The growth properties of an *Escherichia coli* strain carrying a chromosomal deletion of the *ssuEADCB* genes (formerly designated *ycbPONME*) indicated that the products of this gene cluster are required for the utilization of sulfur from aliphatic sulfonates. Sequence similarity searches indicated that the proteins encoded by *ssuA*, *ssuB*, and *ssuC* are likely to constitute an ABC type transport system, whereas *ssuD* and *ssuE* encode an FMNH2-dependent monooxygenase and an NAD(P)H-dependent FMN reductase, respectively (Eichhorn, E., van der Ploeg, J. R., and Leisinger, T. (1999) J. Biol. Chem. 274, 26639–26646). Synthesis of β-galactosidase from a transcriptional chromosomal *ssuE*-lacZ fusion was repressed by sulfate or cysteine and depended on the presence of a functional *cbl* gene, which encodes a LysR-type transcriptional regulator. Electrophoretic mobility shift assays with the *ssu* promoter region and measurements of β-galactosidase from plasmid-encoded *ssuE*-lacZ fusions showed that full expression of the *ssu* operon required the presence of a Cbl-binding site upstream of the −35 region. CysB, the LysR transcriptional regulator for the *cys* genes, was not required for expression of a chromosomal *ssuE*-lacZ fusion although the *ssu* promoter region contained three CysB-binding sites. Integration host factor could also occupy three binding sites in the *ssu* promoter region but had no influence on expression of a chromosomal *ssuE*-lacZ fusion.

When *Escherichia coli* is deprived of sulfate or cysteine, a set of proteins is synthesized *de novo* or at increased levels (1, 2). Some of these sulfate starvation-induced (Ssi) proteins, the sulfate-binding protein (Sbp) and O-acetylserine lyase (CysK), are components of the assimilatory sulfate reduction pathway leading to cysteine (3). Two other proteins whose synthesis is completely repressed when sulfate is present in the growth medium have been identified as the TauD and TauA proteins, which are encoded in the *tauABCD* gene cluster required for the utilization of taurine (2-aminothanesulfonate) as a sulfur source (2). The proteins encoded by *tauABC* probably function as an ABC transporter for taurine, whereas TauD is an α-ketoglutarate-dependent dioxygenase which catalyzes the incorporation of oxygen into taurine and thereby leads to the liberation of sulfite (4).

There is a clear difference between expression of the *cys* genes, required for reduction of sulfate and biosynthesis of cysteine, and that of the *tau* genes. Whereas the *tau* genes are completely repressed by sulfate (5), expression of the *cys* genes is only partly repressed (3). The *cys* genes are positively regulated by the LysR-type (6) transcriptional activator CysB and the inducer N-acetylcysteine (7). Expression of the *tau* genes requires CysB, but also Cbl, another LysR-type transcriptional activator with 41% amino acid sequence identity to CysB (8). *cbl* mutants are unable to utilize taurine as well as a range of other aliphatic sulfonates as sulfur source and many of the Ssi proteins were absent or present in reduced amounts in a *cbl* mutant grown under sulfate-starvation conditions (5). Cbl therefore appears to be a transcription activator for genes whose expression is induced by sulfate starvation.

In this study we report the identification of the *ssuEADCB* genes (for sulfonate-sulfur utilization), whose products include two of the remaining Ssi proteins, Ssi4 and Ssi6. It will be shown that the *ssu* genes require *cbl* for expression and that they encode a set of proteins that enables *E. coli* to utilize aliphatic sulfonates other than taurine as a sulfur source.

**EXPERIMENTAL PROCEDURES**

**Chemicals—**All chemicals used as sulfur sources were of the highest quality available and were obtained from Fluka (Buchs, Switzerland), except for isethionate and lanthionine, which were purchased from Aldrich. Oligonucleotides were obtained from Microsynth (Balgach, Switzerland).

**Bacterial Strains, Plasmids, Phages, and Genetic Procedures—**Strains of *E. coli* and plasmids used in this study are listed in Table I. All strains were grown at 37 °C either in LB medium or sulfate-free minimal medium (2) supplemented with 0.2% glucose and a sulfur source (described in the text) to a final concentration of 0.25 mM. When required, growth media contained ampicillin (100 μg/ml), tetracycline (15 μg/ml), chloramphenicol (20 μg/ml), or kanamycin (50 μg/ml). Amino acids were added at the following concentrations: leucine, isoleucine, and valine at 10 μg/ml and tryptophan at 4 μg/ml. P1 transduction was performed as described (9).

**Mapping and Cloning of Genes Encoding Ssi6 and Ssi4—**Designed on the basis of part of the N-terminal sequence of Ssi6 (LNMFIFLP; see Ref. 1), oligonucleotide 2D6N (5′-TIAA(C/T)ATGTT(C/T)(T/GTTGTT)/(C/T)TICCIACICA-3′) was labeled at its 3′ end using digoxigenin-ddUTP and terminal transferase (Roche Molecular Biochemicals). Total DNA was isolated from *E. coli* MC4100 using the hexadecyltrimethyl ammonium bromide method (10) and digested with the restriction enzymes that had been used to create the *E. coli* chromosomal restriction map (11) with the exception of BglII. Restriction fragments were separated by agarose gel electrophoresis, blotted to a Hybond N membrane (Amersham Pharmacia Biotech) in 20× SSC, and hybridized overnight to 100 pmol of digoxigenin-labeled oligonucleotide 2D6N at 45 °C in 10 ml of hybridization buffer (5× SSC, 0.1% lauroyl-
sarcosine, 0.02% SDS, 1% blocking reagent). The membrane was washed twice in 2 x SSC, 0.1% SDS at room temperature and twice in 0.5 x SSC, 0.1% SDS at 37 °C. Detection of the hybridized probe was performed with dioxidum 3-4-methoxyaminophospho(1,2-dioxiane-3,2-3′-isochloro)tricycl[3.3.1.135]/decan)-4-yi]phenyl phosphate (Roche Molecular Biochemicals) according to the manufacturer. The sizes of the restriction fragments that hybridized to the probe were estimated and rounded to the nearest 100 bp.

Plasmids pME4210, containing the complete ssuEADCB gene cluster plus 326 bp upstream of the ssuE start codon, was constructed as follows. Plasmid pME4204 consists of a 0.33-kb EcoRI/BamHI fragment obtained by PCR with primers JP12 and JP13 (see below). The 24-bp BamHI/MunI fragment from plasmid pME4204 was replaced by the 1.15-kb BamHI/MunI fragment from pME4208. Subsequently the 1.4-kb EcoRI/BamHI fragment from plasmid pME4208 and the 4.2-kb EcoRI/BamHI fragment from plasmid pME4180 were ligated to EcoRI-digested pBluescript KS to yield plasmid pME4210.

To replace two mutations present in the ssuEADCB sequence, the Clai/SalI fragment was substituted by the Clai/SalI fragment from pME4281, which contains an insert generated by PCR amplification from total DNA from E. coli strain EC1250 (14). Since pME4201 contained Clai and SalI sites in the vector backbone, the SacI/HindIII fragment from pME4210 was cloned into pUC19 to give plasmid pME4220. The Clai/SalI fragment from pME4281 was then used to replace the Clai/SalI fragment in pME4220, thereby yielding plasmid pME4221.

**Sequencing and Sequence Analysis**—The dideoxy chain termination method of Sanger et al. (14) was used to sequence DNA fragments cloned into pBluescript KS or pGEM7 using an Applied Biosystems 373 DNA sequencer. Nucleotide and protein sequences were analyzed with GCG (Wisconsin Package Version 9.0, Genetics Computer Group, Madison, WI) and compared with the latest releases of the EMBL, Swiss-
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**FIG. 1. Organization of the *E. coli* ssuEADCB gene cluster.** Relevant restriction sites are shown, and restriction fragments used for construction of plasmids are indicated. The EcoRI sites marked with *+* were constructed by using PCR. The location of the mutations in plasmids is indicated with asterisks. Replacement of the chromosomal ssuEADCB genes by lacZYA gave strain SE40.

Pro, and TREMBL data bases.

The nucleotide sequence described is deposited in the EMBL data base under accession number AJ237695. Construction of a Combined ssuEADCB Deletion and Transcriptional ssuE′-lacZ Fusion—For the simultaneous construction of a deletion in ssuEADCB and a chromosomally encoded transcriptional ssuE′-lacZ fusion, the promoterless lacZYA genes from pRS415 (15) were recovered as a *Bam*II/*Stu*I fragment and cloned in the *Bgl*II/*Stu*I site of pME4210, yielding pME4230. The complete insert from pME4230 was subsequently cloned as *Not*I/*Sal*I fragment in pK03 (16). The resulting plasmid, pME4231, was transferred to strain EC1250 by electroporation. Colonies that appeared at 42 °C were suspended in LB medium and appropriate dilutions plated out on LB medium containing 5% sucrose. The resulting colonies were then replica-plated on LB plates containing chloramphenicol and sucrose. Chloramphenicol-sensitive colonies were selected and tested for growth on butanesulfonate and for expression of *lacZ* on minimal plates containing tryptophan, glutathione, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. One such colony was purified and the strain was designated SE40.

Construction of ssuE′-lacZ Protein Fusions—For the construction of ssuE′-lacZ fusions, PCR was employed to amplify different fragments of the region upstream of ssuE and to introduce *Bam*HI and EcoRI restriction sites in order to facilitate cloning in plasmid pRS552 (15). The forward primers, whose positions at different regions upstream of the transcription start site are given in parentheses, were as follows: JP12 (−287), 5′-CTTCGGTGATGCTAGAATGCC-3′; JP14 (−144), 5′-CAGGAAATTCATGTGATTCAAC-3′; JP15 (−35), 5′-TTTGGGATTCTCTTGCTCTC-3′; JP16 (−98), 5′-TATGGCTGAGTTCTAAGCC-3′; JP21 (−211), 5′-ATACGAAATCCCAATAAGTG-3′; JP24 (−80), 5′-CCCTTCCTTATGTAGATTCAAG-3′; JP25 (−52), 5′-GAACTACAGAATTCATATTGGG-3′ (the nucleotides changed to introduce restriction sites are underlined). The reverse primer used for all fusions was JP13 (+64), 5′-GATGGGATGCTCATACTCTC-3′. The resulting PCR products were digested with EcoRI and *Bam*HI and ligated to plasmid pRS552 which had been cut with the same enzymes. The inserts of all plasmids were sequenced to confirm that no errors had been introduced during the PCR.

For the generation of plasmid pME4234, containing mutations in the HIH-binding site IHF1, PCR was performed with the primers IHF2 (−141) 5′-GTAATTCAGGTTCTCAAC-3′ and T7 (5′-TAATACGACTCACTATAGG-3′) using pME4204 as a template. The product was digested with *Bam*HI and HindIII. In a second PCR reaction, primers IHF1 (5′-AGTGGGATGCTCATACT-3′, complementary to primer IHF2) and T3 (5′-ATATACGACTCACTATAGG-3′) were used with template pME4204, and the product was digested with EcoRI and HindIII. Both fragments were combined and ligated to EcoRI/*Bam*HI-digested pBluescript KS to yield pME4234. The *Bam*HI/EcoRI fragment from pME4234 was subsequently cloned in pRS552 to give pME4240. For construction of pME4242, containing mutations in *HIF*2, primers IHF3 (−181), 5′-ATTAATGGTACATTGAAAT-3′, and IHF4, 5′-CAATGATCTATGAAATAGG-3′ (partially complementary to primer IHF3), were used in the same way as described above for construction of pME4234 but by using ClaI instead of HindIII for digestion of the PCR products. The *Bam*HI/EcoRI fragment from pME4232 was cloned in pRS552 to give pME4233. Plasmid pME4209, containing mutations in both IHF1 and IHF2, was constructed as described above for plasmid pME4232 but by using pME4234 as template DNA for PCR. Plasmid pME4291 was obtained by cloning the EcoRI/*Bam*HI fragment from pME4209 in pRS552.

**Primer Extension Analysis**—For primer extension analysis, strain EC1250 was grown in minimal medium with sulfate or butanesulfonate as sulfur source to an optical density at 600 nm of approximately 0.8. Isolation of total RNA and primer extension analysis was according to Babat et al. (17) using primer JP19 (5′-CCGCGCATATTTCCAGAGGAGCTGTGA-3′; 71 to 45 bp downstream from the translation start; Fig. 3A) and about 100 μg of RNA. The products were loaded on a 6% polyacrylamide gel together with a sequencing reaction done with the same primer and plasmid pME4181 (Table I) by using the fmol cycle sequencing kit (Promega, Madison, WI). **Enzyme Assays**—β-Galactosidase activities were assayed in cells taken from late log-phase cultures according to the method of Miller (9) with o-nitrophenylgalactoside as a substrate.

**DNA Binding Assays**—DNA fragments containing various portions of the *ssu* promoter region were generated by PCR, 5′-labeled with [γ-32P]ATP by using T4 polynucleotide kinase (Promega), and purified by QiAquick spin columns (Qiagen). The primers used for PCR are described above. Proteins used were highly purified CysB from *Salmo nella typhimurium* obtained from N. M. Kredich, partially purified Cbl from *E. coli* (6), and highly purified IHF from *E. coli* (18) obtained from A. Sirko.

**Conditions for DNA-protein binding reactions and the electrophoretic mobility shift assay were as described previously (5).** Briefly, incubation mixtures (20 μl) contained approximately 10 ng of labeled DNA fragment and 2 μg of sonicated calf thymus DNA per ml to reduce nonspecific binding, in a buffer consisting of 40 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 100 mM KCl, 1 mM dithiothreitol, and 100 μg/ml bovine serum albumin. After incubation with given protein (5 min at 37 °C), samples were separated on a 5% acrylamide/bisacrylamide gel containing 1 M urea at 150 V for 4 h.

**RESULTS**

Cloning and Sequence of ssuEADBC—The genes encoding the proteins Ssi4 and Ssi6 were mapped to the chromosome of *E. coli* as described under “Experimental Procedures.” For both genes, the best fit to the restriction map of *E. coli* was obtained at 21.4 min downstream of *pepN* (results not shown). The genes encoding Ssi4 and Ssi6 were located on plasmid pJP30, which consists of a 22-kb chromosomal HindIII fragment cloned in pACYC184 (19). A 5.5-kb SspI/EcoRI fragment from pJP30 was cloned in pBluescript KS to yield plasmid pME4180 (Fig. 1). We sequenced 4499 bp from the SspI site until the end of the *pepN*
gene (20), which corresponds with the genome sequence of E. coli from 996,943 to 992,445 (21). Analysis of the sequence indicated that immediately downstream and opposite to the direction of transcription of pepN five open reading frames were present (Fig. 1). The corresponding genes have been designated ssuE, ssuA, ssuD, ssuC, and ssuB. These genes have previously been designated ycbP, ycbO, ycbN, ycbM, and ycbE, respectively (21).

**Sequence Analysis of ssuEADCB**—The sequence established by us showed two differences with respect to the published sequence of the E. coli genome (21). The T residues at bp 2626 and at bp 2927 were C residues in the sequence of the E. coli genome.

The amino acid sequence of SsuE showed similarity to the NADH-dependent FMN reductase MsuE from Pseudomonas aeruginosa (22) and to SsuE from Pseudomonas putida, involved in utilization of methanesulfonate and aliphatic sulfonates as sulfur sources, respectively. It has been demonstrated recently that the purified SsuE protein from E. coli catalyzes the reduction of flavins by NADH or NADPH (13).

SsuD was similar in sequence to FMNH₂-dependent monoxygenases involved in utilization of sulfonates as sulfur sources (22, 23). In addition, SsuD showed sequence similarity to a class of monoxygenases of diverse substrate range which require FMNH₂ for their activity. The SsuD protein from E. coli has recently been purified and was found to act as an FMNH₂-dependent oxygenase, liberating sulfate from a range of aliphatic sulfonates (13).

The sequences of SsuB and SsuC were similar to ATP-binding proteins and membrane components, respectively, of members of the ABC transporter superfamily (not shown; see Ref. 24). Moreover, the sequences of the proteins encoded by ssuA, ssuB, and ssuC were significantly similar to those of the proteins that constitute putative ABC transporters from E. coli involved in utilization of taurine (2), from Bacillus subtilis involved in utilization of aliphatic sulfonates (SsuA, SsuB, and SsuC respectively; see Ref. 23), and from P. putida involved in utilization of aliphatic and aromatic sulfonates (SsuA, SsuB and SsuC respectively).²

**The ssuEADCB Gene Cluster Is Required for Utilization of Aliphatic Sulfonates as Sulfur Source**—Since the synthesis of both Ssi4 and Ssi6 is up-regulated when sulfate or cysteine are absent from the growth medium (1), it was likely that the ssuEADCB gene cluster is involved in utilization of sulfur sources other than cysteine or sulfate. To study the function and expression of ssuEADCB, the complete gene cluster was deleted and replaced by the promoterless lacZYA genes (Fig. 1; see “Experimental Procedures”). The resulting strain SE40 was unable to utilize a broad range of aliphatic sulfonates as a sulfur source, except for taurine. Sulfur sources that supported growth of the wild type strain EC1250 but not of the mutant SS40 included ethanesulfonate, propanesulfonate, butanesulfonate, pentanesulfonate, hexanesulfonate, ethanedisulfonate, octanesulfonate, decanesulfonate, isethionate, sulfocatechate, MOPS, HEPES, MBS, and PIPES. Both the mutant and wild type strains were unable to use methanesulfonate, cysteate, dodecanesulfonate, or sulfoisuccinate as sulfur source. We were therefore unable to confirm that E. coli utilizes methanesulfonate and cysteate as a source of sulfur (25, 26). The mutant strain was not affected in utilization of sulfur from sulfate, cysteine, cystine, lanthionine, methionine, glutathione, or taurine. The ssuEADCB gene cluster is hence required for utilization of aliphatic sulfonates as a sulfur source except for taurine, for which the desulfurization system is encoded by the

² P. Vermeij and M. A. Kertesz, unpublished observations.
lacking the −35 region was inactive, whereas the fusion containing both −35 and −10 regions resulted in low but nonregulated expression. Apparently, 62 bp preceding the transcription start point were required and sufficient for high level expression of the ssu genes, but wild type regulation of expression by sulfate and cystine was dependent on at maximum 203 bp preceding the transcription start point.

Expression of ssuEADCB Requires Cbl—A chromosomally encoded transcriptional ssuE−lacZ fusion was constructed as described under “Experimental Procedures.” Expression of β-galactosidase from this fusion was measured in strain EC1250 carrying plasmid-encoded ssuE−lacZ fusions. Each value represents the mean of at least three different experiments. ■, cystine; □, butanesulfonate; △, sulfate; □, butanesulfonate + sulfate; □, glutathione.

TABLE II

| Sulfur source (250 μM) | β-Galactosidase activity |
|-----------------------|-------------------------|
| Sulfate               | 4                       |
| Cystine               | 2                       |
| Methionine            | 630                     |
| Glutathione           | 328                     |
| Djenkolic acid        | 128                     |
| Lanthionine           | 3452                    |
| Taurine               | 4747                    |

TABLE III

| Sulfur source | SE40 (wt) | SE45 (cbl) | SE47 (cysB) | SE45pMH176 (cbl/cysB) | SE47pMH176 (cysB) |
|--------------|-----------|------------|-------------|----------------------|-------------------|
| Glutathione  | 392       | 3.2        | <1          | 447                  | 2570              |
| Lanthionine  | 2980      | 5.3        | <1          | 458                  | 1556              |

The synthesis of Cbl is dependent on the presence of CysB (8), and expression of ssu therefore requires indirectly the presence of CysB. To determine whether CysB was also directly involved in expression of ssu, we measured expression of the...
CysB in the expression of the ssu gene cluster (5). CysB might even prevent full expression of the ssu genes, since the levels of β-galactosidase in the cysB mutant containing pMH176 were higher than in the cbl mutant containing the same plasmid (Table III).

**Binding of CysB and Cbl Proteins to ssu Promoter**—In order to characterize further the mechanism of regulation of the ssu operon, we have tested the ability of CysB and Cbl proteins to interact with various portions of the ssu promoter region by an electrophoretic mobility shift assay. The longest DNA fragment used in this assay contained the sequence from position –287 to +64 relative to the transcription start site (the same region was included in plasmid pME4205 used for β-galactosidase assays). As shown in Fig. 4A, both CysB and Cbl formed complexes with this fragment in a protein concentration-dependent manner; binding of Cbl resulted in a single complex, and CysB was able to give several complexes. The picture of these complexes formed by both proteins with the ssu promoter was very similar to that obtained earlier for the –286 to +51 tau promoter fragment where a single binding site for Cbl and at least two binding sites for CysB were identified (5). Therefore we believe that the complexes designated as C1 (Fig. 4A) of either CysB and Cbl with the 351-bp ssu promoter fragment represent occupation of a single binding site by each protein. Similarly as with the tau promoter region (5), acetylserine and thiosulfate stimulated CysB binding to the ssu promoter region that resulted in increased amounts of the C1 complex (Fig. 4A, lanes 5–7). Higher order complexes C2 and C3 were clearly seen after longer exposure of the gel (Fig. 4A, lane 7a) and could represent occupation of additional binding sites by the CysB protein. Acetylserine had no effect on Cbl binding (not shown). Thiosulfate had an opposite effect on Cbl binding than that observed with CysB, a slight reduction of C1 complex formation with Cbl was observed with the ssu promoter fragment (Fig. 4A, lane 11). Essentially a similar picture of complexes formed by CysB and Cbl was obtained with a 208-bp ssu promoter fragment that extended from –144 to +64 relative to the transcription start site, suggesting that all the binding sites for Cbl and CysB were contained within this fragment (not shown). Two more 5'-shortened ssu promoter fragments were also used as promoter probes, one starting from position –98 and the second from position –5 to +64 relative to the transcription start. The former fragment was able to bind CysB and Cbl, and the latter fragment bound only CysB (Fig. 4B). The above results indicate that the Cbl-binding site in the ssu control region lies within the 50-bp sequence preceding the –35 region of the promoter. The ability of CysB to bind to a site lying within the region from –35 to +64 is in accordance with the possible function of this protein as a repressor of ssu expression.

**Integration Host Factor Binds to the ssu Regulatory Region But Has No Influence on Expression from a Chromosomal ssuE-lacZ Fusion**—As shown above, β-galactosidase synthesis from the shorter plasmid-encoded ssuE-lacZ fusions (plasmids pME4243, pME4196, pME4197, and pME4245) was not as strongly repressed by sulfate and cystine as that from the shorter fusions (those present on pME4205 and pME4208; Fig. 3B). We noticed that the shorter fusions lacked one or two putative binding sites for I HF (Fig. 3B) and speculated that these might be important for exerting repression by sulfate and cystine. By using electrophoretic mobility shift assays, the formation of several complexes of I HF with the ssu promoter region was demonstrated (Fig. 5). Binding of I HF to the 275-bp ssu promoter fragment extending from position –211 to +64 resulted in formation of at least 3 complexes, designated C1, C2, and C3 (Fig. 5A, lanes 8–10). The C1 complex could represent an occupation of a single, high affinity binding site by I HF (possibly the IHF1 site) as it was still present when a high excess of unspecific DNA was included in the binding reaction (lanes 11 and 12). Binding of I HF to the 208-bp fragment devoid of the IHF2 site (from –144 to +64) gave a more rapidly migrating specific complex C1 and additional complexes that were lost in the presence of an excess of unspecific DNA (Fig.

![Fig. 4. Binding of Cbl and CysB to the ssu regulatory region in a gel mobility shift assay.](Image)

**A** a gel mobility shift assay. A, the 351-bp DNA fragment used as a probe was obtained by PCR with primers JP12 and JP13, 5'-labeled, and incubated with highly purified CysB protein or partially purified Cbl protein at concentrations indicated (in g/ml). O-Acetylseryne (OAS) was added at 5 mM (lane 5) or 10 mM (lane 6) and thiosulfate (TS) at 5 mM. C1 (primary complexes) and C2 and C3 (higher order complexes seen better after longer exposure of the gel, exemplified by lane 7a) are discussed in the text. B, binding of CysB and Cbl (at concentrations indicated in g/ml) to a 99-bp PCR fragment amplified with primers JP12 and JP13 on template pME4204 (probe I, wild type ssu promoter), template pME4232 (probe II, mutated I HF2 site), or template pME4234 (probe III, mutated I HF1 site). Primary complexes (C1) and higher order complexes (C2 and C3) are discussed in the text. A and B concentrations of purified I HF are indicated in g/ml; FP shows free probe DNA.

![Fig. 5. Binding of I HF to the ssu regulatory region.](Image)

**A** a gel mobility shift assay. A, the 351-bp DNA fragment used as a probe was obtained by PCR with primers JP12 and JP13, 5'-labeled, and incubated with highly purified CysB protein or partially purified Cbl protein at concentrations indicated (in g/ml). O-Acetylseryne (OAS) was added at 5 mM (lane 5) or 10 mM (lane 6) and thiosulfate (TS) at 5 mM. C1 (primary complexes) and C2 and C3 (higher order complexes seen better after longer exposure of the gel, exemplified by lane 7a) are discussed in the text. B, binding of CysB and Cbl (at concentrations indicated in g/ml) to a 99-bp PCR fragment amplified with primers JP12 and JP13 on template pME4204 (probe I, wild type ssu promoter), template pME4232 (probe II, mutated I HF2 site), or template pME4234 (probe III, mutated I HF1 site). Primary complexes (C1) and higher order complexes (C2 and C3) are discussed in the text. A and B concentrations of purified I HF are indicated in g/ml; FP shows free probe DNA.
three different experiments.

We also compared binding of IHF to three ssu promoter fragments of the same size (351 bp) derived from either pME4204 template (wild type, probe I), pME4232 template (probe II), IHF2 site changed by site-directed mutagenesis; see “Experimental Procedures”), or pME4234 (probe III, IHF1 site changed by site-directed mutagenesis; see fragments of the same size (351 bp) derived from either EC2643 (E. coli) and therefore appeared to have a negative effect on expression of the binding sites. The presence of binding sites IHF1 or IHF2, whereas the former plasmids lack one or both pME4205 or pME4208. The latter two plasmids contain both pME4240 or pME4243 (himA) grown with butanesulfonate and carrying plasmid-encoded ssuE\(^{-}\)-lacZ fusions. Each value is the mean of at least three different experiments.

5A, lanes 2–6). The C1 complex may represent occupation of the IHF1 site by IHF, and its relatively fast migration can be explained by the position of this site close to the end of the fragment used as a probe; IHF bound to such site causes less DNA bending than in the case of central location of binding (27).

By using butanesulfonate as sulfur source for growth, \(\beta\)-galactosidase activity from the different plasmid encoded ssuE\(^{-}\)-lacZ fusions was measured in a wild type strain (MC1000) and in an isogenic himA mutant (EC2643), which is unable to synthesize one of the subunits of the heterodimeric IHF protein (Ref. 28; Fig. 6). Whereas activity was not changed in the himA mutant containing plasmids pME4196 and pME4197, it was significantly reduced in the mutant containing plasmid pME4205 or pME4208. The latter two plasmids contain both IHF1 and IHF2, whereas the former plasmids lack one or both binding sites. The presence of binding sites IHF1 or IHF2 therefore appeared to have a negative effect on expression of \(\beta\)-galactosidase from plasmid encoded ssu\(^{-}\)-lacZ fusions in a himA mutant but not in the wild type strain. However, mutation of either binding site IHF1, IHF2, or both IHF1 and IHF2 on plasmid pME4205 also resulted in similarly decreased levels of \(\beta\)-galactosidase in the himA mutant EC2643, indicating that the binding sites themselves are not involved.

Sulfate-, cystine-, butanesulfonate-, or glutathione-grown cells of EC1250 containing a plasmid-encoded ssuE\(^{-}\)-lacZ fusion and mutations in IHF1 (on plasmid pME4240) or in IHF2 (on plasmid pME4233) and both in IHF1 and IHF2 (on plasmid pME4291) had similar levels of \(\beta\)-galactosidase as cells containing the control plasmid pME4205. This indicated that neither IHF1 nor IHF2 has influence on expression of the ssu genes in a wild type background.

No difference in expression of \(\beta\)-galactosidase from the chromosomal ssuE\(^{-}\)-lacZ fusion between wild type and a himA mutant could be observed in the exponential or the stationary growth phase. We therefore conclude that the positive effect of IHF on expression is only seen with plasmid-encoded fusions, although it cannot be excluded that IHF influences expression of the chromosomal ssuE\(^{-}\)-lacZ fusion under conditions that have not been tested.

**FIG. 6.** \(\beta\)-Galactosidase activities (in Miller units) in early exponential phase-grown cells of E. coli MC1000 (wild type) or EC2643 (himA) grown with butanesulfonate and carrying plasmid-encoded ssuE\(^{-}\)-lacZ fusions. Each value is the mean of at least three experiments.

**DISCUSSION**

It has been known for sometime that E. coli can utilize sulfonates as sulfur source under aerobic conditions (2, 25, 26). Sulfonates utilized include not only naturally occurring compounds, like taurine or isethionate, but also industrially produced compounds such as MOPS or HEPES. In this study we have identified the ssuEADCB gene cluster, which encodes proteins for the utilization of sulfur from a wide range of aliphatic sulfonates, and whose expression is repressed by sulfate. Repression by sulfate of organosulfur utilization was also observed in B. subtilis, P. aeruginosa, and Rhodococcus (22, 23, 29, 30). Therefore, sulfate is preferred as sulfur source over organosulfur compounds.

The wide substrate range of the ssuEADCB system contrasts with that of the tauABCD system, for which taurine appears to be the only substrate (2, 4). Since tau mutants are still able to use aliphatic sulfonates (2) and the ssu mutant studied in this work was still able to use taurine, both systems apparently have a complementary substrate range. The key enzyme for the desulfonation of aliphatic sulfonates is the FMNH\(_2\)-dependent oxygenase encoded by ssuD, which has been purified and characterized (13). The reduction of FMN is carried out by the NAD(P)H-dependent FMN reductase, encoded by ssuE (13).

Expression of the ssu genes required the presence of Cbl, which binds just upstream of the –35 region of ssu. Removal of the Cbl-binding site resulted in low levels of \(\beta\)-galactosidase from plasmid-encoded ssuE\(^{-}\)-lacZ fusions. In addition, expression of \(\beta\)-galactosidase was absent from a chromosomal ssuE\(^{-}\)-lacZ fusion in a cbl mutant. These data strongly suggest that the Cbl protein acts as a transcriptional activator for ssu gene expression. It is not surprising that this promoter needs an activator since the –10 sequence only matches 3 out of 6 nucleotides of the consensus sequence. Binding of Cbl in the close vicinity of the –35 region may involve a direct contact of this activator with RNA polymerase, a mechanism suggested for many other LysR-type transcriptional regulators (6).

The role of CysB in the expression of ssu is more difficult to understand. Contrary to its involvement in expression of the tauABCD operon (5), CysB does not seem to be directly required for expression of ssuEADCB. There is, however, at least one CysB-binding site located overlapping the –35 and –10 regions, and occupation of this site by CysB could prevent RNA polymerase from binding to the ssu promoter. The possible function of CysB as a repressor is confirmed by our findings that in the presence of Cbl delivered from a plasmid, a cysB mutant expressed a transcriptional ssuE\(^{-}\)-lacZ fusion at higher levels than a cbl mutant containing this fusion.

Although the direct involvement of CysB at the ssu promoter is not completely clear, CysB is required for expression of cbl (8) and therefore is an indirect activator of the ssu operon. A cysB mutant was unable to synthesize \(\beta\)-galactosidase from a chromosomally encoded ssuE\(^{-}\)-lacZ fusion, unless the cbl gene was expressed from the trc promoter. Activation of transcription of the genes involved in biosynthesis of cysteine from sulfate and
of the cbl gene requires the binding of CysB to activating sites just upstream of the −35 region, the presence of acetylserine as an inducer molecule, and the absence of sulfide or thiosulfate, which function as anti-inducers (7). Cysteine has a negative effect on the synthesis of acetylserine through feedback inhibition of serine transacylase by cysteine. Since expression of cbl is regulated as part of the cys regulon, the presence of cysteine in the growth medium leads to repression of cbl (8). This explains why the ssu genes are not expressed in cysteine-grown cells. In sulfate-grown cells however, cbl is still synthesized, and repression of tau and ssu gene expression by sulfate must be caused by a different mechanism that involves the Cbl protein.

The structure of the C-terminal part of CysB (residues 88–324), which comprises the binding site for the co-inducer acetylserine, has recently been determined (31). The protein contains two αβ domains that enclose a cavity. Unexpectedly, a sulfate ion could be modeled in this cavity, although sulfate has no effect on activation of transcription by CysB (32). It was speculated that this cavity serves as the binding site for both acetylserine and for the anti-inducer thiosulfate. The Cbl protein is very similar in sequence to CysB, and many of the residues that were thought to interact with the inducer and the anti-inducer are conserved. This could indicate that Cbl and CysB recognize the same molecules as inducer and anti-inducer. But it is more likely that the anti-inducer for Cbl is different from that for CysB and that this difference causes repression by sulfate of ssu and tau genes. Sulfate itself could act as anti-inducer for Cbl. Alternatively, it is possible that sulfite, the product of TauD- or SsuD-catalyzed desulfonation of taurine or alkanesulfonates, acts as anti-inducer.

Even though expression of both the tau and ssu genes is repressed by sulfate and regulated by Cbl, the operons are probably regulated in a different manner. This is substantiated by several observations. First, expression of the tau genes requires the direct involvement of CysB, whereas expression of the ssu genes was not dependent on the presence of CysB. Second, binding of Cbl to the tau promoter occurred at −112 to −68 relative to the transcription start (5), which is different from the binding-site at the ssu promoter just upstream of the −35 region. Third, the sequences of both promoter regions did not reveal any similarity, except for their high AT content.

It has previously been observed that IHF mutants showed reduced growth on limiting amounts of inorganic sulfur sources and on djenkolate (33, 34), which supported the assumption that IHF is required for full expression of cysJH in stationary phase but not during exponential phase (34). The presence of several binding sites for IHF in the intergenic region between the ssu operon and yebQ suggested that IHF could also be involved in regulation of ssu, but we were unable to demonstrate it. On the other hand, it has to be considered that IHF may affect the expression of yebQ, which encodes a hypothetical fimbrial-like protein. IHF has previously found to be required for DNA inversion that controls phase variation of type 1 fimbriae in E. coli (35).

In conclusion, we have shown that the ssu genes are required for utilization of sulfur from sulfonates. Our results have demonstrated that there are at least three different proteins that interact with the promoter region of the ssu genes: Cbl, CysB, and IHF. The region from −62 to 0 relative to the transcription start is needed for high level expression, which is probably brought about by transcription activation by the Cbl protein. Full repression by cysteine and sulfate requires also the region from −203 to −62, and although there are binding sites for IHF in this region, IHF does not appear to be involved.

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