Specificity of respiratory pathways involved in the reduction of sulfur compounds by *Salmonella enterica*

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The tetrathionate (Ttr) and thiosulfate (Phs) reductases of *Salmonella enterica* LT2, together with the polysulfide reductase (Psr) of *Wolinella succinogenes*, are unusual examples of enzymes containing a molybdopterin active-site cofactor since all formally catalyse sulfur–sulfur bond cleavage. This is in contrast to the oxygen or hydrogen transfer reactions exhibited by other molybdopterin enzymes. Here the catalytic specificity of Ttr and Phs has been compared using both physiological and synthetic electron-donor systems. Ttr is shown to catalyse reduction of trithionate but not sulfur or thiosulfate. In contrast, Phs cannot reduce tetrathionate or trithionate but allows whole cells to utilize elemental sulfur as an electron acceptor. Mechanisms are proposed by which the bacterium is able to utilize an insoluble sulfur substrate by means of reactions at the cytoplasmic rather than the outer membrane.

Keywords: tetrathionate reductase, thiosulfate reductase, polysulfide reductase, molybdopterin, electron transport

INTRODUCTION

Sulfur in the zero-valent oxidation state is able to form a variety of compounds containing chains of sulfur atoms linked by sulfur–sulfur bonds. These include elemental sulfur (S_8), polysulfides ($^-S-(S)_n-S^-$), sulfane monosulfonates ($^-S-(S)_n-SO_3^-$) and polythionates ($^-O_3S-(S)_n-SO_3^-$), where in each case $n \geq 0$. A range of bacteria are able to use these inorganic sulfur species as respiratory electron acceptors by reductively cleaving the sulfur–sulfur bonds (Barrett & Clark, 1987; Hedderich *et al.*, 1998). Recent studies have revealed that in many cases the enzymes catalysing these reductive cleavage reactions contain a *bis*(molybdopterin guanine dinucleotide)molybdenum (MGD) cofactor at the catalytic site. The most prominent examples of such enzymes are the tetrathionate (Ttr) and thiosulfate (Phs) reductases of the enteric bacterium *Salmonella enterica* LT2 and the polysulfide reductase (Psr) of the rumen bacterium *Wolinella succinogenes*, which catalyse reactions (1)–(3) respectively (Pollock & Knox, 1943; Hensel *et al.*, 1999; Klimmek *et al.*, 1991; Clark & Barrett, 1987).

The use of a molybdopterin cofactor to catalyse these reactions is intriguing since this cofactor normally catalyses oxygen atom transfer, hydrogen atom transfer or a combination of the two (Hille, 1996; Khangulov *et al.*, 1998). The enzymes carrying out sulfur bond cleavage (which might alternatively be styled sulfur atom transfer) therefore operate a previously undescribed biological reaction of the molybdopterin cofactor. Sequence similarities between the MGD-binding subunits of Ttr, Phs and Psr in the polypeptide region that contributes to the enzyme active-site pocket and substrate-access channel support the idea that these proteins form a specificity grouping distinct from other MGD-dependent enzymes (Hensel *et al.*, 1999; Fig. 1).

In this study we have compared the catalytic specificities of the *S. enterica* Ttr and Phs enzymes in the light of the proposal that these enzymes, together with Psr, have similar catalytic mechanisms. Mutant strains lacking either Ttr or Phs, or lacking both these enzymes, were used to allow discrimination between the activity of the two enzymes in whole cells. Some experiments also employed strains lacking the enzyme dissimilatory sulfite reductase (Asr), which reduces sulfite to sulfide (Huang & Barrett, 1990). We have investigated whether

Abbreviation: MGD, *bis*(molybdopterin guanine dinucleotide)molybdenum.

TtrA-like proteins

<i>Salmonella enterica</i>	TtrA	DEGRDAFLRRFALNSFGSKNFGAHGAYCGLAYRAGSGALMG
<i>Yersinia enterocolitica</i>	TtrA	DDGRDAFIQRFAHQAFGTKNLGSHGAYCGLAYRAGSGALMG
<i>Pasteurella multocida</i>	TtrA	PEGROPLLRKFANNSFGTINFASHGSECGLSYRAGSGAFMN
<i>Bordetella parapertussis</i>		NEGRTPLIQRFQAQSFQTVNVSNHGSCGQSFRVGTGAAFG
<i>Desulfitobacterium hafniense</i>		DDGRTPFQAKRFVNNAYGSPNHFHGGICGLISRRVAYLAFLD
<i>Carboxydotherrnus hydrogenoformans</i>		GDRRDFFKERFWSQTLGSLNSLDHGGICGVNGVIGNVQSFN
<i>Dehalococcoides ethenogenes</i>		QGGRVDFIKRFTHDAMGSINAHGHTTVCGSFLQSAKALSA

PhsA/PsrA-like proteins

<i>Salmonella enterica</i>	PhsA	KSGSLSSHLFHLATAFGSPNTFTTHASTCPAGKAI AAKVMMG
<i>Shewanella putrefasciens</i>		KSGSQEKHLFHLATAFGSPNTFTTHASTCPGGYEIAAKAMFG
<i>Wolinella succinogenes</i>	PsrA	RSGWNKTWFHHLAQAYGSPNIFGHESTCPLAYNMAGRDVFG
<i>Carboxydotherrnus hydrogenoformans</i>		HPELAYDYEVIFNQAFGSPNLSAHAPTCYSPRNVAYKTMYG
<i>Desulfovibrio vulgaris</i>		RGGPFMDLHQAFVRGLGSPNYCNHDASCARNVQHACRSVTG
<i>Porphyromonas gingivalis</i>		RGGPFMDLHQAFVRGLGSPNYCNHDASCARNVQHACRSVTG

Fig. 1. Multiple sequence comparison of the polypeptide region that provides an amino acid ligand to the molybdenum atom of MGD-dependent sulfur-sulfur bond reductases. The cysteine residue that is proposed (Hensel *et al.*, 1999) to co-ordinate the molybdenum atom in the sulfur-sulfur bond reductases is boxed.

the catalytic abilities of the active-site cofactors of Ttr and Phs are restricted to their physiological reactions. We have also explored whether these enzymes are able to reduce other inorganic sulfur compounds containing sulfur-sulfur bonds.

METHODS

Bacterial culture conditions. Bacterial strains and plasmids used in this study are described in Table 1. Unless otherwise stated bacteria were cultured on Luria broth (LB) or LB agar at 37 °C (Sambrook *et al.*, 1989). Antibiotics were used at the

following concentrations: tetracycline, 10 µg ml⁻¹; ampicillin, 50 µg ml⁻¹; kanamycin 50 µg ml⁻¹.

For experiments in which tetrathionate induction of the *ttrBCA* operon was desired, *S. enterica* was grown in anoxic liquid culture using the standardized protocol described by Hensel *et al.* (1999). For experiments in which induction of the *phsABC* operon was desired, *S. enterica* was grown to an OD₆₀₀ of 0.4 in anoxic liquid culture supplemented with 50 mM Na₂S₂O₃. In both cases the cells were harvested by centrifugation, washed three times in ice-cold 10 mM sodium phosphate buffer (pH 7.4) and then used immediately for the assay of interest.

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype/characteristics	Source or reference
<i>S. enterica</i>		
LT2a	Parental	B. N. Ames
EB222	<i>phs-101::Mu d1 (Ap^r lac)</i>	Clark & Barrett (1987)
EB231	<i>phs-109::Tn10 (Tet^r)</i>	Clark & Barrett (1987)
EB303	<i>cysI68 asr-1::Tn5 (Kan^r)</i>	Huang & Barrett (1990)
BCB6	<i>ttrA::Tn10 (Tet^r)</i>	Hensel <i>et al.</i> (1999)
APH8	<i>phs-101::Mu d1 asr-1::Tn5</i>	P22 transduction (EB222 × EB303)
APH12	<i>ttrA::Tn10 phs-101::Mu d1 asr-1::Tn5</i>	P22 transduction (APH8 × BCB6)
APH14	<i>ttrA::Tn10 asr-1::Tn5</i>	P22 transduction (EB222 × APH8)
<i>E. coli</i>		
MC4100	F ⁻ Δ lacU169 ara139 rpsL150 relA1 ptsF rbs flbB5301	Casadaban & Cohen (1979)
<i>Plasmids</i>		
pAH26	<i>ttrRSBCA</i> in pSU41	Hensel <i>et al.</i> (1999)
pAH65	pAH26 bearing an Ala for Cys change at <i>ttrA</i> codon 256	This work
pAH66	pAH26 bearing a Ser for Cys change at <i>ttrA</i> codon 256	This work
pNH650	<i>phsABC</i>	E. L. Barrett

For anaerobic growth on solid media, plates were incubated in gas jars under a nitrogen/CO₂ atmosphere generated using the Anaerogen system (Oxoid). Tests for growth on the non-fermentable carbon source glycerol utilized the minimal medium described by Pope & Cole (1982) but lacking the nutrient broth component and supplemented with 30 mM glycerol and the electron acceptor of interest. Dispersed elemental sulfur was introduced into solid media by air oxidation of plates prepared with a polysulfide solution as described by Moser & Nealson (1996).

Genetic techniques. Standard molecular genetic techniques were carried out as described by Sambrook *et al.* (1989). P22-mediated transductions were performed as detailed in Maloy *et al.* (1996) using P22 HT *int*. Point mutations at the Cys256 TGT codon in *ttrA* were constructed using the Quick Change System (Stratagene). The new codons, GCT for the Ala mutation and TCT for the Ser mutation, were first introduced into the *XbaI* fragment of *ttrA* that bears codon 256 and then used to replace the corresponding *XbaI* wild-type fragment in pAH26.

Analytical methods. Trithionate was synthesized as described by Kelly & Wood (1994). Tetrathionate, trithionate and thiosulfate concentrations were determined by cyanolysis according to the protocols of Kelly & Wood (1994). Assays for methyl-viologen-linked activities and for polysulfide reductase activity were carried out in glass cuvettes sealed with a butyl rubber stopper and rendered anoxic by bubbling with oxygen-free nitrogen. For the methyl-viologen-linked measurements the assay solution contained 10 mM sodium phosphate buffer pH 7.4, 2 mM Na₂EDTA and 1 mM methyl viologen. The methyl viologen was reduced by titration with a Ti(III) citrate solution until the A_{600} reached 1.5. The reaction was started by the addition of electron acceptor, and oxidation of methyl viologen radical was monitored at 600 nm [$\Delta\epsilon_{600}$ (MV^{•+} – MV²⁺) = 13 mM⁻¹ cm⁻¹]. Electron acceptors were used at the following concentrations: 500 µM potassium tetrathionate; 500 µM potassium trithionate; 1 mM sodium thiosulfate. Tetrathionate and trithionate reductase assays had to be corrected for the rate of chemical reduction of the substrate by the methyl viologen radical. For measurement of polysulfide reductase activity the assay buffer was 50 mM Tris/HCl, pH 8.3. Polysulfide was added to a concentration of 1 mM from a stock solution prepared from sodium sulfide and sulfur flowers as described by Moser & Nealson (1996). Bacteria were introduced into the cuvette, following which the assay was initiated by addition of 10 mM sodium formate to act as electron donor to the bacterial electron-transfer chain. Reduction of polysulfide was monitored via the decrease in A_{360} using $\Delta\epsilon = 0.38$ mM⁻¹ cm⁻¹ (Klimmeck *et al.*, 1998).

RESULTS AND DISCUSSION

Similarity of active sites probed by mutagenesis of a conserved cysteine residue

A consequence of the proposal that Ttr, Phs and Psr have related mechanisms is that conserved amino acids in the putative active-site regions of these proteins should be important for catalysis. Prominent amongst these residues is a cysteine which is invariant amongst the MGD enzymes involved in sulfur–sulfur bond cleavage (Fig. 1) and which has been suggested to be the amino acid ligand to the active-site molybdenum atom of the MGD cofactor (Hensel *et al.*, 1999). To investigate the importance of this conserved cysteine this residue

was substituted with, separately, serine and alanine in the TtrA catalytic subunit of *S. enterica* tetrathionate reductase. The site-specific mutations were introduced into a plasmid-borne copy of the *ttr* locus and expressed heterologously in *Escherichia coli*. As previously reported (Hensel *et al.*, 1999) the wild-type *ttr* locus confers on *E. coli* the ability to assemble the Ttr complex and to respire tetrathionate. However, *E. coli* cells expressing either of the cysteine mutants were unable to reduce tetrathionate either with physiological electron donors or with the synthetic electron donor methyl viologen radical (MV^{•+}) (Table 2). The conserved ‘active site’ cysteine is thus essential for the production of functional tetrathionate reductase. It was not possible to determine if the variant catalytic subunits had been successfully targeted to the membrane since immunoblotting showed that even the wild-type plasmid-expressed TtrA polypeptide localizes predominantly to the cytoplasmic compartment. Attempts to circumvent this problem by transferring the mutations to the *S. enterica* chromosome were unsuccessful. Nevertheless the effects of the substitution mutations are consistent with the view that the invariant cysteine residue is critically important for the operation of the Ttr catalytic site.

The active sites of *S. enterica* tetrathionate and thiosulfate reductases catalyse distinct reactions

Under normal physiological conditions tetrathionate reductase plays no part in the reduction of thiosulfate (Clark & Barrett, 1987). However, since Ttr and Phs have been inferred to catalyse the same classes of reactions it is possible that the inability of Ttr to mediate physiological thiosulfate reduction is not due to mechanistic reasons but rather to the low potential of the electron acceptor couple [E^0 (S₂O₃²⁻/HS⁻ + HSO₃⁻) = -400 mV] relative to those of the physiological electron donors to Ttr (ubiquinol [E^0 (UQ/UQH₂) = +113 mV] and menaquinol [E^0 (MQ/MQH₂) = -75 mV]). To test this idea we grew a *phs* strain on tetrathionate-containing medium to induce Ttr expression and then measured thiosulfate reductase activity with the low-potential reductant methyl viologen radical [E^0 (MV²⁺/MV^{•+}) = -450 mV] as electron donor. It is generally considered that viologen radicals are capable of donating electrons directly to the active-site molybdenum cofactor of MGD-containing enzymes. The *phs* mutant exhibited no detectable MV^{•+}-linked thiosulfate reductase activity while an activity of 160 nmol MV^{•+} oxidized min⁻¹ (mg protein)⁻¹ was measured for an isogenic *phs*⁺ strain induced for Phs expression. Thus, Ttr is unable to catalyse thiosulfate reduction even when provided with an electron donor of appropriate potential.

Phs does not contribute to physiological tetrathionate oxidation (Hensel *et al.*, 1999). However, it is not clear that Phs is expressed while tetrathionate is present in the growth medium. It was therefore possible that Phs lacks physiological tetrathionate reductase activity not for

Table 2. Testing the involvement of conserved Cys256_{TtrA} in the catalytic activity of *S. enterica* tetrathionate reductase

Strain	Physiological tetrathionate reduction (LB medium)		Tetrathionate reductase activity [nmol MV ^{•+} oxidized min ⁻¹ (mg protein) ⁻¹]
	Tetrathionate consumed (mM)	Thiosulfate produced (mM)	
MC4100	0	0	16
MC4100/pAH26 (<i>ttrRSBCA</i>)	8	16	110
MC4100/pAH65 (<i>ttrRSBCA</i> Cys256 _{TtrA} →Ala)	0	0	7
MC4100/pAH66 (<i>ttrRSBCA</i> Cys256 _{TtrA} →Ser)	0	0	12

mechanistic reasons but because it is not expressed under the appropriate growth conditions. This idea was tested by measuring tetrathionate reductase activity in a *ttr* mutant that had been grown under conditions suitable for the induction of Phs synthesis. No significant tetrathionate reductase activity could be measured in these cells with either MV^{•+} or physiological electron donors even though thiosulfate reductase activity was easily detected. This experiment suggests that Phs is not mechanistically capable of reducing tetrathionate.

Ttr and Phs are capable of oxygen atom transfer

It has previously been reported that Phs is capable of reducing chlorate to chlorite (Riggs *et al.*, 1987). This is a significant observation because the reduction of chlorate involves an oxygen atom transfer rather than the sulfur–sulfur bond cleavage of the physiological thiosulfate reduction reaction. To test whether Ttr is also capable of oxygen atom transfer we cultured *phs* and *phs ttr* mutants in the presence of tetrathionate to induce Ttr expression, and then measured MV^{•+}-linked chlorate reductase activity in whole cells. A specific chlorate reductase activity of 190 nmol MV^{•+} oxidized min⁻¹ (mg protein)⁻¹ was measured for the strain APH8 (*ttr⁺ phs asr*) compared to 70 nmol MV^{•+} oxidized min⁻¹ (mg protein)⁻¹ for strain APH12 (*ttr phs asr*). Thus the Ttr⁺ strain had a chlorate reductase activity almost three times higher than that of the Ttr⁻ strain. The *S. enterica* membrane-bound nitrate reductase is also known to have chlorate reductase activity (Barrett & Riggs, 1982). Control measurements showed that both the Ttr⁺ and Ttr⁻ strains had low, but identical, nitrate reductase activities [29 nmol MV^{•+} oxidized min⁻¹ (mg protein)⁻¹], indicating that the difference in chlorate reductase activity between the strains cannot be ascribed to nitrate reductase. This experiment suggests that Ttr has chlorate reductase activity and that the Ttr molybdopterin cofactor is capable of oxygen atom transfer.

Azide is a specific inhibitor of some molybdopterin-dependent enzymes (e.g. membrane-bound nitrate reductases: Craske & Ferguson, 1986; *E. coli* formate

dehydrogenase-H: Axley *et al.*, 1990). However, azide at a concentration of 100 µmol l⁻¹ did not inhibit the tetrathionate reductase activity of Ttr or the thiosulfate reductase activity of Phs.

Reduction of trithionate by *S. enterica*

It was of interest to determine whether the Ttr enzyme specifically reduces tetrathionate or whether it is also able to cleave polythionate compounds with other chain lengths. Trithionate (⁻O₃S–S–SO₃⁻) can be formed by the facile chemical reaction of tetrathionate and sulfite (reaction 4) and is therefore likely to be present in many tetrathionate-containing environments.

We found that *S. enterica* is capable of anaerobic growth on the non-fermentable carbon source glycerol with trithionate as sole respiratory electron acceptor. This growth was dependent on the Ttr enzyme since *ttr* mutants failed to grow under these culture conditions. In contrast, trithionate-linked growth was unaffected in a *phs* mutant. *E. coli* strain MC4100 was found to be unable to utilize trithionate as terminal electron acceptor. However, introduction of plasmid pAH26 carrying the *ttrRSBCA* cluster allowed growth of this strain on trithionate, confirming that Ttr is responsible for trithionate-dependent growth.

The products of the trithionate reduction reaction were examined in wild-type *S. enterica* LT2a during growth on minimal glycerol medium. For each mole of trithionate consumed approximately one mole of thiosulfate and one mole of sulfite were produced. This suggests that trithionate is reduced by Ttr in a reaction (equation 5) analogous to that of tetrathionate reduction.

The ability of Ttr and of Phs to reduce trithionate was also tested using the non-physiological electron donor MV^{•+}. Cells cultured in the presence of trithionate exhibited similar rates of MV^{•+}-linked trithionate and tetrathionate reduction. This trithionate reductase ac-

Fig. 2. *S. enterica* tetrathionate reductase catalyses trithionate reduction. The figure shows the methyl viologen radical-dependent rate of trithionate reduction by washed whole cells. Strains were precultured anoxically on LB medium either in the presence (filled bars) or absence (striped bars) of tetrathionate.

tivity was absent from a *ttr* but not a *phs* strain, demonstrating that trithionate reduction requires Ttr (Fig. 2). To be sure that Phs does not have trithionate reductase activity the *ttr* mutant was cultured in the presence of thiosulfate rather than trithionate to ensure high Phs expression. Even under these conditions of Phs induction no significant methyl-viologen-dependent trithionate reduction could be measured.

Taken together these data suggest that trithionate is a substrate for Ttr but not Phs. It is not possible to determine by competition experiments whether tetrathionate or trithionate is the preferred substrate of Ttr since if trithionate is metabolized (reaction 5) the product sulfite will react with tetrathionate according to reaction 4 to give a net reaction identical to that of tetrathionate reduction (reaction 1).

Expression of the *ttrBCA* operon requires the TtrSR two-component regulator and is induced by tetrathionate (Hensel *et al.*, 1999; Price-Carter *et al.*, 2001). It is probable that tetrathionate is sensed directly by TtrS since thiosulfate, the product of tetrathionate metabolism, does not induce Ttr expression. In this study we found that Ttr was induced in medium lacking tetrathionate but containing trithionate. This suggests that trithionate is capable of inducing Ttr synthesis and therefore that TtrS may be capable of sensing both trithionate and tetrathionate.

Reduction of elemental sulfur by *S. enterica*

We investigated whether the sulfur-sulfur bonds of elemental sulfur were also amenable to cleavage by *S. enterica*. Solid medium containing dispersed solid sulfur was prepared by air oxidation of agar plates to which an alkaline polysulfide solution had been added at the time of pouring.

S. enterica LT2a was capable of anaerobic growth on LB agar plates containing 50 mM formate and 10 mM elemental sulfur. The opaque elemental sulfur precipitate within the plates cleared around the colonies, indicating that the insoluble sulfur had been converted to another species by bacterial metabolism (Fig. 3a). In the absence of formate, which acts as a direct electron donor to the respiratory electron transfer chain, sulfur clearing still occurred but was not as extensive. Sulfur clearing was also observed on solid minimal medium containing 50 mM formate with 0.4% lactate as carbon source. *E. coli*, which does not utilize inorganic sulfur compounds as respiratory electron acceptors, did not exhibit the sulfur-clearing phenotype (Fig. 3a). Sulfide (up to 5 mM) could be detected in cultures of *S. enterica* growing anaerobically in liquid LB medium in the presence of sulfur powder, suggesting that sulfide is the product of the sulfur dissolution reaction. Significant quantities of tetrathionate or thiosulfate were not detected in these liquid cultures.

To ascertain whether any of the enzymes tetrathionate reductase, thiosulfate reductase or anaerobic sulfite reductase were involved in the sulfur-clearing phenotype, strains with *ttr*, *phs* and *asr* null mutations were cultured on LB-sulfur-formate plates. The sulfur-clearing phenotype was absent in the *phs* mutant (Fig. 3a). In contrast, sulfur clearing was unaffected in *ttr* and *asr* mutants (Fig. 3a). Plasmid pNH650 carrying the *S. enterica phsABC* operon directs functional expression of thiosulfate reductase in *E. coli* (Fong *et al.*, 1993). We found that pNH650 also confers the sulfur-clearing phenotype on *E. coli* (Fig. 3a). This observation confirms the link between sulfur-clearing and *Salmonella* thiosulfate reductase.

Elemental sulfur did not support growth of *S. enterica* in the presence of a non-fermentable carbon source. This observation is congruent with the involvement of Psr in sulfur reduction since *S. enterica* cannot grow by thiosulfate respiration alone. Instead thiosulfate reduction functions to augment growth on fermentable carbon sources (Heinzinger *et al.*, 1995; A. P. Hinsley & B. C. Berks unpublished).

Sulfur clearing by *S. enterica* was prevented by addition of 30 mM tetrathionate to the plates. This tetrathionate inhibition was relieved in a *ttr* mutant, indicating that tetrathionate must be metabolized to suppress sulfur clearing. Addition of 20 mM thiosulfate to the sulfur plates did not inhibit sulfur clearing. This shows that it is not the product of the Ttr reaction that is responsible for suppression of sulfur clearing. Instead the effect of tetrathionate is most likely due to the preferential routing of reducing equivalents to Ttr rather than Phs, a hierarchy previously observed in the differential utilization of tetrathionate and thiosulfate (e.g. Hensel *et al.*, 1999).

Mechanisms for the sulfur-clearing phenotype in which the cells directly reduce elemental sulfur can be ruled out since (i) the sulfur clearing extends far beyond direct contact with the cells (Fig. 3a), (ii) sulfide production

Fig. 3. Metabolism of elemental sulfur in *S. enterica* is associated with thiosulfate reductase activity. (a) Strains were streaked on an LB agar plate containing 50 mM sodium formate and 10 mM sulfur. The plate was then incubated anoxically under a nitrogen/CO₂ atmosphere at 37 °C for 72 h. (b) As (a) except that the agar was supplemented with 50 mM NaMES pH 6.0 and the plate was incubated for 120 h. Utilization of sulfur is manifest as clearing of the opaque sulfur substrate. In this figure the plates have been placed on a black background to allow clearing to be visualized as a dark region around the bacterial streak.

from sulfur can still be measured in liquid culture when either the cells or the sulfur powder are placed inside a sealed dialysis sac and (iii) the thiosulfate reductase active site is in the periplasmic compartment, which the insoluble sulfur substrate cannot access. Two possible models are suggested for the metabolism of sulfur by *S. enterica* using water-soluble species: thiosulphate cycling and polysulfide reduction.

The thiosulfate cycling model (Fig. 4a) assumes that trace quantities of thiosulfate are present in the sulfur plates. This is not unreasonable since the stock polysulfide solution has been oxidized by molecular oxygen. The thiosulfate is metabolized by the normal Phs reaction (overall equation 6). The product sulfite molecule, produced at the periplasmic face of the inner membrane, diffuses away from the cell and reacts by well-established and facile chemistry with elemental sulfur producing thiosulfate (equation 7). Since reaction (7) produces one mole of thiosulfate for each mole of thiosulfate reduced in equation (6) the combination of the two reactions results in the reduction of elemental sulfur to sulfide, with thiosulfate having a catalytic role in the process (equation 8). Possible support for the thiosulfate cycling mechanism comes from the observation that strains that are capable of thiosulfate reduction but are unable to reduce the sulfite product (the *S. enterica asr* mutant and *E. coli* expressing *phs*) reproducibly show larger zones of sulfur clearing

than strains that can reduce both thiosulfate and sulfite (Fig. 3a).

In the polysulfide reduction model (Fig. 4b), which is analogous to the mechanism of sulfur reduction found in *W. succinogenes* (Klimmek *et al.*, 1991; Hedderich *et al.*, 1998), elemental sulfur is converted to water-soluble polysulfides by reaction with sulfide (formal equation 9). The polysulfide is then reduced by Phs using overall equation (10). This mechanism requires either that trace quantities of polysulfide remain in the plates following oxidation or that bacterial metabolism provides small amounts of sulfide to initiate the reaction. Once reaction (10) is operative the sulfide produced will mobilize additional elemental sulfur.

The polysulfide reduction mechanism requires that Phs can operate as a polysulfide reductase. However, we found that washed cells of *S. enterica* that had been induced for Phs expression did not reduce polysulfide with formate as electron donor. Similarly no polysulfide reductase activity could be detected with cells induced for Ttr expression. A caveat with these measurements is

Fig. 4. Two biochemical models for the sulfur-clearing phenotype of *S. enterica*. (a) Thiosulfate cycling model, in which sulfur solid is mobilized by reaction with sulfite. (b) Polysulfide reduction model, in which sulfur solid is mobilized by reaction with sulfide. In both schemes the water-soluble species are depicted as traversing the outer membrane through porins.

that the polysulfide assay is relatively insensitive and might not pick up low-level polysulfide reductase activity [of the order of 10 nmol polysulfide sulfur reduced min^{-1} (mg protein) $^{-1}$ or less] associated with Phs. Nevertheless, our polysulfide reductase activity measurements provide no positive support for the idea that a polysulfide reduction mechanism is operating during sulfur clearing by *S. enterica*. It is also worth noting that addition of 20 mM thiosulfate to the plates did not inhibit the sulfur-clearing reaction (above). Since thiosulfate is a known substrate of Phs, such a high concentration of thiosulfate would be expected to competitively inhibit any polysulfide reductase activity of the enzyme if it existed.

Polysulfides are unstable at low pH, disproportionating to elemental sulfur and sulfide according to equation (9). We considered that an analysis of the pH dependence of the sulfur-clearing phenotype catalysed by *S. enterica* might give insight into the possible involvement of

polysulfides in the process. The ability of *S. enterica* to clear sulfur was tested on LB-formate-sulfur plates buffered by means of a 50 mM concentration of an appropriate Good buffer. A range of pH values in the interval 6.0–9.0 was examined. While sulfur clearing was most efficient at high pH the phenotype was still detectable at pH 6.0 (Fig. 3b). The solubility of polysulfide in a 10 mM solution of sulfide at pH 6.0 and 37 °C is approximately 10 μM (Schauder & Kröger, 1993). Since it is highly unlikely that the concentration of sulfide available in the plates to initiate polysulfide formation is anywhere near 10 mM, the concentration of free polysulfide in the pH 6.0 plates that could act as a potential Phs substrate will be a great deal less than 10 μM . We infer that a polysulfide reduction mechanism alone cannot account for the sulfur-clearing phenotype.

Our observations suggest that *Salmonella* species are capable of utilizing elemental sulfur as an electron acceptor in natural environments. They also raise the

possibility that a thiosulfate cycling system could mediate sulfur respiration in other organisms. It is notable that both the thiosulfate cycling system and the polysulfide-based reactions provide a mechanism by which a substrate that cannot cross the outer membrane can be metabolized by means of reactions taking place at the cytoplasmic membrane (Fig. 4).

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