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Sequence Analysis of the *phs* Operon in *Salmonella typhimurium* and the Contribution of Thiosulfate Reduction to Anaerobic Energy Metabolism

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The *phs* chromosomal locus of *Salmonella typhimurium* is essential for the dissimilatory anaerobic reduction of thiosulfate to hydrogen sulfide. Sequence analysis of the *phs* region revealed a functional operon with three open reading frames, designated *phsA*, *phsB*, and *phsC*, which encode peptides of 82.7, 21.3, and 28.5 kDa, respectively. The predicted products of *phsA* and *phsB* exhibited significant homology with the catalytic and electron transfer subunits of several other anaerobic molybdoprotein oxidoreductases, including *Escherichia coli* dimethyl sulfoxide reductase, nitrate reductase, and formate dehydrogenase. Simultaneous comparison of PhsA to seven homologous molybdoproteins revealed numerous similarities among all eight throughout the entire frame, hence, significant amino acid conservation among molybdoprotein oxidoreductases. Comparison of PhsB to six other homologous sequences revealed four highly conserved iron-sulfur clusters. The predicted *phsC* product was highly hydrophobic and similar in size to the hydrophobic subunits of the molybdoprotein oxidoreductases containing subunits homologous to *phsA* and *phsB*. Thus, *phsABC* appears to encode thiosulfate reductase. Single-copy *phs-lac* translational fusions required both anaerobiosis and thiosulfate for full expression, whereas multicopy *phs-lac* translational fusions responded to either thiosulfate or anaerobiosis, suggesting that oxygen and thiosulfate control of *phs* involves negative regulation. A possible role for thiosulfate reduction in anaerobic respiration was examined. Thiosulfate did not significantly augment the final densities of anaerobic cultures grown on any of the 18 carbon sources tested. On the other hand, washed stationary-phase cells depleted of ATP were shown to synthesize small amounts of ATP on the addition of formate and thiosulfate, suggesting that thiosulfate reduction plays a unique role in anaerobic energy conservation by *S. typhimurium*.

The great majority of *Salmonella* species produce hydrogen sulfide from both thiosulfate and sulfite by means of anaerobically induced dissimilatory pathways which are independent of biosynthetic sulfur assimilation (1, 22). Studies of H_2S production by *Salmonella typhimurium* have shown that two distinct pathways dictate the reduction of thiosulfate and sulfite, respectively, to H_2S (11). Thiosulfate reduction is mediated by a membrane-bound system (11, 22), the essential components of which are encoded by the *phs* (production of hydrogen sulfide) locus at 41.5 min on the *Salmonella* chromosome (11, 41), while sulfite reduction is performed by soluble enzymes (22) encoded by the *asr* (anaerobic sulfite reductase) locus at about 53 min (23, 24). Hydrogen sulfide is not produced from either substrate in the presence of oxygen or nitrate (11), and both pathways are positively affected by reduced sulfur in the growth medium (11, 22). Such regulatory characteristics suggest that both pathways have primarily energetic rather than biosynthetic functions. A physiological connection between the pathways is suggested by the fact that sulfite is produced along with H_2S during thiosulfate reduction and the observation that siroheme mutants, which are unable to reduce sulfite by the *asr*-encoded pathway, produce significantly less H_2S from thiosulfate than does wild-type *S. typhimurium* (2).

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Our previous studies have revealed many similarities between thiosulfate reduction by wild-type *S. typhimurium* and several well-characterized systems for anaerobic respiration in *Escherichia coli*, including nitrate, trimethylamine oxide, and dimethyl sulfoxide reduction. All of these electron transport pathways, including thiosulfate reduction, involve an anaerobically induced, membrane-bound molybdoprotein reductase (11, 14, 38, 42, 43), and all accept electrons from formate (22, 26, 28, 38). However, thiosulfate reduction differs in that it is strongly repressed by glucose because of a specific requirement for cyclic AMP (12). Recently we cloned the *phs* operon and presented indirect evidence that it contains the structural gene for thiosulfate reductase (18). Here we report our studies of the genetic organization of the *phs* region, the results of which are entirely consistent with the earlier suggestion that *phs* contains the structural gene. We have also examined the participation of thiosulfate reduction in energy conservation, a role implied by the significant similarity of the *phs* gene products to other molybdoprotein reductases.

MATERIALS AND METHODS

Strains, culture conditions, and chemicals. The bacterial strains and plasmids used in this study are listed in Table 1. Beef extract, nutrient agar, nutrient broth, peptone, Proteose Peptone, yeast extract, and peptone iron agar were from Difco Laboratories. The minimal medium used in growth experiments was previously described (11). Electron acceptors in minimal media were used at a 6 mM concentration, and electron donors were used at 1% (wt/vol) (5.6 mM for hexoses). Nitrate broth consisted of nutrient broth with 100 mM nitrate; thiosulfate broth consisted of, per liter, 3 g of beef extract, 3 g of yeast extract, 15 g of peptone, 5 g of Proteose Peptone, and 1 g (about 6 mM) of $Na_2S_2O_3$. These media support maximal activities of nitrate reductase and thiosulfate reductase, respectively (2). All incubations were done at 37°C. Anaerobic incubation of

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid(s)	Genotype or phenotype	Source or reference
S. typhimurium		
LT2	Wild type	B. N. Ames
EB478	*S. typhimurium* TT521/pEB41	This study
EB480	Same as LB5000 but with *putPA1303*::Kan^r-*phs-lacZ* (promoter deletion fusion derived from pEB133)	This study
EB481–EB485	Same as EB480 but with promoter deletion fusions derived from pEB137 and pEB139–pEB142, respectively	This study
LB5000	*hsdL6 hsdSA29* (r^- m^+) *metA22 metE551 trpD2 ilv-452*	S. Artz
TT521	*recA1 rpsL srl-202*::Tn*10*	SGSC[a]
E. coli DH5α	F$^-$ *endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1* λ$^-$ *recA1 gyrA96 relA1* Δ(*lacZYA-argF*)U169 ϕ80d*lacZ*ΔM	Bethesda Research Laboratories
Plasmids		
pBR322	Ap^r Tc^r	New England Biolabs
pGEM7Zf(+)	*lacZ* Ap^r	Promega
pJF118EH	p_{tac} *rrnB* Ap^r *lacI*^{q}	19
pRS414–pRS418	pRS414 (36) containing *Hin*dIII in polylinker site	24
pUC19	Ap^r	New England Biolabs
pEB40	pUC19 derivative, *phs* bp 1–5136	18
pEB41	pGEM7Zf(+) derivative, *phs* bp 1–5136	This study
pEB82	pEB41 derivative, *phs* bp 322–5136	This study
pEB90	pEB41 derivative, *phs* bp 143–5136	This study
pEB114	pUC19 derivative, *phs* bp 322–751	This study
pEB123	pEB114 derivative, *phs* bp 599–751	This study
pEB127	pBR322 derivative, *phs* bp 1–5136	This study
pEB128	pUC19 derivative, *phs* bp 599–5136	This study
pEB129	pJF118EH derivative, *phs* bp 599–5136	This study
pEB132–pEB142	pRS414-8 derivatives with *phs* promoter deletions	This study

[a] SGSC, Salmonella Genetic Stock Centre, care of Kenneth Sanderson.

liquid cultures was achieved by filling tubes to the top and stoppering them after inoculation (2%). A_{650} was measured in a Spectronic 21 (Bausch & Lomb, Inc., Rochester, N.Y.).

DNA sequencing. The 5.1-kb *phs* insert in pEB40 (18) was sequenced by using the dideoxynucleotide chain termination method (34) and Sequenase (United States Biochemicals Inc.) in accordance with the manufacturer's directions. Nested deletions for sequencing were constructed from pEB40 and from pEB41, which contains the same insert which is in the opposite orientation in pGEM7Zf(+), by using the Erase-a-Base kit (Promega Biotec Corporation) in accordance with the manufacturer's directions. Both strands were sequenced. Restriction endonucleases were purchased from either Bethesda Research Laboratories or New England Biolabs. T4 DNA ligase was purchased from United States Biochemicals Inc.

Plasmid and strain constructions. Unless otherwise noted, all recombinant DNA procedures were done as described by Maniatis et al. (27). Transformation of *S. typhimurium* was achieved by electroporation (31) with a Gene-Pulser apparatus from Bio-Rad Laboratories.

Translational *phs-lac* fusions with promoter deletions were constructed in plasmid pRS414-8 (24), a derivative of pRS414 (36), as follows. Plasmids pEB82 (insert beginning at bp 322) and pEB90 (insert beginning at bp 143) were among the nested deletions constructed from pEB41 for DNA sequencing. The inserts in pEB82 and pEB90 were cut with *Nsi*I (pGEM vector polylinker site) and *Hin*dIII (bp 751 in ORF1) and ligated with pUC19 cut with *Pst*I and *Hin*dIII, and then further promoter deletions from them were constructed as for DNA sequencing. The promoter deletions were moved into pRS414-8 cut with *Eco*RI and *Hin*dIII, producing translational fusions pEB133, pEB137, and pEB139 to pEB142. In all, *lacZ* was fused to bp 751 in open reading frame 1 (ORF1). The fusions were moved into *S. typhimurium* TT521 by electroporation. The method of Elliot (17) was used to construct single-copy *lac* fusions with same promoter deletions in *S. typhimurium* LB5000, in this case inserted in the chromosomal *putA* gene. Special plasmids and strains required for constructing the single-copy fusions were kindly provided by T. Elliott. Strains EB480 to EB485 contain the *phs*::*lac* fusions carried by pEB133, pEB137, and pEB139 to pEB142, respectively.

For overproducing *phs* proteins in *E. coli* DH5α, we constructed pEB129, which contains the *tac* promoter from expression vector pJF118EH (19) joined to the *phs* insert from pEB128, which contains a promoter deletion extending through bp 559, 5 bp upstream of the putative transcription start site. The promoter deletion in pEB128 was constructed by ligating the truncated promoter in pEB123, a pUC19 derivative with a *phs* insert, bp 751 to 5136, cut from pEB127 with *Hin*dIII and *Sal*I.

Analysis of overproduced *phs* gene products. Products of *E. coli* DH5α cultures carrying either pJF118EH without the *phs* insert or pEB129 were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described (24). The products were also electrophoresed on 8% native polyacrylamide gels and stained for thiosulfate reductase activity by the method of DeVoe et al. (15).

Primer extension. By use of the RNAid Plus kit (Bio 101), RNA was isolated from strain EB478 grown aerobically or anaerobically in minimal medium, with or without thiosulfate. The single-stranded synthetic oligonucleotide primer complementary to the region from bp 643 to 662 (5′-AGCCGATGCCTAC CCCCCTGC-3′) was 5′ end labeled with the DNA 5′ end-labeling system kit (Promega) in accordance with the manufacturer's instructions. Labeled oligonucleotide (5 ng) was added to 80 to 100 μg of RNA and allowed to anneal in the presence of RNAsin (Promega). Reverse transcription was done with the reverse transcription kit from Promega. The extended products were analyzed by electrophoresis on an 8% polyacrylamide–urea gel in parallel with dideoxynucleotide sequencing reactions primed with the same oligonucleotide.

Determination of ATP production facilitated by thiosulfate reduction. *S. typhimurium* LT2 cultures were grown to the early stationary phase in either nitrate broth or thiosulfate broth. Cells were harvested at 15,000 × *g*, washed, and resuspended in 1/50 of a volume of 50 mM phosphate buffer (pH 7.2). Aliquots (0.5 ml) of the cells were then placed in serum cap cuvettes and flushed with argon. The electron donor and acceptor were added as 20-μl aliquots of 1 M solutions. ATP produced in each vial was measured with a luciferase assay kit (Sigma) as described by Cox and Henick-Kling (13).

Nucleotide accession number. The *phsABC* sequence has been assigned GenBank accession no. L32188.

RESULTS

Nucleotide sequence of the *phs* locus. Plasmid pEB40 carries a 5.1-kb insert from the *S. typhimurium phs* region which confers on *E. coli* the ability to produce H_2S from thiosulfate (18). The nucleotide sequence and the deduced amino acid sequence of this 5.1-kb region are shown in Fig. 1. Computer analysis revealed three complete ORFs (ORF1, ORF2, and ORF3) and a fourth, partial ORF (ORF4) which was separated from the others by a sequence resembling a factor-

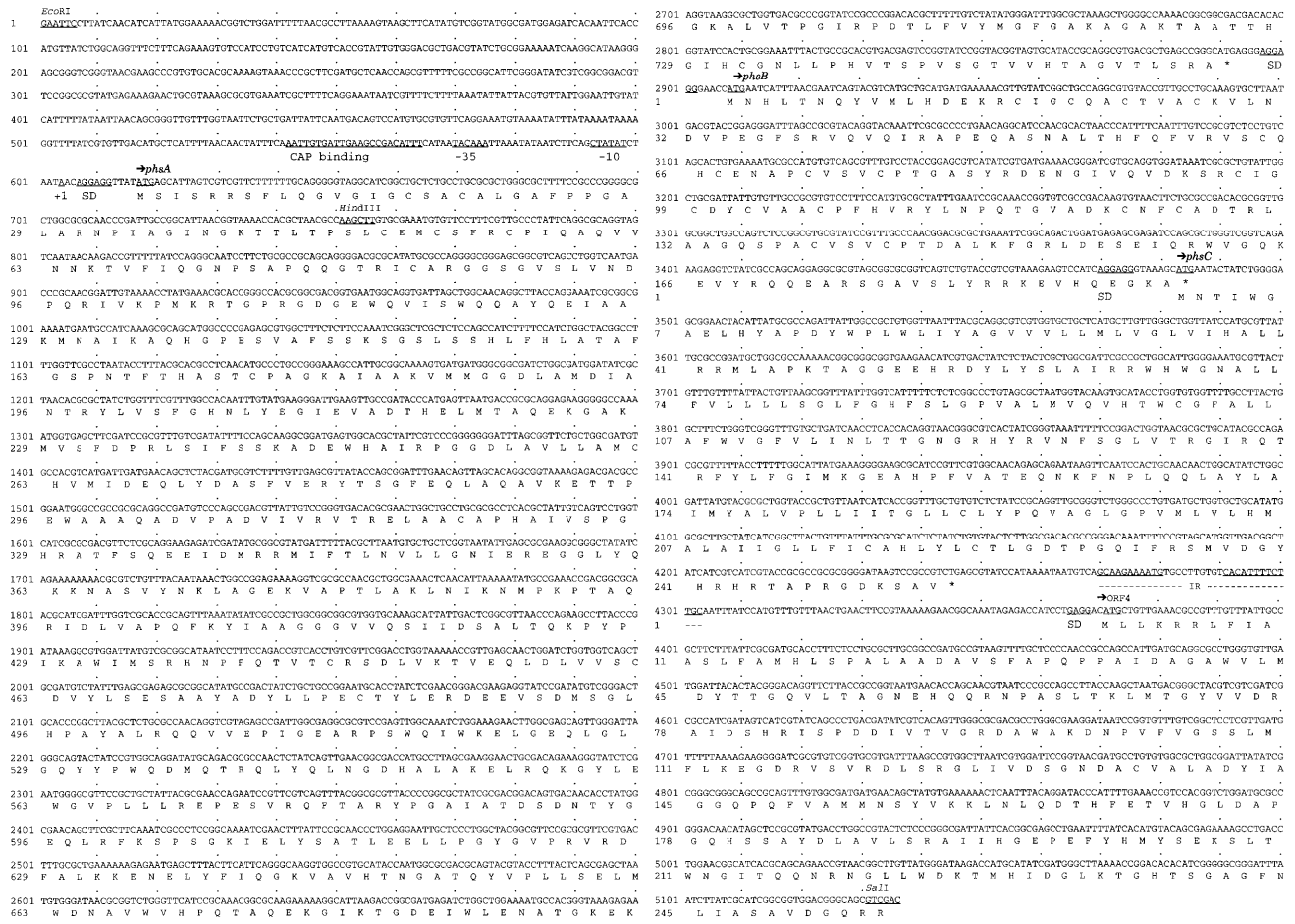

FIG. 1. Nucleotide sequence and deduced amino acid sequence of the *phs* locus. Nucleotides are numbered from the 5′ end, and intervals of 10 bp are marked. The predicted amino acid sequence of each ORF is given in single-letter code under the DNA sequence. The following putative features are underlined: a CAP-binding site, −10 and −35 regions, the transcription start site (+1), ribosome-binding sites (Shine-Dalgarno [SD] sequences), nucleotides that form an inverted repeat (IR) and are thus capable of producing a stem-loop structure, and sites of restriction enzymes noted in Materials and Methods.

independent terminator. All four frames were preceded by Shine-Dalgarno sequences closely resembling the consensus sequence and with appropriate spacing from the AUG codon (20). Each of the nested deletions constructed for sequencing was tested for the ability to confer H_2S production on *E. coli*. These tests revealed that ORF1 and ORF3 are both essential for H_2S production but that ORF4 is not. (The deletions did not permit us to determine whether or not ORF2 is essential.) ORF1, ORF2, and ORF3 encode polypeptides of 758 amino acids (82.7 kDa), 192 amino acids (21.3 kDa), and 254 amino acids (28.5 kDa), respectively. Hydrophobicity analyses (data not shown) indicated that ORF3 is strongly hydrophobic, while the other two predicted polypeptides are primarily hydrophilic. We suggest the designations *phsA*, *phsB*, and *phsC* for the genes representing ORF1, ORF2, and ORF3, respectively.

The *phs* promoter region. Primer extension was used to locate the 5′ end of the *phs* message. In cultures grown anaerobically with thiosulfate, strong signals were detected at bp 604 and 605, immediately preceding the Shine-Dalgarno sequence of *phsA* (Fig. 2). No signals were detected in cultures with air or without thiosulfate. This result suggested the tentative identification of the +1 site at bp 604 and the −10 (CTATAT) and −35 (TACAAA) regions separated by 17 bp indicated in Fig. 1. A putative catabolite gene activator protein (CAP)-binding sequence was located just upstream of the putative −35 sequence, starting at bp 542. The consensus sequence is AA-TG TGA-(6 bp)-TCACA-TT (16), compared with AA-TGTGA-(6 bp)-CCGAC-TT in *phs*. The perfect match of the first half sequence is consistent with the requirement for CAP-cyclic AMP in *phs* expression (12).

To identify potential *cis*-acting regions required for induction by anaerobiosis and thiosulfate, we constructed translational *phs*::*lacZ* fusions by using *phs* inserts representing a series of promoter deletions of increasing sizes. The deletion constructs were also converted into single-copy translational fusions inserted at the *put* gene site in *S. typhimurium* LB5000. The translational fusions were assayed for β-galactosidase in cultures grown with and without air and thiosulfate (Table 2). Chromosomal *phs*::*lac* operon fusions were shown previously to exhibit very low activities under aerobic conditions and/or without an appropriate sulfur compound inducer and 10- to 15-fold induction under anaerobic conditions with the sulfur compound inducer (11). The single-copy translational fusions exhibited higher activities than the previously reported operon fusions, but the induction pattern was quite similar. No activity was obtained in the fusion lacking the entire promoter, indicating that the high activities were not the result of read-through from an upstream promoter. Surprisingly, progressively larger deletions extending up to −71 resulted in

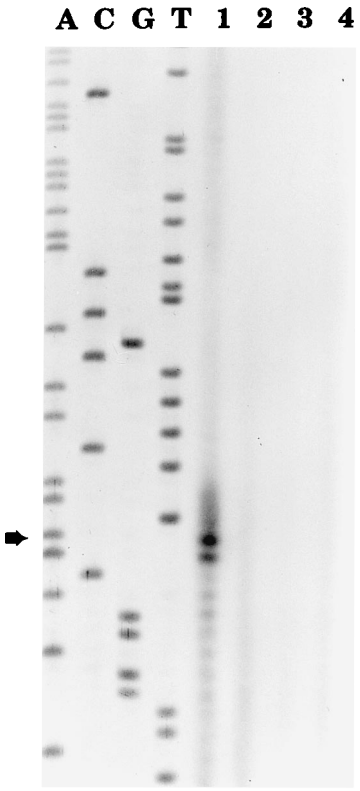

FIG. 2. Determination of the *phs* transcription start site by primer extension analysis. A 21-mer oligonucleotide primer complementary to nucleotides 643 to 662 (Fig. 1) was used to prime the reverse transcriptase reaction. Lanes 1 to 4 contained cDNA synthesized from RNA from *S. typhimurium* TT521/pEB41 grown as follows: lane 1, anaerobic with thiosulfate; lane 2, anaerobic without thiosulfate; lane 3, aerobic with thiosulfate; lane 4, aerobic without thiosulfate. The putative transcription start site is indicated by the arrow (bp 604).

TABLE 2. Effects of *phs* promoter deletions on regulation by air and thiosulfate

Deletion endpoint (bp), fusion location[a]	β-Galactosidase activity[b] expressed by single-copy (and multicopy) translational fusions			
	Aerobic incubation		Anaerobic incubation	
	Without thiosulfate	With thiosulfate	Without thiosulfate	With thiosulfate
322 (−282), EB480 (pEB133)	175 (160)	140 (540)	240 (980)	758 (5,400)
436 (−168), EB481 (pEB137)	270 (590)	280 (1,400)	450 (1,200)	2,100 (4,800)
480 (−124), EB482 (pEB139)	230 (280)	240 (2,100)	380 (1,400)	1,700 (5,600)
497 (−107), EB483 (pEB140)	410 (460)	500 (2,000)	830 (1,600)	3,300 (2,300)
533 (−71), EB484 (pEB141)	420 (<1)	500 (<1)	680 (13)	2,800 (10)
599 (−4), EB485 (pEB142)	<1 (<1)	<1 (<1)	<1 (<1)	<1 (<1)

[a] Each deletion endpoint is given as the starting base pair of the insert and is followed (in parentheses) by the starting site relative to the putative transcription initiation site. The fusion location is given as the strain derived from *S. typhimurium* LB5000 for single-copy fusions and is followed (in parentheses) by the plasmid in the host, *S. typhimurium* TT521, for multicopy fusions.

[b] Cells were grown in minimal medium containing 5 mM galactose with or without 6 mM thiosulfate to an OD_{600} of 0.1 to 0.8. β-Galactosidase activity was assayed by the method of Miller (29) and is expressed in Miller units. Neither *S. typhimurium* LB5000 nor *S. typhimurium* TT521/pRS414-8 (vector without insert) exhibited detectable activity (<1 Miller unit) under any condition. All of the values shown represent results averaged from three or four experiments. For each strain, the ratios of aerobic to anaerobic activities and of activities obtained with and without thiosulfate varied less than 25% among experiments.

progressively higher activities under all conditions. Bp −71 is just upstream of the putative CAP-binding site noted earlier.

Assays of the multicopy fusions yielded results which differed in two important respects. Firstly, the requirement for both thiosulfate and anaerobiosis was attenuated: a severalfold induction occurred both during aerobic growth with thiosulfate and during anaerobic growth without thiosulfate. Secondly, multicopy expression was almost completely prevented by deletions extending into the region between bp −107 and 71, whereas single-copy expression was not abolished until deletions extended somewhere downstream of bp −71. To the level of resolution provided by these deletions, regions for aerobic and thiosulfate regulation were genetically inseparable, and a specific regulatory region could not be identified.

Identification of the *phs* gene products. Proteins encoded by *phs* under *tac* promoter control were examined electrophoretically in extracts of an *E. coli* DH5α host containing either the vector (pJF118EH) alone or the vector with a *phs* insert from bp 599 (−4 from the putative transcription start site) to 5136 (pEB129), grown in the presence or absence of isopropyl-β-D-thiogalactopyranoside (IPTG) (Fig. 3). Three additional proteins were noted in the presence of IPTG for the strain carrying the insert. Their molecular sizes were calculated as 76.8, 26.9, and 17.6 kDa, respectively, which compare favorably with the predicted sizes of ORF1 (82.7 kDa), ORF3 (28.5 kDa), and ORF2 (21.3 kDa). The IPTG-induced extracts contained reduced quantities of several proteins compared with uninduced cultures, suggesting that the overproduction of *phs* products may negatively affect the synthesis of other proteins. Thiosulfate reductase activity was detected in extracts of *E. coli* DH5α/pEB129 subjected to native gel electrophoresis, and the strain also produced H_2S on peptone iron agar (data not shown).

Sequence comparisons and implied functional domains. Computer-assisted analysis of the predicted amino acid sequences revealed significant homology of ORF1 and ORF2 with the catalytic and second subunits, respectively, of other anaerobic oxidoreductases. The analysis revealed no significant homology between ORF3 and any proteins in GenBank. However, we did note that ORF3 is highly hydrophobic and of about the same size as the hydrophobic subunits of the reductase systems which contained subunits homologous to ORF1 and ORF2.

The protein sequences most closely related to ORF1 were those of the catalytic subunits of *E. coli* dimethyl sulfoxide reductase (DmsA) (4), *E. coli* biotin sulfoxide reductase (BisC) (32), *E. coli* nitrate reductase (NarG) (5), the selenocysteine- and molybdenum-containing subunit of *E. coli* hydrogenase-linked formate dehydrogenase (FdhF) (44), the major subunit of the *E. coli* nitrate-linked formate dehydrogenase (FdnG) (3), and the formate dehydrogenase (FdhA) proteins of both *Methanobacterium formicicum* and *Wolinella succinogenes* (7, 35). The greatest similarity was with DmsA (23% identity in a 476-amino-acid overlap). Simultaneous comparison of all eight amino acid sequences (Fig. 4) revealed numerous regions of similarity throughout the sequences, including the five regions

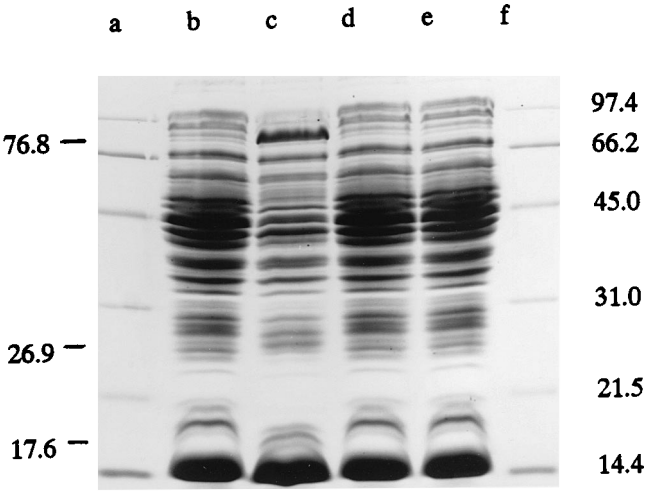

FIG. 3. Overexpression of *phs*-encoded proteins. Proteins synthesized by *E. coli* DH5α containing pEB129 with *phs* genes fused to the *tac* promoter (lanes b and c) or pJF118EH without the *phs* insert (lanes d and e), either uninduced (lanes b and d) or induced by IPTG (lanes c and e), were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with Coomassie blue. The reference proteins in lanes a and f were as follows: phosphorylase (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

identified by Blasco et al. (5) as common to *E. coli* BisC, DmsA, FdhF, and NarG. These similarities place PhsA in the family of anaerobic molybdoenzymes mediating membrane-bound electron transfer. ORF1 is also very close in size (758 amino acids) to the proteins encoded by *dmsA* (785 amino acids), *fdhF* (715 amino acids), and *bisC* (726 amino acids) of *E. coli* and by *fdhA* of *M. formicicum* (684 amino acids) but smaller than the predicted products of *E. coli narG* (1,238 amino acids) and *fdnG* (1,016 amino acids).

Similarly, the 192-amino-acid ORF2 encoded by *phsB* was found to resemble an electron transfer subunit of six different molybdoenzymes, including four of those with a catalytic subunit sequence resembling ORF1. Significant similarity was revealed with *E. coli* DmsB (206 amino acids) (4), FdnH (296 amino acids) (3), and NarH (512 amino acids) (5), as well as with HycB (203 amino acids) (6), HydN (175 amino acids) (25), and also FdhB of *W. succinogenes* (200 amino acids) (7). A simultaneous comparison of all six sequences with the predicted sequence of *phsB* is shown in Fig. 5. The most notable similarities are in the four ferredoxin-like cysteine-containing clusters found in all of them. The first and third cysteine clusters are typical of [4Fe-4S] ferredoxins with the general consensus sequence C-X_2-C-X_2-C-X_3-C (9), but the seven sequences share many residues in addition to the cysteines. The second cluster is more typical of what were once described as [3S-3F] ferredoxins, which share the general consensus sequence C-X_2-C-X_4-C-X_3-C (5, 9) but have been shown in several systems, including NarH (21) and DmsB (10), to coordinate [3Fe-4S] centers instead. The midpoint redox potential of the first and second clusters of both NarH and DmsB is consistent with electron donation from menaquinones (10, 21). The fourth cluster does not follow either consensus sequence, but this cluster, along with the third cluster, in NarH and DmsB has been shown to coordinate [4Fe-4S] clusters typical of bacterial ferredoxins in that the midpoint redox potential is very low (10, 21).

The ORF4 sequence showed very strong homology with *E. coli* penicillin-binding proteins 5 (53% identity over 253 amino

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Ws FdhA 52 PVSKKVKTICTY-CSVGCGIIAEVVDGV(15)GHCCKGADMIDKARSET----RLRYPIEKV-- GG--K
Ec FdnG 41 LRAKEIRNTCTY-CSVGCGLLMYSLGDG(22)ALCPKGPGLLDYVNSEN----RLRYPEYRA-- PGSDK
Mf FdhA  1 MDIKYVPTICPY-CGVGCGMNLVVKDEK(15)KLCPKGNFCYEIIHRED----RLTTPLIKE-- NG--E
Ec FdhF  1   MKKVVTVCPY-CASGCKINLVVDNGK(14)TLCLKGYYGWDPINDTQILTPRLKTPMIRRQ- RGG-K
ORF1    39 GKTTLTPSLCEM-CSFRCPIQAQVVNNK(15)RICARGGSGVSLVNDPQ----RIVKPMKRTGP RGDGE
Ec DmsA 25 SDEKVIWSACTVNCGSRCPLRMHVVDGE(20)RACLRGRSMRRRVYNPD----RLKYPMKRVGA RGEGK
Ec BisC  1                              MENSLQSAVRDQVHSNTRVRFPMVR(13)RGQDE
Ec NarG 45 VRSTHGVN-CTGSCSWKIYVKNGLVTWE(18)RGCPRGASYSWYLYSAN----RLKYPMMR(40)RG---

123 WRKTSWDSAMDKIA-KQLQDLTQKYGPDSVMF(11)YYFRKFAAFF-GTNNLDTIARICHAPTVAGVSNTLGYGGMT
121 WQRISWEEAFSRIA-KLMKA---DRDANPIEK(26)MLTQKFARSL-GMLAVDNQARVCHGPTVASLAPTFGRGAMT
 72 FREATWDEAYDLIASKLGA-----YDPNEIGF(11)YVNQKFARIVVGTHNIDHCARLCHGPTVAGLAASFGSGAMT
 75 LEPVSWDEALNYVAERLSA-IKEKYGPDAIQT(12)YVMQKFARAVIGTNNVDCCARVCHGPSVAGLHQSVGNGAMS
114 WQVISWQQAYQEIAAKMNA-IKAQHGPESVAF(8 )SHLPHLATAF-GSPNTFTHASTCPAGKAIAAVVMMG-GDLA
106 FRRISWEEAYDIIATNMQRLIKE-YGNESIYL(19)TLVARLMNCCGGYLNHYGDYSS--AQIAEGLNYTYG-GWAD
 44 FVRVSWDEALDLIHQQHKR-IREAYGPASIFA(15)TLLQRYMALAGGVTGHLGDYST--GAAQAIMPYVVG-GSEV
157 FVRSSWQEVNELIAASNVYTIKN-YGPDRVAG(12)ASGARYLSLIGGTCLSFYDWY-CDLP-PAS-PQTWG-EQTD

205 NHLA-DMMHSKAIFIGGNPAVNHPV(11)GAKIIVVDPHFSRT---ATKADHYVRLRNGTDVAFMYGMIRH--IVK
215 NHWV-DIKNANVVMVMGGNAAEAHPV(12)DATLIVVDPRFTRT---ASVADIYAPIRSGTDITFLSGVLRY--LIE
151 NSYA-SFEDADLIFSIGANSLEAHPL(11)GAYFIVADPRYTPT---AKQADQYIPFKTGTDVALMNAMMNVI-ISE
159 NAIN-EIDNTDLVFVFGYNPADSHPI(11)GAKIIVCDPRKIET---ARIADMHIALKNGSNIALLNAMGHVI-IEE
196 M----DIANTRYLVSFGHNLYEGIEV(12)GAKMVSFDPRLS---IFSSKADEWHAIRPGGDLAVLLAMCHVM-IDE
194 GNSPSDIENSKLVVLFGNNPGETRMS(15)NARMIIIDPRYTDTG--AGREDEWIPIRPGTDAALVNGLAYVM-ITE
128 Y(18)WSANPLNTLKIAWNASDEQGL(9 )GKKLICIDPMRSETVDFFGDKMEWVAPHMGTDVALMLGIAHT--LVE
237 VPESADWYNSSYIIAWGSNVPQTRTP(11)GTKTVAVTPDYA---EIAKLCDLWLAFKQGTDAAMALAGI(41)LVD

283 NGLE-DKEFIRQRLFGYEEIL     KECEQYTPEVVEEVTGVPAQQLIEITEIFA(5) SLIWGMGLTQHTTGTSN
294 N(20)DFAFEDGLFSGYDAEK(35) EHVSRYTPDVVENICGTPKADFLKVCEVLA(9) TPLYALGWTQHTVGAQN
230 GLE-  DKEFIEKRTKNYEELK      EVVSKYTPEMAEEITQVPADVI----RDIA(9) AIVVSLGITEHSHGVDN
238 NLY-  DKAFVASRTEGFEEYR      KIVEGYTPESVEDITGVSASEIRQAARMYA(5) AILWGMGVTQFYQGVET
269 QLY-  DASFVERYTSGFEQLA      QAVKETTPEWAAAQADVPADVIVRVTRELA(6) IVSPGHRATFSQEEIDM
279 NLV-  DQAFLDKYCVGYDEKT(20) DGVAK-TPEWASQITGVPADKIIKLAREIG(5) FISQGWGPQRHANGEIA
222 NGWH  DEAFLARCTTGYAVFA(7)  DGIAK-TAEWAAEICGVGAAKI----RELA(8) MLMAGWGMQRQQFGEQK
355 ALGQ  ENNP-EWKTVAFNTNG(115)DDVKAYTPAWAEQITGVSRSQIIRIARERFA(10)MIIVGAGLNHWYHLDMN

355 TRLAPILQMILGNIGKRGGGT(83) KLRSLIVVGSGISTAI(35)YILPAATQMET-SGRVA(10)MVMKPLFECR
422 IRTMAMIQLLLGNMGMAGGGV(133)KLKYMVVIDPLVTETSTF(21)LPSTCFAEE-DGSIA(10)KGQDAPGEAR
301 VMQTANLAMLTGNIGRLGTGV(91) NLDFFVVQDIFMTDTAEFADVVLPAACWAEQ-EGTFT(10)KAVDAPGESK
309 VRSLTSLAMLTGNLGKPHAGV(91) DLELVIVQDIFMTKTASAADVILPSTSWGEH-EGVFT(10)KAVEPKWDLK
341 RRMIFTLNVLLGNIERGGGLY(93) QLDLVVSCDVYLSESAAYADYLLPECTYLER-DEEVS(13)QVVEPIGEAR
369 TRAISMLAILTGNVGINGGNS(86) KCELIVVIDCHMTSSAKYADILLPDCTASEQMDFALD(13)QVIKPRFECK
299 HWMIVTLAAMLGQIGTPGGGF(89) KPELVVISECFWTAAAKHADIVLPATTSFERNDLTMT(12)QVVPPRYEAR
546 YRGLINMLIFCGCVGQGSGGGW(189)KLDLVVTLDFRLSSTCLYSDIILPTATWYEK-DDMNT(11)AAVDPAWEAK

546 PDEEILFDLAKRL(35) FQGRTPERLKAHA-ENWHM(210)TRSAHYLAEVQ-PEMFVEIHPETATDLGVKHGDMV
649 NDGEILAGIYHHL(228)PYVGTTYRLTEHF----HT----WTKHALLNAIAQ-PEQFVEISETLAAAKGINNGDRV
469 YDWEIFCDLAKKM(85) PLILTTTRLLFHY----HAA---MTRRAATLDREV-PTGYVEINTEDAAELGIANKEKV
477 TDWQIISEIATRM(84) PMVLSTVREVGHYSCRS------MTGNCAALAALADEPGYAQINTEDAKRLGIEDEALV
514 PSWQIWKELGEQL(109)ELYFIQGKVAVHTNG--------ATQYVPLLSELM-WDNAWWHPQTAQEKGIKTGDEI
536 TIYEMTSELAKRL(112)NKQYPLQLTGFHYKSRVHST---YGNVDVLKAA---CRQEMWINPLDAQKRGIHNGDKV
468 NDFDVFAELSERW(111)AEPEQLQVLSAHPAHRLHSQLN-YSSLRELYAVA--NREPVTIHPDDAQERGIQDGDTV
813 SDWEIYKAIAKKF(247)NQEKALNFLTPHQKWGIHST---YSDNLLMLTLGR-GGPVVWLSEADADKLDGIADNDWI

856  WVHGTNGAKILVKARHSYKVNKTSV(27)IGE-SCNTITSD-AYDINTSTPETKCGL-----CRIEKA
940  TVASKRGF-IRAVAVVTRRLKPLNV(21)KGY-IANTLTPN-VGDANSQTPEYKAFL-----VNIEKA
618  KVKSRRGE-IEIAARVTDDIVKGIV(6) REC-SANILTNAAAIDPKSGMPEYKACA-----VAISKMEGSK
627  WVHSRKGK-IITRAQVSDRPNKGAI(6) WIG-ACNELVTE-NLSPITKTPEYKYCA-----VRVEPI(27)
686  WLENATGK-EKGKALVTPGIRPDTL(17)HGIHCGNLLPHV--------TSPVSGTVHTAG--VTLSRA
714  RIPNDRGE-VHIEAKVTPRMMPGVV(16)KGG-CINVLTTQ------RPSPLAKGNPSHTNLVQVEKV
648  RLWNARGQ-ILAGAVISEGIKPGVI(18)KNG-AVNVLTKD--------LPSSRLGMA----VRVIRR(11)
1128 EVENSNGA-LTARAVVSQRVPAGMT(20)RGG-IHNSVTRI--------TPKPTHMIG(22)VVVRKM(19)
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FIG. 4. Comparison of the predicted ORF1 (PhsA) amino acid sequence with sequences of the catalytic subunit of molybdoprotein oxidoreductases. Partial sequences are as follows: *Ws* FdhA, *W. succinogenes* formate dehydrogenase (7); *Ec* FdnG, *E. coli* nitrate-linked formate dehydrogenase (3); *Mf* FdhF, *M. formicicum* formate dehydrogenase (35); *Ec* FdhF, *E. coli* hydrogenase-linked formate dehydrogenase (44); *Ec* DmsA, *E. coli* dimethyl sulfoxide reductase (4); *Ec* BisC, *E. coli* biotin sulfoxide reductase (32); *Ec* NarG, *E. coli* nitrate reductase (5). Boldface is used for amino acids identical in at least four of the sequences or very similar in at least five of the sequences. Similarities: arginine (R) and lysine (K); aspartate (D) and glutamate (E); aspartamine (N) and glutamine (Q); isoleucine (I), leucine (L), and valine (V); phenylalanine (F) and tyrosine (Y); and serine (S) and threonine (T). Consensus molybdoprotein oxidoreductase sequences suggested by Blasco et al. (5) are underlined.

acids) and 6 (52% identity over 255 amino acids) (8) and substantial similarity (27% identity over 135 residues) to the homologous protein from *Bacillus subtilis* (40). It seems likely that ORF4 is the *S. typhimurium* homolog of these carboxypeptidases in *E. coli* and *B. subtilis.*

Thiosulfate reduction and energy conservation. The similarity of the implied *phs* amino acid sequences to those encoded by *E. coli dmsABC* and other enzyme complexes known to serve as reductases for anaerobic respiration suggested that thiosulfate reductase likewise facilitates anaerobic respiration. Such a function is also consistent with the requirement for heme and menaquinones in the production of H_2S from thiosulfate in vivo (11, 26). We investigated this possibility by looking at two possible contributions of thiosulfate to anaerobic energy metabolism: its effect on the final optical density (OD) obtained in anaerobic liquid cultures provided with a variety of carbon sources and its ability to stimulate ATP synthesis in whole suspended cells.

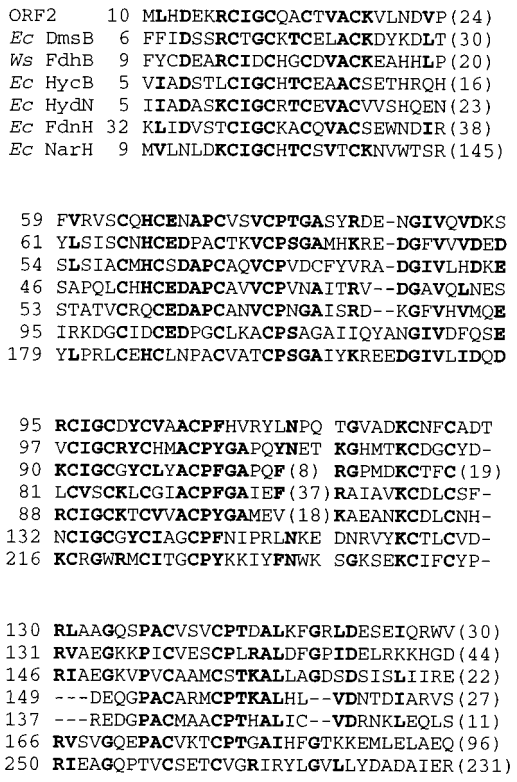

FIG. 5. Comparison of the predicted ORF2 (PhsB) amino acid sequence with sequences of the electron transfer subunit of molybdoprotein oxidoreductases. Partial sequences are as follows: *Ws* FdhB, *W. succinogenes* formate dehydrogenase (7); *Ec* FdnH, *E. coli* nitrate-linked formate dehydrogenase (3); *Ec* HycB, *E. coli* formate hydrogenlyase (6); *Ec* DmsB, *E. coli* dimethyl sulfoxide reductase (4); *Ec* HydC, *E. coli* hydrogenase (25); *Ec* NarH, *E. coli* nitrate reductase (5). Boldface is used for amino acids identical in at least four of the sequences or very similar in at least five of the sequences. Similarities: arginine (R) and lysine (K); aspartate (D) and glutamate (E); aspartamine (N) and glutamine (Q); isoleucine (I), leucine (L), and valine (V); phenylalanine (F) and tyrosine (Y); and serine (S) and threonine (T).

The stationary-phase OD at 650 nm (OD_{650}) was determined for anaerobic liquid cultures grown in minimal media supplemented with thiosulfate, nitrate, or neither, along with each of 18 electron donors known to support aerobic growth in minimal media (Table 3). Thirteen of the electron donors supported anaerobic growth in the absence of an electron acceptor, yielding final OD_{650} values of >0.05. Nitrate increased the final OD obtained with these fermentable substrates by about 1.5- to 2-fold (3-fold in the case of xylose), while thiosulfate increased the final OD by an average of 15%, the greatest increases (20 to 30%) being associated with substrates producing the lowest OD in the absence of an electron acceptor. The remaining five electron donors tested did not support significant growth in the absence of an electron acceptor (final OD_{650}, ≤ 0.025). Nitrate facilitated growth on these five substrates, but thiosulfate had little or no effect. It appears that thiosulfate does not permit growth on nonfermentable substrates, but it may slightly augment yields obtained on fermentable carbon sources used poorly by *S. typhimurium* under anaerobic conditions. These results are not consistent with an electron transport system which facilitates either stoichiometric ATP production by substrate level phosphorylation or the respiratory extrusion of stoichiometric numbers of protons from a physiological electron donor.

Secondly, we compared ATP synthesis by washed stationary-phase cells of *S. typhimurium* provided with formate as the electron donor and either thiosulfate or nitrate as the electron acceptor. Formate is thought to be the most important physiological donor for nitrate reduction (38) and is known to reduce thiosulfate in whole cells (22). The results (Table 4) suggested that thiosulfate does indeed facilitate ATP synthesis in cells that have become ATP depleted after harvest, although the levels obtained were never greater than those found in stationary-phase cells at the time of harvest. Nitrate, in contrast, stimulated the production of ATP to levels significantly higher than those found at the time of harvest.

TABLE 3. Stationary-phase OD_{650} values obtained for wild-type *S. typhimurium* grown anaerobically in minimal medium[a]

Electron donor	Final OD_{650} with following electron acceptor:		
	None	Nitrate	Thiosulfate
D-Glucose	0.21	0.34	0.21
D-Galactitol	0.18	0.32	0.19
D-Mannose	0.18	0.31	0.19
D-Galactose	0.14	0.31	0.15
D-Gluconate	0.14	0.25	0.16
D-Melibiose	0.17	0.31	0.20
D-Fructose	0.17	0.24	0.20
D-Mannitol	0.16	0.31	0.18
L-Arabinose	0.14	0.26	0.16
D-Ribose	0.08	0.21	0.09
Citrate	0.06	0.13	0.08
myo-Inositol	0.05	0.12	0.06
D-Xylose	0.05	0.16	0.07
D-Trehalose	0.03	0.14	0.04
L-Malate	0.01	0.11	0.01
Succinate	0.01	0.01	0.01
Glycerol	0.01	0.18	0.01
Lactate	0.01	0.11	0.01

[a] Minimal medium (11) was supplemented with 1% (wt/vol) electron donor and 6 mM nitrate or thiosulfate. Starting OD_{650} values ranged from 0.004 to 0.008. The values shown represent averaged duplicates which varied from each other by <10%.

DISCUSSION

The results presented here indicate that the previously cloned *phs* plasmid (18), which complements *phs* mutants of *S. typhimurium* and confers on *E. coli* the ability to produce hydrogen sulfide from thiosulfate, contains a functional operon

TABLE 4. ATP synthesis by suspended stationary-phase *S. typhimurium* cells[a]

Time (min) after suspension[b]	Electron donor and acceptor[c]	Mean nmol of ATP/mg of cell protein ± SD	
		Nitrate-grown cells	Thiosulfate-grown cells
0	−	12.9 ± 1.9	6.3 ± 0.4
30	−	7.7 ± 1.8	2.2 ± 0.02
30	+	20.9 ± 1.6	5.8 ± 0.6

[a] Cells were grown anaerobically to the early stationary phase in either nitrate broth or thiosulfate broth.

[b] Time at which suspended cells were lysed. Time zero refers to the time immediately after washed cells had been pipetted into serum cap vials and flushed with argon.

[c] The electron donor (40 μmol of nitrate or thiosulfate) and acceptor (40 μmol of formate) were either omitted (−) or added (+) 2 min prior to lysing. Nitrate was used for nitrate-grown cells, and thiosulfate was used for thiosulfate-grown cells.

consisting of three *phs* genes, for which we suggest the designations *phsA*, *phsB*, and *phsC*. The *phs* products synthesized by *E. coli* DH5α harboring the *tac* expression vector with a *phsABC* insert exhibited thiosulfate reductase activity, confirming the identity of the *phs* operon as the site of the structural gene for thiosulfate reductase. The *phs* promoter region included a sequence closely resembling the CAP-binding consensus sequence, which is consistent with our earlier report that cyclic AMP is required for *phs* expression.

The predicted amino acid sequences corresponding to ORFs of *phsA* and *phsB* exhibited significant homology with the catalytic and electron transfer subunits, respectively, of several other previously characterized anaerobic reductase systems, including dimethyl sulfoxide reductase (4), nitrate reductase (5), nitrate-linked and hydrogenase-linked formate dehydrogenases from *E. coli* (3, 6, 44), and formate dehydrogenase from *W. succinogenes* (7). Many amino acid sequence similarities shared by all members of each group were identified, suggesting a common genetic origin and/or catalytic activity among the various molybdoprotein oxidoreductases. Although the predicted *phsC* sequence did not exhibit significant similarity to any other proteins in the GenBank database, it is about the same size as the third subunit of *E. coli* dimethyl sulfoxide reductase (DmsC) and nitrate-linked formate dehydrogenase (FdnI) and, like them, is highly hydrophobic, thus further confirming the overall similarity among the molybdoprotein enzyme complexes. Evidence has been presented for the identity of FdnI with the *b*-type cytochrome known to be essential for nitrate-linked formate dehydrogenase (3). Thiosulfate reduction similarly requires heme (11). Perhaps PhsC encodes a cytochrome associated with thiosulfate reduction.

These results place thiosulfate reductase within a family of anaerobically induced, molybdoprotein oxidoreductases which includes the *E. coli* anaerobic respiratory nitrate reductase. Although the global anaerobic regulator Fnr does not mediate oxygen regulation of *phs* transcription (11) as it does the transcription of the structural genes for nitrate reductase (37) and several other anaerobic reductases (38), it does prevent H_2S production in vivo, indicating that the H_2S-producing pathway is part of the Fnr regulon even if the reductase itself is not controlled by Fnr. Perhaps Fnr regulation is exerted at the level of the formate dehydrogenase which couples formate oxidation to thiosulfate reduction (22).

As shown for the previously described *phs*::*lac* chromosomal operon fusions (11), full induction of the *phs*::*lacZ* single-copy translational fusions required both anaerobiosis and thiosulfate. In contrast, the multicopy translational fusions were induced by thiosulfate even aerobically and by anaerobiosis even in the absence of thiosulfate. The contrasting single-copy and multicopy results are consistent with a model of regulation involving a repressor(s) inactivated under inducing conditions and titrated out by the multicopy plasmid *phs* insert. A single-regulator model is favored by our recent isolation of *trans*-acting mutations rendering constitutive expression of *phs* with respect to both oxygen control and sulfur control (2). Such a model is also consistent with the fact that regions specific to oxygen control and sulfur control, respectively, were not revealed in this study. However, a complete regulatory model for *phs* must contain additional features. Cyclic AMP also appears to be required for *phs* expression, as suggested by previous physiological studies (12) and supported by both the sequence analysis and the fact that deletions extending beyond −71 (just upstream of the putative CAP-binding site) abolished *phs* expression. In addition, the region between −107 and −71 appears to contribute to *phs* regulation differently from the region upstream of −107. Whereas increasing deletion size up to −107 increased β-galactosidase activity in both single- and multicopy fusions, deletions into the −107 to −71 region nearly abolished multicopy expression while having little effect on single-copy expression. Site-specific mutagenesis is planned in our further studies of *phs* regulation to help define the specific regions necessary for response to the various effectors of thiosulfate reduction.

Although thiosulfate reductase fits the paradigm of anaerobic respiratory systems in the family *Enterobacteriaceae* in terms of subunit amino acid sequences and electron transport chain components, its contribution to energy conservation is still enigmatic. The very small contribution of thiosulfate to anaerobic cell densities does not support a model in which the reduction of thiosulfate facilitates significant oxidative phosphorylation. Redox considerations also cast some doubt on a role for thiosulfate reduction in oxidative phosphorylation, although these considerations do not actually rule it out. The redox potential for the reduction of thiosulfate with formate is very small given that the redox potential for the thiosulfate half reaction ($S_2O_3^{2-}/S^{2-} + SO_3^{2-}$, −402 mV) is close to that of formate (CO_2/HCOOH, −432 mV) (39). However, the calculation of a standard redox assumes a 1 M concentration of all reactants and products, which does not necessarily reflect in vivo conditions, especially in light of the fact that gases (hydrogen and H_2S) are among the products. The observed minor contributions to growth yields (Table 3) may be more consistent with a physiological model for thiosulfate reduction involving either electron sink reactions such as those that characterize nitrite reduction (30) or chemiosmotic processes which increase the proton motive force through symport or antiport systems linked to otherwise non-energy-conserving metabolic reactions, e.g., malolactic fermentation in lactic acid bacteria (13, 33).

The fact that addition of formate and thiosulfate to energy-depleted cells stimulated low levels of ATP synthesis does, nevertheless, suggest some role for thiosulfate reduction in energy conservation. We showed previously that thiosulfate reductase and also sulfite reductase are induced during the stationary phase (11, 22). Perhaps hydrogen sulfide production helps to maintain the energy charge of nongrowing cells through oxidation-reduction reactions which do not underpin oxidative phosphorylation but contribute to the protonic potential in other ways. Experiments exploring the linkage between hydrogen sulfide production and the energy metabolism of *S. typhimurium* are in progress.

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ADDENDUM IN PROOF

An updated computer-assisted sequence comparison has revealed a sequence with significantly greater similarity to ORF1 (PhsA) than to any of those discussed above, namely, the catalytic subunit of polysulfide reductase (PsrA) from *Wolinella succinogenes* (GenBank accession no. P31075). PhsA exhibited 42% identity with PsrA in a 767-amino-acid overlap.

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