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Sequence Analysis and Expression of the *Salmonella typhimurium asr* Operon Encoding Production of Hydrogen Sulfide from Sulfite

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A chromosomal locus of *Salmonella typhimurium* which complements *S. typhimurium asr* (anaerobic sulfite reduction) mutants and confers on *Escherichia coli* the ability to produce hydrogen sulfide from sulfite was recently cloned (C. J. Huang and E. L. Barrett, J. Bacteriol. 172:4100–4102, 1990). The DNA sequence and the transcription start site have been determined. Analysis of the sequence and gene products revealed a functional operon containing three genes which have been designated *asrA*, *asrB*, and *asrC*, encoding peptides of 40, 31, and 37 kDa, respectively. The predicted amino acid sequences of both *asrA* and *asrC* contained arrangements of cysteines characteristic of [4Fe-4S] ferredoxins. The sequence of *asrB* contained a typical nucleotide-binding region. The sequence of *asrC* contained, in addition to the ferredoxinlike cysteine clusters, two other cysteine clusters closely resembling the proposed siroheme-binding site in biosynthetic sulfite reductase. Expression of *lacZ* fused to the *asr* promoter was repressed by oxygen and induced by sulfite. Analysis of promoter deletions revealed a region specific for sulfite regulation and a second region required for anaerobic expression. Computer-assisted DNA sequence analysis revealed a site just upstream of the first open reading frame which had significant homology to the FNR protein-binding site of *E. coli* NADH-linked nitrite reductase. However, *asr* expression by the fusion plasmid was not affected by site-specific mutations within the apparent FNR-binding site.

The reduction of sulfite to sulfide by electrons from hydrogen or an organic substrate constitutes the central energy-conserving step in the metabolism of the sulfate-reducing bacteria (37). Outside this group, dissimilatory sulfite reduction is rare. However, many microorganisms, including members of the *Enterobacteriaceae* family, assimilate sulfate by means of a cysteine biosynthetic pathway in which sulfite reduction is a step (30). The biosynthetic sulfite reductase in *Escherichia coli* and *Salmonella typhimurium* consists of a flavoprotein, encoded by *cysJ*, and a hemoprotein, encoded by *cysI* (30, 38, 40, 51). In both the dissimilatory pathway of the sulfate reducers and the assimilatory pathway of the *Enterobacteriaceae*, siroheme and iron-sulfur clusters are required participants (36, 40). Siroheme biosynthesis is encoded by the *cysG* gene in *E. coli* and *S. typhimurium* (27). Mutations in *cysI*, *cysJ*, or *cysG* all result in cysteine auxotrophy.

S. typhimurium differs from *E. coli* in two aspects of sulfite reduction. Firstly, it produces significant quantities of free hydrogen sulfide from sulfite, a property which, among the *Enterobacteriaceae*, is unique to the genera *Salmonella* and *Edwardsiella* (2, 41). Our studies of H_2S production from sulfite by *S. typhimurium* have shown that it is strictly anaerobic (21), genetically distinct from its biosynthetic analog (21, 24), linked to NADH rather than NADPH oxidation (21), and regulated by available electron acceptors rather than by cysteine (6, 21, 24). Thus, it is essentially a dissimilatory process. Secondly, *S. typhimurium* also differs from *E. coli* in the phenotype of several *cys* mutants. *cysI* and *cysJ* mutants of *S. typhimurium* are prototrophs under anaerobic conditions (1). Furthermore, anaerobic sulfite assimilation in *S. typhimurium* is not under the control of *cysB* (1, 24), which encodes a positive regulator which is otherwise essential for induction of the cysteine biosynthetic pathway (30, 39).

Both aspects of *S. typhimurium* sulfite reduction can be attributed to the presence of chromosomal genes in *S. typhimurium* encoding a unique pathway for sulfite reduction. This was demonstrated in our recent report (24) of the cloning of an *asr* (anaerobic sulfite reduction) chromosomal locus from *S. typhimurium*. Using a *cysI* parent, we isolated Tn*5* insertion mutants that had lost the ability to synthesize cysteine anaerobically and that no longer produced H_2S from sulfite. The cloned *asr* locus restored both properties. It also conferred on an *E. coli cysI* mutant the abilities to produce H_2S from sulfite and to synthesize cysteine anaerobically (24). It did not confer aerobic prototrophy to either organism. Here we report results of our analyses of the sequenced *asr* clone and the key effectors of its transcriptional regulation.

MATERIALS AND METHODS

Strains, culture conditions, assays, and chemicals. The bacterial strains used were *S. typhimurium* EB303 (*cysI68 asr-1*::Tn*5*), isolated in our previous study (24); *S. typhimurium* TT521 (*recA1 rpsL srl-202*::Tn*10*) from the Salmonella Genetic Stock Centre, care of K. E. Sanderson; *E. coli* JM246 (*cysI*) from the *E. coli* Genetic Stock Center, care of Barbara Bachmann; *E. coli* DH5α [F^- *endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1* λ^- *recA1 gyrA96 relA1* Δ(*lacZYA-argF*)U169 φ80d*lacZ*ΔM15], obtained from Bethesda Research Laboratories; and *E. coli* CJ236 (*dut ung*) and MV1190 (*dut*$^+$ *ung*$^+$), both from Bio-Rad Laboratories as part of the Muta-Gene M13 in vitro mutagenesis kit. Plasmid pJF118EH (15) was provided by E. Lanka, and pRS414 (52) was provided by R. W. Simmons. All pEB plasmids used are described in the context of their construction.

Minimal sulfite medium and glucose minimal medium were prepared as described previously (24). The YT medium was

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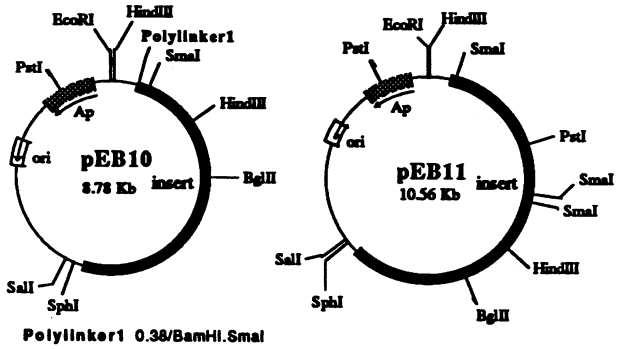


FIG. 1. Plasmids carrying *asr* from *S. typhimurium*. Both were isolated as described previously (24).

that previously described by Davis et al. (11). All other media used were previously described by Miller (35). Ampicillin and kanamycin were added, as needed, at 50 μg/ml in rich media and 25 μg/ml in minimal media. For aerobic conditions, the cultures were shaken in an incubator rotary shaker at 350 rpm. Anaerobic conditions were attained by sparging the medium with oxygen-free argon and completely overlaying the medium with paraffin oil as previously described (21). β-Galactosidase was assayed as previously described by Miller (35).

Restriction endonucleases were purchased from Bethesda Research Laboratories or New England BioLabs. The Klenow fragment of DNA polymerase I, T4 DNA ligase, and avian myeloblastosis virus reverse transcriptase were purchased from United States Biochemical Corporation. Exonuclease III was from Promega Biotec Corporation. Other materials and kits were obtained as noted below.

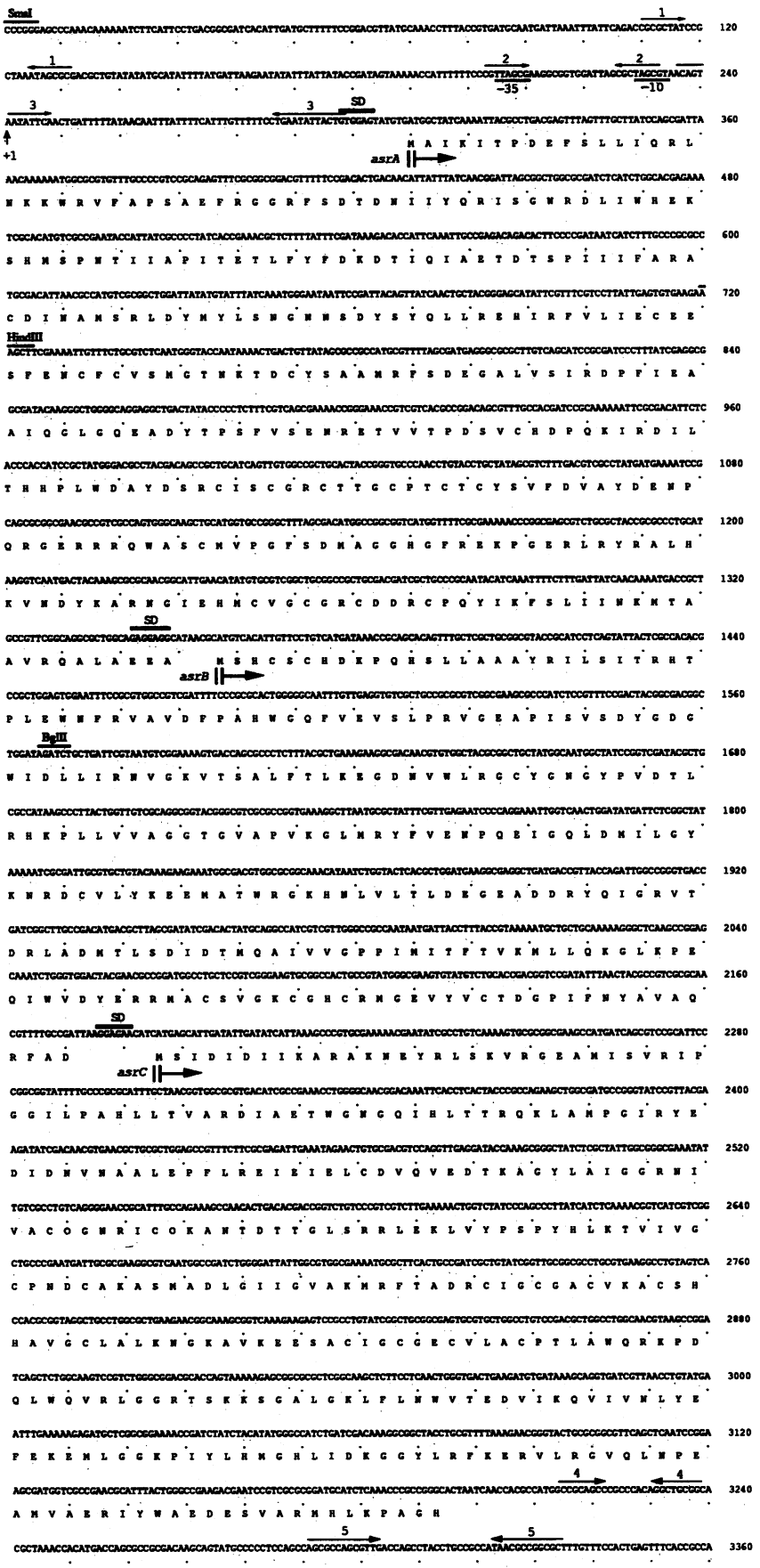
Construction of subclones and deletions. Unless otherwise noted, all recombinant DNA procedures were carried out as previously described by Maniatis et al. (34). Transformation of *S. typhimurium* strains was achieved by electroporation (55) with the Gene-Pulser apparatus from Bio-Rad Laboratories. All *asr* plasmids were derivatives of pEB10 or pEB11 (Fig. 1), two of the pBR322 derivatives capable of complementing *asr* mutants, as described in our previous report (24). The 3.8-kb *Sma*I-*Sal*I fragment of pEB11 was found sufficient for complementation of *S. typhimurium asr* mutant EB303 and *E. coli cysI* mutant JM246. To obtain clones suitable for sequencing, the 3.8-kb *Sma*I-*Sal*I fragment was inserted into the *Sma*I and *Sal*I sites of pUC18, thus generating pEB13 (not shown). To obtain the *asr* insert in the opposite orientation, the 3.8-kb *Sma*I-*Sal*I fragment was end filled with the Klenow fragment and ligated to the *Sma*I site of pUC18, thus generating pEB14 (not shown). To sequence the top strand, *Sst*I and *Sma*I sites on pEB13 were used to construct a series of nested deletions by using exonuclease III as previously described (23). To sequence the bottom strand, the *Pst*I and *Bam*HI sites on pEB14 were used to construct the exonuclease III-generated nested deletions.

For overproducing *asr* proteins in *E. coli*, we constructed pEB15, a hybrid of pEB10 and pEB11 which contained the shortest complete *asr* sequence. It was constructed by joining the 2.1-kb *Bgl*II-*Sal*I fragment from pEB11 to the 6.8-kb *Bgl*II-*Sal*I fragment from pEB10. The resulting insert in pEB15 was cloned into the *Bam*HI-*Sal*I sites of pUC18 to create an *Eco*RI site flanking the insert. The 3.9-kb *Eco*RI-*Sal*I fragment was isolated and inserted into the expression vector pJF118EH (15) to create plasmid pEB16. To remove the sequence upstream of the *asr* open reading frames, deletions were generated from the *Bam*HI site by using exonuclease III and S1 nuclease, and all deletion endpoints were sequenced. Plasmid pEB17, one of the deletion derivatives of pEB16, was selected to examine the overproduction of *asr* proteins in *E. coli*. In this plasmid, the open reading frames were placed just downstream of the *tac* promoter and the sequence upstream of nucleotide 292 was removed by using exonuclease III deletion. Plasmid pEB17 carries the gene for the *lac* repressor (*lacI*^{q}) so that the expression of the open reading frames is completely repressed without the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (15).

Plasmid construction for regulation studies. To study the regulation of *asr* expression, *asr-lac* fusion plasmids were constructed. Plasmid pEB18 was constructed by cutting the 900-bp *Eco*RI-*Hin*dIII fragment (which contains the *asr* regulatory region) from pEB16 and fusing it in frame to the large *Eco*RI-*Hin*dIII fragment of a pRS414 derivative containing a *Hin*dIII octamer linker (pRS414-8). The pRS414-8 plasmid was constructed by inserting a *Hin*dIII octamer linker to the *Bam*HI site of pRS414 (52). For the construction of promoter deletion plasmids pEB22, pEB24, pEB26, pEB28, pEB30, and pEB31, plasmid pEB16 was digested to completion with *Sst*I and *Bam*HI and nested deletions were generated from the *Bam*HI site with exonuclease III. The altered *asr* sequences were excised by digestion with *Eco*RI and *Hin*dIII and ligated with the translational fusion vector pRS414-8 cut with the same enzymes. The deletion endpoints were determined by nucleotide sequence analysis following the cloning of deletion junctions into pUC19. Promoter deletion plasmid pEB29 was constructed by using the *Eco*RI-*Hin*dIII fragment from pEB24. The fragment was cloned into pUC19, linearized with *Eco*RI, and partially digested with exonuclease *Bal*31. The ends were flushed with Klenow fragment, and the Klenow fragment and *Eco*RI linkers were attached by using T4 ligase. The recombinant plasmids were digested with *Eco*RI, and the purified *Eco*RI-cut fragments were ligated and used to transform *E. coli* DH5α. Deletion endpoints were determined by DNA sequencing.

DNA sequencing. DNA sequencing was performed by using the dideoxy-chain termination method (48) with Sequenase (United States Biochemical Co.) and α-^{35}S-dATP (Amersham Corp.). DNA templates for sequencing were prepared as previously described (5). Both strands were sequenced, and on average, each nucleotide was sequenced five times. Analysis of DNA sequence data was carried out by using the University of Wisconsin Genetics Group software package (12).

Transcription analysis. For RNA isolation, strain TT521(pEB18) was grown aerobically or anaerobically in minimal sulfite medium supplemented with ampicillin and 1 mM cysteine to a cell density corresponding to an A_{600} of about 0.3. Total RNA was prepared as previously described by Gilman and Chamberlin (17). The single-stranded synthetic oligonucleotide GCAAACTAAACTCGTCAGGCGT AATTTTG, complementary to a region extending from position 314 to 342 (Fig. 2), was 5′ end labeled with [γ-^{32}P]ATP and polynucleotide kinase. Oligonucleotide was added to 10 μg of total RNA, allowed to anneal, and then extended with reverse transcriptase as follows. Ethanol-precipitated RNA was dried and then dissolved in 10 μl of buffer containing 50 mM Tris (pH 8.3), 100 mM KCl, and 1 mM EDTA. The mixture was heated at 80°C for 5 min and incubated at 42°C for 3 h. Next, 8 μl of 5× elongation buffer


SmaI
1
1
2
2
-35
-10
3
3
SD
+1
asrA
HindIII
SD
asrB
BglII
SD
asrC
4
4
5
5

containing 375 mM Tris (pH 8.3), 100 mM KCl, 40 mM $MgCl_2$, 50 mM dithiothreitol, 5 mM EDTA, 1.6 μl of dNTPs (25 mM each), 0.5 μl of RNasin (Promega Biotec), 9 μl of water, and 16 U of reverse transcriptase was added to the annealing mixture, which was then incubated at 41°C for 45 min. The extended products were analyzed by electrophoresis on an urea–8% polyacrylamide gel in parallel with dideoxynucleotide sequencing reactions primed with the same oligonucleotide.

Analysis of overproduced *asr* gene products. Cultures of *E. coli* DH5α harboring either pJF118EH or pEB17 were grown to mid-log phase (120 Klett units) in YT medium to which IPTG was added to a final concentration of 1 mM. The cultures were incubated for 4 h and harvested by centrifugation. IPTG was omitted from the control cultures. Cells were resuspended in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample application buffer (33) and lysed by being boiled for 5 min. The proteins were then separated on 10% polyacrylamide-SDS gels (33).

Site-specific mutagenesis. Oligonucleotide-directed mutagenesis was performed as previously described by Kunkel et al. (32) by using a Muta-Gene M13 in vitro mutagenesis kit from Bio-Rad Laboratories and 30-mer mutagenic oligonucleotides obtained from Operon Technologies, Inc. The *Eco*RI-*Hin*dIII fragment containing the *asr* 5′ untranslated upstream sequence and a portion of *asrA* was cut from pEB18 and cloned into M13mp18. The resulting plasmid was transfected into *E. coli* CJ236, and single-stranded template DNA (minus strand) was prepared. The 30-mer mutagenic oligonucleotides were treated with T4 polynucleotide kinase and annealed in excess to the template DNA, and the mixture was then treated with T4 DNA polymerase and T4 DNA ligase to complete the second-strand DNA synthesis. The resulting DNA was transfected into *E. coli* MV1190. Single-stranded DNA sequencing was used to screen for the desired mutants. The mutated *asr* sequences were excised from M13 recombinant plasmids by being digested with *Eco*RI and *Hin*dIII and were cloned into the *Eco*RI and *Hin*dIII sites of the translational fusion plasmid pRS414-8 to create plasmids pEB37 and pEB38. The two mutagenic oligonucleotides used resulted in the substitution of 5′ATTTGTTTTTCCTGACTA (pEB37) and 5′ATC CATTTTTCCTGAATA (pEB38) for 5′ATTTGTTTTTCCT GAATA (see Fig. 8).

Nucleotide sequence accession number. The *asrABC* sequence has been assigned GenBank accession no. M57706.

RESULTS

Nucleotide sequence of the *asr* locus. The 3.8-kb *Sma*I-*Sal*I fragment from pEB11 (Fig. 1) was found sufficient to complement *S. typhimurium asr*::Tn*5* mutants and to confer on *E. coli* JM246 (a *cysI* mutant) the abilities to grow prototrophically under anaerobic conditions and to produce hydrogen sulfide from sulfite. The nucleotide sequence of this fragment was determined on both strands by the dideoxy-chain termination method. The nucleotide sequence and the deduced amino acid sequences are shown in Fig. 2. Computer analysis of this sequence revealed three open reading frames. The first open reading frame (ORF1) encoded a 40-kDa polypeptide, the AUG initiator codon for which was preceded by a potential ribosome-binding site (50) located near position 298. Three inverted repeats were found upstream of the putative ribosome-binding site. The second open reading frame (ORF2) encoded a 30.6-kDa polypeptide, the AUG initiator codon for which was preceded by a very strong ribosome-binding site at positions 1344 to 1349 (within the end of ORF1). The third open reading frame (ORF3) encoded a 37-kDa polypeptide, the AUG initiator codon for which was preceded by a potential ribosome-binding site at positions 2176 to 2180 just downstream of the termination codon for ORF2. Two additional inverted repeats were located downstream of ORF3, the second of which constituted a potential *rho*-independent terminator (47). A hydrophobicity analysis of the encoded polypeptides deduced from the DNA sequence (data not shown) indicated that all three deduced peptides were very hydrophilic and thus probably cytoplasmic.

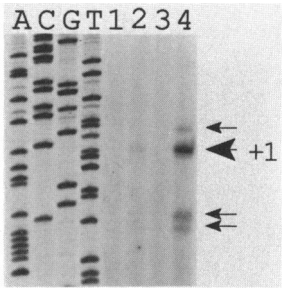


FIG. 3. Determination of the *asr* transcription start site by primer extension analysis. A 29-mer oligonucleotide primer complementary to nucleotides 314 to 342 (see Fig. 2) was used to prime the reverse transcriptase reaction. Lanes 1 to 4 contain products of cDNA synthesized from 8 μg of total RNA from strain TT521 (pEB18) grown as follows: lane 1, aerobic without sulfite; lane 2, aerobic with 1 mM sulfite; lane 3, anaerobic without sulfite; lane 4, anaerobic with 1 mM sulfite. The major transcription start site observed in all such experiments is indicated by a large arrow. Minor start sites are indicated by smaller arrows.

Identification of the *asr* promoter. The *asr* sequence included a 310-nucleotide region upstream of the AUG codon of ORF1 containing three stem-loop structure sequences (Fig. 2). The transcription start site was determined by primer extension analysis (Fig. 3). In cultures grown anaerobically with sulfite, a major primer extension product was found to end at the guanidine residue at position 241, which is 70 nucleotides upstream of the predicted translational initiation site. This indicates that a functional promoter sequence exists within the first 240 nucleotides of the sequenced fragment. No primer extension signal was detected in RNA from cultures grown in the absence of sulfite. A weak signal was present in cultures grown aerobically in the presence of sulfite. Additional minor primer extension products, presumably due to nonspecific primer extension, were sometimes also detected in RNA from the induced cultures. Location of the transcription start site permitted the tentative

FIG. 2. Nucleotide sequence and deduced amino acid sequences of the *asr* locus. Nucleotides are numbered from the 5′ end, and intervals of 10 bp are marked. The predicted amino acid sequence for each open reading frame is given in single-letter code under the DNA sequence. The following features are indicated: transcription initiation site (vertical arrow); the −10 and −35 sequences (underlined); possible ribosome-binding sites (thick overlines designated SD); inverted repeats (overlined with arrows and sequentially numbered); sites for *Sma*I, *Hin*dIII, and *Bgl*II (overlined with enzyme designated); and open reading frames (arrow below N-terminal amino acids).

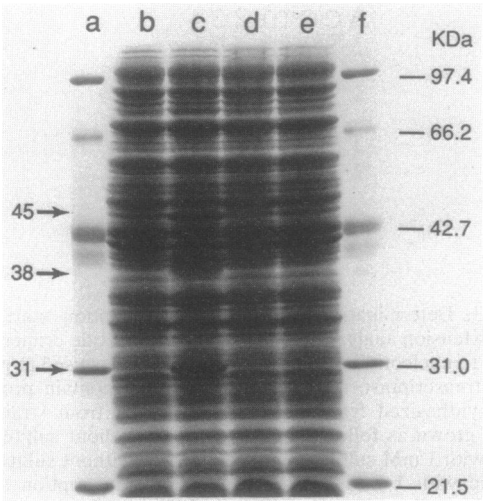


FIG. 4. Overexpression of *asr* proteins. Proteins synthesized by *E. coli* DH5 containing plasmid pEB17 with *asr* genes fused to a *tac* promoter (lanes b and c) or pJF118EH without *asr* insert (lanes d and e), either uninduced (lanes b and d) or induced by IPTG (lanes c and e), were subjected to SDS-PAGE and stained with Coomassie blue. Reference proteins are in lanes a and f as follows: phosphorylase (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

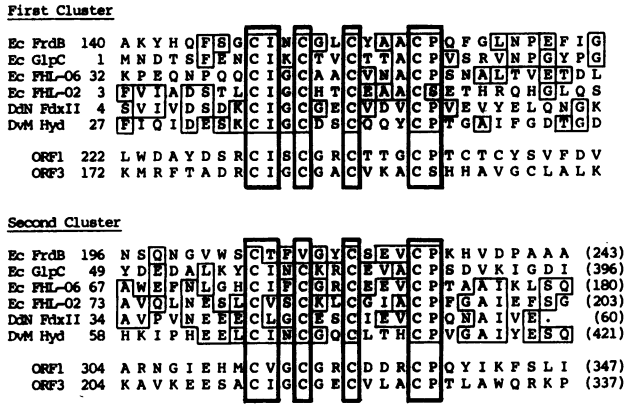


FIG. 5. Comparison of ferredoxinlike sequences in the predicted ORF1 and ORF3 sequences with similar sequences in other proteins. Amino acid positions are indicated, the last number in parentheses indicating the total number of amino acids in the protein. Ec FrdB, *E. coli* fumarate reductase component encoded by *frdB* (8); Ec GlpC, *E. coli* anaerobic glycerol-3-phosphate dehydrogenase component encoded by *glpC* (7); DdN FdxII, *Desulfovibrio desulfuricans* Norway strain ferredoxin II (20); DvM Hyd, *Desulfovibrio vulgaris* Marburg strain hydrogenase (56); Ec FHL-02 and Ec FHL-06, open reading frames 2 and 6, respectively, in an *E. coli* chromosomal locus near min 58 or 59 which is essential for formate hydrogenlyase activity (gene designations not yet assigned) (3). Amino acids shared by any of the six comparison proteins are boxed. Boldface boxes are the highly conserved [4Fe-4S] ferredoxin sequences (16).

identification of the −35 (TTAGCG) and −10 (TAGCGT) regions (22) (Fig. 2).

Identification of the *asr* gene products. Proteins encoded by the *asr* clone were detected by electrophoresis of extracts of an *E. coli* DH5α strain harboring plasmid pEB17, in which *asr* is fused to the *tac* promoter (Fig. 4). The sizes and the number of polypeptides detected were in good agreement with the sequence-predicted proteins and direction of transcription. Three discrete bands corresponding to apparent molecular masses of 45, 38, and 30.6 kDa were detected in electrophoresed extracts from induced cells (Fig. 4, lane c) but not in extracts from uninduced cells (lane b) or in extracts of cells containing vector without the insert (lanes d and e). The observed molecular masses of two of the polypeptides produced from pEB17 were in very close agreement with the sequence data. The discrepancy for the third protein (45-kDa protein detected compared with 40 kDa predicted) may reflect posttranslational modification or other factors affecting mobility on SDS-PAGE, as has been noted for many proteins (18, 44).

Neither sequence analysis nor the electrophoretic identification of the gene products revealed a protein of the size (65 kDa) reported in our previous work (21) to be the major subunit of purified sulfite reductase.

Amino acid sequence and implied functional domains. Computer-assisted analysis of the amino acid sequences did not reveal significant homology with any proteins in the data bases accessed. However, we did identify functional domains suggestive of roles in the reduction of sulfite. The predicted products of both ORF1 and ORF3 contained cysteine arrangements typical of ferredoxin [4Fe-4S] clusters (Cys-X-X-Cys-X-X-Cys-X-X-X-Cys-Pro), with significant homology to ferredoxins of the clostridial family (16) (Fig. 5). Among ferredoxins and other proteins containing ferredoxinlike sequences for which sequences have been published, the most similar to those encoded by ORF1 and ORF3 were the presumed [4Fe-4S] ferredoxinlike regions deduced from the nucleotide sequences of *E. coli frdB* (8) and *glpC* (7), the *Desulfovibrio desulfuricans* (Norway strain) ferredoxin II (16, 20), the *D. vulgaris* Fe-only hydrogenases (56), and two ferredoxinlike proteins encoded in an *E. coli* chromosomal locus essential for formate hydrogenlyase activity in *E. coli* (3). The first cysteine cluster in both the ORF3-encoded protein and one of the *fhl*-encoded proteins differed from the typical [4Fe-4S] clusters in the substitution of a serine residue for the final proline.

The ORF2-encoded sequence contained regions found in nucleotide-binding enzymes, including the flavoprotein peptide (*cysJ* product) of *S. typhimurium* biosynthetic sulfite reductase (Fig. 6). The first region, from Leu-113 to Met-129, contained an arrangement of glycines preceded by several hydrophobic residues similar to that conserved in many procaryotic and eucaryotic nucleotide-binding enzymes (38, 57). The ORF2-encoded region did not share all the features of the consensus flavin adenine dinucleotide-binding fold, which includes the glycine arrangement Gly-X-Gly-X-X-Gly (57). However, the region closely resembled a comparable region in the flavin-binding domain of *Arabidopsis thaliana* nitrate reductase, *Aspergillus nidulans* nitrate reductase, and bovine cytochrome reductase (9), all of which contain flavin adenine dinucleotide and catalyze NADH oxidation. The region showed somewhat less resemblance to the same region in the *S. typhimurium cysI* NADPH-linked protein, rat liver NADPH-cytochrome P-540 oxidoreductase, and spinach ferredoxin-$NADP^+$ oxidoreductase, three proteins which show considerable homology with each other (38). Additional sequences following the glycine motif were also shared by the predicted ORF2 protein and the other enzymes (Fig. 6), including a lysine residue (residue 168 in the ORF2 protein) suggested to participate in nucleotide binding in the

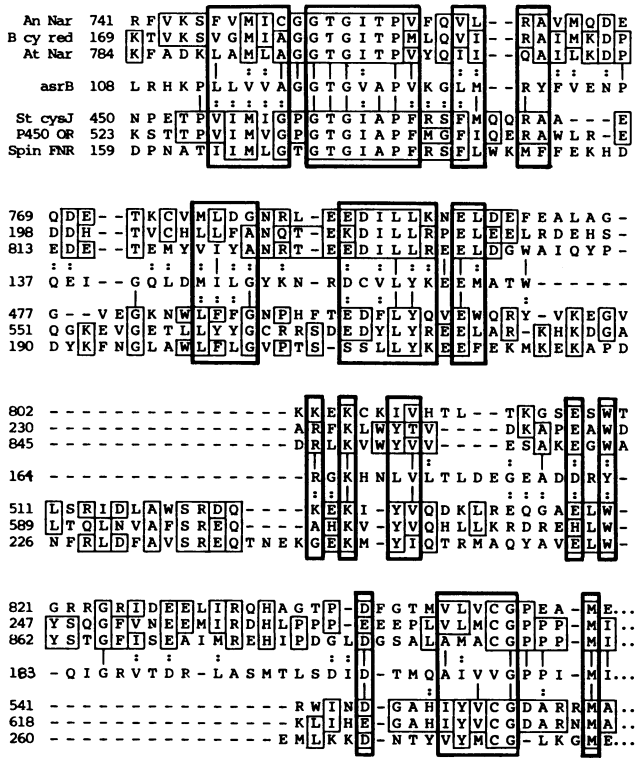


FIG. 6. Comparison of the predicted ORF2 amino acid sequence with sequences of nucleotide-binding enzymes. Partial sequences shown are as follows: An Nar, *Aspergillus nidulans* nitrate reductase (28); B cy red, bovine cytochrome *b* reductase (9); At Nar, *Arabidopsis thaliana* nitrate reductase (9); St cysJ, *S. typhimurium cysJ* protein (38); p450 OR, rat liver NADPH-cytochrome P-450 oxidoreductase (38); Spin FNR, spinach ferredoxin-NADP$^+$ oxidoreductase (38). Amino acids shared by the NADH-linked proteins or the NADP$^+$- or NADPH-linked enzymes are boxed. Boldface boxes indicate conserved regions shared by at least five of the seven proteins.

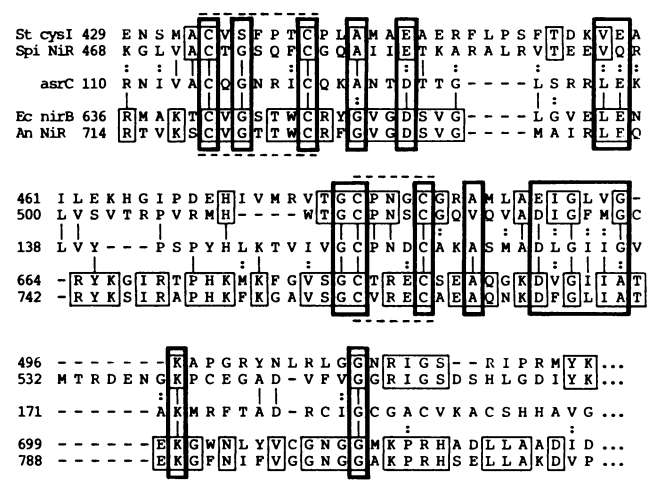


FIG. 7. Comparison of the predicted ORF3 amino acid sequence with similar cysteine-containing regions of four siroheme-binding proteins. Partial sequences shown are as follows: St cysI, *S. typhimurium cysI* protein (40); Spi NiR, spinach nitrite reductase (40); Ec nirB, *E. coli nirB* NADH-linked nitrite reductase (42); and An NiR, *Aspergillus nidulans* nitrite reductase (28). Amino acids shared by *S. typhimurium cysI* and spinach nitrite reductase or by *E. coli nirB* and *A. nidulans* nitrite reductase are boxed. Boldface boxes indicate conserved regions shared by at least four of the five proteins. Dashed lines indicate the cysteine clusters proposed to coordinate siroheme binding.

cysJ protein (38). This lysine was significantly closer to the glycine motif in the NADH-linked proteins than in the NADP- or NADPH-linked proteins. An additional cluster of residues containing Cys-Gly-X_4 or X_5-Met preceded by three of four hydrophobic amino acids was also shared by the seven proteins. This motif was also noted in the *nirB*-encoded NADH-linked nitrite reductase (42) in which it was separated from a nucleotide-binding glycine motif by a much longer stretch of amino acids, including the cysteine clusters typical of siroheme binding (as discussed below). Outside of the glycine motif and this Cys-Gly-containing cluster, no significant similarity was found between the *nirB* amino acid sequence and the predicted ORF2 protein (data not shown). The ORF2-encoded sequence was also found to contain a group of cysteine residues (Cys-240, Cys-245, Cys-248, and Cys-256) that might coordinate metal binding.

The most noteworthy feature of the predicted ORF3 product was the presence of two groups of cysteine-containing clusters (Cys-115 to Cys-121 and Cys-153 to Cys-157) closely resembling the Cys-X_5-Cys-X_{32-36}-Cys-X_3-Cys arrangement that has been proposed to coordinate the binding of a [4Fe-4S] cluster and siroheme in spinach nitrite reductase and biosynthetic sulfite reductase (40) (Fig. 7). The same clusters are also found in two other siroheme-dependent reductases, namely, *E. coli nirB*-encoded NADH-linked nitrite reductase and *Aspergillus nidulans* nitrite reductase (Fig. 7). The ORF3 sequence also contained the conserved alanine (Ala-124) and acidic residue (Asp-127) located just after the first cysteine cluster in the *cysI*-encoded protein and spinach nitrite reductase, two residues also proposed to be of importance in siroheme binding (40). The alanine is replaced by glycine in the *E. coli nirB* and *A. nidulans* nitrite reductases. In general, the latter nitrite reductases showed much more resemblance to each other than to the *cysI* protein and spinach nitrite reductase, and the predicted ORF3 protein deviated significantly from both pairs of the other proteins (Fig. 7). The ORF3-encoded sequence also differed from all of these other siroheme-dependent enzymes in its inclusion of probable [4Fe-4S] binding sites (see above) just beyond the second group of potential siroheme-binding cysteines.

Regulation of *asr* gene expression by oxygen and sulfite. To study regulation of *asr* gene expression by oxygen and sulfite, plasmid pEB18 was constructed such that the *asr* promoter region and the first 131 codons of ORF1 were fused in frame to the eighth codon of the *lacZ* gene (see Materials and Methods). β-Galactosidase was assayed in strain TT521 harboring either pEB18 or the vector without insert (Table 1). Under aerobic conditions, presence of sulfite in the growth medium induced the expression to a significant level. Under anaerobic conditions, slight expression was observed even in the absence of sulfite, while the addition of sulfite increased expression by about 50-fold. The presence of cysteine or nitrate in the growth medium did not diminish expression (data not shown). Regulation by cysteine was not expected because *S. typhimurium* produces hydrogen sulfide from sulfite in complex media (6). However, nitrate does prevent H_2S production, although apparently by a mechanism other than transcriptional regulation.

To define more precisely the 5′ limit of the *asrABC*

TABLE 1. Effects of *asrABC* promoter deletions on regulation by sulfite and air

Plasmid (deletion endpoint[a])	β-Galactosidase activity (U/OD_{600}[b])			
	Aerobic incubation		Anaerobic incubation	
	−Sulfite	+Sulfite	−Sulfite	+Sulfite
pRS414-8 (vector only)	<1	<1	<1	<1
pEB18 (no deletion)	<1	180	60	5,000
pEB22 (−250)	<1	145	45	5,800
pEB26 (−105)	<1	155	53	5,400
pEB28 (−85)	<1	230	57	5,900
pEB29 (−64)	<1	<1	57	200
pEB30 (−41)	<1	<1	4	5
pEB31 (+53)	<1	<1	<1	<1

[a] Deletion endpoints are relative to transcription initiation site (nucleotide position 241 in Fig. 2).

[b] Cells were grown in glucose minimal medium with (1 mM) or without sodium sulfite to an A_{600} of 0.3 to 0.4. OD_{600}, Optical density at 600 nm.

promoter and to locate the nucleotide sequences mediating induction by sulfite and anaerobiosis, we constructed a series of plasmids derived from pEB18 which contained deletions in the *asr* promoter region and then assayed β-galactosidase in cultures grown with or without air or sulfite (Table 1). Removal of the nucleotides upstream of −85 (position 155 in Fig. 2) did not affect induction by sulfite. However, a further deletion extending to −64 severely reduced sulfite induction, and a deletion extending to −41 abolished it. These results indicate that the first inverted repeat appears not to participate in *asr* regulation and that the region between −85 and −64 contains the major portion of a *cis*-acting sequence crucial for *asr* induction by sulfite.

Anaerobic induction in the absence of sulfite was not affected by deletions extending through −64 but was abolished in the plasmid containing a deletion extending through −41. Thus, the sequences essential for regulation by anaerobiosis are downstream of the sulfite regulation area and are probably within the RNA polymerase-binding region.

FNR and anaerobic regulation of *asr*. The FNR protein (the product of the *fnr* gene) serves as a positive regulator for a number of anaerobically induced genes in *E. coli* (14, 26, 53). A counterpart for FNR in *S. typhimurium* has been tentatively identified as the product of the *oxrA* gene (54), but much less is known about *oxrA*-mediated anaerobic regulation. Computer-assisted analysis of the DNA sequence revealed a string of 18 nucleotides just upstream of ORF1 (positions 275 to 282) that was 78% homologous to the proposed FNR protein-binding site in the *nirB* promoter of *E. coli* (26). The homology between *asr* and *nirB* included 7 of the 10 bases proposed by Jayaraman et al. (26) to compose an FNR-binding consensus sequence (Fig. 8). Compared with the FNR box in several FNR-regulated genes (Fig. 8), the *asr* sequence shared many bases with the reported FNR boxes, although it lacked a perfect FNR half-site (5'TTGAT or 5'ATCAA), which is found in almost all FNR boxes (14). The computer-identified "FNR box" in *asr* was also unconvincing because its location was more appropriate for a negative rather than a positive regulator. The location of the "FNR box" in the *asr* operon is similar to its location in the *fnr* gene, which is negatively regulated by its own product (26).

To see if the possible "FNR box" did, however, play some role in *asr* regulation, we examined the effects of two site-specific mutations in the apparent "FNR box" on the

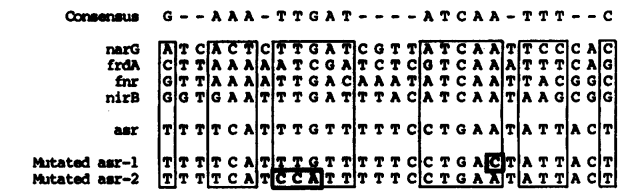


FIG. 8. Homology of *asr* nucleotides 270 to 297 with FNR-binding regions of several FNR-binding proteins in *E. coli* and *asr* site-specific mutations used to determine the importance of this region in *asr* regulation. The FNR-binding consensus sequence and sequences for nitrate reductase (narG), fumarate reductase (frdA), NADH-linked nitrite reductase (nirB), and the *fnr* gene (fnr) are those reported by Eiglmeier et al. (14). The specific alterations in the two mutated *asr* sequences are boxed in boldface.

expression of β-galactosidase by the fusion plasmid. The mutations (Fig. 8) altered highly conserved nucleotides in the consensus FNR box (14, 26), but they did not alter *asr* expression aerobically or anaerobically in the presence or absence of sulfite (data not shown).

DISCUSSION

The results presented here indicate that the previously cloned *asr* plasmid (24), which complements *asr* mutants of *S. typhimurium* and confers on *E. coli* the ability to produce hydrogen sulfide from sulfite, contains a functional operon consisting of three *asr* genes. This conclusion was suggested by the finding that there were three open reading frames preceded by a functional promoter sequence and concluded by an inverted repeat followed by a stretch of thymidine nucleotides, i.e., a typical *rho*-independent terminator sequence (47). We are proposing the gene designations *asrA*, *asrB*, and *asrC* for the three open reading frames. That *asrA*, *asrB*, and *asrC* constitute an *asr* operon is further suggested by the findings that all three genes are translated in vivo and that expression from the promoter is under the same regulation that characterizes hydrogen sulfide production from sulfite by wild-type *S. typhimurium*.

The amino acid sequences predicted by the DNA sequence are consistent with roles for all three proteins in NADH-linked anaerobic sulfite reduction. Although the predicted proteins were smaller than the subunits of biosynthetic sulfite reductase, they contained the key features of a siroheme-linked reductase enzyme complex. Cysteine arrangements proposed to coordinate siroheme binding (40) were present in AsrC, and a region homologous to the conserved nucleotide-binding regions of the flavoprotein of biosynthetic sulfite reductase (38) and other related proteins (Fig. 6) was present in AsrB. The presence of ferredoxinlike cysteine arrangements in AsrA and AsrC is also indicative of participation in electron transfer. Hydrogen and formate can both serve as electron donors for anaerobic sulfite reduction (21). Perhaps AsrA is analogous to the *Desulfovibrio vulgaris* Fe-only hydrogenases that contain similar ferredoxinlike cysteine clusters. This particular type of cysteine arrangement is not found in published sequences of the [Ni-Fe] or [Ni-Fe-Se] hydrogenases of *Desulfovibrio* (56) or other bacteria (49). The nickel-containing hydrogenases are also immunologically unrelated to the Fe-only hydrogenases (29). The clostridia-type ferredoxinlike sequences have been detected, however, in other oxidation-reduction enzymes, including fumarate reductase in *E. coli* (8), succinate dehydrogenase in *E. coli* and *Bacillus subtilis* (10, 43), and

the anaerobic glycerol 3-phosphate dehydrogenase in *E. coli* (7). There were several other cysteine residues in AsrA (Cys-98, Cys-115, Cys-122, Cys-124, Cys-134, Cys-243, Cys-245, and Cys-169) that could conceivably participate in the binding of hydrogen or an additional metal cofactor.

None of the *asr* genes appeared to encode the 65-kDa protein reported earlier to be the siroheme-containing subunit of purified anaerobic sulfite reductase (21). On the other hand, the deduced *asrC* protein did contain regions of significant homology with the proposed siroheme-binding site of biosynthetic sulfite reductase (40), which suggests that *asrC* may encode the siroheme-containing subunit of anaerobic sulfite reductase. The most likely explanation for this contradiction is that the major 65-kDa band observed in the SDS-PAGE of the active fraction obtained from DEAE chromatography was not the sulfite reductase. The sulfite reductase was probably, instead, a less plentiful protein in the active extract that did not show up in the small quantity of active fraction (eluted from a native gel) that was applied to the SDS-PAGE gel and stained with Coomassie blue. Clearly, additional work is necessary to clarify this problem.

Assays of β-galactosidase synthesized by the *asr*::*lacZYA* fusion plasmid revealed that expression was repressed by oxygen and induced by sulfite. Thus, the previously reported physiological regulation by air and sulfite (21) occurs at the transcriptional level. Cysteine had no effect on *asr* expression, which is consistent with our hypothesis that anaerobic sulfite reduction is not primarily a biosynthetic reaction (21, 24), even though the *asr* genes can, under anaerobic growth conditions, compensate for mutations in the biosynthetic pathway (24).

Positive control by sulfite is consistent with the structure of the *asr* promoter. The poor correspondence of the −35 (TTAGCG) and −10 (TAGCGT) sequences to the consensus sequences and the separation of the *asr* −10 and −35 regions by 18 rather than the optimal 17 nucleotides are suggestive of a weak promoter that requires activation for effective transcription (19, 46). Analysis of upstream promoter deletions revealed that a region between −85 and −64 was essential fur sulfite induction. A model consistent with these findings includes a positive regulatory protein, activated by sulfite, which binds to a site between −85 and −64, thereby stimulating transcription by RNA polymerase. However, this model is tenuous in light of its surprising prediction that *E. coli* synthesizes the regulatory protein even though it does not contain the structural genes for dissimilatory sulfite reduction (because the *asr* plasmid conferred on *E. coli* the ability to produce hydrogen sulfide from sulfite).

The region essential for anaerobic expression in the absence of sulfite (−64 to −41) was located just downstream of the sulfite regulatory site and was probably within the RNA polymerase-binding region. Possible explanations for such a location in light of the poor −35 and −10 sequences include transcriptional dependence on (i) a unique RNA polymerase sigma factor (4) that is activated by or induced under anaerobic conditions; (ii) an RNA polymerase altered in nucleotide preference because of interaction with an anaerobically synthesized protein factor, such as the 14-kDa *Bacillus subtilis* switch factor which alters the specificity of σ^K during spore development (31); or (iii) anaerobic alterations in DNA supercoiling with a positive effect on open complex formation (13, 25, 45). DNA supercoiling is known to facilitate expression of many anaerobic genes (13, 25, 58). The anaerobic sigma factor encoded by *ntrA* is probably not involved, because *ntrA* mutants produce wild-type levels of H_2S from sulfite (24a).

Computer-assisted analysis of the DNA sequence revealed a region upstream of ORF1 that had significant resemblance to the proposed FNR box of the *nirB* promoter (26). The FNR box in anaerobically induced promoters of *E. coli* is thought to be the binding site for the FNR protein, which activates transcription of several anaerobic genes. However, unlike the situation for known Fnr-controlled genes involved in anaerobic energy metabolism, the location of the "FNR box" in the *asr* operon was consistent with negative rather than positive regulation. Mutations in the apparent "FNR box" were found to have no effect on *asr* expression. We have similarly observed that mutations in *oxrA*, which is though to be the counterpart of the *E. coli fnr* gene (54), do not prevent the production of H_2S from sulfite. Furthermore, preliminary results of studies of *asr* expression in an *oxrA* chromosomal background revealed somewhat elevated levels of *asr* expression (23a). Mutations in *oxrA* do severely reduce the production of H_2S from thiosulfate (6) but do so without interfering with expression of *phs*, the gene locus for thiosulfate reduction (6). Thus, anaerobic induction of H_2S production from both sulfite and thiosulfate occurs by mechanisms unrelated to those previously described for other anaerobic reductases. Perhaps the lack of Fnr involvement is indicative of the lack of a significant role for the H_2S-producing enzymes in anaerobic energy conservation. In fact, our previous physiological studies failed to reveal a contribution to energy conservation by sulfite reductase (21). At this time, we do not have a model for the function of dissimilatory sulfite reduction by *S. typhimurium*.

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