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Cloning of the *phs* Genetic Locus from *Salmonella typhimurium* and a Role for a *phs* Product in Its Own Induction

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The *Salmonella typhimurium phs* chromosomal locus essential for the reduction of thiosulfate to hydrogen sulfide was cloned, and some features of its regulation were examined. The *phs* locus conferred H_2S production on *Escherichia coli*, suggesting that it contains the structural gene for thiosulfate reductase. H_2S production by the *E. coli* host was, as in *S. typhimurium*, suppressed by nitrate or glucose in the growth medium. The presence of plasmid-borne *phs* genes in a *S. typhimurium chl*$^+$ host containing a chromosomal *phs*::*lacZ* operon fusion was found to significantly increase the relative induction efficiency of β-galactosidase by thiosulfate. These results are consistent with a model for *phs* regulation in which the true inducer is not thiosulfate per se and in which the action of a *phs*-encoded molybdoprotein, possibly the reductase itself, converts thiosulfate into a compound that resembles the true inducer more closely than does thiosulfate.

Salmonella spp. and several other members of the family *Enterobacteriaceae* reduce thiosulfate to hydrogen sulfide under anaerobic conditions (1a). The *phs* gene locus is essential for this activity in *S. typhimurium* (5, 26, 27). The reduction has been shown to involve heme proteins (5), menaquinones (16), and a membrane-bound thiosulfate reductase (TSR) (5, 20) induced by thiosulfate (5) and absent in molybdoprotein-deficient mutants (5, 7). Nitrate in the medium severely represses H_2S formation, as do mutations in *oxrA* (5), the *S. typhimurium* homolog of the *fnr* gene in *Escherichia coli* (13, 24). Thus, thiosulfate reduction resembles several anaerobic respiratory systems such as trimethylamine oxide reduction (3) which are induced by their respective electron acceptors, repressed by nitrate and oxygen (preferred anaerobic electron acceptors), and part of the Fnr regulon (22). However, thiosulfate reduction differs from these other systems in that it is induced by the reduction products of thiosulfate as well as thiosulfate per se (5), is severely repressed by glucose (6), and appears not to facilitate anaerobic growth on nonfermentable carbon sources such as glycerol (1). This lack of an obvious contribution to anaerobic energy conservation is also characteristic of thiosulfate reduction by *Proteus mirabilis* (23). Our studies of *phs*::Mud(*lac*) operon fusions have shown them to lack TSR activity, to be induced by thiosulfate and other sulfur compounds, and to be negatively regulated by air, by glucose, and by growth rate (5, 6). The fusions were not, however, significantly affected by nitrate or by mutations in *oxrA*, suggesting either that *phs* does not contain the structural gene for TSR or that the site of nitrate and Fnr regulation of this system, unlike that of the other anaerobic respiratory systems, is at an earlier point in the electron transport chain.

Our current model for thiosulfate reduction by *S. typhimurium* consists of an anaerobic electron transport chain which shares structural features with known pathways for anaerobic respiration in members of the *Enterobacteriaceae* but which differs from them in terms of function and regulation. As part of our continued studies of this unique electron transport system, we have now cloned the *phs* locus and have completed preliminary studies of its induction by sulfur compounds.

Cloning of the *phs* locus. To date there is no known positive selection for the ability to reduce thiosulfate to sulfide. Thus, we used a cloning strategy involving the screening for H_2S-positive clones among cells that had been transformed with DNA enriched for the *phs* region. The strategy was derived from that of Youderian et al. (28) and employed *S. typhimurium* PY 13745 (*leuA414* r_L^- m_L^+ *hisD9953*::Mu*d*-Q), which was kindly provided by P. Youderian. The Mu*d*-Q inserted in *hisD* in PY13745 is a Mu*d*-P22 hybrid phage lacking the P22 genes for excision. Upon induction by mitomycin (2 mg/ml), the prophage begins packaging from the *pac* site in situ only, resulting in a preparation of phage particles enriched for the adjacent chromosomal DNA, which, in the case of Mu*d*-Q, includes the *phs* locus. The lysate DNA, prepared as described by Youderian et al. (28), was digested with *Eco*RI (from Bethesda Research Laboratories) and ligated with *Eco*RI-cut pUC19 (New England Biolabs) by using T4 ligase (from United States Biochemical Corporation) according to the methods of Maniatis et al. (18). Plasmid DNA was used to transform *S. typhimurium* EB464, an H_2S^- mutant constructed by transferring *phs-109*::Tn*10* from EB231 (5) into LB5000 (*hsdLT hsdSA hsdSB* [r^-m^+] *metA22 metE551 trpD2 leu val*), provided by Stanley Artz, by P22 transduction (9) with P22 *int-4* (21). The transformation procedure was that of Foster et al. (10). Transformants capable of producing H_2S were identified by plating on salmonella-shigella (S-S) agar (Difco Laboratories) supplemented, per liter, with 8 g of tryptone, 4 g of

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FIG. 1. Restriction map of the 5.1-kb genomic fragment (pEB40) of *S. typhimurium.*

yeast extract, 4 g of NaCl, 10 g of agar (all from Difco Laboratories) and 10 μg of ampicillin per ml, and incubating at 37°C for 24 to 48 h. (S-S agar without the complex supplements failed to support the growth of LB5000.) A positive clone selected for further study also conferred H_2S production on the *E. coli* host DH5α [F$^-$ φ80d*lacZ*ΔM15 *endA1 recA1 hsdR17* (r_K^- m_K^+) *supE44* λ$^-$ *thi-1 gyrA96 relA1* Δ(*lacZYA-argF*)*U169*], indicating that the *phs* locus probably contains the structural genes for thiosulfate reductase. In this case, commercial peptone iron (PI) agar (Difco Laboratories) supplemented with 50 μg of ampicillin per ml was used as the detection medium because *E. coli* DH5α failed to grow on the modified S-S agar used for the *S. typhimurium* host.

Subcloning the *phs* locus. Further digestion of the above plasmid with *Sal*I produced a 5.1-kb fragment which also conferred H_2S production on *E. coli* when subcloned into pUC19(pEB38). All smaller inserts obtained were unable to complement *E. coli*, although a 4.4-kb *Hin*dIII-*Sal*I insert (Fig. 1) did complement the *phs*::Tn*10 S. typhimurium* mutant EB464, suggesting that *phs* contains more than one gene, all of which are needed to render *E. coli* H_2S^+ but only some of which are needed to complement the *phs* mutation in the *Salmonella* cloning host. A restriction map of the insertion was constructed by using double digests (Fig. 1).

Because plasmid pEB38 was found to contain part of the vector-borne polylinker cloning site as a result of the subcloning, it was not convenient to use for future exonuclease III deletion studies. Thus, we reisolated the *phs* clone by digesting Mu*d*-Q DNA with *Eco*RI and *Sal*I and ligating with pUC19. Positive clones obtained from this ligation also yielded a 5.1-kb *Eco*RI-*Sal*I insert. A representative selected for further work was designated pEB40.

Parameters affecting *phs* expression in *E. coli*. Both glucose and nitrate were found to repress H_2S production from thiosulfate mediated by pEB40 in *E. coli* DH5α as effectively as in the *Salmonella* host. The glucose effect reflects the fact that many operons in both organisms are subject to catabolite repression mediated by the same cyclic AMP-catabolite activator protein system (4) which was shown responsible for the glucose effect on the expression of H_2S production by *S. typhimurium* (6). The effect of nitrate similarly suggests that its regulation of thiosulfate reduction in *S. typhimurium* involves elements composing a nitrate regulatory system found in both *S. typhimurium* and *E. coli*. The simplest model for nitrate regulation is that thiosulfate reduction is part of a nitrate regulon in *S. typhimurium* which parallels the well-characterized NarL paradigm in *E. coli* (12, 22). To explore this model further, we attempted to test the effect of nitrate on H_2S production mediated by pEB40 in VJS1558, a *narL* mutant of *E. coli* kindly provided by Valley Stewart (*narL215*::Tn*10 recA938*::*cat hsdR514 supE44 supF58 lacY1 galK2 galT22 metB1 trpR55*). Surprisingly, pEB40 was unable to confer H_2S production on VJS1558 under any condition. No explanation for this negative result is apparent in the VJS1558 genotype as compared with that of DH5α (detailed earlier). Perhaps VJS1558 contains an additional unidentified mutation that prevents H_2S production. Strain VJS1558 did produce gas from glucose, indicating that the unidentified defect is not a pleiotropic chlorate resistance mutation.

Sulfur compounds as inducers of *phs* expression. A variety of sulfur compounds were shown previously to induce TSR activity in the wild type and β-galactosidase activity in *phs*::Mu*d*1(Ap^r *lac*) operon fusion strains (5). However, relative efficiency differed in the operon fusion strain and in the wild type. While thiosulfate was the most effective inducer of wild-type thiosulfate reductase, other sulfur compounds such as reduced glutathione, the products of thiosulfate reduction (sulfite and sulfide), and unsupplemented nutrient broth (Difco Laboratories), which contains a variety of organic sulfides and thiosulfates, were all more potent inducers of *phs* expression by the operon fusion strains (5). These results led us to hypothesize that the true inducer of *phs* is not inorganic thiosulfate but is a reduced sulfur-containing compound, possibly an organic sulfide or thiosulfate, resembling thiosulfate in some respects and the products of thiosulfate reduction in other more important respects. In this model, inorganic thiosulfate is a more effective inducer of wild-type TSR synthesis than of β-galactosidase expression by the *phs* operon fusion strain because the latter cannot process it. This is somewhat analogous to induction of the *lac* operon, for which the internal inducer is an *allo* isomer of lactose formed by β-galactosidase (14).

This model predicts that the presence of functional TSR would increase the efficiency of *phs* induction by thiosulfate in the operon fusion strain. To test the effect of functional *phs* gene products on thiosulfate-mediated induction, we constructed a strain carrying a plasmid-encoded *phs* locus in a *phs*::Mu*d*1(Ap^r *lac*) operon fusion chromosomal background. The *Eco*RI-*Pst*I insert was subcloned into the Ap^r gene of pBR322 (United States Biochemical Corporation) and the resulting plasmid (pEB42) was amplified in *E. coli* DH5α H_2S^+ transformants which were selected on anaerobically incubated PI agar supplemented with 20 μg of tetracycline per ml. The effect of a functional *phs* locus on expression of the *phs*::Mu*d*(*lac*) operon fusion was tested in two strains: EB472, a *recA* derivative of *S. typhimurium* EB222 [*phs-101*::Mu*d*1(Ap^r *lac*)] (5), and EB473, a *recA chl* derivative of the same strain. The *recA* mutation was transferred by P22 transduction from SGSC 74 (obtained from the Salmonella Genetic Stock Center) by selecting for the *recA*-linked *srl*::Tn*10* insertion, followed by conversion to tetracycline sensitivity by selection on Bochner's medium (17). The *chl* mutation was selected as a spontaneous mutant of EB472 in the presence of 3% potassium chlorate on anaerobically incubated Luria broth (18) plates. The *chl* phenotype was confirmed by testing for the simultaneous loss of nitrate reduction using MacConkey nitrate plates (2). Transformation of EB472 with pEB42 DNA to produce strain EB474 was achieved by electroporation (25) using a Gene Pulser apparatus from Bio-Rad Laboratories, and transformants were selected on the modified S-S agar described above (except that 20 μg of tetracycline per ml replaced the ampicillin). Similarly, the plasmid was also put into the chlorate-resistant mutant, yielding EB475.

Using the strains shown above, we examined the relative effectiveness of thiosulfate and reduced glutathione as inducers of TSR in the wild type and of *phs* expression in the operon fusion strain. The results (Table 1) were consistent with the above hypothesis in that the introduction of a

TABLE 1. Sulfur induction of β-galactosidase in *phs*::*lac* fusion strains and of TSR in Phs$^+$ strains of *S. typhimurium*

Strain (relevant characteristic or genotype)	β-Galactosidase activity[a] with addition[b]			TSR sp act[c] with addition		
	None	$S_2O_3^{2-}$	GSH	None	$S_2O_3^{2-}$	GSH
LT2 (wild type)				<1	183	44
phs::*lac* fusion strains						
EB222 (parent)	38	191	360			
EB472 (*recA*)	26	124	312			
EB474 (*recA*/pEB42)	29	457	147	595	522	313
EB473 (*recA chl*)	22	127	219			
EB475 (*recA chl*/pEB42)	19	131	246	18	8	6

[a] β-Galactosidase units per optical density (at 600 nm) unit, as described by Miller (19).

[b] Nanomoles of sulfide produced per minute per milligram of protein, as described by Hallenbeck et al. (11).

[c] Sulfur compounds tested: $S_2O_3^{2-}$, 1 mM sodium thiosulfate; GSH, 0.5 mM reduced glutathione, both in M9 minimal medium (8) with 0.5% galactose as the carbon source. These concentrations were found previously to be the minimal amounts producing maximal effect (5).

functional *phs* locus into the *chl*$^+$ operon fusion strain increased the effectiveness of thiosulfate as an inducer from 5-fold to 15-fold over basal levels. The *phs* genes had little effect in a *chl* background, indicating that the *phs* gene product responsible for conversion of thiosulfate into a more effective inducer is probably a molybdoprotein, presumably the reductase itself. The fact that *phs* expression by the operon fusion strain was shown previously to be significantly and consistently greater in complex than in minimal media (5) points to an organic sulfur compound as the most likely candidate for the true inducer. The results also revealed that the presence of a functional *phs* locus, while improving the effectiveness of inorganic thiosulfate as an inducer, actually diminished the effectiveness of reduced glutathione. Perhaps reduced glutathione is rendered unsuitable for efficient induction by a *phs* gene product. In fact, glutathione has been proposed to participate in thiosulfate reduction by *Saccharomyces cerevisiae* (15).

Strains containing *phs* were also assayed for TSR activity (Table 1). While wild-type TSR was completely dependent on a sulfur compound for induction in minimal medium, plasmid-encoded TSR activity in strain EB474 was very high in the absence of any sulfur compound. This suggests that the induction of TSR by a sulfur compound may represent the inhibition of a repressor, which is titrated out by the multicopy plasmid. The induction of *nar* genes as well as that of the other well-characterized systems for anaerobic respiration in *E. coli* involves, instead, a positive regulator (22).

Our preliminary studies of the regulation of *phs* reported above reinforce our earlier speculations that thiosulfate reduction differs from other better-characterized systems for anaerobic respiration in several respects. Additional studies are planned to determine the physiological significance of the ability to produce H_2S and the details of its unique regulatory picture.

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