

Hydrogen Sulfide Production and Fermentative Gas Production by *Salmonella typhimurium* Require F_0F_1 ATP Synthase Activity

KYLE C. SASAHARA, NINA K. HEINZINGER,[†] AND ERICKA L. BARRETT*

Department of Food Science and Technology, University of California, Davis, California 95616-8598

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A previously isolated mutant of *Salmonella typhimurium* lacking hydrogen sulfide production from both thiosulfate and sulfite was shown to have a single mutation which also caused the loss of fermentative gas production and the ability to grow on nonfermentable substrates and which mapped in the vicinity of the *atp* chromosomal locus. The implication that F_0F_1 ATP synthase might be essential for H_2S and fermentative gas production was explored. The *phs* plasmid conferring H_2S production on wild-type *Escherichia coli* failed to confer this ability on seven of eight *E. coli atp* point mutants representing, collectively, the eight genes encoding the subunits of F_0F_1 ATP synthase. However, it did confer some thiosulfate reductase activity on all except the mutant with a lesion in the ATP synthase catalytic subunit. Localized mutagenesis of the *Salmonella atp* chromosomal region yielded 500 point mutants unable to reduce thiosulfate to H_2S or to produce gas from glucose, but differing in the extents of their ability to grow on succinate, to perform proton translocation as measured in a fluorescence quenching assay, and to reduce sulfite to H_2S . Biochemical assays showed that all mutants were completely devoid of both methyl viologen and formate-linked thiosulfate reductase and that *N,N'*-dicyclohexylcarbodiimide blocked thiosulfate reductase activity by the wild type, suggesting that thiosulfate reductase activity has an absolute requirement for F_0F_1 ATP synthase. Hydrogenase-linked formate dehydrogenase was also affected, but not as severely as thiosulfate reductase. These results imply that in addition to linking oxidation with phosphorylation, F_0F_1 ATP synthase plays a key role in the proton movement accompanying certain anaerobic reductions and oxidations.

Salmonella typhimurium and almost all other serotypes of *Salmonella* which cause human gastroenteritis produce hydrogen sulfide from both thiosulfate and sulfite (2). Two separate operons, which have been designated *phsABC* (production of hydrogen sulfide) and *asrABC* (anaerobic sulfite reduction), encode the reductases mediating H_2S production from thiosulfate and sulfite, respectively (9, 11, 13, 14). The *asrABC* products constitute a soluble system (10, 14), while the *phsABC* products constitute a membrane-bound system with many similarities to reductases known to facilitate anaerobic respiration (11). Both reductases are induced by anaerobiosis and their respective substrates (5, 13), both reach their highest levels in stationary phase (5, 10), both require heme for activity (5); both accept electrons from formate (10), and neither mediates significant production of H_2S in the presence of nitrate or mutations in the global regulator *oxrA*, although nitrate and *oxrA* are not effectors of the operons (5, 13). (Nitrate and *oxrA* may exert their effects on the formate dehydrogenase end of the electron transport chains.) Although thiosulfate reduction resembles other reductions shown to facilitate anaerobic respiration in terms of electron transport components and the reductase structure, thiosulfate is one of the least energetically favorable terminal electron acceptors; its reduction only slightly increases cell yields and is accompanied by only very low levels of ATP synthesis (11), levels seemingly inadequate for bona fide growth-supporting anaerobic respiration. We have hypothesized that the physiological role of electron transport to thiosulfate differs from those of other anaerobic electron transport systems.

Our previous isolation of H_2S^- mutants yielded one isolate,

strain EB299, which failed to produce H_2S from both thiosulfate and sulfite and which did not appear defective in heme synthesis, because it retained the ability to reduce nitrate (5). Very surprisingly, this mutant grew aerobically, but not anaerobically, on glucose minimal plates, and it also failed to produce gas from glucose in liquid media. In our continued investigation of the physiological significance of H_2S production, we have examined the phenotype of strain EB299 in more detail. Here, we show its primary defect to be in F_0F_1 ATP synthase and we show that all ATP synthase mutants are, in fact, defective in the production of both H_2S and fermentative gas.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. The bacterial strains used are listed in Table 1. All bacteria were cultivated at 37°C. Aerobic cultures were grown on roller drums or a rotary shaker at 250 rpm. Anaerobic cultures were grown in standing flasks that were filled to the top. Anaerobic conditions for solid media were achieved in sealed GasPak jars (BBL Microbiology Systems) with a catalyst generating a 95% H_2 and 5% CO_2 atmosphere.

Nutrient broth, yeast extract, peptone iron agar, and triple sugar iron agar were from Difco Laboratories. The following other media were formulated as previously described: formate hydrogen lyase broth (3), thiosulfate reductase broth and nitrate broth (11), sulfite tubes (5), Luria broth (19), and M9 minimal agar base (7), to which carbon sources were supplemented at a 1% (wt/vol) concentration. Tetracycline was added to selection media at 20 μ g/ml for complex media and 10 μ g/ml for minimal media as noted. The 9-amino-6-chloro-2-methoxyacridine (ACMA) used for fluorescence-quenching experiments was from Molecular Probes. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), *N,N'*-dicyclohexylcarbodiimide (DCCD), and all other chemicals were from Sigma.

Genetic methods. *Escherichia coli atp* strains were made competent with calcium and transformed with *phs* plasmid pEB40 (9) by the methods described by Maniatis et al. (18). Hydroxylamine-localized mutagenesis was performed as described by Hong and Ames (12) with P22 HT105int-201 grown on Tn10 strain (16) AK3119 or AK3241. Tetracycline-resistant mutants were selected with wild-type LT2 as the recipient on Luria-Bertani-galactose with 20 μ g of tetracycline per ml.

Qualitative tests and enzyme assays. Isolates were screened for H_2S production by stabbing colonies in peptone iron agar (thiosulfate reduction) and sulfite agar (sulfite reduction) and for their ability to grow on succinate with aerobically

* Corresponding author. Phone: (916) 752-8079. Fax: (916) 752-4759. E-mail: elbarrett@ucdavis.edu.

[†] Present address: Department of Cellular and Molecular Physiology, Hershey Medical Center, Hershey, PA.

TABLE 1. Bacterial strains used

Strain	Genotype or phenotype	Reference or source
<i>S. typhimurium</i>		
LT2	Wild type	B. N. Ames
AK3119	<i>zib-3119::Tn10</i>	SGSC ^a
AK3241	<i>zic-3241::Tn10</i>	SGSC
EB299	<i>Mud1(Ap^r lac) H₂S⁻ ATP synthase⁻</i>	5
EB600	<i>zic-3241::Tn10 ATP synthase⁻</i>	This study
EB613	<i>zic-3241::Tn10 ATP synthase⁻</i>	This study
EB625	<i>zic-3241::Tn10 ATP synthase⁻</i>	This study
<i>E. coli</i>		
AN724	<i>ilv argH pyrE entA recA</i>	L. Hatch
AN730	<i>ilv argH pyrE entA recA uncA401</i>	L. Hatch
AN727	<i>ilv argH pyrE entA recA uncB402</i>	L. Hatch
AN802	<i>ilv argH pyrE entA recA uncC424</i>	L. Hatch
AN818	<i>ilv argH pyrE entA recA uncD409</i>	L. Hatch
AN943	<i>ilv argH pyrE entA recA uncE429</i>	L. Hatch
AN1440	<i>ilv argH pyrE entA recA uncF469</i>	L. Hatch
AN1273	<i>ilv argH pyrE entA recA uncG428</i>	L. Hatch
AN2015	<i>ilv argH pyrE entA recA uncH241</i>	L. Hatch

^a SGSC, Salmonella Genetic Stock Centre (care of K. E. Sanderson); Tn10 insertion described by Kukral et al. (16).

incubated succinate minimal plates. Previously described methods were used to test for gas production (3) and for nitrate reduction (5).

Crude extracts, which were used for all enzyme assays, consisted of whole cells freeze-thawed for three cycles in liquid nitrogen and an 85°C water bath. Glycerol was added to a final concentration of 20%, and the mixture was frozen in liquid nitrogen and stored at -70°C. Inverted vesicles were prepared and fluorescence quenching experiments were performed according to the method described by Ames et al. (1). Anaerobic assay conditions were achieved by bubbling scrubbed argon through the assay mixture. Argon was scrubbed by passing through an oxygen-moisture trap (R & D Separations) and a tower of BASF copper catalyst (Kontes Glass Co.) for 2 min. In reductase assays, benzyl and methyl viologen were pre-reduced by adding 10 to 100 μl of 0.25 M Na₂S₂O₄ dissolved in 0.52 M NaHCO₃. A Shimadzu UV 160U spectrophotometer was used for all assays unless otherwise noted.

Hydrogenase-linked formate dehydrogenase (FDH_H), nitrate-linked formate dehydrogenase (FDH_N), and nitrate reductase assays were described by Lester and DeMoss (17), except that a pH of 6.4 rather than 7.2 was used for the FDH_H assay and methyl viologen rather than benzyl viologen was used for the nitrate reductase assay. Thiosulfate reductase was assayed as described previously (10). ATPase activity was assayed as phosphate released from ATP by the method described by Simoni and Shandell (23). For all of the above assays, the amounts of protein were determined by the Bradford method (4). β-Galactosidase assays were performed by the Miller method (19). The generation of ΔpH achieved by inverted vesicles upon the addition of ATP was measured as the quenching of ACMA with a Hitachi F-2000 fluorescence spectrophotometer.

RESULTS

Characterization of *Mud1 (Ap^r lac)* fusion strain EB299.

Further physiological studies of H₂S⁻ strain EB299 revealed that it required both a fermentable sugar and an electron acceptor for growth; although it grew on glucose minimal medium aerobically, it was unable to grow on succinate, malate, or lactate aerobically and was unable to grow on glucose anaerobically in the absence of an electron acceptor such as nitrate, trimethylamine oxide, or tetrathionate. When the electron acceptors were added to liquid cultures, the final densities obtained with strain EB299 were 40 to 60% lower than those obtained in wild-type cultures. Transductional correction of any of these growth deficiencies, e.g., by selection for the ability to grow aerobically on succinate, restored the ability of all transductants to grow under all of the conditions described above as well as the ability to make H₂S from both thiosulfate and sulfite and the ability to make gas from glucose. These results strongly suggested that all of the defects could be traced

TABLE 2. Hydrogen sulfide production and thiosulfate reductase activities in *E. coli atp* mutants transformed with *phs*-containing plasmid pEB40

Recipient strain (mutation site) ^a	Subunit affected	Hydrogen sulfide production ^b	TSR sp act ^c
AN724	Wild type	+++	96
AN727 (<i>uncB</i>)	F ₀ a	-	90
AN943 (<i>uncE</i>)	F ₀ c	-	38
AN1440 (<i>uncF</i>)	F ₀ b	-	45
AN2015 (<i>uncH</i>)	F ₁ δ	-	66
AN730 (<i>uncA</i>)	F ₁ α	-	80
AN1273 (<i>uncG</i>)	F ₁ γ	-	41
AN818 (<i>uncD</i>)	F ₁ β	-	2
AN802 (<i>uncC</i>)	F ₁ ε	+	98

^a Recipient strains arranged according to gene order in the *E. coli atp* operon. Each contains nitrosoguanidine-induced point mutations in the indicated genes.

^b Hydrogen sulfide tested by stabbing peptone-iron agar.

^c TSR, thiosulfate reductase. Specific activity is expressed as nanomoles of sulfide produced per minute per milligram of protein with methyl viologen as the electron donor.

to single mutation in a locus encoding something essential for the coupling of phosphorylation with respiration as well as for H₂S and H₂ production.

Conjugational and transductional mapping placed the mutation site in the vicinity of the *Salmonella atp* locus. The *atp* locus in *Salmonella* has not been extensively studied, but it is located at a site homologous to the *E. coli* locus, and the *E. coli atp* locus has been shown to encode all of the subunits of F₀F₁ ATP synthase (24). The mutation in strain EB299 was 41% cotransduced with Tn10 in strain AK3119 and 30% with Tn10 in strain AK3241, which are located, respectively, at approximately min 81 and 82 on the edition VII map (22). The *uncA* gene, which is now designated *atp*, is located at min 82 on this map. (It is located at min 84.6 on the edition VIII map [21].)

Expression of β-galactosidase by strain EB299 was assayed in cultures grown aerobically and anaerobically in unsupplemented nutrient broth or in nutrient broth supplemented with nitrate, thiosulfate, trimethylamine oxide, or glucose. Relatively strong expression (450 to 900 Miller U) was found under all conditions examined. Kasimoglu et al. (15) obtained similar results with an *atpI::lacZ* fusion, namely, a narrow range of high expression levels under all growth conditions examined.

The results described above suggested that strain EB299 contains a mutation in the *atp* operon. They suggested further that ATP synthase may be required not only for the coupling of respiration with phosphorylation, but also for production of H₂S and gas from glucose. The following experiments were designed to test the latter hypothesis.

Expression of *phs* in an ATP synthase⁻ background. We showed previously that *S. typhimurium* on plasmid pEB40, which contains the complete *phs* operon, confers on *E. coli* the ability to produce H₂S from thiosulfate (9). If F₀F₁ ATP synthase is required for H₂S production, then *phs* should not confer H₂S production on an *E. coli* host lacking it. We transformed a series of eight *E. coli atp* mutants containing nitrosoguanidine-induced point mutations in each of the F₀F₁ ATP synthase subunits, respectively, with plasmid pEB40 and then assayed the transformants for H₂S production and thiosulfate reductase activity (Table 2). Only the *atp*⁺ host and the host mutated in *atpC* (encoding the ε subunit of the F₁ portion [not at the catalytic site]) produced any H₂S, and the *atpC* host produced very little compared to its *atp*⁺ parent. On the other hand, all transformants except the *uncD* mutant exhibited significant thiosulfate reductase activity, which was measured as

TABLE 3. Effect of DCCD on formate dehydrogenase and on thiosulfate and nitrate reductases in vesicles of wild-type *S. typhimurium*

Enzyme ^a	Sp act ^b	
	-DCCD	+DCCD
FDH _H	115	34
FDH _N	327	434
Thiosulfate reductase	19	<0.4
Nitrate reductase	43	15

^a FDH_H, formate as donor and benzyl viologen as acceptor; FDH_N, formate as donor and phenazine methosulfate-dichlorophenolindophenol as acceptor; thiosulfate reductase, methyl viologen as donor and thiosulfate as acceptor; nitrate reductase, methyl viologen as donor and nitrate as acceptor.

^b Specific activity expressed as nmol oxidized or reduced artificial donor or acceptor (see footnote a) per minute per milligram of protein. Vesicles were either preincubated for 20 min with DCCD added at a concentration of 28 μM or were assayed without DCCD.

sulfide produced (captured as zinc sulfide) with methyl viologen as the electron donor. The *uncD* gene encodes the β subunit, in which the actual catalytic site is located. These results support an essential role for catalytic ATP synthase activity in the reduction of thiosulfate and a requirement for the complete F₀F₁ complex in the formation and/or release of H₂S by whole cells.

Effect of DCCD on H₂S production by *Salmonella*. If a functional F₀F₁ ATP synthase is essential for thiosulfate reduction to H₂S and for the production of gas from glucose via formate hydrogen lyase in *Salmonella*, then DCCD, which binds to and thereby inactivates the F₀ portion, should interfere with these activities. We assayed the effect of DCCD on thiosulfate reductase and formate hydrogen lyase in vesicles prepared from wild-type *S. typhimurium* and compared the results with those obtained for enzymes involved in nitrate respiration, which is an activity which did not appear deficient in qualitative tests of strain EB299. The assays (Table 3) showed that DCCD had no effect on FDH_N, while it reduced the activities of nitrate reductase and formate hydrogen lyase by 66 and 72%, respectively, and completely eliminated thiosulfate reductase activity. DCCD is known to inhibit a number of membrane proteins. Thus, its effect in these assays could represent either a direct effect on the oxidoreductase or an indirect effect of the inactivation of the F₀F₁ ATP synthase.

Isolation of additional *atp* mutants. Additional *atp* mutants of *S. typhimurium* were isolated to see if the EB299 phenotype represented a class of mutations rather than a single aberrant strain. About 500 point mutations were isolated by localized mutagenesis of the DNA in the vicinity of Tn10 insertions

known to be linked to *atp*. Tetracycline-resistant transductants obtained by using mutagenized P22 phage of the Tn10 strains were screened for H₂S from thiosulfate. All H₂S⁻ isolates were then screened for several other phenotypic characters.

Three classes of H₂S⁻ (from thiosulfate) mutants were obtained, all of which failed to produce gas from glucose, while retaining the ability to reduce nitrate to nitrite in qualitative tests. They differed with respect to growth on succinate and their abilities to produce H₂S from sulfite. Class I mutants (411 isolates) retained the ability to grow slowly on aerobic succinate minimal medium but could not produce H₂S from sulfite. Class II mutants (110 isolates) could not grow on succinate minimal medium but still produced H₂S from sulfite. Class III mutants (11 isolates), like strain EB299, were negative for both succinate utilization and H₂S from sulfite. All mutations were transductionally linked to the *atp*-linked Tn10, but there was no correlation between cotransduction frequencies and mutant class obtained. A number of representatives of each mutant class and also strain EB299 were assayed for ATP synthase activity. All of them exhibited activities that were less than 2% of the wild-type activity (see representative assays in Table 4), indicating that they were, in fact, defective in this enzyme. It is interesting that so many isolates were negative for H₂S from thiosulfate and gas from glucose, while retaining their ability to grow on succinate, suggesting that H₂S and gas production may be more sensitive to small perturbations in F₀F₁ ATP synthase than is the coupling of oxidation to phosphorylation.

Proton translocation by the *atp* mutants. Inverted membrane vesicles of representative mutants were assayed for proton translocation with the fluorescent probe ACMA (Fig. 1). Quenching of the fluorescence of free ACMA occurs when ATP is hydrolyzed by the F₁ portion of the complex on the vesicle surface and protons are pumped through the F₀ channel. Subsequent addition of the protonophore CCCP allows the dye accumulated inside the vesicle to move back out, restoring fluorescence which was quenched through proton translocation. The class I mutant translocated protons almost as effectively as the wild type, the class II mutant was somewhat less effective, and the class III mutant was completely devoid of translocation activity. These results are consistent with the mutant phenotypes.

Anaerobic reductase and dehydrogenase activities of the *atp* point mutants. Assays of the specific reductases and dehydrogenases were performed to pinpoint the enzyme activities affected by the loss of ATP synthase activity (Table 4). In crude extracts of class I and III mutants, loss of ATP synthase activity was associated with a loss of methyl viologen-linked thiosulfate reductase, but not methyl viologen-linked nitrate reductase. In class II mutant extracts, both reductases were negatively af-

TABLE 4. Enzyme activities and other characteristics of representative *atp* mutants

Strain	H ₂ S from S ₂ O ₃ ²⁻	H ₂ S from SO ₃ ²⁻	Gas ^b	Growth on succinate	Enzyme sp act ^a						
					ATPase	FDH _H	FDH _N	TSR with formate as donor	TSR with MV as donor	NaR with formate as donor	NaR with MV as donor
LT2 (wild type)	+	+	+	+	5.3	24	39	17	36	0.14	754
EB299	-	-	-	-	0.12	<1	<1	<0.3	<0.9	<0.002	594
EB600 (class I)	-	-	-	+	0.14	2.5	<1	<0.3	<0.8	<0.002	333
EB613 (class II)	-	+	-	-	0.08	2.5	<1	<0.3	8.0	0.14	101
EB625 (class III)	-	-	-	-	0.10	5	<1	<0.3	<0.9	<0.002	130

^a ATPase, micromoles of P_i released; FDH_H, nanomoles of benzyl viologen reduced; FDH_N, nanomoles of dichlorophenolindophenol reduced; thiosulfate reductase (TSR), nanomoles of sulfide produced with formate or methyl viologen (MV); nitrate reductase (NaR), nanomoles of nitrite produced with formate or methyl viologen. All assays were performed with crude extracts.

^b Production of gas during fermentation of glucose.

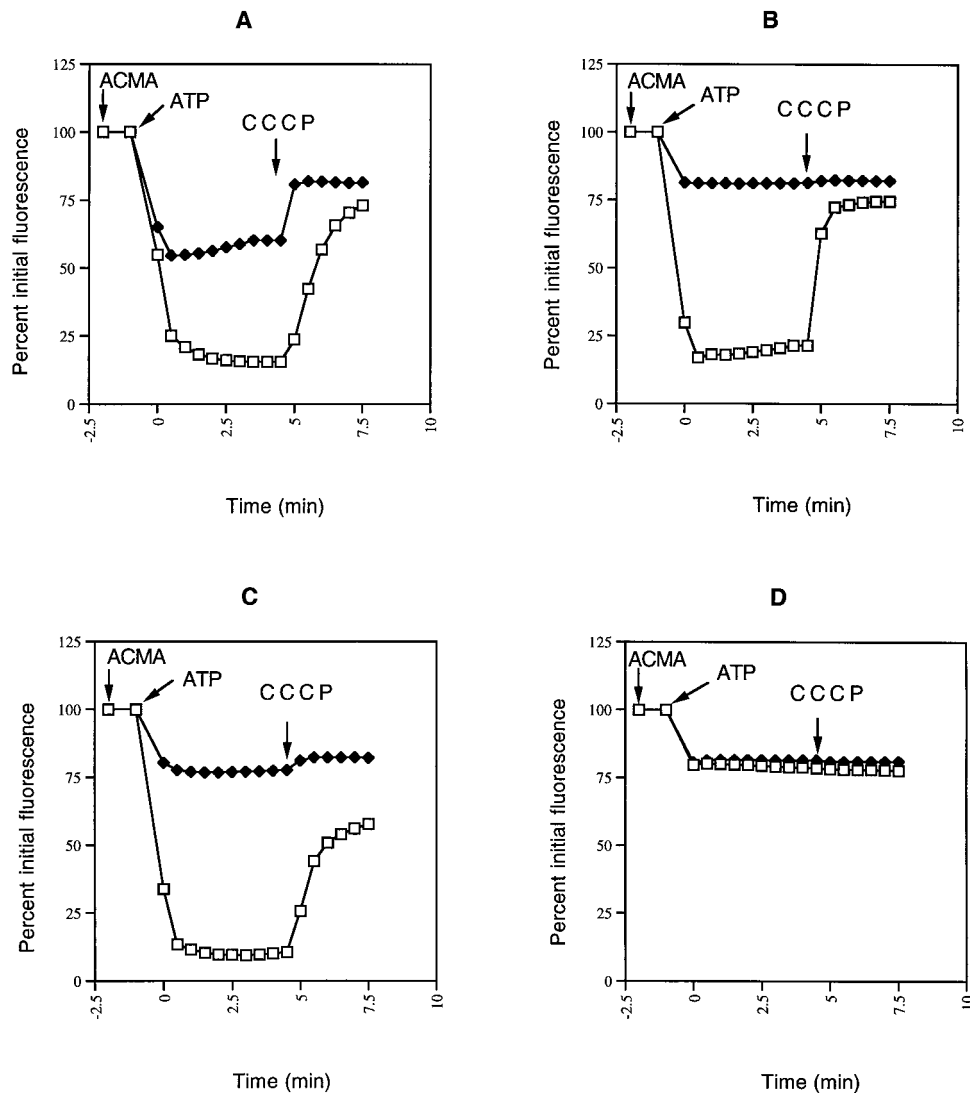


FIG. 1. ATP-dependent ACMA quenching by inverted vesicles, with (◆) or without (□) preincubation of the vesicles with DCCD. (A) Wild-type LT2; (B) class I mutant EB600; (C) class II mutant EB613; (D) class III mutant EB625.

fected to the same extent. In all mutants, FDH_H and FDH_N were severely impaired. The lack of the latter enzyme probably explains the fact that class I and III mutants were unable to reduce nitrate with formate. Surprisingly, class II mutants did retain formate-linked nitrate reduction, although they had no detectable FDH_N activity (with phenazine methosulfate-dichlorophenolindophenol as the acceptor). Apparently, the defect in this class is leaky with respect to several oxidoreductase activities, including sulfite reductase, as shown in the initial mutant characterization. The assay results do not provide any clues regarding the specific sites of mutations in the three respective classes. They suggest instead that the classes represent different levels of mutation severity in the context of separate and unique interactions between native F₀F₁ ATP synthase and each of the various oxidoreductases included in the study.

DISCUSSION

The results described above strongly suggest that a fully functional F₀F₁ ATP synthase is an absolute requirement for the production of H₂S from thiosulfate and gas (H₂ and CO₂)

from glucose. Two lines of evidence strongly support this conclusion: (i) the observation that the *phs* operon fails to confer H₂S production on *E. coli atp* mutants and (ii) the isolation of more than 500 point mutants defective in F₀F₁ ATP synthase, all of which failed to produce H₂S from thiosulfate and gas from glucose, even though all of them produced nitrite from nitrate in qualitative tests (and thus did not have a pleiotropic mutation affecting all molybdoenzymes). The finding that DCCD interferes with thiosulfate reduction and, to a lesser extent, FDH_H activity is also consistent with the requirement for F₀F₁ ATP synthase in H₂S and gas production, although direct inhibition of these enzymes by DCCD could not be ruled out by the experimental results. Assays of specific reductases and dehydrogenases in the mutant *atp* background revealed electron transfer from either formate or methyl viologen to thiosulfate to be completely blocked. In the same background, both FDH_H and FDH_N were severely impaired but not inactive. The complete loss of fermentative gas production in the mutant *atp* background could be the result of a more serious impairment in the activity of the associated hydrogenase.

The role of F_0F_1 ATP synthase in maintaining the protonic potential of bacterial cell membranes in the context of ATP synthesis and hydrolysis is now well-established (see reference 8 for a review). However, this complex has not previously been implicated as an essential part of proton movement associated with oxidation-reduction enzymes in the absence of phosphorylation. Perhaps the proton movement accompanying thiosulfate reduction to H_2S and formate oxidation to H_2 and CO_2 resembles that of proton-dependent transport systems, such as some of the proton-dependent multidrug efflux systems (20), more than it resembles proton export via electron transport chains. One possible model for the role of F_0F_1 ATP synthase in thiosulfate reduction as well as sulfite reduction (a soluble system) would include the separate transport of S^{2-} and H^+ to the exterior to form H_2S , with the proton movement relying on the F_0F_1 complex. Similarly, hydrogen gas might be formed outside the cell after protons are exported via the F_0F_1 complex and electrons are brought to the exterior via membrane electron transport.

Three phenotypically distinct classes of mutants defective in F_0F_1 ATP synthase and thiosulfate reductase were isolated, but the respective phenotypes did not suggest specific defects in F_0F_1 ATP synthase subunits. Analysis of the phenotypes at this stage is difficult because the *atp* operon in *Salmonella* has not been analyzed and specific interactions of F_0F_1 ATP synthase with anaerobic oxidoreductases have not been previously described. The range of defects regarding the anaerobic enzymes assayed suggests that ATP synthase may interact with several anaerobic enzymes in different ways. The thiosulfate reductase activities of the mutants isolated in this study were lower than the activities determined for most of the *E. coli atp* mutants transformed with the *phs* plasmid. This difference may reflect the multicopy nature of the *phs* plasmid and/or different regulatory systems operating in the *E. coli* background compared to the *Salmonella* background.

The requirement by thiosulfate reductase for F_0F_1 ATP synthase is yet another feature that distinguishes thiosulfate reduction from other anaerobic reductions which involve structurally similar membrane-bound electron transport systems, such as nitrate and trimethylamine oxide reductions. Unlike these other systems, thiosulfate reduction is extremely sensitive to catabolite repression (6) and does not support growth on nonfermentative substrates (11). We have hypothesized that the production of H_2S as a toxic molecule may instead play a specific role in gastroenteritis (2). Experiments to test this hypothesis are in progress.

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