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The Alternative Electron Acceptor Tetrathionate Supports B_{12}-Dependent Anaerobic Growth of *Salmonella enterica* Serovar Typhimurium on Ethanolamine or 1,2-Propanediol

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Synthesis of cobalamin de novo by *Salmonella enterica* serovar Typhimurium strain LT2 and the absence of this ability in *Escherichia coli* present several problems. This large synthetic pathway is shared by virtually all salmonellae and must be maintained by selection, yet no conditions are known under which growth depends on endogenous B_{12}. The cofactor is required for degradation of 1,2-propanediol and ethanolamine. However, cofactor synthesis occurs only anaerobically, and neither of these carbon sources supports anaerobic growth with any of the alternative electron acceptors tested thus far. This paradox is resolved by the electron acceptor tetrathionate, which allows *Salmonella* to grow anaerobically on ethanolamine or 1,2-propanediol by using endogenously synthesized B_{12}. Tetrathionate provides the only known conditions under which simple *cob* mutants (unable to make B_{12}) show a growth defect. Genes involved in this metabolism include the *ttr* operon, which encodes tetrathionate reductase. This operon is globally regulated by OxrA (Fnr) and induced anaerobically by a two-component system in response to tetrathionate. *Salmonella* reduces tetrathionate to thiosulfate, which it can further reduce to H_2S, by using enzymes encoded by the genes *phs* and *asr*. The genes for 1,2-propanediol degradation (*pdu*) and B_{12} synthesis (*cob*), along with the genes for sulfur reduction (*ttr*, *phs,* and *asr*), constitute more than 1% of the *Salmonella* genome and are all absent from *E. coli*. In diverging from *E. coli*, *Salmonella* acquired some of these genes unilaterally and maintained others that are ancestral but have been lost from the *E. coli* lineage.

Virtually all *Salmonella* isolates synthesize B_{12} de novo under anaerobic conditions (27, 34, 43). The ability to synthesize and import B_{12} requires more than 35 known genes (48)—approaching 1% of the genome. However, mutations that eliminate B_{12} synthesis from otherwise wild-type strains cause no growth defect under the standard aerobic or anaerobic lab conditions used thus far. Since evolutionary maintenance of such a large fraction of the genome requires selection, it seems inescapable that natural conditions must exist under which endogenously synthesized B_{12} is important to growth of salmonellae. *Salmonella enterica* serovar Typhimurium makes B_{12} de novo only in the absence of oxygen (27). Degradation of ethanolamine or 1,2-propanediol requires B_{12} and provides a carbon and energy source, but growth on these compounds has been observed only under aerobic conditions requiring exogenous B_{12} (28, 46). These paradoxical aspects of B_{12} metabolism have been reviewed (47).

The B_{12} paradox may be resolved by the finding, described here, that the electron acceptor tetrathionate supports anaerobic use of ethanolamine or 1,2-propanediol as the sole carbon and energy source by using endogenously synthesized B_{12}. Under anaerobic conditions, tetrathionate supports considerably better growth on these carbon sources than the other alternative electron acceptors tested. Tetrathionate plus either ethanolamine or 1,2-propanediol provides the only known conditions under which B_{12} synthesis is essential to the growth of wild-type *Salmonella*.

While tetrathionate metabolism has not been studied extensively, its reduction is likely to follow the pathway diagrammed in Fig. 1 (4, 36). The enzymes encoded by the *ttr* operon reduce tetrathionate to thiosulfate (9, 21), which can be reduced further to sulfite plus hydrogen sulfide by enzymes encoded by the *phs* operon (11, 20). Sulfite can be reduced to hydrogen sulfide by the dissimilatory anaerobic sulfite reductase encoded by the *asr* genes (17, 25, 26). *Salmonella* also has an assimilatory sulfite reductase (CysJI), which acts with or without oxygen (32). It is not clear where in nature tetrathionate might be encountered, but it seems likely to occur in bacterial communities that include sulfate-reducing bacteria; it has been detected in humid soils that support growth of such bacteria (52).

Taken together, the three sulfur-reducing enzyme systems (encoded by *ttr*, *phs*, and *asr*) and the genes for 1,2-propanediol degradation (*pdu*) and de novo B_{12} synthesis (*cbi*) comprise more than 1% of the *Salmonella* genome and seem to act as part of a logical pattern of metabolism. All of these genes are characteristic of *S. enterica* and absent from *Escherichia coli*. This suggests that B_{12}-dependent anaerobic catabolism of small molecules, supported by reduction of sulfur compounds, may be central to the life of salmonellae. A model is described for evolution of this system during the divergence of *Salmonella* and *Escherichia coli*. (Throughout the rest of this article, propanediol refers to 1,2-propanediol.)

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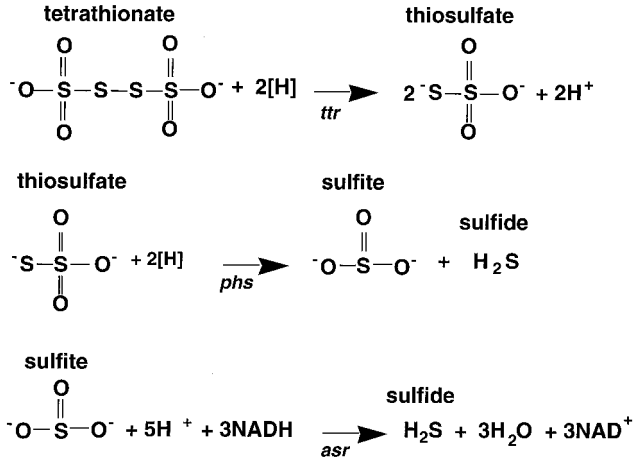

FIG. 1. Reduction of tetrathionate to sulfide. The tetrathionate reductase (Ttr) described here performs the initial reduction to thiosulfate ($S_2O_3^{2-}$). This area of metabolism has been reviewed by Barrett and Clark (4).

MATERIALS AND METHODS

Bacterial strains and crosses. All strains are derived from *S. enterica* serovar Typhimurium strain LT2**.** Key strains and their sources are listed in Table 1. The transposable elements TPOP1 and TPOP2 were described previously (44). All transductional crosses were mediated by the high-frequency transducing mutant of phage P22 (HT105, *int*) (51). Growth of phage and procedures for crosses have been described previously (7). Standard methods for cell culture have been described previously (1, 6).

Chemical reagents and growth media. Standard aerobic cell culture was conducted in Difco nutrient broth supplemented with 0.1 mM NaCl (14). Minimal medium was the No-carbon-E (NCE) medium (13), supplemented with trace metals (0.3 μM $CaCl_2$, 0.1 μM $ZnSO_4$, 0.045 μM $FeSO_4$, 0.2 μM $Na_2Se_2O_3$, 0.2 μM Na_2MoO_4, 2 μM $MnSO_4$, 0.1 μM $CuSO_4$, 3 μM $CoCl_2$, and 0.1 μM $NiSO_4$). Unless otherwise indicated, carbon sources were provided at the following concentrations: glucose, 11 mM; glycerol, 43 mM; ethanolamine (Aldrich Chemical Co.), 25 mM in solid media, 10 mM in liquid; and 1,2-propanediol and potassium acetate (Aldrich Chemical Co.), 25 mM in solid media, 50 mM in liquid. Tetrathionate (Sigma Chemical Co.) was added at the final concentrations indicated in the figure legends and tables; for growth on plates and for assay of induction of the *ttrBCA* genes, it was added at a final concentration of 10 mM. For growth in liquid culture, it was added at 40 mM, since under these conditions a higher growth yield was obtained by adding the fourfold excess. Trimethylamine *N*-oxide, potassium nitrate, fumaric acid, and dimethyl sulfoxide were added at concentrations of 5, 10, 15, and 20 mM. Cyanocobalamin (Sigma Chemical Co.) was added at a final concentration of 200 nM unless otherwise specified. $AdoB_{12}$ (5′-deoxyadenosylcobalamine) and Cbi (cobinamide dicyanide; Sigma Chemical Co.) were each added at a final concentration of 15 nM. Antibiotics were added to nutrient broth medium at the following concentrations: kanamycin, 50 μg/ml; tetracycline, 20 μg/ml; and chloramphenicol, 20 μg/ml. In minimal medium, tetracycline was added to a final concentration of 10 μg/ml.

Cell growth and enzyme assays. Anaerobic conditions (37°C) for petri dishes were provided by an anaerobic chamber (Forma Anaerobic System Model 1024) with a gas mixture of CO_2, H_2, and N_2 (5:6:89). For anaerobic liquid cultures, media were preincubated in the anaerobic chamber for 12 to 24 h. Cells for these cultures were pregrown aerobically to stationary phase in NCE glycerol, washed twice in NCE, and then diluted 10,000-fold into the liquid media inside the anaerobic chamber. The anaerobic culture tubes were then crimp capped, and the medium and headspace were flushed with nitrogen (7). Incubation was at 37° with shaking. Cultures that contained $AdoB_{12}$ or Cbi were prepared and incubated in the dark. Turbidity was monitored in a spectrophotometer at 650 nm. Chlorate sensitivity was tested anaerobically on agar plates containing minimal E medium supplemented with 11 mM glucose with or without 0.2 mM potassium chlorate. β-Galactosidase activity from liquid cell cultures was assayed as described previously (37).

Sulfur assay. Elemental sulfur was detected in cell cultures with a modified version of a method of cyanolysis (19). Soluble forms of sulfur were removed by pelleting cells and washing pellets with water. The cell pellet was dried, and elemental sulfur was dissolved in acetone by overnight incubation at 37°C with shaking. Sample dilutions were made in acetone. Elemental sulfur was detected by cyanolysis; thiocyanate derivatives were formed by adding 0.1 ml of 0.1 M KCN to a 1-ml sample at room temperature. To detect thiocyanates, 0.1 ml of an aqueous $Fe(NO_3)_3$ solution (Aldrich Chemical Co.) [0.25 M $Fe(NO_3)_3$ in 3 M HNO_3] was added and the samples were read at 460 nm in a Beckman DU 640 spectrophotometer. Final sulfur concentrations were estimated by comparing absorbance to a standard curve prepared with elemental sulfur.

Isolation of insertion mutations in and near the *ttr* locus. Insertion mutants unable to reduce tetrathionate were obtained by using MudJ (Kn^r-*lac*) or Tn*10*dTc; these mutants were identified by their failure to produce acid on MacConkey indicator medium (Difco) containing 10 mM tetrathionate. Mutants defective in synthesis of the molybdopterin cofactor (required for tetrathionate reductase) were identified on the basis of their resistance to chlorate and have been described previously (54). Of 18 *ttr*::MudJ insertions isolated (from 10,000 random insertion mutants), 4 expressed the *lacZ* gene from the *ttr* promoter. Other insertions of the MudJ (Kn^r-*lac*) element were obtained by screening a random insertion pool for clones whose β-galactosidase level was induced by tetrathionate (white colonies on nutrient broth–X-Gal [5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside] plates, blue colonies on nutrient broth–X-Gal tetrathionate plates).

Insertions of Tn*10*dCm linked to the *ttr* operon were obtained by transducing a *ttr*::MudJ insertion mutant with a P22 lysate grown on a pool of random Tn*10*dCm insertion mutants; chloramphenicol-resistant (Cm^r) transductants were screened for those that had become Lac$^-$ by loss of the recipient MudJ insertion. These clones had inherited a donor fragment that carried both a wild-type *ttr* operon and a linked Tn*10*dCm element; the mutant *ttr* region of the recipient was replaced, leaving a *ttr*$^+$ strain with a nearby Tn*10*dCm insertion. The same strategy was used to obtain insertions of the Tn*10*dTc (TPOP2) element (44) in and near the *ttr* operon. Additional TPOP insertions within the *ttr* coding sequence were obtained by transducing a strain carrying the distal MudJ insertion (*ttrA120*) with phage grown on a pool of random TPOP insertion mutants. Insertions upstream of the recipient MudJ insertion were identified because they allowed tetracycline (instead of tetrathionate) to induce expression of β-galactosidase.

Determination of the *ttr* operon sequence and insertion sites. Starting with strains carrying multiple insertions in the *ttr* operon, fragments of the operon were amplified by PCR using primers complementary to known sequences at the ends of the particular inserted elements. The amplified fragments were sequenced according to the method of Sanger et al. (49) at the University of Utah Health Sciences DNA Sequence Facility. This information facilitated the design of primers that were used to amplify and sequence the regions between the *ttr* operon and nearby genes and to determine the insertion site of transposable elements.

Construction of *ttrR* and *ttrS* and *prpRBCDE* deletion-substitution (swap) mutations. The linear transformation method was that of Murphy (38) and used cells expressing recombination genes of phage lambda. The ability of these lambda enzymes to support targeted recombination with very short homologous sequences has recently been demonstrated (12, 61). Methods were optimized for *Salmonella* by Eric Kofoid (personal communication). The transformation recipient was a wild-type *Salmonella* strain carrying plasmid pPT223 (TT22236), which includes the *lam*, *bet,* and *exo* genes of phage lambda expressed from a *lac* promoter; this plasmid was constructed and supplied by Poteete and Fenton (42). Cells were pregrown in Luria broth with isopropyl-β-D-thiogalactopyranoside (2 mM) to induce the plasmid recombination genes and washed three times in 10% glycerol prior to electroporation.

For the *ttrR* swap, the 5′ end of primer 1 included 40 bp just outside of the downstream end of the *ttrR* coding sequence, followed by a sequence adjacent to the promoter of the chloramphenicol resistance gene of pACYC184. Primer 2 had at its 5′ end 40 bases complementary to a sequence centered on the translational start codon of *ttrR* (which overlaps *ttrS*), followed by 20 bp homologous to the region immediately outside of the pACYC184 chloramphenicol resistance gene. These primers were used to amplify the chloramphenicol resistance gene (using *Taq* polymerase [Promega]), and the resulting linear fragment was electroporated into a recipient strain as described above. The resulting Cm^r recombinants carried the resistance determinant in place of all of the *ttrR* gene except for the upstream 30 bp, which were left in place because they include the downstream end of the overlapping *ttrS* gene and its stop codon.

To construct the *ttrS* swap, all of the *ttrS* coding sequence was eliminated except for the distal 44 bases needed for initiation of the overlapping *ttrR* gene; the deletion was replaced by the intact Cm^r gene from Tn*10*dCm plus the downstream end of the Tn*10* element and 10 bases downstream of the *ttrS130*

TABLE 1. Bacterial strains

Strain	Genotype[a]	Source or reference[b]
TT22362	Wild-type *S. enterica* serovar Typhimurium strain LT2	Lab collection
TT22336	*cbiD24*::MudJ	3
TT22338	*oxrA*::Tn*10*	Charles Miller
TT22340	*phs-213*::MudJ	Justin Tingey, unpublished data
TT22341	*eut-336* (*eutS-K*)::Tn*10*dTPOP1	31
TT22342	*aceA112*::MudJ	SGSC
TT22343	*aceB113*::MudJ	SGSC
TT22344	*pdu-12*::MudA	60
TT22474	*prp-54*(*prpR-E*)::Cm(swap)	This study
TT20444	*phs208*::Tn*10*dGn *ttrB122*::MudJ	N. Patrick Higgins
TT22347	*oxrA2*::Tn*10 ttrB123*::MudJ	This study
TT22467	*oxrA2*::Tn*10 ttrB123*::MudJ *ttrS130*::Tn*10*dCm	This study
TT22468	*oxrA2*::Tn*10 ttrS130*::Tn*10*dCm	This study
TT22469	*ack-408*::Tn*10*	SGSC
TT18691	*ttrB122*::MudJ	This study
TT18694	*ttrB123*::MudJ	This study
TT18683	*ttrA121*::MudJ	This study
TT20590	*ttrB122*::MudJ *arcA201*::Tn*10*dTc	1; this study
TT20464	*ttrB122*::MudJ *cya-961*::Tn*10 metE205 ara-9*	Peter Postma; this study
TT20469	*ttrB112*::MudJ *crp-773*::Tn*10 metE205 ara-9*	Peter Postma; this study
TT22372	*ttrS133*(del)::Cm(swap)	This study
TT22373	*ttrS133*(del)::Cm^r, *ttrB123*::MudJ	This study
TT18665	*ttrS108*::Tn*10*dTc	This study
TT20433	*ttrS108*::Tn*10*dTc *ttrB123*::MudJ	This study
TT20369	*ttrS130*::Tn*10*dCm	This study
TT22348	*ttrS130*::Tn*10*dCm *ttrB123*::MudJ	This study
TT18675	*ttrS117*::Tn*10*dTc	This study
TT20440	*ttrS117*::Tn*10*dTc *ttrB123*::MudJ	This study
TT22349	*ttrS108*::Tn*10*dTc *ttrS130*::Tn*10*dCm	This study
TT22350	*ttrS108*::Tn*10*dTc *ttrS130*::Tn*10*dCm *ttrB123*::MudJ	This study
TT22351	*ttrS130*::Tn*10*dCm *ttrS117*::Tn*10*dTc	This study
TT22352	*ttrS130*::Tn*10*dCm *ttrS117*::Tn*10*dTc *ttrB123*::MudJ	This study
TT22353	*ttrP127*::Tn*10*dTPOP2	This study
TT22354	*ttrP127*::Tn*10*dTPOP2 *ttrA121*::MudJ	This study
TT22355	*ttrP128*::Tn*10*dTPOP2	This study
TT22356	*ttrP128*::Tn*10*dTPOP2 *ttrA121*::MudJ	This study
TT22470	*ttrR132*(del)::Cm(swap)	This study
TT22471	*ttrR132*(del)::Cm(swap) *ttrB123*::MudJ	This study
TT22472	*ttrR132*::Cm(swap) *ttrS126*::Tn*10*dTPOP2	This study
TT22473	*ttrR132*::Cm(swap) *ttrS126*::Tn*10*dTPOP2 *ttrA120*::MudJ	This study
TT22359	*ttrB129*::Tn*10*dTPOP2	This study
TT22360	*ttrB129*::Tn*10*dTPOP2 *ttrA120*::MudJ	This study
TT22339	*ttrC131*::Tn*10*dTPOP2	This study
TT22361	*ttrC131*::Tn*10*dTPOP2 *ttrA121*::MudJ	This study

[a] Swap refers to a resistance determinant that replaces a substantial amount of the target gene sequence.
[b] SGSC, *Salmonella* Genetic Stock Center.

insertion site. This was done to reconstruct any possible promoters associated with insertion (*ttrS130*::Tn*10*dCm). The first 40 bases of primer 3 are homologous to the 40 bases immediately outside the upstream end of the *ttrS* gene predicted by Hensel et al. (GenBank accession number AJ224978). The next 20 bases of this primer are the same Cm^r sequence used in primer 1 described above. The first 40 bases of primer 4 correspond to the *ttrS* sequence immediately 5′ to the Shine-Dalgarno sequence of the *ttrR* gene. The next 20 bases of this primer were designed for the amplification (from *ttrS130*::Tn*10*dCm) of the entire Cm^r marker, the adjacent Tn*10* material, and 10 bases of downstream *ttrS* sequence. This same technique was used to replace the entire *prp* operon (23) (*prpRBCDE*) with a Cm^r cassette from pACYC184.

Nucleotide sequence accession number. The accession number for the *ttr* operon sequence and the location of insertions in this sequence are available from GenBank (accession number AF282268).

RESULTS

Fermentation of 1,2-propanediol and ethanolamine. Ethanolamine is degraded by B_{12}-dependent conversion to acetyl coenzyme A (acetyl-CoA), which can enter the tricarboxylic acid (TCA) cycle and the glyoxalate shunt; this is diagrammed in Fig. 2 and has been reviewed previously (47). Propanediol is converted to propionyl-CoA, joined to oxaloacetate (the 2-methyl-citrate pathway), and converted to succinate plus pyruvate, which can be converted to acetyl-CoA and enter the standard TCA cycle (23, 24, 56, 60). In the absence of any electron acceptor, ethanolamine and propanediol might be fermented, providing an ATP source by conversion of acetyl-CoA (or propionyl-CoA) to acetyl-PO_4 (or propionyl-PO_4) and thence to acetate (or propionate) plus ATP. The latter reactions could be performed by the Ack and Pta enzymes or by similar enzymes encoded by the *eut* and *pdu* operons (5, 31). Excess reducing equivalents generated by conversion of acetaldehyde (or propionaldehyde) to acetyl-CoA (or propionyl-CoA) could, in principle, be balanced by reducing some aldehyde to ethanol (or propanol) and excreting the alcohol. This scheme might allow ethanolamine and propanediol to provide

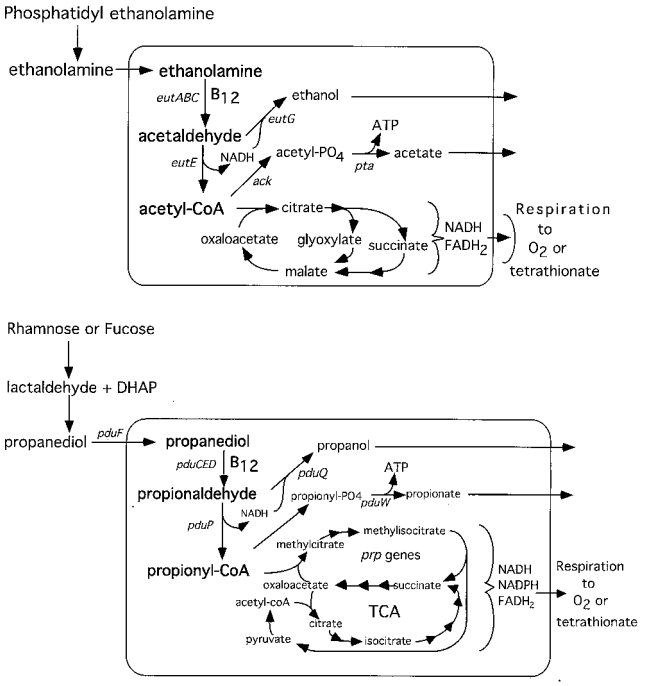

FIG. 2. Metabolism of ethanolamine and of propanediol. The upper part of this diagram outlines the known metabolism of ethanolamine and indicates the proposed role of various proteins encoded by the ethanolamine (*eut*) operon (31). The lower part of the diagram outlines the known metabolism of propanediol as described in references 5 and 24.

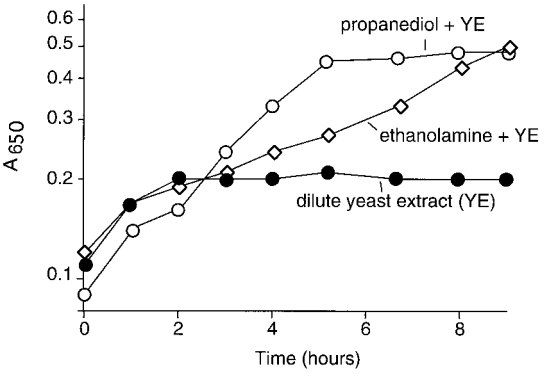

FIG. 3. Stimulation of anaerobic growth by ethanolamine or propanediol. Cells of wild-type serovar Typhimurium, strain LT2, were grown anaerobically on minimal NCE medium supplemented with 0.2% yeast extract (YE) to provide a carbon source with or without 80 mM propanediol or 98 mM ethanolamine as an energy source.

an energy source without respiration (fermentative growth). When this was tested, *Salmonella* seemed unable to ferment either ethanolamine or propanediol for use as both a carbon and energy source (see below).

Fermentative growth on ethanolamine or propanediol envisioned above depends on excretion of carbon. Perhaps fermentation might provide energy if some additional source of carbon was provided. To test this, dilute yeast extract was provided at a concentration (0.2%) that could not support anaerobic growth alone. Growth with this added carbon source was stimulated by propanediol or ethanolamine (Fig. 3).

The fermentative growth on ethanolamine or propanediol (facilitated by added carbon sources) was eliminated by mutations which block B_{12} synthesis (*cob*); addition of B_{12} overcame the effect of the *cob* mutation (data not shown). As can be seen in Fig. 3, the growth rate was lower on ethanolamine than on propanediol; this deficit was corrected by addition of B_{12} (data not shown). This B_{12} limitation is expected, since the B_{12} synthesis genes (*cob*) are induced by propanediol but not by ethanolamine (7, 10, 45). Thus, when ethanolamine alone is provided, the level of endogenous B_{12} may limit growth. Mutations in the *eut* operon eliminated the ethanolamine stimulation, and mutations in the *pdu* operon eliminated growth on propanediol. Thus, the inferred fermentative use of ethanolamine and propanediol as energy sources seems to involve the standard degradative pathways. (Data on use of endogenous B_{12} are given below.)

Qualitative tests of respiratory electron acceptors. *Salmonella* is unable to use propanediol or ethanolamine as the sole carbon and energy source under anaerobic conditions even with the alternative respiratory electron acceptor fumarate, dimethyl sulfoxide, or trimethylamine *N*-oxide; very slight growth was seen with nitrate (data not shown). This result was seen both on solid media and when growth was scored qualitatively (as plus or minus) in anaerobic tubes. As expected, all of these electron acceptors allowed anaerobic growth on glycerol. However, only tetrathionate allowed strong anaerobic growth on all three carbon sources. No electron acceptor was required for growth on the fermentable carbon source glucose.

Growth of wild-type strains with tetrathionate. Growth was initially measured in anaerobic liquid cultures by monitoring the increase in optical density (OD) at 650 nm. Tetrathionate supported anaerobic growth on acetate, demonstrating that it is truly serving as a respiratory electron acceptor (Table 2). As can be seen in Fig. 4A and B, tetrathionate supported anaerobic growth on ethanolamine plus B_{12} and on propanediol. (Reasons for supplying B_{12} for ethanolamine tests are described below.) In the absence of an electron acceptor, none of these cultures reached an OD in excess of 0.01. Similarly no growth was seen on tetrathionate alone, in the absence of added carbon source. In agreement with previous qualitative results, some growth was seen with nitrate as an electron acceptor.

Late in anaerobic growth on tetrathionate, a precipitate formed. To be sure that the OD measurements reflected cell growth and not this precipitate, we monitored the increase in viable cells (CFU) and the increase in visible cells (examined with a microscope). These measurements (Fig. 4C and D) generally reflected the OD measurements through most of the growth period but revealed some important differences. This precipitate was not observed during growth on nitrate.

On ethanolamine, the OD increase for the first 40 h on tetrathionate reflected the increase in cell number or viable counts; thereafter, OD increased without a parallel increase in cell number. This late discrepancy was not seen during growth on NO_3. Some of the late OD increase was due to a precipitate that was visualized with a microscope both as smaller refractile granules associated with 5 to 10% of the cells in the culture (Fig. 5B) and as larger, extracellular, granules (Fig. 5C). How-

TABLE 2. Effect of mutations on anaerobic growth of *S. enterica* serovar Typhimurium

Strain[a]	Relevant genotype	Relevant phenotype	Growth under indicated conditions[b]							
			Glycerol		Acetate		Ethanolamine[c]		Propanediol	
			Solid[d]	Liquid[e]	Solid[d]	Liquid[e]	Solid[d]	Liquid[e]	Solid[d]	Liquid[e]
TT22362	Wild type	Ttr$^+$ Eut$^+$ Pdu$^+$	+	1.5	+	4.3	+	4.3	+	5.4
TT22341	*eut-336* (*eutS-K*)	Deletion of *eut* operon	+	1.7	+	5.5	−	—[f]	+	14
TT22342	*aceA112*::MudJ	Isocitrate lyase negative	+	1.6	−	21	−	20	+	4.1
TT22343	*aceB113*::MudJ	Malate synthase A negative	+	1.5	−	31	−	44	+	4.1
TT22469	*ack-408*::Tn*10*	Acetate kinase negative	+	1.5	±	26	±	10	+	5.4
TT22344	*pdu-12*::MudA	Propanediol negative	+	1.5	+	4.8	+	4.9	−	—[f]
TT22474	*prp-54*(*prpR-E*)	Propionate negative	+	1.5	+	4.0	+	3.8	−	83
TT22359	*ttrB129*::Tn*10*dTPOP2	Tetrathionate reductase negative	−	19	−	24	−	13	−	12
TT22338	*oxrA*::Tn*10*	OxrA negative (Fnr$^-$)	−	10	−	10	−	17	−	17
TT22340	*phs-213*::MudJ	Thiosulfate reductase negative	+	1.6	+	4.2	+	4.1	+	5.4

[a] See Table 1.
[b] The growth media were NCE minimal medium supplemented with tetrathionate and the indicated carbon and energy sources. Growth rates were estimated by monitoring optical density and microscopic counts. All strains were able to grow anaerobically on solid media with glucose; TT22362 (wild type), TT22359 (*ttrB*), and TT22338 (*oxrA*) were also grown in liquid cultures with glucose; in each case, the doubling time was measured to be 1.2 h.
[c] Ethanolamine liquid cultures also contained 0.2 μM cyano-B_{12}.
[d] Anaerobic growth phenotype for strains grown on solid media after 72 h with 10 mM tetrathionate and the indicated carbon source as described in Materials and Methods. Symbols: +, wild-type growth; ±, slight growth; −, no visible growth.
[e] Doubling times (in hours) calculated for the indicated strain grown anaerobically with 40 mM tetrathionate on the indicated carbon source in liquid culture as described in Materials and Methods.
[f] A slight drop in optical density was seen with time.

ever, some of the OD increase reflected the accumulation of biomass, since cells grown on tetrathionate form long chains which are counted as single cells in both the microscopic and viable-cell enumerations (Fig. 5A and B). During growth on ethanolamine plus tetrathionate, 10 to 20% of the cell units are present as chains; these are short at the earlier stages of growth and increase to an average length of 10 cells (range, 6 to 14) as cells enter stationary phase. Cells in these chains appear more

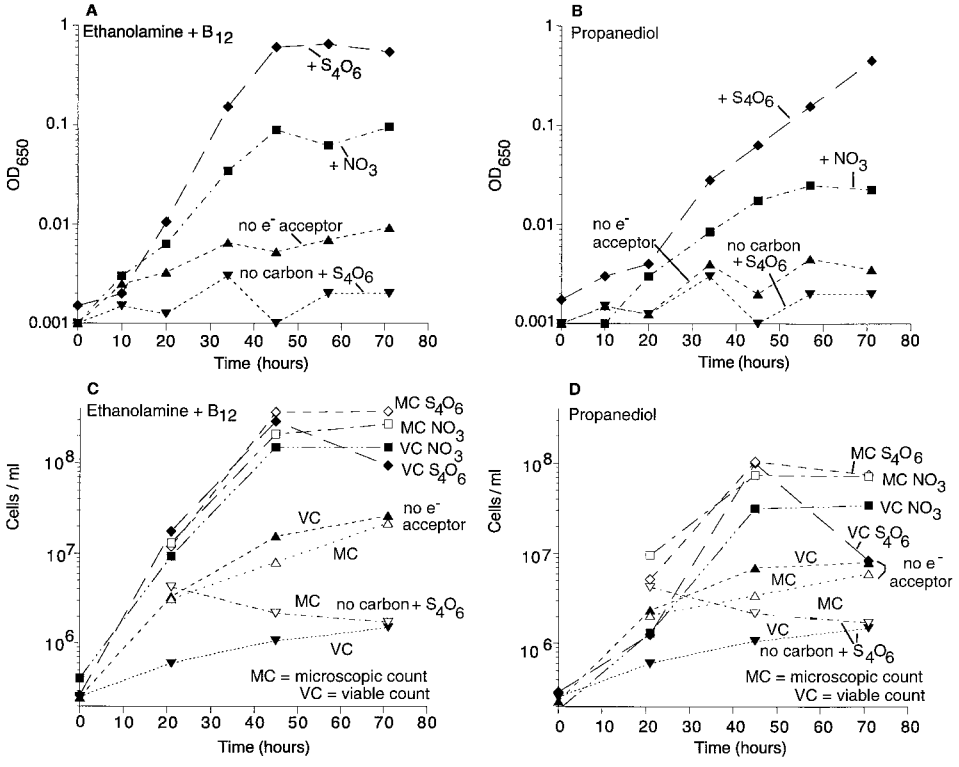

FIG. 4. Anaerobic growth on ethanolamine plus B_{12} or on propanediol with the electron acceptor tetrathionate (S_4O_6) or nitrate (NO_3). Additions to NCE minimal medium were as follows: sodium tetrathionate (40 mM) or potassium nitrate (10 mM), ethanolamine (10 mM), and B_{12} (0.2 mM) (A and C) or propanediol (50 mM) (B and D). Growth was monitored on the basis of absorbance at 650 nm (A and B), viable cell counts (filled symbols in panels C and D), and microscopic counts (open symbols in panels C and D) using a Petroff-Hausser bacterial cell counter (C and D). The data for cells grown with S_4O_6 but no carbon source are replotted in graphs A and B (for turbidity) and C and D (for microscopic and viable cell counts).

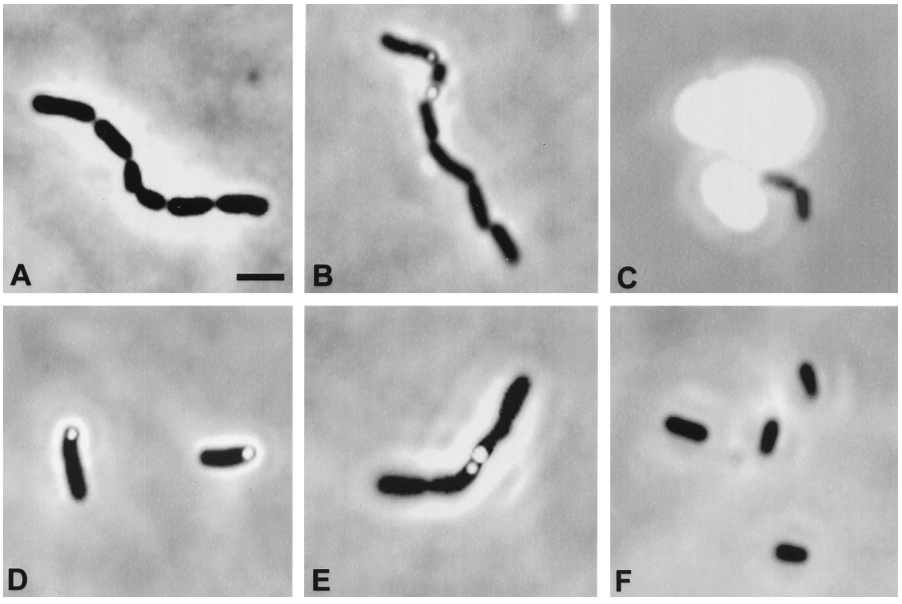

FIG. 5. Cell chain and granule formation during growth of wild-type serovar Typhimurium on ethanolamine or propanediol plus tetrathionate. Cells were viewed on a Zeiss Axioplan phase-contrast microscope. The scale bar in panel A represents 2 μm, and all photos are at the magnification indicated in panel A. Photographs are from stationary-phase cultures grown as described in the legend to Fig. 4. Panels A to C show cells (A and B) and refractile granules (B and C) observed in an ethanolamine-tetrathionate culture after 44 h of incubation at 37°C. (D and E) Cells and refractile granules observed in propanediol-tetrathionate culture after 67 h of incubation at 37°C. (F) Cells grown on propanediol plus NO_3. Neither cell chains nor refractile granules were seen in either ethanolamine- or propanediol-grown cultures when nitrate was the electron acceptor.

rounded and distinct than in typical rod-like cell filaments (with or without septa) formed by *Salmonella* during SOS induction. Thus, OD measurements of growth on ethanolamine with tetrathionate appear to overestimate cell number (because of the precipitate), and the cell counts underestimate biomass accumulation due to the presence of chains. The precipitate that is seen late in the growth experiment appears to be elemental sulfur (see below).

On propanediol, roughly 90% of the OD increase that is observed after 40 h of growth is not paralleled by an increase in cell number. The increase seems to be due to changes in absorption or refractivity of cells that are forming short chains (four to six cells long) and accumulating cell-associated refractile granules (see below and Fig. 5D and E). Approximately 80% of the cells in these cultures had associated refractile granules.

Neither cell chains nor refractile granules were seen during growth on ethanolamine or propanediol when nitrate was used as an electron acceptor. Cells grown on propanediol and nitrate are shown in Fig. 5F.

A surprising aspect of the data shown in Fig. 4 is the observation that cells growing with tetrathionate are dying (but not lysing) late in the growth period. This loss of viability was seen for all carbon sources whenever tetrathionate was used as the electron acceptor; it was not seen during growth on nitrate. The loss of viability is temporally associated with appearance of the small cell-associated refractile granules that appear to be within cells but that could be on their surface (Fig. 5B, D, and E).

In Fig. 4D it can be seen that tetrathionate provides only about a 10-fold increase in viable cell number on propanediol. This growth yield is increased severalfold when glutamate is added (data not shown). We propose that accumulated intermediates in the methyl-citrate pathway (for propanediol degradation) inhibit the TCA cycle, limiting synthesis of glutamate and related amino acids.

Nature of the granules appearing in tetrathionate-grown cultures. A variety of bacteria are known to produce elemental sulfur granules during growth involving oxidation or reduction of sulfur compounds (36, 50). This suggested that *Salmonella* might deposit sulfur to form the granules that appear within and outside of cells during growth on tetrathionate. To test this, we assayed elemental sulfur by the method of Hazeu et al. (19) as described in Materials and Methods. The method detects sulfur that can be removed by centrifugation (inside or outside of cells); it does not detect dissolved sulfides. The assays revealed that, late during anaerobic growth on tetrathionate, elemental sulfur was accumulating in cells and as precipitable material to a concentration of about 1 mM (based on the volume of the culture).

Anaerobic respiration of ethanolamine and propanediol requires endogenous cobalamin. Tetrathionate appears to provide conditions that allow *S. enterica* serovar Typhimurium to use ethanolamine and propanediol under anaerobic conditions, the conditions under which cobalamin is synthesized. Mutants blocked in B_{12} synthesis were used to show that anaerobic growth on these carbon sources did, in fact, require endogenous B_{12}. These experiments revealed some unexpected things.

Wild-type cells (Fig. 6A) grow anaerobically on ethanolamine plus tetrathionate without any added cobalamin. Growth rate is stimulated twofold by added cyano-B_{12} or by the intermediate cobinamide (Cbi). The limited growth on endogenous B_{12} may reflect the fact that the B_{12} synthetic

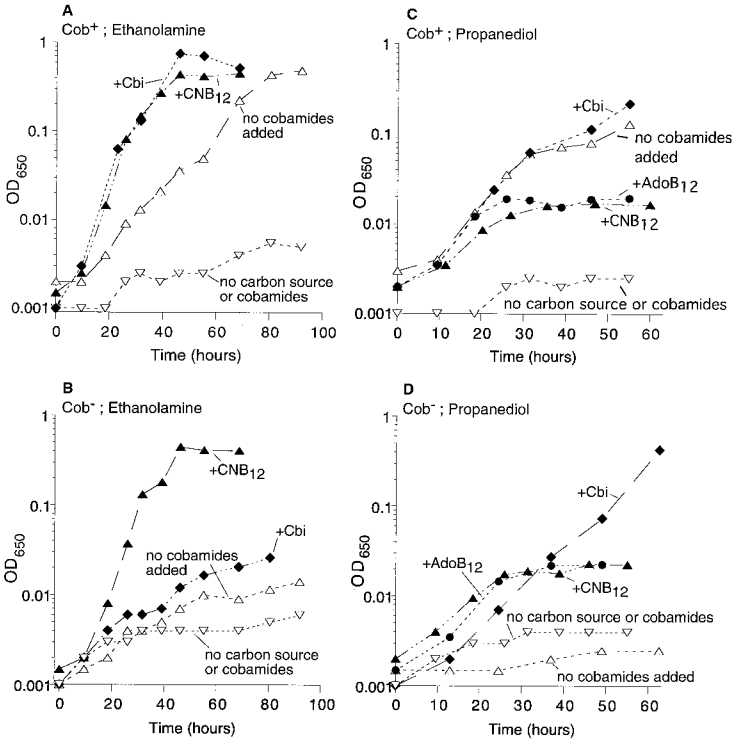

FIG. 6. Effect of cobamides on anaerobic growth on ethanolamine or propanediol. Wild-type serovar Typhimurium (A and C) and a mutant with an insertion in *cbiD* (Cob^-) (B and D) were grown anaerobically on ethanolamine plus tetrathionate (A and B) or propanediol plus tetrathionate (C and D). Additions were as follows: cyano-B_{12} (0.2 μM in panels A, B, and D; 15 nM in panel C), cobinamide dicyanide (15 nM), and $AdoB_{12}$ (15 nM).

operon is induced by propanediol but not by ethanolamine; thus, during growth on ethanolamine, B_{12} synthesis relies on a repressed *cob* operon. A mutant blocked prior to Cbi in B_{12} synthesis did not grow without added cobamides (Fig. 6B). Growth was stimulated by cyano-B_{12} but not by the intermediate Cbi. This probably reflects the fact that ethanolamine does not induce the *cob* operon (see below), and the *cob* mutation used (*cbiD24*) is a polar insertion that reduces the expression of genes required for conversion of Cbi to $AdoB_{12}$. These growth experiments used OD_{650} to monitor growth; turbidity increases in these experiments, corresponded closely to microscopic cell counts.

On propanediol, which induces the *cob* operon, wild-type cells grew best without added B_{12} or with added Cbi (Fig. 6C). Surprisingly, their growth yield was inhibited by exogenous cyano-B_{12}. Growth was monitored with cyano-B_{12} added to make final concentrations of 2, 15, and 200 nM. Inhibition is seen even at cyano-B_{12} levels (2 and 15 nM) insufficient to repress the *cob* operon (2), making it unlikely that B_{12} represses a function encoded by the *cob* operon and essential to propanediol catabolism (Fig. 6C). It seemed possible that excess added cyano-B_{12} might be inhibiting propanediol dehydratase (as seen in vitro), but added $AdoB_{12}$ also reduced growth yield (Fig. 6C). A mutant blocked in cobalamin synthesis showed no growth on propanediol without cobalamin (Fig. 6D), and growth was fully restored by adding Cbi. Added cyano-B_{12} or $AdoB_{12}$ corrected growth only to the level seen for inhibited wild-type cells (Fig. 6D).

All of the inhibitory effects noted above involve addition of forms of B_{12} with dimethyl benzimidazole (DMB) as the lower ligand. Recently, Keck and Renz reported that *Salmonella* cannot synthesize DMB anaerobically and makes pseudo-B_{12} (adenine as lower ligand) under these conditions (29). Based on this finding, the best growth seen here is supported by endogenous pseudo-B_{12}. The growth inhibition data suggest that anaerobic propanediol utilization is inhibited by DMB-containing cofactors. Consistent with this possibility, addition of DMB inhibits anaerobic growth of wild-type cells on propanediol plus tetrathionate (data not shown). The strong stimulation by Cbi may reflect its conversion to pseudo-B_{12}. This conversion may be less sensitive to the polarity effects that impaired use of Cbi during ethanolamine growth.

Defining the pathways of ethanolamine and propanediol respiration by mutant phenotypes. The present view of the aerobic degradative pathways for ethanolamine and propanediol is outlined in Fig. 2. However, the anaerobic respiration of these compounds has not been investigated. Table 2 presents effects of various mutations on anaerobic growth using tetrathionate as an electron acceptor in solid and liquid media. The results on solid media are extremely clear—strong growth on tetrathionate and no visible growth seen for conditions indicated by a minus sign. In liquid medium, some increase in turbidity and cell number was measured for conditions that produced no growth on plates. The difference may reflect the observation that cells are dividing to form smaller and smaller cells under these starvation conditions and may do

so with very little increase in biomass (dividing down). This increases turbidity and cell number but is not apparent on the plates.

Anaerobic growth on ethanolamine requires genes of the *eut* operon and the glyoxalate shunt (*aceAB*); this is also true for growth under aerobic conditions (Tom Fazzio, personal communication). The *ack* and *pta* genes (converting acetyl-CoA to acetate and producing ATP) are required for aerobic use of ethanolamine (Tom Fazzio, personal communication). However under anaerobic conditions with tetrathionate, an *ack* mutation caused only a partial loss of growth ability (Table 2), suggesting that the *ack* and *pta* genes are not absolutely required anaerobically.

Anaerobic growth on propanediol requires enzymes encoded by the *pdu* operon (which convert propanediol to propionyl-CoA) and some proteins from the *prp* operon, presumably those that convert propionyl-CoA to succinate plus pyruvate (23, 24, 57). A deletion of the entire *prp* operon (*prp-54*) eliminated growth on propanediol but made cells sensitive to growth inhibition by propionate on other carbon sources (J. Tittensor, unpublished results); we suspect that this is due to accumulated propionyl-CoA, which has previously been seen to inhibit growth (59). Thus, the failure of *prp* mutants to use propanediol could be due to lack of the *prp* pathway or to inhibition by accumulated propionyl-CoA. The glyoxalate shunt (*aceA* and *aceB* genes) is not required for use of propanediol.

Mutants unable to reduce tetrathionate (*ttr* [described below]) cannot use tetrathionate to support growth on any of the tested carbon sources whose utilization requires an electron acceptor (Table 2). Growth is also eliminated by *oxrA* (*fnr*) mutations, since this regulator is required for induction of the *ttr* operon (see below) (21). The pathway for tetrathionate reduction, illustrated in Fig. 1, suggested a requirement for subsequent steps in sulfur reduction (*phs*, *asr*). These subsequent steps are not required under the conditions tested here, since a *phs* insertion mutant grows normally.

Mutations (*ttr*) causing a defect in tetrathionate reduction. The results below confirm and support those of Hensel et al. (21, 22), which were reported while this work was in progress. A large set of mutants unable to reduce tetrathionate was isolated by using MudJ, Tn*10*dTc, Tn*10*dCm, and TPOP elements (see Materials and Methods). These mutations all affected a single region (*ttr*) whose chromosomal position was confirmed by transductional linkage to markers near the previously determined position of *ttr* mutants (9). The region includes an operon of three structural genes for enzymes, all of which are required for tetrathionate reduction, and two genes that are essential to expression of the three-gene operon. These regulatory proteins are homologous with proteins that are part of known two-component regulatory systems (21). Our mutant set included insertions in all genes except the small *ttrR* gene, for which a deletion was constructed as described below. All sequenced insertion mutations are described in GenBank under accession number AF282268. Insertions near the *ttr* operon were isolated to obtain mutations in immediately adjacent open reading frames; the *ttr*$^+$ phenotypes of the *rkh* and *nth* mutants ensured that these adjacent genes are not essential to tetrathionate reduction.

In Fig. 7, a map of the *Salmonella ttr* region is compared to the analogous region of the *E. coli* chromosome, which lacks *ttr*. It should be noted that the *Salmonella ttr* operon is part of a larger block of genes absent from *E. coli* and is near another such region (SPI2) that is unique to *Salmonella*. A small open reading frame shared by both *S. enterica* and *E. coli* is located between these two regions, suggesting that distinct genetic events account for the presence of the flanking sequence blocks.

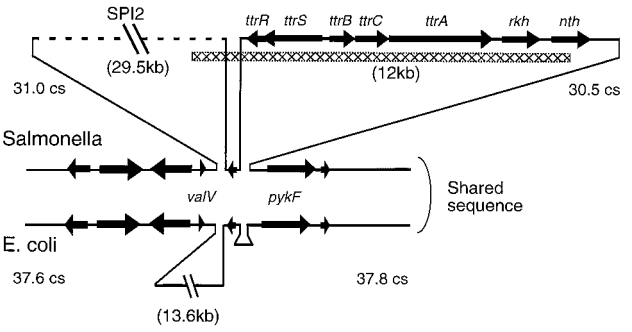

FIG. 7. Map of the Sallmonella *ttr* region and analogous region of the *E. coli* chromosome. Regions present in only one genome are represented as raised triangles. The map is not to scale; the sizes of various fragments, in kilobases, are shown in parentheses. The SPI2 region is represented by a dashed line to indicate its foreign evolutionary origin. Genes are represented by arrows that point in the direction of transcription. The hatched box represents the region of the *Salmonella* chromosome that was sequenced during the course of this work.

Global regulation of the *ttr* operon. By use of fusions to the *lac* operon formed by *ttr*::MudJ insertions, conditions for induction of the operon were tested. Results in Tables 3 and 4 show that, under aerobic conditions, tetrathionate causes a slight induction (5- to 10-fold), but its major inductive effect (100- to 900-fold) occurs anaerobically. The requirement for anaerobic conditions is mediated by the OxrA protein, homologous to Fnr of *E. coli* (16). An insertion in the *oxrA* gene eliminated anaerobic induction of a *ttr*::MudJ fusion by tetrathionate (Table 3) and prevented anaerobic growth on ethanolamine and propanediol (Table 2). Neither the ArcA global regulator (also responding to anaerobic conditions) nor the Crp-cyclic AMP system (responding to carbon starvation) affected *ttr* operon induction.

When *Salmonella* grows in anaerobic nitrate medium, synthesis of other anaerobic respiratory enzymes is transcriptionally down-regulated by a pair of two-component sensor-response regulatory systems—NarX-NarL and NarQ-NarP. This regulation has been studied extensively and is reviewed in references 53, 54, and 55. Nitrate reduced *ttr* operon expression three- to fivefold (Table 3). For other promoters regulated by NarL-NarP, variable numbers of binding sites [the heptad repeat: TAC(c/t)N(a/c)T] are found within the first 200 bases upstream of the transcriptional start sites (55, 58). A search of the *ttr* control region (between the divergent *ttrS* and *ttrB* genes) revealed none of these sequence elements. Several poor matches are located near the region of overlap between the regulatory genes *ttrR* and *ttrS*, a region not thought to have promoters (see below). It is unclear how *ttr* operon transcription is down-regulated by nitrate. The small effect of nitrate on

TABLE 3. Global control of *ttr* expression

Strain	Relevant mutation[a]	β-Galactosidase levels in *ttr-lac* fusion strains grown as indicated[b]									
		Aerobic				Anaerobic					
		Glucose		Glycerol		Glucose				Glycerol	
		Alone	+ tetrathionate	Alone	+ tetrathionate	Alone	+ nitrate	+ tetrathionate	+ nitrate and tetrathionate	+ nitrate	+ nitrate and tetrathionate
TT18691	*ttrB1221*::*MudJ*	2	20	4	34	7	4	1,729	310	5	1,010
TT20444	*phs-213*::MudJ	2	25	4	63	9	5	2,505	559	4	1,298
TT20590	*arcA201*::Tn*10*dTc	3	24	3	46	4	3	1,530	240	3	628
TT20464	*cya-961*::Tn*10*	3	20	4	42	6	6	1,578	414	4	952
TT20469	*crp-773*::Tn*10*	3	21	4	45	5	5	1,496	573	4	1,152
TT18694	*ttrB123*::Mud-*lac*	1	20	4	44	6	3	1,775	376	5	863
TT22347	*oxrA2*::Tn*10* (*fnr*)	2	24	3	37	4	6	52	46	NT[c]	NT

[a] The first six strains carry the *ttrB122*::MudJ insertion, which fuses transcription of *lac* operon to the *ttr* promoter. The last two strains carry *ttrB123*::MudJ; this insertion lies 123 bases downstream of *ttrB122*::MudJ and also fuses transcription of the *lac* operon to the *ttr* promoter.

[b] The level of β-galactosidase activity is presented in units defined by Miller (37). Carbon source concentrations and growth conditions are as described in Materials and Methods; nitrate was used at 20 mM, and tetrathionate was used at 10 mM.

[c] NT, not tested.

ttr expression may suggest that tetrathionate serves as an electron acceptor whose quality is comparable to that of nitrate.

Proximal inducers of the *ttr* operon. Tetrathionate was the most effective inducer of the compounds tested (Table 4). The inducer seems to be tetrathionate per se, rather than its reduction products, because the strains used in these tests carry a *ttr*::MudJ insertion and are unable to reduce tetrathionate. Furthermore, mutations in the *phs* genes, which prevent further reduction of thiosulfate (Fig. 1), had little effect on induction by tetrathionate (Table 3). Sulfite (SO_3^{2-}) and thiosulfate ($S_2O_3^{2-}$) caused very little induction, even at high concentrations (Table 4); the small effects seen could reflect internal production of tetrathionate.

Mechanism of proximal *ttr* control. The two regulatory genes (*ttrS* and *ttrR*), which overlap by 26 bases (see Fig. 8), encode proteins similar to those of two-component regulatory systems (40) and are responsible for the tetrathionate-specific regulation of the *ttr* operon (21). The TtrS protein resembles sensor kinases, which (in other systems) act to phosphorylate another protein in response to a regulatory stimulus. The TtrR protein resembles these responsive regulatory proteins. Alignment of the TtrS sequence with that of known sensor kinases reveals two functional domains. The C-terminal sensory kinase domain (residue 325 to the end) includes all of the motifs required for autokinase activity (N box, G1 box, F box, and G2 box). The N-terminal domain of TtrS (residues 1 to 358) shows little homology to any gene in GenBank but contains the H box (autophosphorylation site) and is most likely involved in sensing tetrathionate. To examine the role of these proteins, we assayed the effects of mutations in the *ttrR*, *ttrS,* and promoter regions on transcription of the structural genes (*ttrBCA*). Results are shown in Table 5; normal regulation is shown in lines 1 and 10. These data support the model presented by Hensel et al. (21) and outlined in Fig. 8.

Since no *ttrR* mutants emerged during our search for mutants, a deletion was constructed which removes almost all of the *ttrR* gene but retains all *ttrS* sequences (see Materials and Methods). This mutant is unable to induce operon expression, suggesting that the response regulator works as a positive effector for transcription (Table 5, line 2). A similar conclusion was reached by Hensel et al. on the basis of very different experiments (21).

Insertions of Tn*10*dTc at either of two sites within the *ttrS* gene (Fig. 8) prevent operon induction and cause a complete Ttr^- growth phenotype (Table 5, lines 3 and 4 and footnote *d*). One sensor kinase insertion (*ttrS117*) (Table 5, line 3) disrupts the downstream kinase domain (close to the conserved G2 box); the other (*ttrS108*) (Table 5, line 4) is located in the upstream sensor domain (Fig. 8).

Surprisingly, a Tn*10*dCm insertion (*ttrS130*) in the middle of the *ttrS* gene caused constitutive operon expression (independent of tetrathionate), and cells remained Ttr^+ (Table 5, line 5 and footnote *d*; Fig. 8). This insertion is upstream of the kinase domain; it appears to cause tetrathionate-independent induction of the *ttrBCA* operon via TtrR, since expression requires anaerobic conditions and OxrA protein (Table 5, line 8). The constitutive phenotype is independent of the sensor domain since expression is unaffected by adding the upstream sensor insertion (*ttrS108* in Table 5, line 6). A double mutant with the constitutive Tn*10*dCm element and the downstream

TABLE 4. Inducers of *ttr* operon transcription

Growth conditions	β-Galactosidase levels in a *ttr-lac* fusion strain grown in minimal medium with the following addition[a]					
	None	Sulfite (1 mM)	Sulfite (10 mM)	Thiosulfate (6 mM)	Thiosulfate (18 mM)	Tetrathionate (10 mM)
Glucose + O_2	2	2	2	4	5	20
Glycerol + O_2	3	3	NT	3	25	34
Glucose − O_2	5	7	92	55	15	1,729

[a] The level of β-galactosidase activity is presented in units defined by Miller (37). The strain tested (TT18691) carries the insertion *ttr-122*::MudJ, which fuses *lac* operon transcription to the *ttr* promoter. Sulfite, thiosulfate, and tetrathionate were used as sodium salts at the concentrations indicated. NT, not tested.

TABLE 5. Mutations that affect proximal regulation of the *ttr* operon

Strain	Relevant genotype[a]		β-Galactosidase level in cells grown as indicated[b]			
			Aerobic		Anaerobic	
	ttr mutation tested[d]	*lac* fusion	Medium alone	Medium plus tetrathionate	Medium alone	Medium plus tetrathionate
			− Tc (+Tc)	− Tc (+Tc)	− Tc (+Tc)	− Tc (+Tc)
TT18694	*ttrB123*::MudJ	A	1	23	3	1,775
TT22471	*ttrR132*(del)::Cm(swap)	A	3	3	3	4
TT20440	*ttrS117*::Tn*10*dTc	A	1	2	6	12
TT20433	*ttrS108*::Tn*10*dTc	A	1	2	7	7
TT22348	*ttrS130*::Tn*10*dCm	A	2	2	815	297
TT22350	*ttrS130*::Tn*10*dCm *ttrS108*::Tn*10*dTc	A	2	2	325	106
TT22352	*ttrS130*::Tn*10*dCm *ttrS117*::Tn*10*dTc	A	2	2	5	5
TT22467	*ttrS130*::Tn*10*dCm *oxrA2*::Tn*10*	A	3	3	19	11
TT22373	*ttrS133*(del)::Cm(swap)[c]	A	4	4	1,545	141
TT18683	*ttrA121*::MudJ	B	3	25	13	1,734
TT22356	*ttrP128*::Tn*10*dTPOP2	B	34 (1,438)	10 (1,455)	17 (724)	12 (1,676)
TT22354	*ttrP127*::Tn*10*dTPOP2	B	9 (216)	6 (139)	10 (51)	8 (163)

[a] All strains carried a *ttr*::MudJ insertion that fuses *lac* transcription to the *ttrBCA* promoter. Strains designated as A carry *ttrB123*::MudJ; those designated B carry *ttrA121*::MudJ.

[b] The level of β-galactosidase activity is presented in units defined by Miller (37). Enzyme levels in parentheses were observed in cells grown in the presence of the antibiotic tetracycline (Tc), which induces promoters within the TPOP transposon.

[c] All of the coding sequence unique to *ttrS* was removed and replaced by the chloramphenicol resistance gene and distal Tn*10*dCm transposon, followed by the 10 bases adjacent to the distal end of this Tn*10*dCm element in strain *ttrS130*::Tn*10*dCm.

[d] Each of the *ttr* control mutations was tested for its effect on anaerobic acid production and growth in strains carrying a wild-type *ttrBCA* operon. The wild-type strain (LT2), as well as TT20369 (*ttrS130*::Tn*10*dCm), TT22349 (*ttrS108*::Tn*10*dTc *ttrS130*::Tn*10*dCm), and TT22372 [*ttrS133*(del)::Cm(swap)], formed red patches (produced acid) when grown on MacConkey-tetrathionate (10 mM) medium. All other strains formed white patches. Strains with an insertion in the promoter region (*ttrP128*::Tn*10*dTPOP2 or *ttrP127*::Tn*10*dTPOP2) formed red patches when grown on this medium in the presence of the antibiotic tetracycline (Tc), which induces promoters within the TPOP transposon. Growth was tested anaerobically on solid medium with propanediol or ethanolamine as the sole carbon source and 10 mM tetrathionate; LT2, as well as TT20369 (*ttrS130*::Tn*10*dCm), TT22349 (*ttrS108*::Tn*10*dTc, *ttrS130*::Tn*10*dCm), and TT22372 [*ttrS133*(del)::Cm(swap)], produced visible patches on this media. All other strains failed to grow under these conditions. One of the strains with an insertion in the promoter region (*ttrP128*::Tn*10*dTPOP) produced a visible patch when grown on this medium in the presence of tetracycline.

Tn*10*dTc insertion, in the kinase domain, showed no operon expression (Table 5, line 7).

These results could be explained by a promoter within the Tn*10*dCm element that expresses a shorter kinase domain, which can activate TtrR without tetrathionate; alternatively, this promoter might transcribe *ttrR*, and the excess TtrR protein could be nonspecifically phosphorylated or could induce transcription without phosphorylation. Consistent with the idea of a promoter within the inserted material, the chloramphenicol resistance gene within the element is transcribed toward *ttrR,* and outward transcription from Tn*10*dCm elements has been seen in several operons. To determine whether the kinase domain of TtrS is essential for this constitutive phenotype, the entire *ttrS* gene was deleted and replaced with the Tn*10*dCm sequences likely to include a promoter. The insertion included the chloramphenicol resistance determinant, the

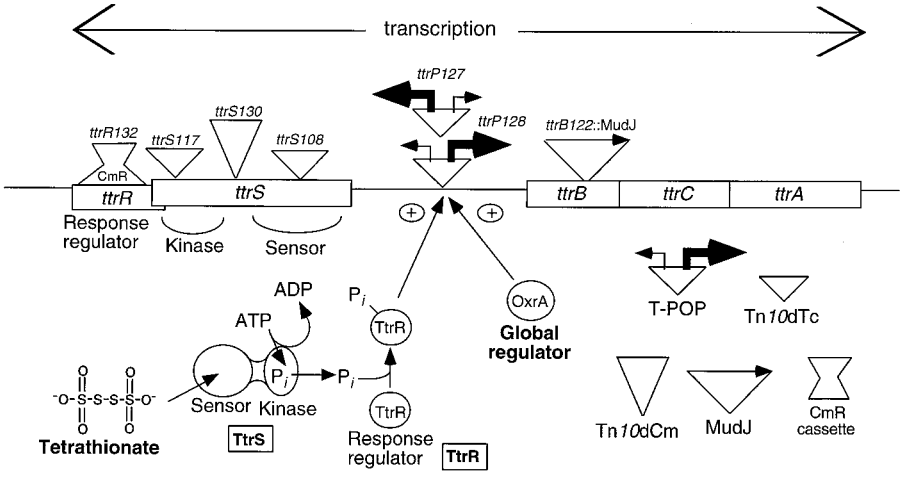

FIG. 8. Proximal regulation of the *ttrBCA* operon. The key to inserted elements is shown on the lower right. In the model presented below the map, tetrathionate is sensed by the TtrS protein, which autophosphorylates and then transfers the phosphate group to activate TtrR. Activated TtrR cooperates with the global regulator OxrA (Fnr) to positively regulate expression of the *ttrBCA* operon.

downstream region of the Tn*10*dCm element, and 10 bases from the *ttrS* gene immediately distal to insertion *ttrS130*. This inserted material (derived from *ttrS130*::Tn*10*dCm) resulted in a better constitutive phenotype without the *ttrS* (kinase domain) than that seen for the parent *ttrS130*::Tn*10*dCm element in the presence of this domain (Table 5, compare lines 5 and 9). This rules out dependence on the kinase domain and supports the idea that constitutive expression is due to overproduction of the TtrR protein. It is not understood why tetrathionate reduces expression of the *ttrBCA* operon in all of the strains carrying insertion *ttrS130*::Tn*10*dCm or parts thereof. The effect does not seem to depend on the sensor domain of TtrS, since it is seen in the swap strain lacking almost all *ttrS* coding sequence and in strains with an upstream *ttrS* insertion.

Two TPOP2 insertions within the regulatory region were found to be in opposite orientations at the same site 20 bp upstream of the *ttrBCA* operon transcriptional start site. The two ends of TPOP have outward-directed, tetracycline-inducible promoters of different strengths (44). The insertion that directs the stronger (*tetA*) promoter toward the structural genes, when induced by tetracycline, can provide a Ttr^+ phenotype as judged by both the acid production (on MacConkey medium) and anaerobic growth tests (Table 5, line 11 and footnote *d*). Induction of the other insertion, which directs the 10-fold-weaker (*tetR*) promoter across the *ttr* operon, provides acid production from tetrathionate but not anaerobic growth on ethanolamine or propanediol (Table 5, line 12 and footnote *d*). As expected, expression of *ttrBCA* in these two insertion mutants requires neither the global regulatory input (OxrA) nor a proximal inducer; induction is seen both aerobically and anaerobically. This demonstrates that tetrathionate use requires only expression of the *ttrBCA* genes; the two-component regulatory system does not appear to activate any unlinked genes required for use of tetrathionate.

DISCUSSION

The alternative electron acceptor tetrathionate allows *Salmonella* to grow anaerobically on ethanolamine or propanediol by using endogenously synthesized B_{12}. These are the only conditions we know under which wild-type *Salmonella* requires B_{12} for growth. Almost 2% of the *Salmonella* genome (88 genes) is dedicated to the metabolism discussed here. Synthesis and import of B_{12} requires at least 30 genes; the *eut*, the propanediol (*pdu*), and the proprionate (*prp*) operons contain 17, 23, and 5 genes, respectively. Operons for sulfur reduction are *ttr* (five genes), *phs* (five genes), and *asr* (three genes). We presume that the natural environment of *Salmonella* must frequently include anaerobic conditions with tetrathionate, ethanolamine, and/or propanediol; these conditions select for maintenance of the B_{12} synthesis (*cob*) genes. The importance of B_{12} to propanediol use is supported by the fact that propanediol induces the *cob* operon (7, 45). This complex of abilities is found in virtually every *Salmonella* isolate; most are absent from *E. coli*. Aspects of this complex are used to enrich for and identify salmonellae in natural isolates and distinguish them from *E. coli* (8, 21, 39, 41, 43). The metabolism described here appears to be an important aspect of a *Salmonella*-specific lifestyle.

The mechanism of tetrathionate reduction has not been studied extensively in *Salmonella* (4, 15). By analogy with similar systems in other bacteria, we presume that reduction of tetrathionate can support electron transport and generate a proton gradient. *Salmonella* can grow anaerobically on tetrathionate plus glycerol, and use of glycerol as a carbon source is known to require electron transport (15). Furthermore, tetrathionate allows anaerobic growth on acetate, the catabolism of which provides no means of substrate-linked phosphorylation. Surprisingly, tetrathionate can serve as an electron acceptor even in strains with mutations in synthesis of both ubiquinone and menaquinone (Tom Fazzio, personal communication), suggesting that tetrathionate reductase may accept electrons directly from NADH or $FADH_2$. It is not clear why tetrathionate should be superior to nitrate in supporting anaerobic growth on ethanolamine or propanediol or acetate.

Cell chains and lethality were noted at late stages of growth on tetrathionate. We suspect that these phenomena reflect toxic effects of thiosulfate, sulfite, or sulfide in the medium or sulfur accumulation within cells. *Salmonella* possesses the *phs* and *asr* systems, which can reduce thiosulfate completely to sulfide. A mutational block in the first step (*phs*) neither relieves nor exacerbates the toxicity. However, the effects of such mutants may be masked by the assimilatory thiosulfate reductase (CysM), which converts thiosulfate to sulfite, or by rhodanese, which can, in principle, convert thiosulfate to sulfite plus sulfide. While little work has been done on rhodanese in *Salmonella*, the activity has been reported in *E. coli* (18), and genomes of both *E. coli* and serovar Typhimurium include rhodanese homologues. Under natural conditions, toxic accumulations might be diluted more than was possible in the growth tubes used for these experiments.

A surprising aspect of anaerobic growth on tetrathionate was the appearance of sulfur granules. We presume that the sulfur granules are generated by the chemical reaction of sulfide ions with tetrathionate, which has been described previously (30). It is not known whether *Salmonella* enzymes contribute to this process. However, serovar Typhimurium (but not *E. coli*) can reduce mineral sulfur, and *Salmonella* mutants are known which fail in this (K. Nealson and D. Lies, personal communication; M. Price-Carter, unpublished results).

Most of the activities mentioned here are found in *Salmonella* but not in *E. coli*. (The ethanolamine operon is shared by both species.) We suggest that the *Salmonella* pattern evolved by acquisition of foreign genes and mutational loss of ancestral genes. The ability to synthesize B_{12} and catabolize propanediol was acquired by *Salmonella* (but not *E. coli*) as a single DNA fragment from an organism having a guanosine-plus-cytosine content and codon usage atypical for *Salmonella* (47). This occurred about 70 million years ago, perhaps 30 million years after the divergence of salmonellae and *E. coli* (33–35). In contrast, the *ttr* operon was probably carried by the common ancestor of *E. coli* and *Salmonella* and unilaterally lost from the *E. coli* lineage in the course of their divergence. The guanosine-plus-cytosine content and codon usage of *ttr* are typical of ancestral genes shared by *Salmonella* and *E. coli* (21). Tetrathionate reduction is found in many other enteric bacteria, suggesting that it was present in the common ancestor of enteric lineages (11). The *phs* and *asr* genes also seem likely to be ancestral genes lost from the *E. coli* lineage. The pathogenicity island SPI2, close to the *ttr* operon on the *Salmonella* chromo-

some (Fig. 7), is clearly of foreign origin and appears to have been added to the genome of *Salmonella* but not to that of *E. coli* (22). The evolution of the genes described here exemplifies the divergence of *Salmonella* and *E. coli* by genomic flux—lineage-specific events of gene loss and acquisition (35).

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