A Cluster of Four Genes Encoding Enzymes for Five Steps in the Folate Biosynthetic Pathway of *Streptococcus pneumoniae*

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Two genes, sulB and sulC, in a folate biosynthetic gene cluster of Streptococcus pneumoniae were identified after determination of the DNA sequence between two previously reported genes, sulA and sulD, in a cloned segment of chromosomal DNA containing a mutation to sulfonamide resistance. The gene products, SulB and SulC, correspond to polypeptides of 49 and 21 kDa, respectively. SulC has GTP cyclohydrolase activity and catalyzes the first step in the folate biosynthetic pathway. SulB apparently has dihydrofolate synthetase activity in that it complements a folC mutant of Escherichia coli and thus catalyzes the last step in the pathway. Prior work showed that SulA, a dihydropteroate synthase, and SulD, a bifunctional enzyme, catalyze three intervening steps. Mapping of the mRNA transcribed from the operon was consistent with its beginning at a promoter with a -35 site (gTGtCc) and an extended -10 site (T-TG-TAaAAT) and its termination at the end of a hairpin structure, which would give a transcript 3,745 nucleotides in length. SulC showed a considerable conservation of sequence by comparison with proven or putative GTP cyclohydrolases from four unrelated species, with 38 to 53% of the residues being identical. A similar comparison of SulB with dihydrofolate synthetases showed an identity of only 26 to 37%. Overall, comparisons of the five folate biosynthetic enzymes in different species suggest that S. pneumoniae is related more closely to other gram-positive bacteria, less closely to eucaryotes, and least closely to the gram-negative E. coli. The varied arrangements of folate biosynthetic genes in different species imply an early evolutionary period of fluidity in genomic rearrangement.

Folate derivatives are essential cofactors in the biosynthesis of purines, pyrimidines, and amino acids in living cells. Whereas animals, including human beings, require folate in their diet, most bacterial species synthesize their own and are thus subject to inhibition by compounds, such as sulfonamides, that block folate biosynthesis. Sulfonamides and other folate antagonists are important chemotherapeutic agents used effectively against bacterial infections, human cancer, and more recently against *Pneumocystis* pneumonia resulting from AIDS (45).

The folate biosynthetic pathway depicted in Fig. 1 was elucidated by studies of cell extracts and purified enzymes from several bacterial species (32, 36), including Streptococcus pneumoniae (31). It begins with GTP, which is converted by GTP cyclohydrolase (GTPCH) to dihydroneopterin triphosphate. [Pterin is the trivial name for 2-amino-4-hydroxypteridine, and neopterin is the trivial name for 6-(D-erythro-1',2',3'-trihydroxypropyl)pterin.] The enzyme first breaks the five-member ring of guanine to release formate and an intermediate that recyclizes to give a pteridine ring. Removal of the phosphate residues from dihydroneopterin triphosphate must occur, presumably by the action of a phosphatase, but the nature of this enzyme has not been demonstrated in S. pneumoniae or any other system. Dihydroneopterin aldolase (DHNA) then acts on the product to give 6-hydroxymethyl-7,8-dihydropterin. The latter is converted to 6-hydroxymethyl-7,8-dihydropterin pyrophosphate by 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase (PPPK) and ATP. Dihydropteroate synthase (DHPS) catalyzes the linkage to p-aminobenzoate to give 7,8-dihydropteroate. Finally, the addition of glutamate to dihydropteroate

to produce dihydrofolate is catalyzed by dihydrofolate synthetase (DHFS).

Genetic and molecular analysis of this pathway in S. pneumoniae began with the cloning of a 10-kb chromosomal fragment containing a marker for sulfonamide resistance (sul) in the vector plasmid pLS1 (18), with S. pneumoniae as the host species (38). Part of this fragment was transferred to an Escherichia coli expression system and shown to produce four polypeptides, SulA, SulB, SulC, and SulD (20, 22). The sulfonamide resistance mutation, sul-d, was located within sulA. The 34-kDa product of sulA, SulA, was shown to have DHPS activity (20). The sul-d mutation was sufficient to confer resistance to sulfonamides, but it did so only when present, cis, in the full gene cluster (20). SulD, a 31-kDa polypeptide coded for by *sulD*, was shown to be a bifunctional protein carrying both PPPK and DHNA activities (22, 23). On the basis of these findings, putative genes encoding DHPS, PPPK, and DHNA were identified in Bacillus subtilis (37) and Pneumocystis carinii (43, 44). Later, genes for PPPK and DHPS were independently identified in E. coli (7, 8, 40).

An *E. coli* gene, *folC*, encoding DHFS was cloned earlier (3, 4), and human and bacterial homologs were found (9, 26, 29, 41). Rat cDNA encoding GTPCH was cloned and sequenced (12), and a homolog in *B. subtilis* was identified (1). In the present work, we report the sequences of *sulB* and *sulC*, show that these genes encode the pneumococcal DHFS and GTPCH, compare the various homologs of these enzymes, and map the mRNA produced by the folate biosynthetic operon in *S. pneumoniae*. We also compare the arrangements of these genes in various species.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used are listed in Table 1. **Growth and transformation of bacteria.** Cultures of *S. pneumoniae* were grown and transformed as previously described (17). Transformants were se-

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FIG. 1. Pathway for folate biosynthesis, beginning with guanosine triphosphate and proceeding clockwise. Identified enzymes include GTPCH, DHNA, PPPK, DHPS, and DHFS. The phosphatase(s) that presumably removes the phosphate residues of 7,8-dihydroneopterin triphosphate (Pase) has not been identified. Brackets indicate a presumed reaction intermediate. Triphosphate residues are indicated as $(P)_3$.

lected in agar medium containing tetracycline at 1.0 μ g/ml. In general, *E. coli* was grown in L broth and transformed according to the method of Kushner (16). Transformants were selected with ampicillin at 50 μ g/ml. To test the abilities of various plasmids to complement the *folC* defect in strain SF4, cultures were grown in L broth to an optical density at 600 nm of 0.4 and then diluted and plated in E medium (42) with and without methionine at 50 μ g/ml. All transformations were carried out with plasmid DNA at 1.0 μ g/ml.

DNA sequence determination. Purified pLS830 was prepared from cells of S.

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant feature(s)	Refer- ence
S. pneumoniae 708	Derivative of R6, wild-type sul (sul-s)	21
E. coli BL21(DE3)	Inducible T7 RNA polymerase	39
E. coli SF4	F^- folC strA recA Tn10:srlC	4
Plasmids		
pLS830	Contains cloned sul segment (sul-d)	21
pET-5	Contains T7 RNA polymerase promoter	33
pLS23	Intact pLS830 sul fragment in pET-5	22
pLS24	Deletion of pLS23 in sulA	22
pLS27	Deletion of pLS23 spanning sulBC	22
pLS29	Deletion in pLS23 of 4 bp in sulB	22
pLS30	Deletion in pLS23 of 4 bp in sulC	22
pLS31	Deletion in pLS23 of 4 bp in sulD	22

pneumoniae 708 by a modification of the procedure of Currier and Nester (6). Trace amounts of RNA were removed from the plasmid samples by treatment of 0.1 mg of DNA dissolved in 0.3 ml of buffer (10 mM Tris-HCI [pH 7.6]–1 mM EDTA) with 4.5 μ l of pancreatic RNase (4 mg/ml; heated for 15 min at 90°C) for 30 min at 37°C. After shaking of the solution with an equal volume of buffer-equilibrated phenol, the DNA in the aqueous phase was precipitated with ethanol and dissolved in 0.2 ml of buffer. This material was then fractionated by gel filtration on a Bio-Gel A-1.5m column (0.9 by 20 cm). Fractions (1.0 ml) containing plasmid DNA were pooled. After plasmid cleavage with restriction enzymes, the DNA fragments were treated with alkaline phosphatase and labeled at their 5' ends with [γ -³²P]ATP and T4 polynucleotide kinase (27). Nucleotide sequences on both strands of DNA were determined by the chemical method of Maxam and Gilbert (27).

Preparation of cell extracts. Cultures of *E. coli* BL21(DE3) containing various plasmids were grown with shaking at 37°C in M9 medium with ampicillin at 0.2 mg/ml until an optical density at 600 nm of 0.5 was reached, at which time IPTG (isopropyl-β-D-thiogalactopyranoside) was added to 0.5 mM, and the culture was incubated for 90 min more. For protein purification, cells from 1 liter of culture were centrifuged, washed, and suspended in 10 ml of buffer containing 20 mM Tris-HCl (pH 7.6), 50 mM NaCl, 1 mM EDTA, and 3 mM β-mercaptoethanol. For enzyme assays, cells from 25 ml of an induced culture were suspended in 5 ml of buffer containing 0.1 M Tris-HCl (pH 8.5), 0.5 M NaCl, and 1 mM dithiothreitol. The cells were disrupted by passage through a French pressure cell at 20,000 lb/in², and the crude extract was clarified by centrifugation for 20 min at 20,000 × g. The protein was determined by the method of Lowry et al. (25).

Determination of amino-terminal polypeptide sequence. SulC protein purified by gel filtration as described (22) was subjected to preparative electrophoresis in the presence of sodium dodecyl sulfate on a 10 to 20% polyacylamide gradient gel prepared from "ultrapure" acrylamide (International Biotechnologies). The polypeptide band was visualized by placing the gel in 4 M sodium acetate at 4° C for 30 min (13), and the band of interest was excised. The polypeptide was

ATGACGTAGCTAGCTAGTCACGAGTGGCAGTGGAAATIGCCTCTGCCATICGTCTGGCTGATGAAGGCGGAAAATTTAGATTAAAACAATATAAATAA
AAGAAATTGAAAACAATCAATGGATTGCTAACTACCGGACGGA
CCATCTCAAACTCAAGGTCCTCCATATCGGAGGAACTAACCGCAAGGGCTCGACTATTGCTTTTTGAAAAAGATGCTAGAAAAGCTAGGGCTGAGAGTT - 1400 HisLeuLysLeuLysValleuHisIleGlyGlyThrAsnGlyLysGlySerThrIleAlaPheLeuLysLysMetLeuGluLysLeuGlyLeuArgVal
GGCGTGTTTAGCTCGCCCTATCTCATTCATTCACACAGACCAGATTAGCATCAATGGGGAATCGATCTCAGAAGCGAGGCTAGAAGCTCTCATGGCAGACT-1500 GlyValPheSerSerProTyrLeuileHisTyrThrAspGlnileSerIleAsnGiyGluSerIleSerGluAlaArgLeuGluAlaLeuMetAlaAsp
ATCAGTCTTTGCTGGAGGGGAGAAGCGGTCGCCAATTTACAGGGCACAACCGAGTTTGAGATTATCACAGGCCTGGCCTATGACTACTTTGCCTCAGAGCA-1600 TyrGlnSerLeuLeuGluGlyGluAlaValAlaAsnLeuGlnGlyThrThrGluPheGluIleIleThrAlaLeuAlaTyrAspTyrPheAlaSerGluGln
AGTAGATGTGGCCATCATGGAAGTTGGCATGGGTGGACTTTTTGATAGTÄCCAATGTCTGTCAGCCCATTTTGACAGGAATTACAACTATTGGCTTGGAT-1700 ValAspValAlaIleMetGluValGlyMetGlyGlyLeuPheAspSerThrAsnValCysGlnProIleLeuThrGlyIleThrThrIleGlyLeuAsp
CATGTGGCCTTACTTGGTGACACCTTGGAGGCCATAGCAGAGCAGGAGGAGGAGTATTATCAAACAAGGGATGCCCTTGGTAACAGGGGGTATTGCTCCAG-1800 HisValAlaLeuLeuGlyAspThrLeuGluAlaIleAlaGluGlnLysAlaGlyIleIleLysGlnGlyMetProLeuValThrGlyArgIleAlaPro
AAGCCTTGGGTTGTGATTGACCGCATTGCGGAAAGGGAAAGATGCGCCGAGACTTGCCTACGGGACAGATTATCAGGTTCGTCATCAAGAAAGTGTGGTGAC-1900 GlualaLeuAlaValIleAspArgIleAlaGluGlyLysAspAlaProArgLeuAlaTyrGlyThrAspTyrGInValArgHisGInGluSerValValThr
AGGGGAAGTCTTTGACTATÀCAAGTGCTGTCAGACAAGGTCGCTTCCAGÀCTAGCCTGCTTGGTTTGTACCAAATAGAGAATGCTGGGATGGCCATAGCT - 2000 GlyGluValPheAspTyrThrSerAlaValArgGlnGlyArgPheGlnThrSerLeuLeuGlyLeuTyrGlnIleGluAsnAlaGlyMetAlaIleAla
TTACTTGATACTTTTTGTCAAGAAGATGGTCGAGAGCTAGCAAGCA
TCGTGTCAAGAGATCCCTTGATGATTTTGGATGGAGCCCATAATCCCCATGCTATCAAGGCCTTGTTGGTAACCTTGCAGAACGTTTTGCGGATTATCA-2200 IleValSerArgAspProLeuMetIleLeuAspGlyAlaHisAsnProHisAlaIleLySAlaLeuLeuValThrLeuGInGluArgPheAlaAspTyrHis
TAAGGAAATCCTCTTCACTTGTATCAAAACCAAAGCCTTGGAGGATATGTTGGACTTGCTGGGAGCCATGCCAGATACCGAGCTTACTCTAACACATTTT-2300 LysGluileLeuPheThrCysIleLysThrLysAlaLeuGluAspMetLeuAspLeuLeuGlyAlaMetProAspThrGluLeuThrLeuThrHisPhe
GCGGATAGTCGGGCGACGGATGAAAGCGTGCTGAAAGAGGCAGCTAAGTCTAGAAATCTCAGCTACCAAGATTGGCATGATTTTCTAGAGCAGAATGTGA-2400 AlaAspSerArgAlaThrAspGluSerValLeuLysGluAlaAlaLysSerArgAsnLeuSerTyrGlnAspTrpHisAspPheLeuGluGlnAsnVal
CAGATAAAAAAGAAGAAGAAGAAACAAACAAGTTAGGATTGTCACAGGTTCCTTGTATTTCTTGAGCCAAGTGAGGGTCTATCTGATGGAG <u>AGGAAGAAC</u> GAGAA-2500 ThrAspLysLysGluGluLysGlnThrValArglleValThrGlySerLeuTyrPheLeuSerGlnValArgValTyrLeuMetGluArgLysAsnGluAsn Start SulC>
TGGATACACAAAAGATTGAAGCAGCTGTAAAAATGATTATCGAGGCTGTAGGAGAGGACGCTAATCGCGAGGGTTTGCAGGAAACACCTGCTCGTGTAGC-2600 GlyTyrThrLysAspxEnd SulB MetAspThrGlnLysIleGluAlaAlaValLysMetIleIleGluAlaValGlyGluAspAlaAsnArgGluGlyLeuGlnGluThrProAlaArgValAla
* * * * * * * * * * * * * * * * * * *
GATATCTTTTTCCATACCATGTGTGAACACCACTTCTTGCCATTTTATGGTAGAGCGCACATTGCCTACATTCCAGATGGTCGTGGCAGGCTTGTCTA-2800 AspilePhePheHisThrMetCysGluHisHisPheLeuProPheTyrGlyArgAlaHisIleAlaTyrIleProAspClyArgValAlaGlyLeuSer
AGCTAGCCCGTACGGTTGAAGTTTATTCGAAAAAACCACAAATTCAAGAACGTTGGAATATCGAAGTGGCCGATGCCTTGATGGACTATCTAGGTGCTAA-2900 LysLcuAlaArgThrValGluValTyrSerLysLysProGlnileGlnGluArgLeuAsnileGluValAlaAspAlaleuMetAspTyrLeuGlyAlaLys
AGGAGCCTTTGTTATCATTGAGGCGGAACATATGTGTATGAGTATGCGTGGTGTTAGAAAACCAGGCACTGCAACCTTGACGACAGTAGCTCGTGGTCTA-3000 GlyAlaPheVallleileGluAlaGluHisMetCysMetSerMetArgGlyValArgLysProGlyThrAlaThrLeuThrThrValAlaArgGlyLeu

TTTGAAACAGATAAGGACCTCCGCGCCCAAGCTTATCGTTTAATGGGGCTATAAAAGAATCCGTTTCAAGCGGATTTTTCTAG<u>AAAGGA</u>ATCATTATGG-3100 PheGluThrAspLysAspLeuArgAspGlnAlaTyrArgLeuMetGlyLeu<--End SulC Start SulD-->Met

FIG. 2. Nucleotide sequence of a 2-kb portion of the *sul* segment of the *S. pneumoniae* chromosome containing *sulB* and *sulC*. The sequence of one DNA strand is shown with its 5' end at 1101 and its 3' end at 3100, with numbering extending from the reported *sulA* sequence (20). The predicted amino acid sequences of the SulB and SulC polypeptides are shown together with putative mRNA ribosome binding sites for SulC and SulD (underlined). Determination of the amino-terminal sequence of the purified SulC polypeptide revealed the residues marked with asterisks.

electroeluted with an ISCO sample concentrator with a chamber solution of 50 mM NH₄HCO₃ and 0.1% sodium dodecyl sulfate. The solution was lyophilized, and the residue was dissolved in 0.4 ml of water. This was repeated four times to remove NH₄HCO₃. Approximately 1 nmol of polypeptide was subjected to amino-terminal sequence determination in a gas-phase sequenator (Applied Biosystems).

Enzyme assay. GTPCH was assayed as described by Babitzke et al. (1). The mixtures contained, in a volume of 0.5 ml, 0.1 M Tris-HCl (pH 8.5), 25 mM EDTA, 1 mM dithiothreitol, 1 mM GTP, and 0.1 ml of the crude extract. After incubation at 42°C for 30 min, 0.5 ml of an aqueous suspension containing 20 mg of activated charcoal was added and the mixture was filtered through a type HA Millipore filter (pore size, 0.45 μ m). After it was washed with 5 ml of water and 5 ml of 5% ethanol, the neopterin triphosphate product was eluted from the charcoal by 3.1% NaOH in 50% ethanol. To increase the fluorescence yield, samples (1.0 ml) of the NaOH-containing extracts were neutralized by the addition of 0.1 ml of 1 M Tris-HCl (pH 7.4) and 0.14 ml of 4 N HCl. With excitation at 265 nm, the fluorescence emission at 450 nm was measured in a SimoAminco 500 spectrofluorometer and compared with a neopterin standard under the same conditions.

Mapping of mRNA endpoints. RNA was prepared as previously described (24) from *S. pneumoniae* 708 and 708(pLS830). The 5' end of *sul* mRNA was determined by primer extension with 5'-GGCTTTACTTGACATAT-3', which is complementary to nucleotides 258 to 242 in the *sul* DNA (20). One picomole of this

 TABLE 2. GTPCH activity in extracts of *E. coli* expressing various sul genes^a

Plasmid	<i>sul</i> genotype ^b	GTPCH activity (nmol/h/mg of protein)
pET-5	None	2
pLS23	ABCD	32
pLS24	BCD	40
pLS27	AD	3
pLS29	ACD	37
pLS30	ABD	1
pLS31	ABC	20

^a Cultures of BL21(DE3) containing the indicated plasmids were induced, extracts were prepared, and samples containing approximately 50 μg of protein were assayed for GTPCH activity as indicated in Materials and Methods. ^b Intact pneumococcal *sul* genes carried by the plasmids are indicated.

TABLE 3.	Complemen	tation of ar	n E. coli	mutant	deficient	in
DHI	S by various	mutants of	the sul	gene clu	uster ^a	

Plasmid	sul	Colonies (no.) on synthetic medium							
	genotype	Without Met	With Met						
None	None	0	45						
pLS23	ABCD	0	309						
pLS24	BCD	86	141						
pLS27	AD	0	159						
pLS29	ACD	0	91						
pLS30	ABD	85	184						
pLS31	ABC	66	80						

^{*a*} Cultures of strain SF4 containing various plasmids were grown in L broth and diluted and plated in E medium \pm methionine at 50 µg/ml.

^b Intact pneumococcal sul genes carried by the plasmids are indicated.

oligonucleotide was annealed with 15 μ g of total RNA in 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 1 mM MgCl₂, 1 mM dithiothreitol, and 33 U of RNase inhibitor (Promega) in 14 μ l for 5 min at 65°C. After addition of dATP, dGTP, and dTTP, each to 100 μ M, and dCTP to 10 μ M, 15 μ Ci of [α -³²P]dCTP and 20 U of avian myeloblastosis virus reverse transcriptase were added (volume now 20 μ l) and the mixture was incubated at 42°C for 30 min. The products were examined on a sequencing gel and compared with a dideoxynucleotide-terminated DNA sequencing ladder obtained with the same oligonucleotide.

To map the 3' end of the mRNA, pLS23 was first cut with AvaI and SacI. The 604-bp fragment was isolated and cut with HinPI; the two resulting fragments

were labeled at their 3' ends with $[\alpha$ -³²P]dCTP by the Klenow fragment. Further digestion of the *Hin*PI-*Ava*I fragments by *Taq*I gave a 199-bp *Hin*PI-*Taq*I fragment labeled at the 3'-*Hin*PI end and including the 3' end of *sulD*. The labeled strand was used as a probe for nuclease S1 mapping as previously described (24) but with the following modifications. This probe (containing 18,000 cpm) was hybridized by heating for 10 min at 85°C and then for 3 h at 46°C with 150 µg of RNA from strain 708(pLS830) in 50 µl of 50% formamide containing 40 mM PIPES [piperazine-*N*,*N'*-bis(2-ethanesulfonic acid)] (pH 6.3), 0.4 M NaCl, and 1 mM EDTA. The hybridized sample was treated with 100 U of nuclease S1 for 20 min at 30°C and processed as described previously (24). The length of the protected mRNA was determined by comparison with a sequencing ladder from an unrelated DNA fragment.

Nucleotide sequence accession number. The DNA sequence data described in the paper have been deposited in GenBank with the accession number U16156.

RESULTS

DNA sequence of the *sulB* and *sulC* genes. The DNA sequence of a 2-kb segment of the cloned *sul* chromosomal region of *S. pneumoniae* is shown in Fig. 2. Beginning at bp 1101 of the previously reported *sulA* sequence (20), it links that gene to *sulD*, with bp 3100 corresponding to bp 933 of the previously reported *sulD* sequence (22) (add 2,167 to the numbering in reference 22 to match the present base pair designation). Analysis of the newly reported segment revealed two open reading frames in the same orientation between *sulA* and *sulD*. Called *sulB* and *sulC*, they are capable of encoding polypeptides of 49 kDa and 21 kDa, respectively (Fig. 2). High expression of the *sul* gene segment in *E. coli* previously showed



FIG. 3. Mapping of endpoints of the folate pathway mRNA. (A) Mapping of the 5' endpoint by primer extension. Lanes A, C, G, and T show DNA sequencing ladders primed by the oligonucleotide used for primer extension on RNA from *S. pneumoniae* 708 (lane 1) and 708(pLS830) (lane 2). The arrow marks the primer extension product with the 5'-labeled oligonucleotide. The sequence at the bottom indicates the start site and the promoter, with the -10 site and two possible -35 sites boxed. Uppercase letters indicate agreement with the consensus sequence (and also the mRNA start). (B) Mapping of the 3' endpoint by S1 nuclease digestion. Lane 3, RNA from *S. pneumoniae* 708(pLS830) hybridized to the 3'-labeled *HinPI-TaqI* restriction fragment and treated with S1 nuclease. Lanes A, C, G, and T, sequencing ladders for unrelated DNA (lengths from the beginning of the primer are shown on the right). F, full-length restriction fragment. Unlabeled arrows indicate termination points, with half-arrows corresponding to minor bands. At the left is depicted a segment of DNA following the *sulD* gene showing the ability of the mRNA to fold into a hairpin.

	1 2	
	X X iX Xged r gL TP R A XX X GX X f demVXvKDI X s	mCE
Spn	1-MDTQKIEAAVKMIIEAVGEDANREGLQETPARVARMY-QEIFSGLGQTAEEHLSK-SFEIIDDNMVVEKDIFFHT	MCE-76
Bsu	1-MKEVNKEQIEQAVRQILEAIGEDPNREGLLDTPKRVAKMY-AEVFSGLNEDPKEHFQT-IFGENHEELVLVKDIAFHS	MCE-79
Rra	22-ASRPAEKSRPPEAKGAQPADAWKAGRPRSEEDNELNLPNLAAAYSSILRSLGEDPQRQGLLKTPWRAATAM-QFFTKGYQETISDVLNDAIFDEDHDEMVIVKDIDMFS	MCE-132
Dme	91-TNGSSPDSDGHEKCTFHHDLELDHKPPTREALLPDMARSYRLLLGGLGENPDRQGLIKTPERAAKAM-LYFTKGYDQSLEDVLNGAVFDEDHDEMVVVKDIEMFS	MCE-198
Eco	1-MPSLSKEAALVHEALVARGLETPLRPPVHEMDNETRKSLIAGHMTEIMQLLNLDLADDSLMETPHRIAKMYVDEIFSGLDYANFPKITLIENKMKVDEMVTVRDITLTS	:: TCE-112
	2 3 4 5	
	HHXvpfxGBX XOYXP VXGLSKlaRXVeXXObB QXQERLt ia l v VxXea HmCm mRGv k TXTs X GXF d R eXX xX	
Spn	77-HHFLPFYGRAHIAYIP-DGRVAGLSKLARTVEVYSKKPQIQERLNIEVADALMDYL-GAKGAFVIIEAEHMCMSMRGVRKPGTATLTTVARGLFETDKDLRDQAYRLMG	L-184
Bsu	80-HHLVPFYGKAHVAYIPRGGKVTGLSKLARAVEAVAKRPQLQERITSTIAESIVETL-DPHGVMVVVEAEHMCMTMRGVRKPGAKTVTSAVRGVFKDDAAARAEVLEHIK	RQD - 190
Rra	133-HHLVPFVGRVHIGYLP-NKQVLGLSKLARIVEIYSRRLQVQERLTKQIAVAITEAL-QPAGVGVVIEATHMCMVMRGVQKMNSKTVTSTMLGVFREDPKTREEFLTLIR	s-241
Rra Dme	133-HHLVPFVGRVHIGYLP-NKQVLGLSKLARIVEIYSRRLQVOERLTKQIAVAITEAL-OPAGVGVVIEATHMCMVMRGVQKMNSKTVTSTMLGVFREDPKTREEFLTLIR 199-HHLVPFYGKVSIGYLP-CNKILGLSKLARIVEIFSRRLQVOERLTKQIAVARDSGPCNPAGVAVVVEGVHMCMVMRGVQKINSKTVTSTMLGVFRDDPKTREEFLNLVN	S-241 : SK-308

FIG. 4. Sequence comparison of SulC and homologous proteins. Numbers indicate positions in the protein sequence, with 1 corresponding to the N terminus. Dashes within the sequences indicate gaps giving optimal alignment. Spn, *Streptococcus pneumoniae*; Bsu, *Bacillus subtilis* (11); Rra, *Rattus rattus* (12); Dme, *Drosophila melanogaster* (28); Eco, *Escherichia coli* (14); :, identical residues; ,, similar residues (based on the groups A, G, and P; D, E, N, and Q; F, I, L, M, V, W, and Y; H, K, and R; and S and T). Above the Spn sequence are indicated residues invariant among all five species (uppercase) or any four (lowercase). X and x represent hydrophobic residues A, F, I, L, M, V, W, and Y; O represents a residue (A, G, or P) with a small side chain. Boxes of conserved residues are indicated by numbered overlines.

the production of polypeptides of these sizes, which we shall call SulB and SulC, in addition to polypeptides SulA and SulD. The SulC polypeptide was purified, and its amino-terminal sequence was determined. The results show that SulC begins at the predicted site in that 11 of the 12 predicted amino acid residues were observed (Fig. 2). No product corresponding to the Glu residue was observed in the seventh step. Insufficient amounts of SulB were obtained for amino-terminal analysis. A Shine-Dalgarno sequence for ribosome binding is present before the start of *sulC* (Fig. 2); no such sequence is associated with *sulB*. At the position complementary to the Shine-Dalgarno sequence, the nucleotide sequence of *S. pneumoniae* 16S rRNA is identical to that of *E. coli* (2).

Identification of SulC function. When the sulC sequence was determined and the protein it encoded was compared with those in the GenBank database, a high degree of similarity was found with the GTPCH protein of the rat (12) and the protein produced by the *mtrA* gene of *B. subtilis* (11), with 43 and 53% identities of amino acid residues, respectively (see Fig. 4). The mtrA gene product was subsequently shown to have GTPCH activity (1). We have determined that the sulC product has GTPCH activity by expressing the protein from plasmids carrying the sul segment in E. coli. Only plasmids carrying an intact sulC gene expressed this enzyme activity, which was measured in extracts of E. coli containing various deleted derivatives of pLS23 (Table 2). These deletions removed either part of sulA (pLS24), parts of both sulB and sulC (pLS27), or 4-bp segments within each of the genes sulB (pLS29), sulC (pLS30), and sulD (pLS31). Only pLS27 and pLS30 failed to express the pneumococcal GTPCH.

Identification of SulB function. The *sulB* sequence encoded a protein that showed similarity (28% identity) to the previously determined *E. coli* DHFS coded for by the *folC* gene (3). We have not been successful in demonstrating DHFS activity in extracts of cells carrying this gene, but we were able to show that plasmids containing an intact *sulB* gene could complement a mutant *folC* gene in *E. coli*. The *E. coli* mutant strain SF4, as a result of a defect in DHFS, requires methionine for growth (4). We transferred plasmids containing the entire *sul* segment or various deleted derivatives into strain SF4 and tested colony-forming ability in the presence and absence of methionine (Table 3). Plasmids pLS24, pLS30, and pLS31, which are defective in genes other than *sulB*, complemented SF4 in the absence of methionine; plasmids pLS27 and pLS29, which are defective in *sulB*, did not. We cannot explain the behavior of pLS23, which contains an intact *sul* segment but did not complement the SF4 mutation. Otherwise, all the data support a DHFS function for the *sulB* product.

Mapping of the sul transcription product. From the DNA sequence, a consensus procaryotic promoter was observed upstream from sulA (20) and a palindrome corresponding to a potential terminator was evident downstream from sulD (22). The palindrome was positioned between *sulD* and a convergent open reading frame, so it could serve as a terminator for mRNAs transcribed in the two directions. The 5' end of the sul mRNA was mapped by primer extension and shown to start at A-210 in the reported DNA sequence (20) for mRNA transcribed both from the chromosome and from plasmid pLS830 (Fig. 3A). This start site corresponds to a promoter with a -10site close to consensus (34) and with all three bases corresponding to extended -10 sites (15), as shown in Fig. 3A. Two possible -35 sites, at a distance of 17 or 18 nucleotides from the -10 TAaAAT box, both show consensus bases in three of the six positions. The 3' end of the sul mRNA was determined by S1 nuclease digestion of RNA from S. pneumoniae containing pLS830 hybridized with a 3'-end-labeled DNA segment extending from positions 3843 to 4041, as numbered in the present work. Several closely spaced bands were obtained, with the three heaviest indicating termination of the mRNA at the end of the hairpin structure (bp 3955) deduced from the DNA sequence (Fig. 3B). These results are consistent with transcription of the sul genes as a polycistronic mRNA of 3,745 \pm 2 nucleotides in length.

Comparison of the SulC and SulB proteins with their homologs. The protein sequence of SulC is compared in Fig. 4 with those of its rat homolog and the MtrB protein of *B. subtilis.* All three proteins have been shown to function as GTPCH. In addition, two apparently homologous proteins

X XG Xssp XX X e i Xn XXhX GTnGKgs ΧL G х XE хх -LRVGVFSSPYLIHY-TDQISINGESISEARLEALMADYQSLLEGEAVAN-113 1-MKEIENNQWIANYRTDQPHFGLERMVELLALRGNPHLKLKVLHIGGTNGKGSTIAFLKKMLEKLG-Spn MFTAYQDARSWIHGRLKFGVKPGLGRMKQLMARLGHPEKKIRAFHVAGTNGKGSTVAFIRSMLQEAG -LTVGLYTSPFIMRF-NERIMIDHEPIPDAALVNAVAFVRAALERLQQQQ-113 Lca Hsa 9-AASPLASWLSYLENLHSKTIDLGLERVSLVAARLGVLKPAPFVFTVAGTNGKGTTCRTLESILMAAG-Eco .: :.. :. 1-MDDISGRQTLPRINRLLEHVGNPQDSLSILHIAGTNGKETVSKFLTSILQHPGQQRQRVLIGRYTTSSLLNAKEEDISINNEAIS---LIEYSRIEKELIEADSSLK-115 Sce

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Bsu 116-YGQP	::::::::::::::::::::::::::::::::::::::	CAFLY	:: Faefh	:: : KVDFV1	FETGL	:: .GGR	:::::: FDSTNVVE	-PL	:: :: LTVIT	:.:: SIGH	::. DHMI	.::. NILGN	:.: : TIEEI	: [: A A	.:: GEKA	:::: GIIK	.:.: EGIP	.::. •IVTA	:: VTQPE	: : :: \-LQVI	. :: RHEAE	. :: RHAAP	FQSLH-	225
Ica 114-ADEN	:::::::: /TEFEFIT#	 Algywyi	: : . FRQ-R	::	: :. /IEVGI	:: GGD	::::: TDSTNVIT	:. -PV	:.: VSVLT	: TEVALI	:: DHQI	.:: Kllgh	:: :: TITAI	: [: AI	:: KHKA	:::: GIIK	::: RGIF	· : : .	 NLVPD/	: :. \-AAV\	AAKVA	TTGSO		223
Hea 130-CVSM	: :.:	MAEHVI	: : FLQ-F	::.::		::	:.::.: YDCTN11R	::	:		:: DHT:	::: SII GD	:. : TVFKI		: AI	:. WOKGI	::.: GI F K	:.:	.: AFT-	: :. VI OPFI	. ::.	RDRAD	DISCP	YI CP-	238
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Spn	223-TDYQVRHQESVV1	-GEVFDYTSAVR	-QGRFQTSLLGL	YQIENAGMAI	ALLDTFCQEDG	RELASN	-DFLGQALEETSW	PGRLEIVSRDP	LMILDGAHNPHAIKALLVT-3	26
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ßsu	226-DACVIFNEEALPA	-GEQFSFKTEEK	CYEDIRTSLIG	HQRQNAALSI	-LAAEWLNKEN	IAHISD	-EALRSGLVKAAW	PGRLELVQEHP	PVYLDGAHNEEGVEKLAET - 3	29
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Lca	224-RDFSVPKAKLHG	-GQRFTYEDQDGI	RISDLEVPLVGD	YQQRNMAIAI	-QTAKVYAKQT	EWPLTP	-QNIRQGLAASHW	PARLEKISDTP	LIVIDGAHNPDGINGLITA-3	27
		:			: .	:: :	.: :: . :			
Hsa	239-MLEALEEGGPPL1	LGLEGEHQRSNA	ALALQLAHCWLG	RODRHGAGEP	KASRPGLLW	OLPLAPVFQP	TSHMRLGLRNTEW	PGRTQVLRRGP	-LTWYLDGAHTASSAQACVRW-3	49
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Eco	219-VEWNYSV-TDHDW	4	AFSDAHGTLE	NLPLPLVPQP	-NAATALAALR	ASGLEVSE	-NAIRDGIASAIL	PGRFQIVSESP	RVIFDVAHNPHAAEYLTGR-3	16
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Sce	210DYNVHTDYNV	/HTDTWG	TLEVRLPYSEE	YQIFNLRVAI	AVLD-FLSKEK	KVCISK	-DQLSQGLISVDW	PRSLHRLDYCYESTSGK	KIALLLDNANNAKAARNLACH-3	14

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Bsu	330·	-MKQ	RFANSRISV	/VFSA	\LK	۶KP	QNMIKE	LETIA	HA I	HFASF	FDFPRA	SLAK	DLYD	ASEIS	NKSWSI	EDPDD	VIKFI	ESK	KGSNE	EIVLI	ITGSL	YFI	SDIRKI	RLK-43	30	
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Sce	315-	LRŤ	TYGDTPLTF	VIAI	TTO	KK/	SPLLDF	L - IRPO	DYVIVT	RFGSV	VGMPW	IQSL	EPVN	LLAFI	K-377	39	97-LE	TSG	LKTIN	/PVI\	/CGSL	YIC	KELLRI	LHNCHL	.PV-430)

FIG. 5. Sequence comparison of SulB and homologous proteins. Numbers indicate positions in the protein sequence, with 1 corresponding to the N terminus. Dashes within the sequences indicate gaps giving optimal alignment. Spn, *Streptococcus pneumoniae*; Bsu, *Bacillus subtilis* (26, 29); Lca, *Lactobacillus casei* (41); Hsa, *Homo sapiens* (9); Eco, *Escherichia coli* (3); Sce, *Saccharomyces cerevisiae* (GenBank accession no. Z28131); ; identical residues; ., similar residues (based on the groups A, G, and P; D, E, N, and Q; F, I, L, M, V, W, and Y; H, K, and R; and S and T). Above the Spn sequence are indicated residues conserved among all six examples (uppercase) or any five (lowercase). X and x represent hydrophobic residues A, F, I, L, M, V, W, and Y; O and o represent residues (A, G, and P) with a small side chain. Boxes of conserved residues are indicated by numbered overlines.

identified by nucleic acid sequences in *E. coli* and *Drosophila melanogaster* are included in the comparison. Homologous proteins identified by nucleic acid sequencing of human and mouse nucleic acids are very similar to the rat protein and will not be considered separately. It is evident from Fig. 4 that a very high degree of sequence conservation exists for this protein. The Spn and Bsu proteins have 53% of their amino acid residues in common. Among the five homologs, 37 amino acid

residues, or 20%, are invariant. An additional 20% have only hydrophobic side chains. These figures are considerably higher than those that would be found among exemplars of other proteins in these not closely related species, as witnessed for SulB (see below). The basis for such conservation may be the necessity for a protein of relatively small size to carry out multiple reactions. As shown in Fig. 1, the enzyme recognizes GTP, breaks a bond in a ring, removes a formate group, and

Streptococcus pneumoniae:		4 DHPS	5 DHFS	1 GTPCH D	2 3 HNA PPPK
	P 🗔	sulA	XsulB	XX sulC	sulD
Bacillus subtilis:		4 DHPS	2 3 DHNA PPPK	1 GTPCH	5 DHFS
pab trpG p	X pabC	sul	orf1 orf2	mtrA, B	folC
Pneumocystis carinii:			2 3 DHNA PPPK <i>fas</i> (ABCD)	4 DHPS	
Escherichia coli: F	3 HPPK <i>folK</i> 4cs	1 GTPCH <i>folE</i> 48cs	5 DHFS <i>folC</i> 50cs		4 DHPS folP 72cs

FIG. 6. Folate biosynthetic gene arrangements in different species. Genes or gene segments corresponding to enzyme domains are drawn to scale as boxlike arrows, with the orientation indicated by the arrowhead. Genes not shown adjacent are far apart in the genome. Gene designations and enzyme designations are given below and above the arrows, respectively. The numbers refer to the order of the steps in the folate pathway. The chromosomal locations of the *E. coli* genes were determined, and they are indicated in centisomes (cs). P and T refer to experimentally verified transcription promoters and terminators.

reforms a ring. Presumably, there is not much variability that can be tolerated in the structural basis for this performance. In keeping with multiple functions, there are five boxes of especially highly conserved regions, as indicated in Fig. 4. The first of these was hypothesized to correspond to a pterin-binding site (12), but possible functions of the other boxes are unknown.

In Fig. 5, the SulB sequence is compared with those of five homologous proteins, of which three (Eco, Hsa, and Lca) have been shown to exhibit DHFS activity and folylpoly(γ -glutamate) synthetase activity as well (9, 14, 41). The Bsu and Sce homologs were identified by nucleic acid sequencing. This group of proteins shows significant homology over their entire lengths (except for an insert of approximately 100 amino acid residues in Hsa). Six boxes of especially high conservation are evident (Fig. 5).

DISCUSSION

Because the 5' and 3' endpoints of sul mRNA correspond to the only promoter and transcription terminator evident in the nucleotide sequence, we presume that the operon is expressed as a single transcript. In matching the consensus promoter sequence, the sul promoter is weak at its -35 site (3 of 6 nucleotides match) and strong at its -10 site (5 of 6 nucleotides match), and it shows an extended -10 sequence (15). Good matches to the Shine-Dalgarno sequence (35) are found upstream of all the genes except sulB. Not surprisingly, the sulB gene product was found in the least amount when the operon was expressed in minicells of B. subtilis (unpublished results) or on an *E. coli* plasmid (22). Whether its expression is closer to that of the other genes under natural conditions in the chromosome of S. pneumoniae is not known. The proximity of the SulB start site to the termination of SulA might allow enhancement of SulB production by translational coupling (30). The homologous E. coli gene, folC, is also expressed relatively poorly in maxicells (3), and the corresponding enzyme is present at low levels in all cells studied (41).

Our findings indicate that all five enzymatic activities that have been demonstrated to function in folate biosynthesis are coded for by a single gene cluster in *S. pneumoniae*. Inasmuch as one of the protein products, SulD, carries two of these activities, it is conceivable that one of the other proteins may harbor another activity, such as the phosphatase needed to convert the product of GTPCH action to dihydroneopterin. The syncretion of two activities in SulD that are coded for as separate polypeptides in *B. subtilis* (37) and *E. coli* (40) is carried even further in the case of *P. carinii*, in which the polypeptide product of a single gene, *fas*, also contains a DHPS region and an additional segment of unknown function in addition to the two activities of SulD (44). It was shown that the *fas* product can express its multiple activities, like SulD, without prior cleavage (43).

The clustering of the genes for folate biosynthesis, presumably to help regulate their expression, is more marked for S. pneumoniae than for the other species in which these genes have been identified. Figure 6 depicts the arrangement of the genes in the different species. Although the three genes encoding DHPS, DHNA, and PPPK are found together in B. subtilis, the intervening genes in S. pneumoniae that encode DHFS and GTPCH are found elsewhere and separately. In addition, the three former genes are part of a larger cluster that includes two genes for p-aminobenzoate synthesis and the trpG gene, which has a role in aromatic synthesis (37). Interestingly, GTPCH is coded for by mtrA, which forms an operon with *mtrB*, the product of which acts as repressor of the *trp* operon and possibly as a translational repressor of trpG expression (1). In P. carinii, as mentioned, three activities are encoded together, but the genes for the other activities have not been located. In the case of E. coli, which encodes PPPK on a separate polypeptide, none of the four genes identified so far are linked. In particular, the DNA immediately upstream of the PPPK homolog does not encode DHNA, as in the other three species, but corresponds to the pcnB gene (19), which encodes a poly(A) polymerase (5).

The five genes or gene segments encoding the different activities show various degrees of conservation, as indicated in Table 4, which lists the percentages of identical amino acids between *S. pneumoniae* and the other species. For example, with *B. subtilis*, which showed the greatest similarity to pneumococcal proteins, the degrees of identity ranged from 28%

	Percent residues identical in S. pneumoniae												
Species	GTPCH	DHNA ^a	$PPPK^b$	DHPS	DHFS								
Bacillus subtilis	53	28	38	41	37								
Lactobacillus casei					34								
Pneumocystis carinii ^c		20	36	41									
Homo sapiens	42				31								
Drosophila melanogaster	40												
Escherichia coli	38		32	35	28								
Saccharomyces cerevisiae					26								

 TABLE 4. Extent of homology between folate biosynthetic proteins of S. pneumoniae and those of other species

^a 119 N-terminal amino acid residues of SulD.

^b 151 C-terminal amino acid residues of SulD.

^c Corresponding segments of Fas protein (44).

for DHNA to 53% for GTPCH. Another gram-positive bacterium, Lactobacillus casei, gave a 34% identity with S. pneumoniae, compared with 37% for B. subtilis, for the single protein examined (DHFS). For each protein, E. coli usually showed the least identity, averaging nine percentage points below the identity values for *B. subtilis*. The identity values for eucaryotic proteins were in between, averaging seven percentage points below those for B. subtilis. Although these data are admittedly very limited, they suggest that the gram-positive bacteria diverged from the gram-negative bacteria earlier than they did from the progenitor of eucaryotes. At the time when the bacterial and eucaryotic progenitors separated, presumably more than a billion years ago, a prevalence of introns in their genomes (as hypothesized in reference 10) may have fostered a fluidity in gene rearrangement that accounts for the variety of folate biosynthetic gene organization that we see today.

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