JOURNAL OF BACTERIOLOGY, June 1990, p. 3358–3366
0021-9193/90/063358-09$02.00/0

Vol. 172, No. 6

Sulfate and Thiosulfate Transport in *Escherichia coli* K-12: Identification of a Gene Encoding a Novel Protein Involved in Thiosulfate Binding

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Received 16 October 1989/Accepted 22 February 1990

The sequence of 1,973 nucleotides encompassing the region at and directly adjacent to the CysB-dependent promoter controlling expression and synthesis of the sulfate-thiosulfate transport system of *Escherichia coli* has been determined. The transcription start site has been mapped by primer extension. One open reading frame representing the first gene of the presumed sulfate transport operon was identified and designated *cysP*. The deduced amino acid sequence of the CysP polypeptide indicates the presence of a signal peptide. Expression of the *cysP* gene in the T7 promoter-polymerase system revealed the location of the gene product in the periplasm. Construction of a *cysP* insertional mutant and assays of binding and uptake of sulfate and thiosulfate by this mutant allowed the identification of the *cysP* gene product as a thiosulfate-binding protein. The TGA termination codon of *cysP* was found to overlap the putative ATG initiation codon of the next open reading frame, inferred as being essential for the sulfate transport system, and it was designated *cysT*. Preliminary sequence data from the corresponding region of the *Salmonella typhimurium* chromosome showed strictly homologous counterparts of the *E. coli cysP* and *cysT* genes.

Escherichia coli and *Salmonella typhimurium* possess a sulfate transport system which is controlled in parallel with cysteine-biosynthetic enzymes and which is a part of the cysteine regulon (19); gene expression of this region requires sulfur limitation and the positive regulator CysB, the product of the *cysB* gene. Mutations impairing sulfate transport are localized in the "cysA" region, which maps at min 49 of *S. typhimurium* (8, 9, 25) and min 52 of the *E. coli* chromosome (4, 18). In *E. coli* a second locus, *cysZ*, situated about 10 kilobases (kb) from "cysA," has been reported to be essential for sulfate transport (34). Early genetic studies on the "cysA" locus in *S. typhimurium* showed the presence of three complementation groups (25). Furthermore, these studies indicated that integrity of the whole three-cistron region is required for both sulfate and thiosulfate transport (8). Subsequently, the specific sulfate-binding protein (SBP) was isolated from *S. typhimurium* (32) and studied in detail (16, 29, 33, 35). This analysis raised the possibility that the sulfate transport system might have similarities with other well-characterized binding-protein-dependent transport systems (1), where a single operon contains genes encoding the binding protein and several membrane-bound components. However, a direct relationship between SBP and the genetics of sulfate transport remains to be demonstrated because (i) none of the three "cysA" cistrons of *S. typhimurium* has been shown to encode this activity, and (ii) transport-defective mutants with a specific lesion in SBP could not be isolated despite a thorough search (29).

We have cloned the region containing the "cysA" and *cysM* (the latter specifying the *O*-acetylserine sulfhydrylase B) loci of *E. coli* (40) and identified the CysB-dependent promoter and two complementation groups within the "cysA" locus (M. Hryniewicz, A. Sirko, and D. Hulanicka, Acta Biochim. Polon., in press). The sequence analysis of this region presented in the accompanying paper (41) revealed the presence of four open reading frames (ORFs) coding for three membrane components of the sulfate transport system and of an ORF for *cysM*. Here, we report the identification of an unknown locus, designated *cysP*, specifying the first gene of the region encoding the sulfate-thiosulfate transport system. It is expressed from a CysB-dependent promoter and encodes a thiosulfate-binding protein.

(The nucleotide sequence data reported here will appear in the EMBL, GenBank, and DDBJ nucleotide sequence data bases under accession no. M32101.)

MATERIALS AND METHODS

Bacterial strains and phages. All strains used in this study were derivatives of *E. coli* K-12 and *S. typhimurium* LT2 and are listed in Table 1. P1 *vir* phage was used for transductions by the method of Miller (24). Phage M13 derivatives mp10, mp11, mp18, and mp19 (48) were used to generate single-stranded templates of subcloned DNA fragments.

Media. Media used for bacterial cultures were described by Miller (24) and consisted of LB for plasmid transformation, 2×YT for M13 DNA transformation and phage production, and M9 for pulse-labeling of plasmid-encoded proteins. Sulfate-free minimal medium (SF) (14) was supplemented with 0.5% glucose and 1 mM L-djenkolic acid or 0.1 mM L-cystine as sole sulfur sources. These two compounds cause derepression and repression of the cysteine regulon, respectively (19). Amino acids were added to 0.2 mM, ampicillin to 50 μg/ml, and chloramphenicol to 30 μg/ml final concentrations when required. Solid media contained 1.5% agar or 0.6% agar for overlays.

Plasmids. Plasmid pGP1-2 (carrying the gene for the T7 polymerase cloned into pBR322 under the inducible control

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TABLE 1. Bacterial strains

Strain	Genotype or relevant characteristics	Source or reference
E. coli		
JM101	F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q}ZΔM15/Δ(*pro-lac*) *thi supE*	48
JC7623	*recB recC sbcB15*	20
K38	HfrC *phoA4 pit-10 tonA22 ompF627 relA1* (λ)	22
EC1119	Δ*trpE5 leu-6 thi hsdR hsdM*$^+$ *hsdM*$^+$ *cysA15*	Stock collection
EC1250	F$^-$ *araD139* Δ*lacU169 rpsL thi fla trp-3*	17
EC2256	EC1250 *cysT329*::*lac imm*$^{\lambda p1(209)}$	12
EC2275	EC1250 *cysB trp*$^+$	Stock collection
EC2297	EC1250 *cysP*	This work
EC2299	EC2297(pMHH3222)	This work
S. typhimurium TL117	Δ(*proAB47*)	7

of the bacteriophage λ p_L promoter) and PT7 vector, for placing the target gene under control of the T7 promoter, were described before (42) and were gifts of S. Tabor. Plasmids containing DNA fragments from the 52-min region of the *E. coli* chromosome are shown in Fig. 1a. Plasmid pAMH2 has been described previously (40); it carries an approximately 7.5-kilobase (kb) chromosomal DNA fragment containing the entire "cysA"-*cysM* region on a mini-Mu-*lac* replicon. A 2.3-kb *Eco*RV fragment spanning the region of the CysB-dependent promoter was subcloned into the *Hin*cII site of pUC18 to create plasmid pMHH1822. This plasmid was subsequently used as a source of restriction fragments for DNA sequencing (Fig. 1b). As the handling of some strains with pMHH1822 appeared to be difficult, presumably because of overproduction of the *cysP* gene product, the 2.1-kb *Hin*dIII-*Bam*HI fragment (*Hin*dIII site from the polylinker of pUC18) was recloned into pBR322 to create pMHH3222. pASB1 was a derivative of pT7-6 containing the same chromosomal DNA fragment as pMHH3222 (the entire *cysP* region) inserted into the multiple cloning site of the vector in the same orientation as the T7 promoter (not shown). pASB2 was constructed by insertion of the 1.3-kb *Hae*II fragment of pACYC184 (6), containing the *cat* gene, into the *Asu*II site of pMHH1822. Plasmid pAPH1 (Fig. 1c), containing the region spanning "cysA"-*cysM* of *S. typhimurium*, according to a previously published restriction map (15), was obtained by cloning an 8-kb chromosomal *Cla*I fragment into the *Cla*I site of pBR322. Positive clones were selected on the basis of recovery of a Cys$^+$ phenotype in transformants of *E. coli* mutant strain EC1119 (*cysA15*).

Recombinant DNA techniques. Plasmid DNA preparations, restriction enzyme digestions, ligations, gel electrophoresis of DNA fragments, and ^{32}P-labeling of DNA probes (nick translation) were performed by standard methods (23).

Southern hybridization. Transfer of restriction fragments from agarose gels onto nitrocellulose sheets and hybridization with radiolabeled DNA fragments were done as described before (2). Hybridization was performed at 65°C in the following buffer: 1% crystalline bovine serum albumin, 1 mM EDTA, 0.5 M $NaHPO_4$ (pH 7.2), and 7% sodium dodecyl sulfate (SDS). Filters were washed twice with low-stringency wash buffer and then five to eight times with high-stringency wash buffer at room temperature.

DNA sequencing. Techniques used for cloning and sequence determination in recombinant M13 phages have been described (38, 43, 48). The sequencing procedures followed the protocols recommended in the M13 Sequencing Kit (Amersham) for Klenow reactions and United States Biochemical Corp. for Sequenase reactions. Most sequencing reactions were done with the 17-mer universal primer; in some instances DNA synthesis was initiated at sites internal to a subcloned fragment by using a synthetic primer.

5′-End mapping of *cysP* mRNA. Transcript mapping was performed with avian myeloblastosis virus reverse transcriptase (13). Synthetic primers 5′-TACCGTTTGGTGAT TTGGAAGTTGAAAAGG-3′ and 5′-TGAGTTCTTTTTCA GTAAGTTAACGGCCAT-3′ were supplied by the Centre for Molecular and Macromolecular Studies (Łódź, Poland). Total RNA was isolated by the diethylpyrocarbonate method (2) from *E. coli* EC1250 grown on SF medium supplemented with either djenkolic acid or cystine. The primers were labeled with [γ-^{32}P]dATP by using polynucleotide kinase (23). Hybridization of the labeled primer (10^6 cpm) to RNA and extension of the primer with reverse transcriptase were performed by the method of Ausubel et al. (2). The reaction products were analyzed on denaturing polyacrylamide gels containing 7 M urea and compared with a sequence ladder of M13mp18 template DNA.

Analysis of plasmid-encoded proteins. The principle for exclusive labeling of plasmid-encoded proteins with T7 RNA polymerase-T7 promoter system was that described by Tabor and Richardson (42). *E. coli* K38, containing both pGP1-2 and the pT7-derived recombinant plasmid pASB1, was grown at 30°C in LB medium with 40 μg of ampicillin and kanamycin per ml. At an OD_{590} of 0.5, 0.2-ml portions of the culture were harvested, washed with 5 ml of M9 medium, and suspended in 1 ml of M9 medium supplemented with thiamine (20 μg/ml) and 18 amino acids (0.01% [wt/vol] each) lacking cysteine and methionine. Cells were grown with shaking at 30°C for 60 min, and then the temperature was shifted to 42°C for 15 min, rifampin was added at a final concentration of 200 μg/ml, and incubation was continued for 10 min. The temperature was lowered to 30°C for 20 min, and samples were pulsed with 10 μCi of [^{35}S]methionine for 5 min. Subsequently, cells were centrifuged, suspended in 120 μl of disaggregation buffer (60 mM Tris hydrochloride [Tris-HCl, pH 6.8], 1% SDS, 1% 2-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue), heated to 95°C for 3 min, and applied to 12.5% SDS–polyacrylamide gels. Polyacrylamide-SDS gel electrophoresis was performed (21), and the radiolabeled proteins were visualized by autoradiography.

Isolation of periplasmic proteins. Periplasmic proteins were isolated from cultures grown on SF medium with djenkolic acid as a sulfur source and were released by osmotic shock (46). Protein concentration was estimated by the method of Whitaker and Granum (45).

Assays of sulfate and thiosulfate uptake and binding. The

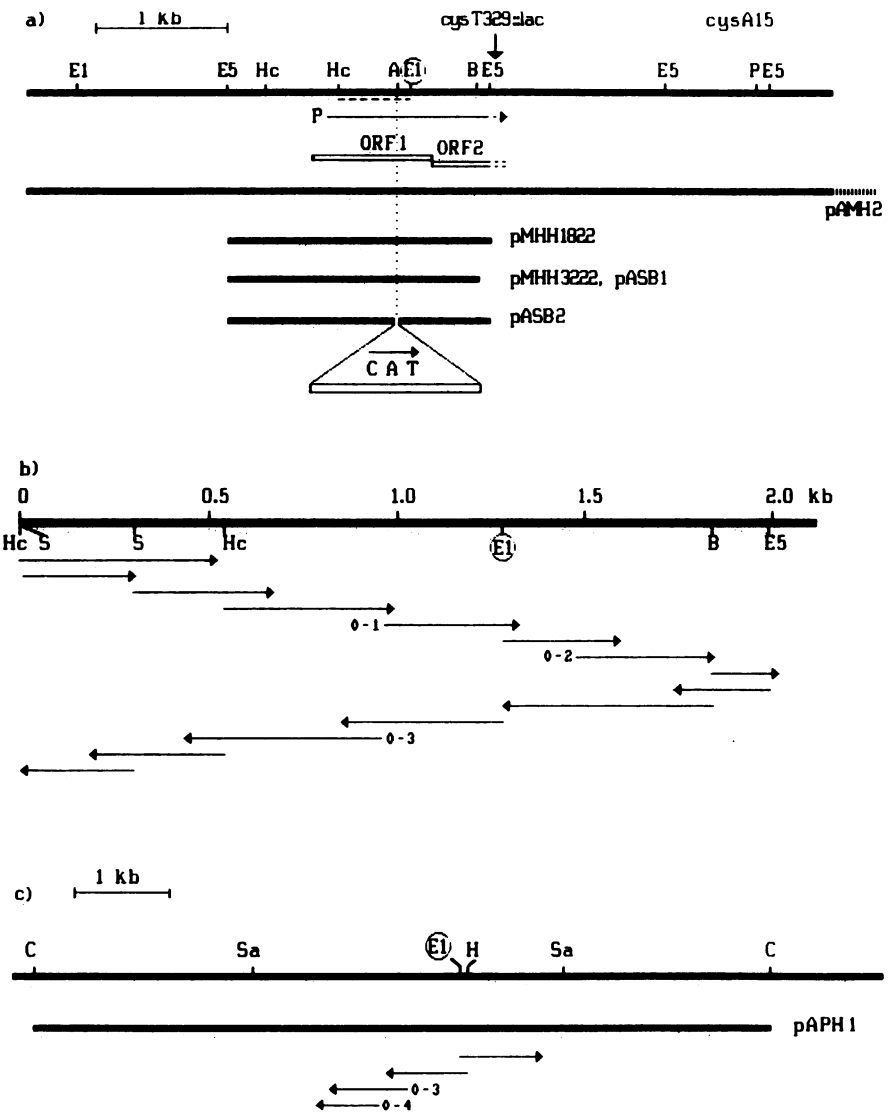

FIG. 1. Restriction maps of the "cysA" region of the (a) *E. coli* (40) and (c) *S. typhimurium* (15) chromosomes. Plasmids used in this study containing cloned fragments of chromosomal DNA are shown below the restriction maps. pMHH1822 is derivative of pUC18, pASB1 is a derivative of pT7-6, and pMHH3222 and pAPH1 are derivatives of pBR322. They are aligned with respect to the corresponding restriction sites on the chromosomal maps. Plasmid pASB2 contains the 1.3-kb insert of pACYC184 spanning the *cat* gene cloned in the *Asu*II site of pAMH1822. Positions of previously localized mutations *cysT329*::*lac* and *cysA15* are given above the *E. coli* map. The position of the CysB-dependent promoter is shown by P, and the direction of transcription is shown by a horizontal arrow. The dashed line shows the 0.72-kb fragment used as a probe for the hybridization experiments. The sequencing strategy of the *E. coli* (b) *S. typhimurium* (c) fragments is shown; segments marked by 0-1, 0-2, 0-3, and 0-4 were sequenced by using synthetic oligonucleotides as primers specific for that particular sequence. Abbreviations: A, *Asu*II; B, *Bam*HI; C, *Cla*I; E1, *Eco*RI; E5, *Eco*RV; H, *Hin*dIII; Hc, *Hin*cII; P, *Pst*I; S, *Sma*I; Sa, *Sal*I; the *Eco*RI site homologous in *E. coli* and *S. typhimurium* is circled.

indirect assay of sulfate and thiosulfate uptake by whole cells and the assay of binding activity of osmotic shock fluids were performed by the method of Pardee et al. (33) with minor modifications. For the uptake assay, *E. coli* cells were grown in SF medium with djenkolic acid. At an OD_{650} of 0.4 to 0.6, cells were harvested and resuspended in SF medium with glucose (2 mg/ml) and chloramphenicol (30 μg/ml). Cells (1.7 to 3.4 mg of protein per ml) were incubated in a final volume of 0.2 ml with 0.01 mM sodium [^{35}S]sulfate (0.14 μCi) or 0.01 mM sodium [^{35}S]thiosulfate (0.014 μCi) for 3 min at 37°C. The incubation was terminated by a 30-s centrifugation in an Eppendorf centrifuge; samples of the supernatant were transferred to GF/C fiberglass filters, placed in scintillation vials, dried, and counted in a scintillation counter. The protein content of the cell suspension was estimated by assuming that 10^9 cells contained 150 μg of protein (24). Binding activity of cell-free shock fluids was measured by incubation of 0.2-ml samples of shock fluid (concentrated about 50 times) with 25 μl of 4 μM sodium [^{35}S]sulfate (0.067 μCi) or sodium [^{35}S]thiosulfate (0.007 μCi) for 3 min at 37°C. Incubation was terminated by addition of 0.5 ml of an aqueous suspension of AG1-X8 resin (0.3 g/ml). The resin was allowed to settle for 2 min, and samples from the supernatant were analyzed for radioactivity bound by proteins present in the shock fluid.

Chemicals. Antibiotics were from Sigma Chemical Co.

L-[^{35}S]methionine (>800 Ci/mmol), sodium [outer^{35}S]thiosulfate (10.4 mCi/mmol), and [α-^{35}S]dATP (400 Ci/mmol) were purchased from Amersham Corp. Carrier-free sodium [^{35}S]sulfate was from the Centre for Nuclear Studies (Swierk, Poland), and [γ-^{32}P]dATP was from the Institute of Biochemistry and Biophysics (Warsaw, Poland). Anion-exchange resin AG1-X8, Cl^- form, 100–200 mesh, was from Bio-Rad Laboratories. Restriction endonucleases and other enzymes for DNA manipulations were from Bethesda Research Laboratories and U.S. Biochemical or Amersham (England). Other chemicals were of analytical-grade purity.

RESULTS

DNA sequence of the 52-min region of *E. coli* chromosome spanning the CysB-dependent promoter. The restriction map of the chromosomal region containing genes essential for sulfate transport along with the previously localized CysB-dependent promoter is shown in Fig. 1a. This DNA fragment contains the genes necessary for complementation of the *cys-329*::*lac* and *cysA15* mutations (40; Hryniewicz et al., in press). The nucleotide sequence was determined by the chain termination method (38) for both strands of the 2-kb (*Hin*cII-*Eco*RV) DNA insert present in plasmid pMH1822 (Fig. 1a). The sequencing strategy used is outlined in Fig. 1b and the DNA sequence is given in Fig. 2.

Deduced protein products. Two large ORFs were identified on the same DNA strand of the sequenced region (Fig. 2). The first (ORF1), extending from positions 559 to 1572, codes for a 338-amino-acid polypeptide with a deduced M_r of 37,614. The predicted initiation codon for ORF1 was ideally separated from a well-defined ribosome-binding site (39). ORF1 was located directly downstream from the region containing the CysB-dependent promoter, mapped within the 0.56-kb *Hin*cII fragment (Fig. 1a) that is between nucleotide positions 1 and 565 (Hryniewicz et al., in press). In agreement with our previous data concerning the organization of the "cysA" locus in *E. coli*, ORF1 was expected to specify the first gene in the sulfate transport operon and was designated *cysP*. The NH_2-terminal portion of the CysP polypeptide contained a 25-amino-acid sequence with features typical of signal sequences of *E. coli*, including a positively charged N-terminus followed by a hydrophobic region and an alanine residue at a potential site of proteolytic cleavage (44). The sequence distal to the ORF1 termination codon contained a region of dyad symmetry, but it did not include the stretch of T's typical of rho-independent termination signals (37). The presumed termination codon of *cysP*, TGA at position 1573, overlapped the putative ATG codon of the next ORF (ORF2), which extended to the end of the DNA strand sequenced and lacked any in-frame translation termination codons. The restriction mapping of the *cys-329*::*lac* mutation (EC2256), isolated by in vivo mutagenesis with Mu dl(Ap *lac*) and characterized as giving rise to a sulfate permease-deficient phenotype (12), localized the fusion joint to just downstream of the *Eco*RV site (Fig. 1a), that is, in the region specified by ORF2. As the *cys-329*::*lac* mutant was a cysteine auxotroph, ORF2 was presumed to specify the essential component of the sulfate transport system, which has been proposed to be different from that specified by the *cysA15* mutation (40). ORF2 was designated *cysT* (and consequently, the *cys-329* mutation was named *cysT329*), and its location in the sulfate transport operon was confirmed by further sequencing of the whole region (41).

Mapping of the transcription start. Several putative sequences showing similarity to the canonical −35 and −10 promoter elements (11) were found in the 5′-flanking region of the *cysP* gene. To determine which of the putative promoters is actually functional in vivo, the mRNA start site was determined by primer extension. Two different 5′-^{32}P-labeled oligodeoxynucleotides were used as primers: the first extended from and was complementary to nucleotide positions 451 to 480, and the second extended from nucleotide positions 559 to 588 (Fig. 2). Total RNA was isolated

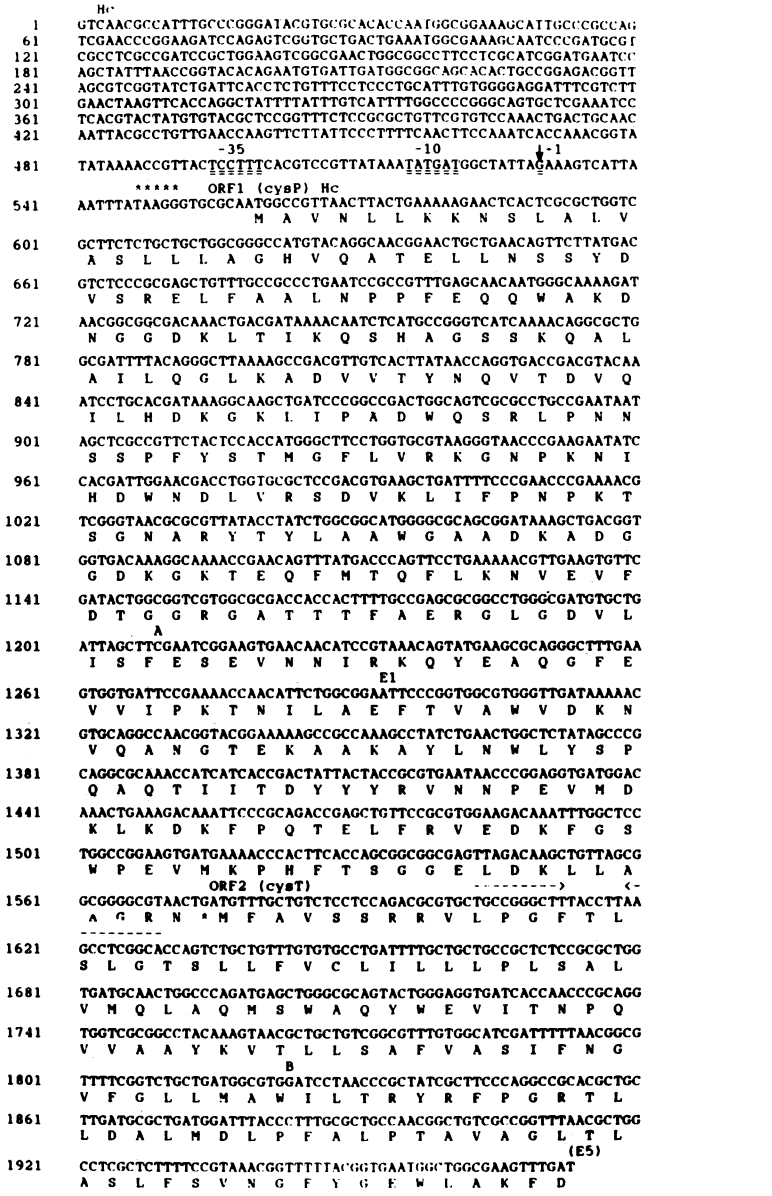

FIG. 2. Nucleotide sequence of the DNA fragment spanning the CysB-dependent promoter and the gene which lies adjacent. The sequence starts at the *Hin*cII site (base 3) and ends just after the *Eco*RV site (base 1980). Relevant restriction sites for *Hin*cII (Hc), *Sma*I (S), *Asu*II (A), *Eco*RI (E1), *Bam*HI (B), and *Eco*RV (EV) are shown above the sequence. Double dashed lines below the sequence show the −35 and −10 elements of the promoter; the vertical arrow indicates the position of the transcription start; and **** indicates the Shine-Dalgarno (39) sequence. An inverted repeat in the 3′-flanking region is indicated by arrows. The deduced amino acid sequence is shown below the open reading frames.

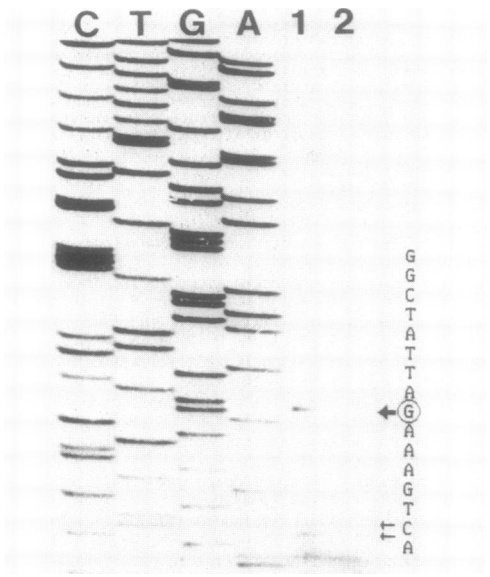

FIG. 3. Primer extension analysis of in vivo transcription start for the *cysP* gene. RNA was isolated from strain EC1250 grown in SF minimal medium with (lane 1) djenkolic acid or (lane 2) cystine as the sulfur source. Identical amounts of the primer and RNA were used for each reaction. Product sizes were estimated by comparison with M13mp18 sequence initiated from a 17-mer sequencing primer (−40). The relevant portion of the *E. coli* sequence is presented, and the nucleotide whose size corresponds to that of the extension product (indicated by the large arrow) is circled. It is not known whether the minor products indicated by the small arrows represent additional transcription start sites or whether they are artifacts of the primer extension reaction.

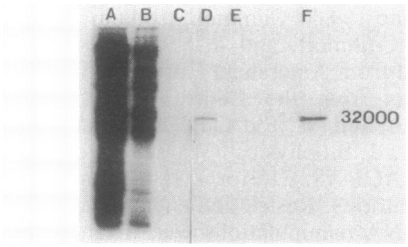

FIG. 4. Autoradiograph of [^{35}S]methionine-labeled proteins from *E. coli* K38 transformed with pASB1 and pGP1-2. Lane A, Induction of the T7 polymerase (42°C) without rifampin; lane B, without induction of the T7 polymerase (30°C), without rifampin; lane C, induction of the T7 polymerase (42°C), with rifampin; lane D, shocked fluid; lane E, crude extract of osmotically shocked cells; lane F, crude extracts of unshocked cells. Experiments whose results are presented in lanes D, E, and F were performed with induction of the T7 polymerase and with rifampin (see also Materials and Methods). After the labeling of proteins, the cells were divided into two portions. Bacteria from one portion were osmotically shocked; the shocked cells and the unshocked cells from the second portion were sonicated. Samples in lanes D, E, and F correspond to the same amounts of bacterial cells. Molecular weight standards were: bovine serum albumin (68,000), ovalbumin (45,000), aldolase (40,000), trypsin inhibitor (21,500) and lysozyme (14,300).

from the wild-type strain of *E. coli* grown on either nonrepressive (djenkolic acid) or repressive (cystine) sulfur sources.

Extension of the first of the primers (nucleotides 451 to 480) used did not give any specific product (not shown). Extension of the second primer gave a product of 59 nucleotides (Fig. 3) with the RNA isolated from the culture grown on djenkolic acid but not with that from the culture grown on cystine as a sulfur source. This result suggested the G at position 530 as a possible candidate for the transcription start site of the *cysP* gene.

Identification of the *cysP* gene product in the T7 promoter-polymerase system. The nature of the *cysP* gene product was investigated in the system developed by Tabor and Richardson (42) for exclusive expression of target genes placed on a plasmid under the control of the T7 promoter. A labeled product with an approximate M_r of 32,000 was observed (Fig. 4) in the extract of whole cells harboring plasmid pASB1 as well as in the fluid released by osmotic shock; there was only a small residual portion of this product in the extract of shocked cells. The periplasmic location of the CysP polypeptide is consistent with the presence of a signal peptide typical of proteins transported through cytoplasmic membranes (44). The molecular weight of the CysP polypeptide estimated by SDS-gel electrophoresis (M_r of 32,000) is lower than that predicted from the amino acid composition of the mature form of CysP (M_r of 35,057). Such anomalous migration in SDS gels has also been observed for the histidine-binding protein (27).

Analysis of the *cysP* gene from the *S. typhimurium* chromosome. Comparison of the deduced amino acid sequence of *E. coli* CysP protein with that of SBP of *S. typhimurium* (16, 35) showed 45% similarity (Fig. 5). This value seemed to be rather low for genes from such closely related organisms as *E. coli* and *S. typhimurium* (see also Discussion). Thus, in order to check whether the *S. typhimurium* genome contains a counterpart to the *E. coli cysP* gene, Southern blots of *Eco*RV-digested chromosomal DNAs from both *E. coli* and *S. typhimurium* were hybridized with a ^{32}P-labeled probe from the coding region of *cysP* (0.72-kb *Hin*cII-*Eco*RI fragment; Fig. 1a). The same 2.3-kb fragment (containing the internal *Eco*RI site in *E. coli*) was detected in *E. coli* and *S. typhimurium* (Fig. 6). The above result suggested that these regions in *E. coli* and *S. typhimurium* are homologous. The 8-kb *Cla*I fragment (Fig. 1c) presumed to contain the entire *cysP* region of *S. typhimurium* was chosen for cloning in pBR322 to create plasmid pAPH1 (Fig. 1c). The strategy for sequencing this portion of the chromosomal insert present in this plasmid is outlined in Fig. 1c. The sequence estimated for one DNA strand of this region (Fig. 7) showed 86% similarity with ORF1 (*cysP*) and also with the sequenced portion of ORF2 (*cysT*) of *E. coli*; the similarity on the amino acid level was even higher (data not shown). These results indicated that the *cysP* gene of *E. coli* has a homologous counterpart in *S. typhimurium* and codes for a gene product distinct from the characterized sulfate-binding protein (16, 35).

Construction of a *cysP* mutant. A means of determining the precise function of the CysP protein was to construct a defined *cysP* mutant. For this purpose, plasmid pASB2 was constructed by insertion of the *cat* gene into the coding region of *cysP* (Fig. 1a). Crossing of this *cysP* insertional mutation back onto the chromosome was performed by the technique of Winans et al. (47). pASB2 DNA was linearized by restriction cleavage with *Pst*I and introduced to a *recBC sbcB* strain (JC7623) by transformation. Chloramphenicol-resistant, ampicillin-sensitive colonies were selected, and phage P1 grown on one of the clones obtained was used to transduce the EC1250 strain to chloramphenicol resistance. One of the transductants (EC2297) was verified as a *cysP* insertional mutant by Southern analysis of *Eco*RV-cleaved

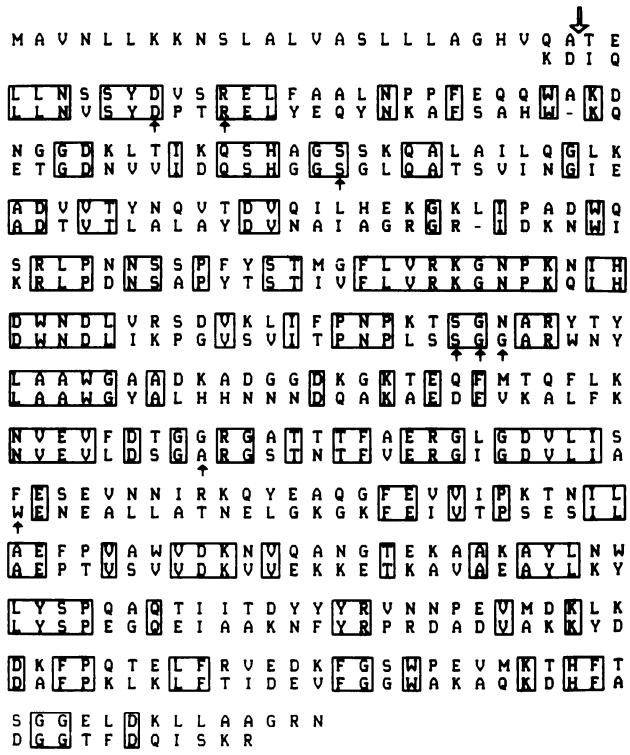

FIG. 5. Comparison of the amino acid sequences of the CysP polypeptide from *E. coli* (upper sequence) and the SBP from *S. typhimurium* (lower sequence). The sequences are aligned for maximum identity. Identical amino acid positions between two sequences are boxed. An arrow at amino acid position 26 of CysP shows the potential site for cleavage of the signal peptide (44). Amino acids essential for sulfate binding by SBP (35) are marked by vertical arrows below the SBP sequence. The SBP sequence is essentially consistent with that published by Isihara and Hogg (16) with the amendments given by Pflugrath and Quiocho (35).

chromosomal DNA from EC2297 and by comparison with the pattern generated by cleavage of chromosomal DNA of the parental strain (EC1250). The *Hin*cII-*Eco*RI 0.72-kb fragment (Fig. 1a) from the coding portion of *cysP* was used as a hybridization probe. This probe detected the predicted 2.3-kb band in the case of strain EC1250 and a 3.6-kb band for strain EC2297 (Fig. 6). These data confirm the insertion site of the *cat* gene fragment and are in agreement with the predicted length of the *cysP-cat* gene hybrid.

The insertional mutation in *cysP* did not result in the cysteine auxotrophy observed for other known sulfate transport mutants (e.g., *cysA15* and *cysT329::lac*). However, the rate of growth of the *cysP* strain was about twofold lower in liquid medium with either sulfate (0.04 mM) or thiosulfate (0.02 mM) as the sulfur source compared with the wild-type strain (data not shown).

Uptake and binding of sulfate and thiosulfate by the *cysP* mutant. The reduced growth rate of the *cysP* mutant on minimal medium with sulfate or thiosulfate as the sole sulfur source suggested that the CysP protein might be involved in binding of either one or both of these anions. To check this possibility, assays of sulfate and thiosulfate binding and uptake were performed in the wild-type strain, sulfate transport-deficient mutants, and a *cysP* mutant. As shown in Table 2, the binding of thiosulfate by crude osmotic shock fluid of the *cysP* strain (EC2297) was reduced more than 10-fold compared with the wild-type strain, whereas the binding of sulfate was practically unchanged. The presence of the *cysP*$^+$ gene on a multicopy plasmid in the *cysP* mutant (EC2299) resulted in a significant increase in thiosulfate-binding activity (more than 100 times), but sulfate-binding activity was unaffected. The above findings strongly suggest that the major function of the CysP polypeptide is the binding of thiosulfate.

Indirect assay of uptake of both anions by whole cells confirmed the function of the *cysP* gene product as a thiosulfate-binding protein. Uptake of thiosulfate by the *cysP* mutant was decreased about seven times, whereas sulfate uptake was diminished only twofold compared with wild-type activity. Furthermore, the EC2299 strain [*cysP* (*cysP*$^+$)] showed the wild-type level of thiosulfate uptake; however, the *cysP* plasmid had no effect on sulfate uptake, consistent with the findings in the binding experiments. A possible explanation for the decrease in sulfate uptake by the *cysP* mutant might be an effect of the 1.3-kb fragment insertion on the expression of the genes encoding other components of the transport system common to both anions. The very low levels of binding and uptake of thiosulfate in the *cysB* mutant (EC2275) indicated that *cysP* gene expression requires a functional CysB protein.

DISCUSSION

The genetic region of *E. coli* heretofore designated "cysA" is in fact composed of several genes specifying distinct components of the sulfate-thiosulfate transport system (see accompanying paper [41]). Earlier studies allowed us to approximately localize the CysB-dependent promoter governing the expression of the downstream genes within the restriction map of this region (Hryniewicz et al., in press). In this study we have presented the sequence analysis of the region encompassing this promoter and the gene lying directly adjacent to it and we have identified its gene product.

The DNA sequence of the region directly adjacent to the promoter revealed the presence of a 1,014-bp open reading frame, designated *cysP*, encoding a protein of 338 amino acid residues (Fig. 2) with a predicted molecular weight of 37,614. As the NH_2-terminal portion of the deduced amino acid sequence showed the features typical of a signal peptide (44), the mature CysP protein may in fact consist of 313 amino

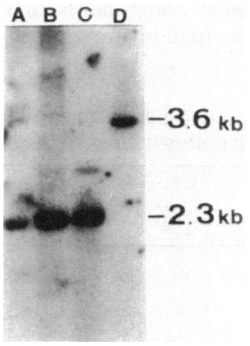

FIG. 6. Autoradiograph of the chromosomal DNA of *S. typhimurium* TL117 (lane A), the chromosomal DNA of *E. coli* EC1250 (lane B) and EC2297 (lane D), and pAMH2 DNA (lane C) hybridized to the probe spanning the coding region of the *cysP* gene from *E. coli* (*Hin*cII-*Eco*RI fragment). All DNAs were digested with *Eco*RV before loading on the gel. The sizes of observed bands are indicated. The molecular weight standard was λ DNA cut with *Bst*EII.

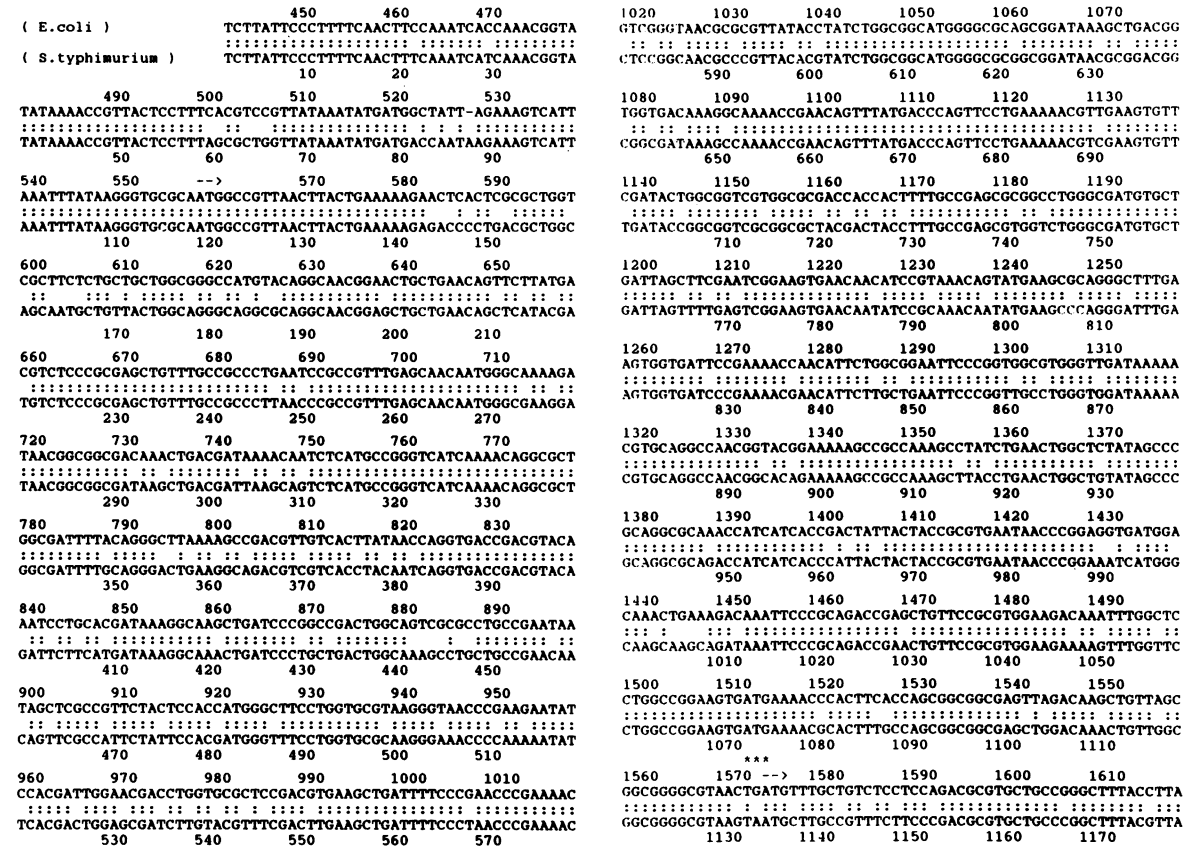

FIG. 7. Comparison of the *E. coli cysP* DNA sequence with the homologous region of the *S. typhimurium* chromosome. Identical nucleotides are indicated by a colon between the two sequences. Nucleotide positions of *E. coli* are numbered as in Fig. 2. The initiation codons of *cysP* and *cysT* are marked by arrows, and the stop codon of *cysP* is marked by asterisks above the *E. coli* sequence.

acids with a predicted M_r of 35,057. The periplasmic location of the mature CysP polypeptide, inferred by the presence of a signal peptide, was confirmed experimentally by specific expression of the *cysP* gene in the T7 promoter-polymerase system (Fig. 4). The suggested cleavage site between Ala and Thr (Fig. 5) conforms to the rules for procaryotic signal peptidase sites (44).

In most well-characterized periplasmic binding-protein-dependent transport systems, the single operon contains genes for all transport components, and the gene for the binder is usually the first in the cluster (for a review, see reference 1). Thus, our initial assumption was that the *cysP* gene product was identical to the SBP which was isolated from *S. typhimurium* and characterized as a sulfate binder and shown to be regulated in parallel with sulfate permease (29). However, the deduced amino acid sequence of mature CysP protein showed only 45% sequence similarity with that of *S. typhimurium* SBP (16, 35), a value relatively low compared with other pairs of predicted homologous gene products from these organisms, like *cysB* and *cysK*, which share, respectively, 95% (30) and 96% (5) sequence similarity. Therefore, the corresponding region of the *S. typhimurium* chromosome whose restriction map was established previously (15) was cloned and sequenced. The sequence analysis of this region revealed the presence in *S. typhimurium* of a strictly homologous counterpart of the *E. coli cysP* gene (over 86% sequence homology on the DNA level). The deduced amino acid sequence of CysP from both *E. coli* and *S. typhimurium* is distinct from that of the previously characterized SBP (16, 35).

TABLE 2. Sulfate and thiosulfate uptake and binding activities in strains with different mutations in the "cysA" region[a]

Strain	Relevant genotype	Cys phenotype	Sulfate		Thiosulfate	
			Uptake	Binding	Uptake	Binding
EC1250	Wild type	+	1,900	119	1,900	300
EC2275	*cysB*	−	80	38	110	46
EC1119	*cysA15*	−	100	ND[b]	140	ND
EC2256	*cysT329*	−	170	ND	290	ND
EC2297	*cysP*	+	740	132	260	5
EC2299	*cysP*(*cysP*$^+$)	+	650	128	1,500	2,200

[a] Uptake and binding activities are expressed as picomoles of [^{35}S]sulfate or [^{35}S]thiosulfate removed from the incubation mixture by 1 mg of whole-cell protein (uptake) or bound by 1 mg of protein of crude osmotic shock fluid at 37°C (binding).

[b] ND, Not determined.

Assays of sulfate and thiosulfate binding and uptake by *E. coli* strains with different lesions in the sulfate transport system, including a *cysP* insertional mutant (Table 2), allowed us to establish that the major activity of CysP protein is the binding of thiosulfate. Therefore, this protein has been designated a thiosulfate-binding protein. It is worth noting that some amino acid residues essential for sulfate binding by SBP of *S. typhimurium* as estimated by Pflugrath and Quiocho (35) are conserved in the thiosulfate-binding protein

```
                                                                  -35
S.typh. cysJIH   AACAGGTTAGTTCATTTGGTTATTTGTTATT TCCAACC-CTTCTTTAAT
E.coli  cysJIH   AACAGGTTAGTCGATTTGGTTATTAGTTATC GCTATCC-CGTCTTTAAT
S.typh. cysK     ACCATTATTTCCCATCAGCATATA-GATATGCGAAATCC-TTACTTCCCC
E.coli  cysK     GTCATTATTTCCCTTCTGTATATA-GATATGCTAAATCC-TTACTTCCGC
S.typh. cysP     TTCAACTTT-CAAATC-ATCAAAC-GGTATA-TAAAACCGTTACTCCTTT
E.coli  cysP     TTCAACTTC-CAAATC-ACCAAAC-GGTATA-TAAAACCGTTACTCCTTT
(conserved)      ..CA...T......T......A...G.TAT.....A.CC....CT.....

                                                          -10
S.typh. cysJIH (cont.)  TGTTATTCCTCTCACCGTTAACCTTATCCTC
E.coli  cysJIH (cont.)  CCACACCGTTTGCCCCGTTAACCTTACCTT
S.typh. cysK   (cont.)  ATATCTGGCTGGAAGG--TATGCTGGGAAG
E.coli  cysK   (cont.)  ATATTCTCTGAGCGGG--TATGCTACCTGTTG
S.typh. cysP   (cont.)  AGCGCTGGTTATAAA---TATGATGACCAATAA
E.coli  cysP   (cont.)  CACGTCCGTTATAAA---TATGATGGCTATTAG
(conserved)             ................TA...T
```

FIG. 8. Comparison of the *cys* promoter regions of *E. coli* and *S. typhimurium* (*S. typh*). Sequences are aligned with respect to −35 and −10 regions and have been adjusted by inclusion of gaps. The *cysK* and *cysJIH* data are from reference 31.

sequence (Fig. 5). Thus, it is an attractive possibility to consider the sulfate-thiosulfate transport system as a porter interacting with two binding proteins whose functions may partially overlap. Precedents for such a situation exist; e.g., the *liv* system, where two binding proteins, one being specific for leucine and the other for leucine, isoleucine, and valine, interact with the same membrane-bound components (1). Such functional overlap would explain both the failure to isolate transport-defective mutants with specific defects in SBP activity (29) and the cysteine bradytrophy of our *cysP* mutant. Genetic analysis of the sulfate-binding protein will reveal whether this assumption holds true.

The presence in *S. typhimurium* of a gene homologous to *cysP* of *E. coli*, as well as unchanged sulfate-binding activity of the *E. coli cysP* mutant (Table 2), indicates the presence of a separate gene encoding the SBP. Our attempts to find the open reading frame encoding SBP of *E. coli* in the region extending about 1,500 bp upstream of *cysP* (where it would be expected, by analogy to the *liv* system) were, however, unsuccessful.

The primer extension analysis defined the −35 and −10 elements of the promoter as TTACTC and TATGAT, respectively (Fig. 2). The deviation of the −35 region from the consensus sequence (10, 11) was also observed for two other CysB-dependent promoters governing the expression of the *cysK* and *cysJIH* genes (31) in both *E. coli* and *S. typhimurium*. Comparison of the −35 upstream region with that published by Ostrowski and Kredich (31) for the *cysK* and *cysJIH* promoters did not reveal any clear homology; however, some nucleotide sequence arrangements seem to be similar (Fig. 8). The second ORF (*cysT*) found in the region directly downstream of *cysP* and inferred to be essential for sulfate transport by identification of a *cysT329*::*lac* mutation (12; Hryniewicz et al., in press) also has a strictly homologous counterpart in *S. typhimurium*. Overlapping of the TGA and ATG at the *cysP-cysT* junction (28) and the apparent requirement of the *cysP* promoter for maximum expression of the *cysT329*::*lac* fusion (Hryniewicz et al., in press) suggests that *cysP* and *cysT* form an operon. A sequence of dyad symmetry was found in the 3′ nontranslated region of the *cysP* gene; however, it does not include the usual run of T's typical of rho-independent terminator signals (36, 37). It was reported that alterations reducing the number of terminal uridines in RNA transcript weaken the termination response (36). The ability of dyad symmetry regions to function as a barrier to degradation of polycistronic transcripts was reported for the region of *trp t-trp t′* in the tryptophan operon (26) and the intercistronic region between *papA* and *papH* loci (3) of *E. coli*; thus, this potential stem-loop structure may have such a function.

The presence of two separate complementation groups specified by two sulfate transport-defective mutants (Hryniewicz et al., in press) suggests that the region downstream of the *cysP* gene encodes other components of the sulfate transport system. The sequence analysis of this region and the identification of gene products are presented in the accompanying paper (41).

ACKNOWLEDGMENTS

We thank G. Sawers for his help in correcting the manuscript and R. Mertz for kindly supplying oligonucleotides.

Part of this research was supported by the Polish Academy of Sciences within project 3.13.

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