Anaerobic Cysteine Degradation and Potential Metabolic Coordination in *Salmonella enterica* and *Escherichia coli*

Melissa Loddeke,^a Barbara Schneider,^a Tamiko Oguri,^{a*} Iti Mehta,^a Zhenyu Xuan,^{a,b} Larry Reitzer^a

Department of Biological Sciences, University of Texas at Dallas, Richardson, Texas, USA^a; Center for Systems Biology, University of Texas at Dallas, Richardson, Texas, USA^b

ABSTRACT *Salmonella enterica* has two CyuR-activated enzymes that degrade cysteine, i.e., the aerobic CdsH and an unidentified anaerobic enzyme; *Escherichia coli* has only the latter. To identify the anaerobic enzyme, transcript profiling was performed for *E. coli* without *cyuR* and with overexpressed *cyuR*. Thirty-seven genes showed at least 5-fold changes in expression, and the *cyuPA* (formerly *yhaOM*) operon showed the greatest difference. Homology suggested that CyuP and CyuA represent a cysteine transporter and an iron-sulfur-containing cysteine desulfidase, respectively. *E. coli* and *S. enterica* $\Delta cyuA$ mutants grown with cysteine generated substantially less sulfide and had lower growth yields. Oxygen affected the CyuR-dependent genes reciprocally; *cyuP-lacZ* expression was greater anaerobically, whereas *cdsH-lacZ* expression was greater aerobically. In *E. coli* and *S. enterica*, anaerobic *cyuP* expression required *cyuR* and cysteine and was induced by L-cysteine, D-cysteine, and a few sulfur-containing compounds. Loss of either CyuA or RidA, both of which contribute to cysteine degradation to pyruvate, increased *cyuP-lacZ* expression, which suggests that CyuA modulates intracellular cysteine concentrations. Phylogenetic analysis showed that CyuA homologs are present in obligate and facultative anaerobes, confirming an anaerobic function, and in archaeal methanogens and bacterial acetogens, suggesting an ancient origin. Our results show that CyuA is the major anaerobic cysteine-catabolizing enzyme in both *E. coli* and *S. enterica*, and it is proposed that anaerobic cysteine catabolism can contribute to coordination of sulfur assimilation and amino acid synthesis.

IMPORTANCE Sulfur-containing compounds such as cysteine and sulfide are essential and reactive metabolites. Exogenous sulfur-containing compounds can alter the thiol landscape and intracellular redox reactions and are known to affect several cellular processes, including swarming motility, antibiotic sensitivity, and biofilm formation. Cysteine inhibits several enzymes of amino acid synthesis; therefore, increasing cysteine concentrations could increase the levels of the inhibited enzymes. This inhibition implies that control of intracellular cysteine levels, which is the immediate product of sulfide assimilation, can affect several pathways and coordinate metabolism. For these and other reasons, cysteine and sulfide concentrations must be controlled, and this work shows that cysteine catabolism contributes to this control.

KEYWORDS *Escherichia coli*, *Salmonella enterica*, cysteine catabolism, metabolism

Cysteine and sulfide are metabolically related and reactive, and both can have either cytotoxic or beneficial effects. Cysteine can impair bacterial growth (1, 2), inhibit several enzymes (e.g., threonine deaminase and homoserine dehydrogenase I) (3), enhance oxidative stress (4, 5), stimulate swarming motility, and increase antibiotic resistance (6, 7). Sulfide inhibits several enzymes (e.g., cytochrome oxidases and other metalloenzymes) (8, 9), ameliorates antibiotic-induced oxidative stress (10), may serve

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Address correspondence to Larry Reitzer, reitzer@utdallas.edu.

* Present address: Tamiko Oguri, Center for Pharmaceutical Biotechnology, Chicago, Illinois, USA.

as a regulatory signal for intestinal tract bacteria (8), and reacts with sulfhydryls to form persulfides (RSSH), which may modify regulatory proteins and affect gene expression (11, 12).

Intracellular levels of cysteine are controlled by synthesis (13), import and export (14), and possibly degradation (15). Cysteine catabolism is the least studied, yet several enzymes have been proposed to degrade cysteine in *Escherichia coli* and *Salmonella enterica*. CdsH is a pyridoxal-5'-phosphate-dependent cysteine desulfhydrase that degrades cysteine aerobically to pyruvate, ammonia, and sulfide in *S. enterica* but not *E. coli* (15–18). *E. coli* has at least five enzymes with cysteine desulfhydrase activity as a secondary activity, namely, TnaA, MetC, CysK, CysM, and MalY (19), but their total activity (27 nmol min⁻¹ mg protein⁻¹ at 37°C) is much less than that of CdsH in *S. enterica* (260 nmol min⁻¹ mg protein⁻¹ at 23°C) (17, 19).

The products of cysteine catabolism are often depicted as pyruvate, ammonia, and sulfide; however, cysteine catabolism is actually a two-step process. Cysteine desulfhydrase degrades cysteine to sulfide and the toxic intermediate 2-aminoacrylate (an enamine); while 2-aminoacrylate may spontaneously decompose, RidA degrades 2-aminoacrylate to pyruvate and ammonia (20). A different two-step pathway of cysteine catabolism, in which aspartate transaminase deaminates cysteine to 3-mercaptopyruvate, which SseA (a mercaptopyruvate sulfur transferase) further cleaves to sulfide and pyruvate (10), has also been proposed.

Loss of CdsH in *S. enterica* eliminated aerobic cysteine degradation but had no effect on anaerobic cysteine degradation (15). The activator CyuR, which is homologous to the leucine-responsive regulatory protein (Lrp), is required for synthesis of both CdsH and the anaerobic enzyme (15). The goals of this work were to identify and to characterize the regulation and function of the CyuR-regulated gene for the anaerobic cysteine-catabolizing enzyme in *S. enterica* and *E. coli*.

RESULTS

CyuR activation of the major anaerobic pathway of cysteine catabolism in both *E. coli* and *S. enterica*. Anaerobic cysteine degradation was readily detected as cysteine-dependent sulfide production in *S. enterica* with a stab culture assay (Fig. 1A) and an overlay assay (Fig. 1B). Neither assay detected cysteine-dependent sulfide production in wild-type *E. coli* (Fig. 1A) (15), and production was instead detected with lead acetate strips placed above the medium inside closed microcentrifuge tubes, when the culture contained at least 0.5 mM cysteine (Fig. 1C).

Loss of CyuR eliminated anaerobic cysteine-dependent sulfide production in both *E. coli* and *S. enterica* (Fig. 1), and *E. coli cyuR* on a plasmid largely restored sulfide production from *S. enterica* (Fig. 2B). The reason for only partial restoration is unknown, but it could result from a non-wild-type level of CyuR. Previous results showed that aerobic CdsH expression in *S. enterica* requires CyuR (15), and *E. coli cyuR* restored CdsH activity in an *S. enterica* $\Delta cyuR$ mutant (Fig. 2A). These results show that the *E. coli* and *S. enterica* CyuRs are interchangeable and that CyuR is likely to regulate the same anaerobic cysteine-catabolizing enzyme in the two organisms.

Transcript profiling of potential *cyuR*-regulated genes in *E. coli*. The gene for the CyuR-regulated anaerobic enzyme was analyzed initially from *E. coli* because of the available microarray chip. To identify CyuR-regulated genes, transcripts were analyzed from an *E. coli* $\Delta cyuR$ strain and a parental wild-type *E. coli* strain with *cyuR* on a plasmid. Quantitative reverse transcription-PCR (qRT-PCR) analysis indicated that the level of CyuR in the plasmid-containing strain was ~55 times higher than that in a wild-type strain (M. Loddeke, unpublished observation). Cells in exponential phase were grown aerobically at 37°C in Luria-Bertani (LB) broth and challenged with 5 mM cysteine for 30 min (see Materials and Methods for more details). Although the goal was to identify the gene for an anaerobic enzyme, we assumed that overexpression of *cyuR* in an aerobic environment would permit detection of an anaerobically expressed gene. In addition, a secondary goal was to identify other CyuR-regulated genes involved in the metabolism of S-containing compounds.

FIG 1 Anaerobic cysteine-dependent sulfide generation. (A) Sulfide detection with the stab method. (B) Sulfide detection from *S. enterica* strains with the overlay method. (C) Sulfide detection from *E. coli* strains with the lead acetate strip method, in triplicate. The *S. enterica* strains are TR10000 (wild type), TO1 ($\Delta cdsH$), TO2 ($\Delta cdsH \Delta metC$), TO3 ($\Delta cyuR$), TO20 ($\Delta cyuP$), and TO21 ($\Delta cyuA$), and the *E. coli* strains are W3110 (wild type), ML2 ($\Delta cyuR$), ML3 ($\Delta cyuP$), and ML4 ($\Delta cyuA$).

Plasmid-borne *cyuR* increased the expression of 20 genes at least 5-fold and decreased the expression of 17 genes at least 5-fold (see Table S1 in the supplemental material). The two most upregulated genes were the adjacent *yhaO* (94-fold) and *yhaM* (56-fold) (Table S1); qRT-PCR analysis confirmed this regulation and showed a 40-fold difference for these transcripts with and without overproduced CyuR (M. Loddeke, unpublished observations). The other genes activated at least 5-fold were four genes of aromatic amino acid metabolism (both genes of the *tyrA-aroF* operon, *aroP*, and *aroL*) and several genes of carbohydrate metabolism (*lacI*, two genes of the *srlA* operon, and all of the genes of the *uidABC* and *dhaKLM* operons). Six of the repressed genes degrade glucarate and galacturate, i.e., *gudP* and the adjacent *garPLRK* and *garD* operons. Nine of the downregulated genes are in the maltose regulon, i.e., *malEFG*, *malk-lamB-malM*, *malPQ*, and *malS*. Except for *yhaO* and *yhaM*, which are described below, none of the *cyuR*-regulated genes with a 5-fold change in expression has an obvious connection to cysteine or sulfur metabolism or has C-S lyase activity (i.e., degrades cysteine) as a secondary activity. A possible explanation for most of the apparent CyuR control is that overexpressed CyuR artifactually interacts with other regulators; an interfering interaction with two activators, CdaR and MalT, could explain repression of 15 of the 17 most repressed genes.

The highly regulated *yhaO* and *yhaM* genes are shown to participate in cysteine catabolism, and we propose the designations *cyuP* (cysteine utilization permease) for

FIG 2 Complementation of a *S. enterica* $\Delta cyuR$ strain with a plasmid containing *E. coli* *cyuR*. Strains TR10000 (wild type [WT]) and TO3 ($\Delta cyuR$) were used. (A) Aerobic cysteine-dependent sulfide production. Cysteine desulfhydrase (CdsH) activity in aerobic *S. enterica* extracts was measured in a nondenaturing gel. The presence of the inducer cysteine and a plasmid containing *cyuR* from *E. coli* is indicated. (B) Anaerobic sulfide generation from *S. enterica*, measured with the agar overlay method.

yhaO homologs and *cyuA* (cysteine utilization A) for *yhaM* homologs for both *E. coli* and *S. enterica*. Alternative names exist for *cyuP* (*cdsA* and *dlsT*), *cyuA* (*cdsB*), and *cyuR* (*cutR* and *decR*) (21–23). Nonetheless, we propose the *cyu* designation because the *cds* designation (for CDP-diglyceride synthetase) and the *cut* designation (for copper utilization) are already in use, and the *dec* designation (for detoxification of cysteine) does not suggest expression of these genes and the phenotype of mutants lacking these genes when cysteine is not toxic (described below).

Suggestion from homology that *E. coli* CyuP and CyuA specify a cysteine transporter and a cysteine desulfidase, respectively. *E. coli* CyuP is a putative 422-residue protein with 87% amino acid identity with *Y. ruckeri* CdsA, which transports cysteine (21). CyuP is different from the two previously described cysteine/cystine transporters in *E. coli*. The *E. coli* TcyJ-TcyL-TcyN system transports both cystine and diaminopimelate, is sensitive to osmotic shock (24–27), and probably corresponds to the osmotic shock-sensitive CTS-1 in *S. enterica* (28). The *E. coli* TcyP transporter is specific for cystine and is resistant to osmotic shock (24, 25). Regulation is a major distinguishing feature of these systems; cysteine limitation and CysB control TcyJLN and TcyP (24, 26, 28, 29), whereas exogenous cysteine and CyuR regulate CyuP.

E. coli CyuA is a putative 436-residue protein with homology to YhaN from *S. enterica* (84% amino acid identity), CdsB from *Yersinia ruckeri* (67% identity), and MJ1025 from the archaea *Methanocaldococcus jannaschii* (25% identity) (21, 30). The *M. jannaschii* CyuA homolog catalyzes the same reaction as cysteine desulfhydrase, but the former was called cysteine desulfidase because it contains an iron-sulfur cluster instead of pyridoxal-5'-phosphate (30). The *M. jannaschii* cysteine desulfidase contains four conserved cysteines that bind the iron-sulfur cluster, and these cysteines are present in the *E. coli* and *S. enterica* homologs (30).

Roles of CyuA and CyuP in anaerobic cysteine catabolism. (i) Cysteine-dependent sulfide production. *E. coli* and *S. enterica* $\Delta cyuA$ mutants produced little sulfide when grown anaerobically for 1 day in LB broth with cysteine (Fig. 1B and C), although the

FIG 3 Anaerobic growth of *E. coli* strains with and without cysteine. Anaerobic growth cultures contained 25% LB medium without exogenous cysteine (A) or with 10 mM cysteine (B). The results are from one experiment. This experiment was performed three times with similar results. The strains used were W3110 (wild type) (●), ML2 ($\Delta cyuR$) (▽), ML3 ($\Delta cyuP$) (■), and ML4 ($\Delta cyuA$) (◇).

E. coli mutants did produce sulfide after 2 days (data not shown). A quantitative assay showed that the *S. enterica* $\Delta cyuA$ mutant produced only 43% of the sulfide produced by a wild-type strain in LB broth with cysteine. An *E. coli* $\Delta cyuP$ mutant failed to produce sulfide from cysteine, in contrast to an *S. enterica* $\Delta cyuP$ mutant, which did produce sulfide (Fig. 1B and C).

An alternative cysteine catabolic pathway proposes that aspartate transaminase deaminates cysteine and generates 3-mercaptopyruvate, which is further degraded to pyruvate and sulfide by SseA, a 3-mercaptopyruvate sulfur transferase (10). However, an *E. coli* $\Delta sseA$ mutant and a $\Delta aspC \Delta tyrB$ double mutant, which lacked all aspartate transaminase activity, generated wild-type levels of sulfide both anaerobically and aerobically after 1 day (data not shown).

(ii) Anaerobic cysteine sensitivity. Anaerobic growth with and without cysteine was examined in broth instead of glucose-containing minimal medium because glucose represses anaerobic sulfide production from cysteine (15). Growth inhibition was observed in 25% LB broth (see Materials and Methods for medium composition) but not in 100% LB broth (data not shown). Cysteine at 10 mM inhibited the growth of *E. coli* $\Delta cyuR$ or $\Delta cyuA$ mutants but had no effect on $\Delta cyuP$ or wild-type strains (Fig. 3). Cysteine at ≥ 5 mM inhibited the growth of *S. enterica* $\Delta cyuR$ and $\Delta cyuA$ mutants (Fig. 4D and E), but 2 mM cysteine did not inhibit growth (data not shown). Cysteine actually stimulated the growth of *S. enterica* wild-type, $\Delta cyuP$, and $\Delta cdsH$ strains (Fig. 4A to C). The growth stimulation did not result from cysteine catabolism, since neither pyruvate, ammonia, nor sulfide stimulated growth; sulfide was actually inhibitory (Fig. 4F).

Transcriptional regulation of *cyuPA* expression. (i) **Experimental approach.** To examine *cyuA* expression, β -galactosidase was assayed in strains with a *cyuP-lacZ* fusion. The *cyuP* promoter probably controls *cyuA* expression, because (i) these genes are separated by only 25 bases in *E. coli*, which suggests that *cyuP* and *cyuA* probably form an operon, and (ii) a global analysis of *E. coli* transcription start sites indicates a

FIG 4 Anaerobic growth of *S. enterica* strains with and without cysteine. Anaerobic growth cultures contained 25% LB medium with the indicated supplement. The results are from one experiment. This experiment was performed three times, with similar results. The strains used were TR10000 (wild type), TO1 ($\Delta cdsH$), TO3 ($\Delta cyuR$), TO20 ($\Delta cyuP$), and TO21 ($\Delta cyuA$). (A to E) Mutants grown with no exogenous cysteine (●), 5 mM cysteine (▽), or 10 mM cysteine (■). (F) Wild-type *S. enterica* grown with no cysteine (●), 5 mM cysteine (▽), 5 mM pyruvate (■), 5 mM Na_2S (◇), or 5 mM $(NH_4)_2SO_4$ (▲). (G to I) Comparison of the wild-type strain (●) and $\Delta cyuP$ (◇), $\Delta cyuA$ (▲), $\Delta cyuR$ (▽), and $\Delta cdsH$ (■) mutants with 0, 5, and 10 mM cysteine.

cyuP transcription start site that is 159 bases from the start codon but no start site for *cyuA* (31). Transcriptional and translational *cyuP-lacZ* fusions were integrated into *E. coli* and *S. enterica* chromosomes, but no difference was observed between the two types of fusions, and only results from the transcriptional fusions are shown.

(ii) Dependence on CyuR and cysteine. Anaerobic *cyuP-lacZ* expression required cysteine and CyuR in both *E. coli* (Fig. 5A) and *S. enterica* (Fig. 5B); the numbers above the bars indicate the ratios of results with and without cysteine. Expression of *E. coli cyuP-lacZ* was 5 times higher in stationary-phase cells ($7,000 \pm 500$ units) (Fig. 5A) than in exponential-phase cells ($1,200 \pm 120$ units) (Fig. 6A), and cysteine induced expression to essentially the same extents (24-fold and 20-fold, respectively). It should be noted that induction occurred in a medium in which cysteine is not toxic (100% LB broth).

Further confirmation of control by cysteine comes from observations of elevated *cyuP-lacZ* expression in $\Delta cyuA$ and $\Delta ridA$ mutants, which have impaired cysteine catabolism and presumably higher intracellular cysteine levels. For a $\Delta cyuA$ mutant grown in 2 mM cysteine, *cyuP-lacZ* expression was 50% higher in *E. coli* (Fig. 5A) and 4-fold higher in *S. enterica* (Fig. 5B). Even without exogenous cysteine, *S. enterica cyuP-lacZ* expression was 6.3-fold higher in a $\Delta cyuA$ mutant (670 ± 75 units) than

FIG 5 β -Galactosidase activity in anaerobic *E. coli* and *S. enterica* strains with *cyuP-lacZ* fusions. Cells were grown anaerobically in LB broth with and without 2 mM cysteine. The numbers over the bars represent the ratios of activity with versus without cysteine. (A) *E. coli* mutants. Cells were harvested for measurement of β -galactosidase activity after overnight incubation (in stationary phase). The strains used were ML6, ML7, IM35, IM36, IM37, IM38, and IM40. (B) *S. enterica* mutants. Cells were harvested in late exponential phase (~ 40 Klett units). The strains used were BLS143, BLS145, BLS147, and BLS149. WT, wild type.

in a wild-type strain (106 ± 4 units), which suggests that intracellular cysteine levels were higher in the $\Delta cyuA$ mutant. Similar results were observed for a mutant without *RidA*, which degrades the enamine/imine intermediates in the two-step conversion of cysteine to pyruvate (20). Loss of *ridA* in *E. coli* increased *cyuP-lacZ* expression 1.6-fold ($P = 0.02$) in medium with 2 mM cysteine (Fig. 5A) and 2.5-fold without exogenous cysteine (720 ± 20 units for the $\Delta ridA$ mutant versus 290 ± 25 units for the wild-type strain). The probable explanation is that loss of *RidA* results in enamine/imine accumulation, which in turn inhibits *CyuA* and increases intracellular cysteine levels. The results obtained with the $\Delta cyuA$ and $\Delta ridA$ mutants not only confirm control by cysteine but also indicate that *CyuA* and *RidA* modulate the intracellular cysteine concentrations. In contrast to these results, loss of the aerobic *CdsH* did not affect *cyuP-lacZ* expression (Fig. 5B).

(iii) Aerobic versus anaerobic regulation of *cyuPA* and *cdsH*. Anaerobic *cyuP-lacZ* expression was 3 and 28 times higher than aerobic expression in *E. coli* and *S. enterica*, respectively (Fig. 6A and B). For comparison, expression of the *cyuR*-regulated *cdsH* was

FIG 6 β -Galactosidase activity under aerobic versus anaerobic conditions in strains with *lacZ* fusions. Cells were grown in LB broth without cysteine (black bars) or with 1 mM cysteine (gray bars); 0.5- to 1.5-ml samples were removed for measurement of β -galactosidase activity in late exponential phase (100 Klett units for aerobic cultures and 40 Klett units for anaerobic cultures). (A) *E. coli cyuP-lacZ* strain (ML6). (B) *S. enterica cyuP-lacZ* strain (BLS143). (C) *S. enterica cdsH-lacZ* strain (TO11).

examined in *S. enterica*; in contrast to *cyuP-lacZ* expression, *cdsH-lacZ* expression was 6 times higher aerobically than anaerobically (Fig. 6C). One possible explanation for the greater anaerobic versus aerobic expression of *cyuP-lacZ* is the participation of a coregulator that differentially controls anaerobic and aerobic genes. Loss of ArcA, ArcB, and SoxR did not impair anaerobic expression (Fig. 5A), whereas loss of SoxR unexpectedly increased *cyuP-lacZ* expression 50% ($P = 0.05$). This limited analysis did not identify a potential coregulator if it exists.

(iv) Induction of *cyuP-lacZ* expression by amino acids and sulfur-containing compounds. Of the L-amino acids tested, only L-cysteine induced expression in both *E. coli* and *S. enterica* (Fig. 7A and C). This finding confirms a recent report that L-cysteine is the only L-amino acid that induces *cyuP* expression (22). A few D-amino acids were

tested for induction, since CyuP-dependent D-serine transport was shown to be important for the virulence of enterohemorrhagic *E. coli* (23). D-Cysteine induced expression about 3 times less well than L-cysteine in both *E. coli* and *S. enterica*, but D-alanine and D-serine did not induce expression (Fig. 7A and C).

In *E. coli*, the following sulfur-containing compounds induced expression at the indicated expression levels, relative to L-cysteine induction: N-acetylcysteine (40%), homocysteine (21%), cystine (17%), S-nitrosocysteine (14%), and glutathione (11%) (Fig. 7B). In *S. enterica*, cystine (34%), N-acetylcysteine (11%), and homocysteine (9%) induced expression (data not shown). Sulfide and djenkolate did not induce *cyuP* expression in either organism (data not shown). The induction by these compounds does not imply direct binding to CyuR, because the inducing compound could indirectly affect intracellular cysteine levels.

Glutathione induces *E. coli cyuP-lacZ* expression about 4-fold over background levels (Fig. 7A). With the assumption that glutathione degradation to cysteine is the mechanism of induction, expression was examined in a mutant lacking the periplasmic γ -glutamyltranspeptidase (Ggt), which is the major (but not only) enzyme of glutathione utilization (32). Unexpectedly, loss of Ggt increased glutathione-independent and glutathione-dependent *cyuP-lacZ* expression (Fig. 7A). Both results suggest that Ggt-independent glutathione catabolism generates more cysteine than Ggt-dependent catabolism.

Presence of CyuA in anaerobes and possible ancient origin. Eighty-four organisms have CyuA homologs. These organisms are in the following phyla, with the numbers of representatives in parentheses: *Euryarchaeota* (5 representatives), *Actinobacteria* (3 representatives), *Bacteroidetes* (7 representatives), *Firmicutes* (16 representatives in class *Clostridia* and 5 representatives in other classes), *Fusobacteria* (2 representatives), *Spirochaetes* (3 representatives), *Synergistetes* (4 representatives), *Thermotogae* (5 representatives), *Proteobacteria* (31 representatives), and other bacteria (3 representatives) (Table S1). Excluding the *cyuA*-containing betaproteobacteria and gammaproteobacteria, 61 of 62 organisms are obligate anaerobes; the single exception is a microaerotolerant anaerobe. Among the *cyuA*-containing betaproteobacteria and gammaproteobacteria, 20 of 22 organisms are facultative anaerobes and the remaining 2 organisms are obligate anaerobes. No strict aerobe has a CyuA homolog. This organismal distribution strongly suggests an anaerobic function for CyuA homologs.

A rooted phylogenetic tree of CyuA homologs is shown in Fig. 8. CyuA homologs appeared in five archaea. The CyuA homologs from three archaea form an outgroup (group C), and the CyuA homologs from two archaea are grouped with bacterial homologs. These observations are consistent with some gene transfer between kingdoms. The bacterial CyuAs are separated into two groups, with group A containing the deltaproteobacterial homologs and group B containing the betaproteobacterial and gammaproteobacterial homologs (Fig. 8). Some firmicutes have multiple *cyuA* paralogs, such as *Pelosinus fermentans* JBW45, which has four paralogs. CyuA homologs are found in archaeal methanogens and bacterial acetogens, which are considered some of the most ancient organisms (33). This distribution may suggest an origin for CyuA that predates the split between archaea and bacteria.

DISCUSSION

Anaerobic cysteine catabolic pathways and differences between *E. coli* and *S. enterica* sulfur metabolism. CyuR-regulated CyuA is the major anaerobic cysteine-catabolizing enzyme in both *E. coli* and *S. enterica*, at least under the conditions tested. Neither CdsH in *S. enterica* nor a transaminase/mercaptopyruvate sulfur transferase pathway in *E. coli* obviously contributed to anaerobic cysteine catabolism. The failure of CdsH to contribute to anaerobic cysteine catabolism is surprising, because CdsH is expressed anaerobically, although not optimally (Fig. 6C). For unknown reasons, CdsH is not active during anaerobic growth, even when overexpressed (see Fig. 4C of reference 15).

FIG 7 Specificity of *cyuP* induction. β -Galactosidase activity was measured in strains with *cyuP-lacZ* fusions. (A) Amino acid induction in *E. coli* ML6 (wild type) and IM39 (Δggt). (B) Induction by sulfur-containing compounds in *E. coli* ML6. (C) Induction in *S. enterica* BLS143. Cells were grown anaerobically in LB broth with 2 mM levels of the indicated supplements, with the exceptions of Na₂S (0.5 mM), N-acetylcysteine (N-Ac-Cys) (5 mM), diaminopimelate (DAP) (5 mM), djenkolate (DJA) (5 mM), homocysteine (Homocys) (5 mM), and cystine (10 mM). Sno-Cys, S-nitrosocysteine.

FIG 8 Rooted phylogenetic tree of CyuA homologs in 60 bacteria and 5 archaea. Three archaea form an outgroup (group C), and the bacteria are separated into two major groups (groups A and B), with one archaeon in each group; the archaeal homologs are in bold. The *E. coli* and *S. enterica* homologs are underlined. The bootstrapping values from 100 samplings are shown at each fork. Clades with bootstrapping values of 100 are marked with filled circles. The accession numbers for these proteins are presented in Table S2 in the supplemental material.

A comparison of anaerobic cysteine catabolism in *E. coli* and *S. enterica* suggests several subtle differences; CyuA is more active in *S. enterica* (Fig. 1), CyuP is the only anaerobic cysteine transporter in *E. coli* but not in *S. enterica*, which must have an additional transporter (Fig. 3 and 4), and the *E. coli* Δ cyuA mutant is more sensitive to cysteine (compare Fig. 3 and Fig. 4). More generally, *S. enterica* has a greater capacity than *E. coli* to metabolize sulfur-containing compounds anaerobically; *S. enterica*, but not *E. coli*, contains reductases for sulfite, thiosulfate, and tetrathionate, which allows their utilization as alternative anaerobic electron acceptors (13). Its greater capacity to utilize a variety of sulfur-containing compounds suggests that *S. enterica* is a specialist in anaerobic sulfur metabolism that endows *S. enterica* with unusual capabilities, such as pathogenic outgrowth in the intestinal tract (34).

Function of anaerobic cysteine catabolism and potential to coordinate metabolism. At least three possible functions can be proposed: (i) cysteine as a carbon or energy source, (ii) detoxification of cysteine at concentrations that inhibit growth, and (iii) modulation of intracellular cysteine levels at nontoxic concentrations. The evidence to support utilization of cysteine as a carbon or energy source is CyuA repression by compounds associated with energy generation, including oxygen (electron acceptor), glucose (carbon and energy sources), and nitrate (alternative electron acceptor) (Fig. 6) (15). Evidence against this possibility is that pyruvate, which is the energy-generating product of cysteine catabolism, did not stimulate anaerobic growth.

The second proposed function of anaerobic cysteine catabolism is detoxification of cysteine at concentrations that inhibit growth. The evidence for this function is

enhanced cysteine sensitivity in a $\Delta cyuA$ mutant (this work and reference 22). Cysteine sensitivity requires at least 5 mM exogenous cysteine (this work), which is probably not often encountered in nature.

The third possible function of cysteine catabolism is modulation of intracellular cysteine levels. The elevated *cyuP-lacZ* expression in $\Delta cyuA$ and $\Delta ridA$ mutants of both *E. coli* and *S. enterica* supports this possibility (Fig. 5). The mutants presumably have elevated intracellular levels of cysteine, which binds to CyuR and increases *cyuP-lacZ* expression. If cysteine catabolism modulates intracellular cysteine levels, then what is the function of this modulation? A possible function of this modulation is control of cysteine-inhibitable enzymes. Cysteine inhibits several enzymes of amino acid synthesis (3), and this inhibition is relevant *in vivo*. Cysteine impairs the growth of $\Delta cyuA$ mutants in 25% LB broth but not in 100% LB broth, which has higher amino acid concentrations. Even subtoxic cysteine concentrations are likely to inhibit several enzymes, at least partially, and elevated expression of those enzymes could compensate for the inhibition. Cysteine catabolism would minimize the need to increase the expression of those enzymes. By this logic, the catabolism of cysteine, the product of sulfur assimilation, affects the synthesis of other amino acids and integrates sulfur assimilation with several pathways of amino acid synthesis. The modulation of intracellular cysteine levels to integrate metabolism explains the reduced growth yield of $\Delta cyuA$ mutants; with high cysteine levels, energy is diverted for synthesis of the inhibited enzymes (Fig. 3 and 4). Proteins that coordinate metabolism, as proposed for CyuA, may be similar to products of quasiessential genes, i.e., nonessential but required for optimal and robust growth (35).

Evidence that CyuA is an ancient enzyme. CyuA homologs are present in archaeal methanogens and eubacterial acetogens, which are considered two of the most ancient organisms (33), and their presence might suggest that CyuA homologs existed before the divergence of archaea and bacteria, in the last universal common ancestor. The methanogens and acetogens do not share pathways of cysteine synthesis, which implies that the catabolic pathway was present before the anabolic pathways (36); perhaps a primitive (nonenzymatic?) pathway of cysteine synthesis existed before the divergence of archaea and bacteria. Regardless of the early mechanism of cysteine synthesis, the fluctuating concentrations of environmental sulfide, which was readily available in a proposed primordial environment (33), probably resulted in fluctuating concentrations of cysteine and the potential to inhibit other pathways, such as those of threonine or methionine synthesis. It is proposed that ancient CyuA homologs not only minimized cysteine toxicity but also coordinated sulfide assimilation with synthesis of some amino acids.

What does it all mean? We have presented evidence that CyuA is the major enzyme that degrades cysteine during anaerobic growth in both *E. coli* and *S. enterica*; analysis of gene expression and phylogenetic relationships confirm an anaerobic function. Because of cysteine's ability to inhibit several enzymes of amino acid synthesis, cysteine catabolism has the potential to modulate the synthesis of some amino acids. The presence of CyuA homologs in the most ancient organisms suggests that cysteine catabolism was an ancient mechanism of metabolic coordination.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* and *S. enterica* strains used in this study are listed in Table 1. The deletion alleles for *E. coli* strains were obtained from Keio Collection strains (37) and then transduced into various strains with phage P1. All results were reported from a strain W3110 background. *S. enterica* gene deletions were constructed in strain TT22971 by the one-step inactivation method, as described previously (38), and alleles with the antibiotic resistance cassettes were transduced into TR10000 with P22. For both *E. coli* and *S. enterica* strains, the antibiotic resistance cassettes were removed as described previously (38). The primers for the *cyuP/yhaO* deletion were GAATTTTATGGAATCGGCAAGTAATACCAG CGTAATCCTCATTCCGGGATCCGTCGACC and AATGCCAGGAACGGAGAAACGCATAGCAGCAGACCGGTAA GTGTAGGCTGGAGCTGCTTC. The primers for the *cyuA/yhaN* deletion were TCCATTGTGGCAAAGTTTAT TCTGGCCGTACAGGAAGAAATCCGGGGATCCGTCGACC and ATAATCTCGATGATCTGCTTGTCTGGTCTGCTG CATTGAGCGTGTAGGCTGGAGCTGCTTC. The subsequent *S. enterica* strains were shown to be phage-free by cross-streaking against P22-H5 on green agar plates (39).

TABLE 1 Strains and plasmids

Strain or plasmid	Relevant genotype or phenotype	Source or reference
<i>S. enterica</i>		
TR10000	<i>S. enterica</i> serovar Typhimurium LT2	48
TO1	TR10000 $\Delta cdsH$	15
TO2	TR10000 $\Delta cdsH \Delta metC::cat$	15
TO3	TR10000 $\Delta cyuR$	15
TO11	TR10000 $att\lambda::[\Phi(cdsH'-lacZ) Kan^r]$; <i>cdsH</i> region from –138 to +57 ^a	15
TO20	TR10000 $\Delta cyuP$ ($\Delta 16-432/443$) ^b	This study
TO21	TR10000 $\Delta cyuA$ ($\Delta 21-416/436$) ^b	This study
BLS143	TR10000 $att\lambda::[\Phi(cyuP'-lacZ) Kan^r]$; <i>cyuP</i> region from –381 to +44 ^a	This study
BLS145	BLS143 $\Delta cdsH$	This study
BLS147	BLS143 $\Delta cyuR$	This study
BLS149	BLS143 $\Delta cyuA::cat$	This study
<i>E. coli</i>		
MG1655	K-12 reference strain	Laboratory stock
ML1	MG1655 $\Delta cyuR$	This study
W3110	K-12 <i>lacL8 lacI^q</i>	Laboratory stock
ML2	W3110 $\Delta cyuR$	This study
ML3	W3110 $\Delta cyuP$	This study
ML4	W3110 $\Delta cyuA$	This study
BW25113	K-12 reference strain	38
JW2505	BW25113 $\Delta sseA::kan$	37
JW3467	BW25113 $\Delta gor::kan$	37
ML5	W3110 $\Delta aspC$	This study
PL41	W3110 $\Delta aspC \Delta tyrB$	This study
JOEY281	MG1655 $att\lambda::[\Phi(cyuP'-lacZ) Kan^r]$; <i>cyuPA</i> region from –306 to +100 ^a	Stephen Spiro
ML6	W3110 $att\lambda::[\Phi(cyuP'-lacZ) Kan^r]$	This study
ML7	ML6 $\Delta cyuR$	This study
IM35	ML6 $\Delta arcA::kan$	This study
IM36	ML6 $\Delta arcB::kan$	This study
IM37	ML6 $\Delta ridA::kan$	This study
IM38	ML6 $\Delta soxR::kan$	This study
IM39	ML6 $\Delta ggt::kan$	This study
IM40	ML6 $\Delta cyuA::kan$	This study
Plasmids		
pCP20	FLP (FRT-specific) recombinase; Amp ^r , Cam ^r	38
pcyuR	Same as JW0437; pCA24N carrying <i>cyuR</i>	49
pBLS18	<i>lacZ</i> transcriptional fusion vector; Kan ^r	50
pBLS45	$[\Phi(cyuP'-lacZ)] Kan^r$ in pBLS18; <i>cyuP</i> region from –381 to +44 ^a	This study

^aThe fusions are transcriptional fusions. The promoter region fused to *lacZ* is indicated with +1 being the A of the ATG start codon. The transcriptional fusions were first generated in pBLS18 except for the *E. coli cyuP'-lacZ* fusions (JOEY281, ML6, and pBLS45), which were derived using pRS415 as an intermediate (51). Kan^r, kanamycin resistant.

^bThe numbers indicate the residues deleted relative to the total residues for the indicated protein.

^cFLP, flippase; FRT, flippase recombinase target; Amp^r, ampicillin resistant; Cam^r, chloramphenicol resistant.

Bacterial growth. (i) Media. Cultures for enzyme assays were grown in LB broth with a variety of supplements, whereas cells for analysis of growth characteristics were grown in a modified LB broth that was called 25% LB broth. The 25% LB broth contained 0.25× LB medium with 5.25 g/liter K₂HPO₄, 2.25 g/liter K₂HPO₄, and 0.025 g/liter MgSO₄. When added, supplements were from concentrated stock solutions except for the more insoluble supplements, which were added directly to the cultures. Supplemental amino acids were L-amino acids, unless otherwise indicated.

(ii) Overnight cultures. Prior to experiments, cells were streaked for single colonies from frozen stocks to check for uniformity. All experiments for growth analysis or enzyme assays involved cells from three separate single colonies. Shaking overnight cultures were grown in 20-ml culture tubes with 4 ml of LB broth without supplements. The cultures were placed in a test tube rack on a rotating shaker (240 rpm) at 37°C. The overnight cultures were probably microaerobic or anaerobic.

(iii) Culture conditions for growth and enzyme assays. Overnight cultures were diluted so that the initial turbidity was ≤5 Klett units (optical density at 600 nm [OD₆₀₀] of ≤0.03), and cultures for enzyme assays were grown to at least 50 Klett units, in order to establish steady-state aerobic or anaerobic growth. Anaerobic cultures were grown in 9-ml screw-cap tubes containing 8.5 ml of

medium and were incubated in a 37°C water bath in a test tube rack, without agitation. The tubes were inserted directly into a colorimeter to measure turbidity. Aerobic cultures contained 10 ml of medium in 125-ml sidearm flasks, which allowed turbidity measurements, and were shaken at 240 rpm at 37°C. Turbidity was measured using a Scienceware Klett colorimeter with a KS-54 filter (100 Klett units represent an OD₆₀₀ value of about 0.7).

Nonstandard chemical. S-Nitrosocysteine was synthesized and quantified as described previously (40, 41).

Enzyme assays and sulfide detection. Aerobic cysteine degradation was qualitatively assessed with an activity stain that detected sulfide production (15, 42). Sonicated cell extracts were run into a nondenaturing gel and incubated with cysteine, pyridoxal-5'-phosphate, and BiCl₃. Bismuth sulfide forms a black precipitate at the site of the enzyme.

Three different qualitative methods (assays 1 to 3) and one quantitative method (assay 4) detected anaerobic cysteine-dependent sulfide production. The qualitative methods produced a black metal sulfide precipitate. Assay 1 was a stab culture assay in which cells were stabbed into tubes filled with LB broth containing 0.6% agar, 0.1% FeSO₄·7H₂O, and 5 mM cysteine (15). Assay 2 was an overlay plate assay with the following layers, from top to bottom: LB broth with 0.6% agar, a filter disc with 5 μl of 1 M cysteine and 5 μl of 10% FeSO₄·7H₂O, 0.5 ml of cells from an overnight culture mixed with LB broth with 0.6% agar, and LB broth with 1.5% agar (15). Assay 3 detected sulfide from lead acetate strips (Fisher Scientific), which were sealed on top of 1.7-ml Eppendorf centrifuge tubes. Cells were grown in tubes containing 1.3 ml of LB broth and 0.5 mM cysteine, without shaking (10, 15). Assay 4 measured the sulfide-dependent reaction with N,N'-dimethyl-p-phenylenediamine to form methylene blue, which was measured at 670 nm (43).

β-Galactosidase assays were performed as described previously (44). Specific activity is presented as nanomoles per minute per milligram of total protein. Values are the mean ± standard deviation from three separate determinations.

Growth and RNA isolation for microarray analysis. For RNA isolation, 4-ml overnight cultures (see above) were grown in LB broth and then diluted 100-fold into 25 ml of LB broth. The cultures were grown aerobically and, at an OD₆₀₀ value of 0.3, the cells were challenged with 5 mM cysteine for 30 min. Cells (500 μl) were then incubated with 2.5 ml of RNeasy Protect (Qiagen), the mixture was centrifuged (14,000 × g for 5 min at 4°C), and total RNA was purified using the RNeasy minikit and an on-column DNase kit (Qiagen).

RNA was extracted from ML2 (Δ*cyuR* strain) and W3110 (wild-type strain) with the *pcyR* plasmid. Ten micrograms of RNA was used for the microarray analysis. The cDNA synthesis, labeling, and hybridization to an Affymetrix *E. coli* gene chip were performed by the Core Microarray Facility at the University of Texas Southwestern Medical Center (Dallas, TX), and the data were processed with RMA and LIMMA from the Bioconductor package (45–47). The complete results have been submitted to NCBI.

Phylogenetic analysis of CyuA in archaea and bacteria. The protein sequences were collected from the Clusters of Orthologous Groups (COGs) database (<ftp://ftp.ncbi.nih.gov/pub/COG/COG2014/static/byCOG/COG3681.html>). Only organisms without multiple paralogs were selected for tree construction. The rooted unweighted pair group method using average linkages (UPGMA) tree was constructed with PHYLIP software (version 3.69) (<http://evolution.genetics.washington.edu/phylip.html>). One hundred bootstrapping samples were used to estimate the robustness of the tree topology.

Accession number(s). The transcript profiling results in this study were submitted to the NCBI GEO database under accession number GSE90066.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00117-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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