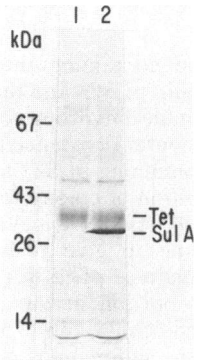

FIG. 5. Expression of the SulA polypeptide in minicells of *B. subtilis*. Extracts of minicells of CU403*divIVB1*(pLS1) (lane 1) and CU403*divIVB1*(pLS83) (lane 2), labeled with [^{35}S]methionine and containing ~5 μg of protein, were subjected to electrophoresis in a 5 to 25% polyacrylamide gradient gel in the presence of sodium dodecyl sulfate. Shown on the autoradiogram are positions of protein size standards (top to bottom: bovine serum albumin, chicken ovalbumin, chymotrypsinogen and lysozyme) from a stained parallel lane. Positions of the Tet and SulA polypeptides encoded by the plasmids are indicated.

Figure 6 compares proteins synthesized by BL21(DE3) carrying pLS26 with those produced by the same strain carrying only the pAR2192 vector. After various periods of induction in the presence and absence of rifampin, cells were treated with a pulse of [^{35}S]methionine, and extracts were subjected to gel electrophoresis. The gel was stained with Coomassie blue and exposed for autoradiography. In the presence of rifampin (lanes 1 and 5), which inhibits the bacterial but not the phage RNA polymerase (34), almost all of the incorporated pulse label was found in plasmid-encoded polypeptides. In the case of pAR2192 it was in the 29-kDa β-lactamase and its 31-kDa precursor; in the case of pLS26 an additional strongly labeled band corresponded to the 34-kDa dihydropteroate synthase polypeptide. In the absence of rifampin (Fig. 6b, lanes 2 to 4 and 6 to 8), label was present in other protein bands as well. When the gel was stained for protein (Fig. 6a), a distinct band was visible at 34 kDa only in the strain containing pLS26. The dihydropteroate synthase polypeptide accounted for approximately 25% of the protein present.

Purification and characterization of dihydropteroate synthase. Dihydropteroate synthase was purified by agarose

TABLE 2. Dihydropteroate synthase activity in bacterial extracts

Bacterial strain	Plasmid	Dihydropteroate synthase activity (U/mg of protein)
S. pneumoniae 708 (*sul*-s)	None	1.2
S. pneumoniae 772 (*sul*-d)	None	1.8
S. pneumoniae 708 (*sul*-s)	pLS83	13.5
E. coli BL21(DE3)	pSM23	0.6
E. coli BL21(DE3)	pLS26	1,480

gel (Bio-Gel A-0.5m) filtration of an extract of induced BL21(DE3)(pLS26) labeled with [^{35}S]methionine in the presence of rifampin (Fig. 7a). Three peaks of radioactivity were observed, one at the breakthrough (fraction 16), another at fraction 29, and a third at fraction 36. A single peak of dihydropteroate synthase activity corresponded to the radioactivity peak at fraction 29. The polypeptide content of selected fractions was examined by gel electrophoresis (Fig. 7b). The radioactivity peak at fraction 36 contained the 29-kDa β-lactamase polypeptide. The peak at fraction 29, which corresponded to the peak of dihydropteroate synthase activity (Fig. 7a), contained the 34-kDa polypeptide (Fig. 7b). From the stained gel it appeared that the enzyme constituted at least 80% of the protein in this fraction. Comparison of the position of elution of dihydropteroate synthase with the elution behavior of β-lactamase in this column and of other proteins subjected to similar gel filtration (unpublished data) indicated a molecular weight for the enzyme between 70,000 and 100,000. This means that the native protein is at least a dimer of the 34-kDa polypeptide.

The enzyme polypeptide was purified by gel electrophoresis, and the sequence of amino acid residues at its amino terminal end was determined (Fig. 4). The sequence corresponded to that predicted for initiation at the first ATG codon in the open reading frame. Seven of 10 residues were accurately identified. The initial methionine residue was apparently removed after translation.

Nature of the mutation conferring resistance to sulfonamides. Comparison of the DNA sequences of the *sul*-d mutant and the wild-type alleles revealed a single difference, an insertion of 6 additional nucleotides in the mutant allele (underlined in Fig. 4). This insertion corresponded to a duplication of the preceding sequence and resulted in a repeat of an Ile-Glu dipeptide in the amino acid chain.

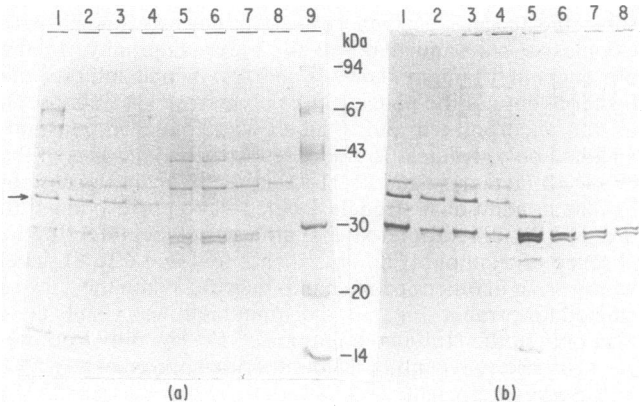

FIG. 6. Plasmid-specific expression and hyperproduction of dihydropteroate synthase in *E. coli*. (a) Gel stained for protein with Coomassie blue. (b) Autoradiogram of dried gel. Lanes 1 to 4, extracts of BL21(DE3)(pLS26); lanes 5 to 8, extracts of BL21(DE3)(pAR2192); lane 9, protein size standards (top to bottom: rabbit phosphorylase, bovine serum albumin, chicken ovalbumin, bovine carbonic anhydrase, soybean trypsin inhibitor, and bovine lactalbumin). Extracts prepared from cells incubated with IPTG for 30 min followed by 30 min with rifampin (lanes 1 and 5) and with IPTG alone for 120 min (lanes 2 and 6), 60 min (lanes 3 and 7), or 30 min (lanes 4 and 8) and subsequently labeled with [^{35}S]methionine for 10 min were subjected to electrophoresis in a 10 to 20% polyacrylamide gradient gel in the presence of sodium dodecyl sulfate. Samples contained ~5 μg of total cell protein. Arrow, DHPS polypeptide.

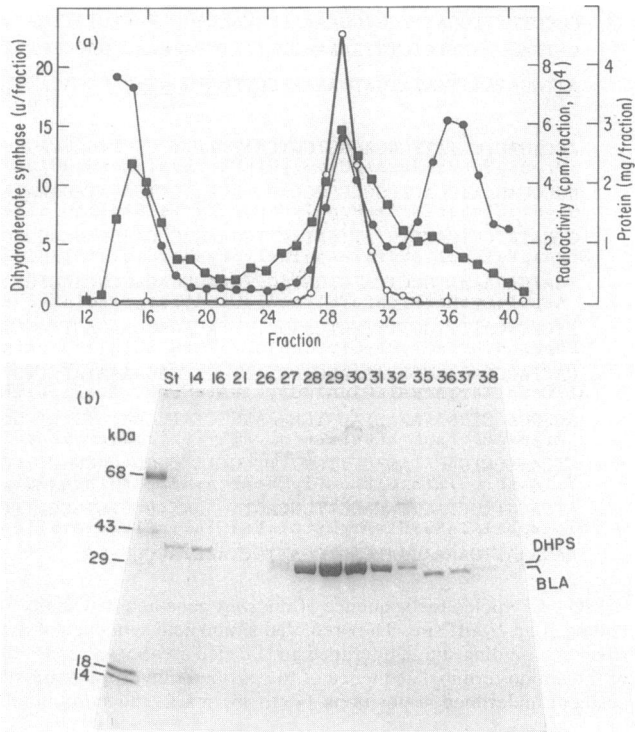

FIG. 7. Purification of dihydropteroate synthase. (a) Fractionation by gel filtration. A mixture of 2 ml of a crude extract of *E. coli* BL21(DE3)(pLS26), induced with IPTG for 2 h, which contained 40 mg of protein, and 0.02 ml of a crude extract of the same strain induced with IPTG for 30 min, treated with rifampin for 30 min, and labeled with [^{35}S]methionine for 10 min, which contained 3.7×10^5 cpm, was applied to a column (1.9 by 66 cm) of Bio-Gel A-0.5m and eluted with gel filtration buffer. Fractions of 4 ml were collected. Symbols: ●, [^{35}S]methionine radioactivity; ○, dihydropteroate synthase activity; ■, protein. (b) Protein content of fractions. Samples containing 12 μl of the fractions indicated and protein size standards (lane St), which included (top to bottom) bovine serum albumin, chicken ovalbumin, bovine carbonic anhydrase, β-lactoglobulin, and lysozyme, were subjected to electrophoresis in a 10 to 20% polyacrylamide gradient gel in the presence of sodium dodecyl sulfate. The gel was stained with Coomassie blue. DHPS, Dihydropteroate synthase; BLA, β-lactamase.

Although the gene containing the *sul*-d mutation was present in its entirety in pLS83 and the plasmid gave rise to expression of the mutant dihydropteroate synthase product, pLS83 itself did not confer resistance to sulfonamides. Cells of *S. pneumoniae* containing pLS83 showed wild-type sensitivity, that is, inhibition of colony formation with sulfanilamide at 40 μg/ml (19). Its DNA, nevertheless, could transform the chromosome to give resistance to 200 μg/ml. Furthermore, a derivative, pLS830 (Fig. 2), in which the downstream deleted portion of the original chromosomal segment was restored, could confer resistance to 2 mg/ml (19). It appears that the sulfonamide resistance conferred by the *sul*-d mutation required something more than the mutant dihydropteroate synthase enzyme. It is conceivable that the SulA polypeptide also functions in another drug-sensitive step of folate biosynthesis.

DISCUSSION

The *sul*-d mutation originally arose in a cluster of three linked mutations, b, a, and d, following prolonged selection

with sulfanilamide (13). In the light of intercistronic recombination frequencies in transformation (16), the relatively low level of linkage (<0.5) between d and the other two mutations (13) suggests that the latter were located outside the *sulA* gene. The *sul*-d mutation itself consisted of a 6-nucleotide repeat. It could have arisen by slippage of a nascent strand on the template during replication. Its effect is to repeat an Ile-Glu sequence in a region of the SulA polypeptide that can be predicted to compose an α-helical stretch of secondary structure (4). The mutation would thus extend the helical stretch by two residues, which could significantly alter tertiary structure at the ends of the helical segment. Prior studies have shown that the dihydropteroate synthase enzyme in the *sul*-d mutant has a twofold-higher K_m for PAB and a sevenfold-higher K_i for sulfanilamide than the wild type (24, 25). This is a relatively modest effect on the enzyme activity, and as indicated below, it does not appear to account for the sulfanilamide resistance of the mutant.

When it was originally observed that pLS83 failed to confer drug resistance unless a deleted portion was restored to give pLS830, it was suggested either that the affected gene extended into the deleted region or that a promoter necessary for expression of the gene was missing (19). The present results rule out both possibilities, in that the gene containing the mutation was intact in pLS83 and its promoter was present and functional. The essential region that was deleted was downstream from the promoter and *sulA*. It is possible that the SulA polypeptide is also a component of another enzyme in the folate pathway. This hypothetical enzyme might be critically inhibited by either sulfonamides or their hydroxymethyldihydropterin conjugates, which can be formed by dihydropteroate synthase action (28) and which could act as dihydropteroate analogs in a subsequent reaction step. A possible candidate for this step is the conversion of dihydropteroate to folate by the addition of glutamate. An enzyme catalyzing such a reaction was reported for *E. coli* and other organisms (11) but has not yet been demonstrated in *S. pneumoniae*. An alternative possibility is that folate biosynthesis in *S. pneumoniae* proceeds by the prior coupling of PAB and glutamate, with the product subsequently conjugated to the pterin, a reaction that may also be carried out by dihydropteroate synthase (24). The former coupling could be critically sensitive to sulfonamides.

Supporting the idea that the sulfonamide resistance of the *sul*-d mutant did not result from its altered dihydropteroate synthase enzyme are the rather minor differences in relative affinity, mentioned above, of the enzyme for both the substrate and drug. Added support comes from the observation that another PAB analog, *p*-nitrobenzoate, inhibits *sul*-d strains (14), although this analog has no effect on either wild-type or mutant dihydropteroate synthase activity in vitro (24). The susceptibility of the *sul*-d mutant to *p*-nitrobenzoate, which cannot be conjugated to the pterin by dihydropteroate synthase, suggests also that free, and not conjugated, sulfonamides inhibit the critical enzymatic step. Preliminary data indicate that several open reading frames, possibly in the same operon, lie downstream from *sulA*. A product of a downstream gene might join SulA to form the enzyme critically sensitive to sulfonamides and *p*-nitrobenzoate.

We speculate, then, that the sensitivity of *S. pneumoniae* to sulfonamides derives from their inhibition not of dihydropteroate synthesis but of another step in the folate pathway. The SulA polypeptide, which by itself constitutes the dihydropteroate synthase, can mutate to give sulfonamide resistance, perhaps by critically affecting some other unspecified step, for example, by serving as a subunit of an enzyme complex catalyzing that step. In the *sul*-d mutant this enzyme would be sensitive to *p*-nitrobenzoate. This step could be sensitive to either free sulfonamides or their pterin conjugates.

In *E. coli*, introduction of a plasmid-encoded dihydropteroate synthase that is 100-fold more resistant to sulfonamides in vitro than the chromosomally encoded enzyme confers sulfonamide resistance in vivo (35). If the conjugated sulfonamide is the ultimate inhibitor in *E. coli* as well, then resistance to sulfonamides may be achieved either by altering the dihydropteroate synthase to prevent sulfonamide conjugation, as in this case, or by altering the enzyme catalyzing a different step, as suggested for the case of *sul*-d.

Formation of the hypothetical enzyme complex consisting of SulA and a downstream product appears to depend on a *cis*-configuration of the genes. Inasmuch as pLS83 does not confer sulfonamide resistance, its SulA product must not interact with the downstream product expressed by the corresponding chromosomal gene. One possible mechanism for explaining this lack of complementation could be a requirement for the newly synthesized SulA and downstream polypeptides to interact prior to folding of the proteins into their final tertiary structure. The required folding to give the complex may depend on synthesis of the polypeptides from the same RNA. An alternative mechanism for the *cis*-effect could be an occasional frameshift in translation of *sulA* prior to its normal termination to fuse part of SulA to the product of a downstream gene to give an extended polypeptide that would function as the hypothetical enzyme. A third possibility is that balanced production of SulA and the downstream product is required for sulfonamide resistance.

A point of interest relates to the integration efficiency of the *sul*-d mutation in transformation. The DNA mismatch repair system known as Hex can recognize the configuration of certain mismatches in the heteroduplex transformation intermediate and eliminate the donor contribution to reduce integration efficiency (reviewed in reference 5). The *sul*-d transformation occurs with high efficiency and for this reason has been used as a standard of reference in the investigation of this topic (16). Thus, the *sul*-d/*sul*-s insertion/deletion mismatch of 6 nucleotides is not recognized at all by the Hex system. Inasmuch as deletions of 1 or 2 nucleotides are subject to mismatch repair (6, 17), the limit of Hex recognition must occur for a disparity in length between 2 and 6 nucleotides.

ACKNOWLEDGMENTS

We thank Marshall Elzinga and Jeanne Wysocki for carrying out the amino-terminal sequence determination. J. J. Dunn and F. W. Studier kindly provided us with the *E. coli* BL21(DE3)(pAR2192) expression system and information on its use prior to publication. We appreciate helpful discussion with R. D. Hotchkiss.

Research at Brookhaven National Laboratory was done under the auspices of the U.S. Department of Energy Office of Health and Environmental Research; research at the Centro de Investigaciones Biológicas was under the auspices of the Consejo Superior de Investigaciones Científicas. This work was supported in part by U.S. Public Health Service grants AI-14885 and GM-29721 to S.A.L., by CSIC-CAICYT grants 618-501 to M.E. and FIS86-713 to P.L., and by cooperative research award CCB-8402-036 to M.E. and S.A.L. from the U.S.-Spain Joint Committee for Scientific and Technological Cooperation.

LITERATURE CITED

1. **Angier, R. B., J. H. Boothe, B. L. Hutchings, J. H. Mowat, J. Semb, E. L. R. Stokstad, Y. SubbaRow, C. W. Waller, D. B. Cosulich, M. J. Fahrenbach, M. E. Hultquist, E. Kuh, E. H. Northey, D. R. Seeger, J. P. Sickels, and J. M. Smith, Jr.** 1946. The structure and synthesis of the liver *L. casei* factor. Science **103**:667–669.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant DNA. Nucleic Acids Res. **7**:1513–1523.
3. **Brown, G. M.** 1962. The biosynthesis of folic acid. II. Inhibition by sulfonamides. J. Biol. Chem. **237**:536–540.
4. **Chou, P. Y., and G. D. Fasman.** 1978. Prediction of the secondary structure of proteins from their amino acid sequence. Adv. Enzymol. **47**:45–148.
5. **Claverys, J. P., and S. A. Lacks.** 1986. Heteroduplex deoxyribonucleic acid base mismatch repair in bacteria. Microbiol. Rev. **50**:133–165.
6. **Claverys, J. P., V. Mejean, A. M. Gasc, and A. M. Sicard.** 1983. Mismatch repair in *Streptococcus pneumoniae*: relationship between base mismatches and transformation efficiencies. Proc. Natl. Acad. Sci. USA **80**:5956–5960.
7. **Currier, T. C., and E. W. Nester.** 1976. Isolation of covalently closed circular DNA of high molecular weight from bacteria. Anal. Biochem. **76**:431–441.
8. **Espinosa, M., P. Lopez, and S. A. Lacks.** 1984. Transfer and expression of recombinant plasmids carrying pneumococcal *mal* genes in *Bacillus subtilis*. Gene **28**:301–310.
9. **Espinosa, M., P. Lopez, M. T. Perez-Urena, and S. A. Lacks.** 1982. Interspecific plasmid transfer between *Streptococcus pneumoniae* and *Bacillus subtilis*. Mol. Gen. Genet. **188**:195–201.
10. **Futterman, S.** 1957. Enzymatic reduction of folic acid and dihydrofolic acid to tetrahydrofolic acid. J. Biol. Chem. **228**: 1031–1038.
11. **Griffin, M. J., and G. M. Brown.** 1964. The biosynthesis of folic acid. III. Enzymatic formation of dihydrofolic acid from dihydropteroic acid and of tetrahydropteroylglutamic acid compounds from tetrahydrofolic acid. J. Biol. Chem. **239**:310–316.
12. **Higgins, R. C., and M. E. Dahmus.** 1979. Rapid visualization of protein bands in preparative SDS-polyacrylamide gels. Anal. Biochem. **93**:257–260.
13. **Hotchkiss, R. D., and A. H. Evans.** 1958. Analysis of the complex sulfonamide resistance locus of pneumococcus. Cold Spring Harbor Symp. Quant. Biol. **23**:85–97.
14. **Hotchkiss, R. D., and A. H. Evans.** 1960. Fine structure of a genetically modified enzyme as revealed by relative affinities for modified substrate. Fed. Proc. **19**:912–925.
15. **Kushner, S. R.** 1978. An improved method for transformation of *Escherichia coli* with ColE1 derived plasmids, p. 17–23. *In* H. W. Boyer and S. Nicosia (ed.), Genetic engineering. Elsevier/North-Holland, Amsterdam.
16. **Lacks, S.** 1966. Integration efficiency and genetic recombination in pneumococcal transformation. Genetics **53**:207–235.
17. **Lacks, S. A., J. J. Dunn, and B. Greenberg.** 1982. Identification of base mismatches recognized by the heteroduplex-DNA-repair system of *Streptococcus pneumoniae*. Cell **31**:327–336.
18. **Lacks, S. A., P. Lopez, B. Greenberg, and M. Espinosa.** 1986. Identification and analysis of genes for tetracycline resistance and replication functions in the broad-host-range plasmid pLS1. J. Mol. Biol. **192**:753–765.
19. **Lopez, P., M. Espinosa, and S. A. Lacks.** 1984. Physical structure and genetic expression of the sulfonamide-resistance plasmid pLS80 and its derivatives in *Streptococcus pneumoniae* and *Bacillus subtilis*. Mol. Gen. Genet. **195**:402–410.
20. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
21. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Martinez, S., P. Lopez, M. Espinosa, and S. A. Lacks.** 1986. Cloning of a gene encoding a DNA polymerase-exonuclease of *Streptococcus pneumoniae*. Gene **44**:79–88.
23. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
24. **Ortiz, P. J.** 1970. Dihydrofolate and dihydropteroate synthesis by partially purified enzymes from wild-type and sulfonamide-resistant pneumococcus. Biochemistry **9**:355–361.
25. **Ortiz, P. J., and R. D. Hotchkiss.** 1966. The enzymatic synthesis of dihydrofolate and dihyropteroate in cell-free preparations from wild-type and sulfonamide-resistant pneumococcus. Biochemistry **5**:67–74.
26. **Reeve, J. N., N. H. Mendelson, S. I. Coyne, L. L. Hallock, and R. M. Cole.** 1973. Minicells of *Bacillus subtilis*. J. Bacteriol. **114**:860–873.
27. **Richey, D. P., and G. M. Brown.** 1969. The biosynthesis of folic acid. IX. Purification and properties of the enzymes required for the formation of dihydropteroic acid. J. Biol. Chem. **244**: 1582–1592.
28. **Roland, S., R. Ferone, R. J. Harvey, V. L. Styles, and R. W. Morrison.** 1979. Characteristics and significance of sulfonamides as substrates for *Escherichia coli* dihydropteroate synthase. J. Biol. Chem. **254**:10337–10345.
29. **Rosenberg, M., and D. Court.** 1979. Regulatory sequences involved in the promotion and termination of RNA transcription. Annu. Rev. Genet. **13**:319–353.
30. **Shine, J., and L. Dalgarno.** 1975. Determinant of cistron specificity in bacterial ribosomes. Nature (London) **254**:34–38.
31. **Shiota, T., C. M. Baugh, R. Jackson, and R. Dillard.** 1969. The enzymatic synthesis of hydroxymethyldihydropteridine pyrophosphate and dihydrofolate. Biochemistry **8**:5022–5028.
32. **Shiota, T., M. N. Disraely, and M. P. McCann.** 1964. The enzymatic synthesis of folate-like compounds from hydroxymethyldihydropteridine pyrophosphate. J. Biol. Chem. **239**: 2259–2266.
33. **Stassi, D. L., P. Lopez, M. Espinosa, and S. A. Lacks.** 1981. Cloning of chromosomal genes in *Streptococcus pneumoniae*. Proc. Natl. Acad. Sci. USA **78**:7028–7032.
34. **Studier, F. W., and B. A. Moffatt.** 1986. Use of T7 RNA polymerase to direct selective, high-level expression of cloned genes. J. Mol. Biol. **189**:113–130.
35. **Swedberg, G., and O. Skold.** 1980. Characterization of different plasmid-borne dihydropteroate synthases mediating bacterial resistance to sulfonamides. J. Bacteriol. **142**:1–7.
36. **Wolf, B., and R. D. Hotchkiss.** 1963. Genetically modified folic acid synthesizing enzymes of pneumococcus. Biochemistry **2**:145–150.
37. **Woods, D. D.** 1940. The relationship of *p*-aminobenzoic acid to the mechanism of the action of sulphanilamide. Br. J. Exp. Pathol. **21**:74–90.