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Minimal Functions and Physiological Conditions Required for Growth of *Salmonella enterica* on Ethanolamine in the Absence of the Metabolosome

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During growth on ethanolamine, *Salmonella enterica* synthesizes a multimolecular structure that mimics the carboxysome used by some photosynthetic bacteria to fix CO_2. In *S. enterica*, this carboxysome-like structure (hereafter referred to as the ethanolamine metabolosome) is thought to contain the enzymatic machinery needed to metabolize ethanolamine into acetyl coenzyme A (acetyl-CoA). Analysis of the growth behavior of mutant strains of *S. enterica* lacking specific functions encoded by the 17-gene *e*thanolamine *ut*ilization (*eut*) operon established the minimal biochemical functions needed by this bacterium to use ethanolamine as a source of carbon and energy. The data obtained support the conclusion that the ethanolamine ammnonia-lyase (EAL) enzyme (encoded by the *eutBC* genes) and coenzyme B_{12} are necessary and sufficient to grow on ethanolamine. We propose that the EutD phosphotransacetylase and EutG alcohol dehydrogenase are important to maintain metabolic balance. Glutathione (GSH) had a strong positive effect that compensated for the lack of the EAL reactivase EutA protein under aerobic growth on ethanolamine. Neither GSH nor EutA was needed during growth on ethanolamine under reduced-oxygen conditions. GSH also stimulated growth of a strain lacking the acetaldehyde dehydrogenase (EutE) enzyme. The role of GSH in ethanolamine catabolism is complex and requires further investigation. Our data show that the ethanolamine metabolosome is not involved in the biochemistry of ethanolamine catabolism. We propose the metabolosome is needed to concentrate low levels of ethanolamine catabolic enzymes, to keep the level of toxic acetaldehyde low, to generate enough acetyl-CoA to support cell growth, and to maintain a pool of free CoA.

Salmonella enterica serovar Typhimurium LT2 (hereafter referred to as *S. enterica*) can use ethanolamine as a sole source of carbon, nitrogen, and energy (9, 27, 28). In this bacterium, the genetic information needed to use ethanolamine as a carbon, energy, and nitrogen source is encoded in a 17-gene operon known as the *e*thanolamine *ut*ilization operon (*eut*) (Fig. 1) (17, 35). The *eut* operon encodes proteins homologous to the shell proteins of the carboxysome structure found in cyanobacteria (2, 24). The carboxysome is proposed to concentrate carbon dioxide, the substrate for the ribulose-1,5-biphosphate carboxylase/oxygenase (RubisCO) enzyme. Carboxysome-like structures have been visualized in *S. enterica* cells growing on 1,2-propanediol (5). We refer to these structures as metabolosomes to reflect their broad involvement in metabolism.

An understanding of the physiological role of metabolosomes in *S. enterica* is lacking. Penrod and coworkers recently demonstrated that the EutH protein facilitates the translocation of charged ethanolamine across the inner membrane at a low pH (23). It is not clear, however, whether ethanolamine is transported into the cytosol or directly into the metabolosome where the enzymatic machinery for the degradation of ethanolamine is presumably located.

In principle, the biochemistry underpinning ethanolamine catabolism is simple. Two reactions convert ethanolamine into acetyl coenzyme A (Ac-CoA). First, ethanolamine is converted to acetaldehyde and free ammonia by the adenosylcobalamin (AdoCbl; also known as coenzyme B_{12})-dependent ethanolamine ammonia-lyase (EC 4.3.1.7) (EAL) encoded by the *eutBC* genes of the operon (13). Second, acetaldehyde is oxidized to acetate and activated to Ac-CoA in a single step presumably catalyzed by the acetaldehyde dehydrogenase (EutE) enzyme (35). Ammonia generated by the EAL enzyme is used by *S. enterica* as a source of nitrogen, and Ac-CoA is used to make energy via the tricarboxylic acid cycle and glyoxylate bypass and is a building block for primary and secondary metabolism (34). Eut enzymes catalyze two additional reactions. First, acetaldehyde is reduced to ethanol in a reaction catalyzed by the EutG enzyme with an unclear involvement of the EutJ protein (23). Second, the Ac-CoA is converted to Ac-phosphate (Ac-P) by the EutD phosphotransacetylase (6). Eventually, Ac-P is used to conserve energy via substrate level phosphorylation catalyzed by acetate kinase, yielding ATP and acetate, which is excreted into the medium (34). Excreted acetate is later recaptured by Ac-CoA synthetase (Acs) (33).

The EAL enzyme is prone to suicidal inactivation by ethanol and other compounds (1, 36), and its reactivation requires EutA activity to exchange AdoCbl for hydroxycobalamin from the active site of EAL in the presence of Mg-ATP (21). The EutT enzyme synthesizes AdoCbl (7), which is sensed by the activator EutR protein, triggering transcription of the operon (26, 31).

The physiological role of the metabolosome during ethanolamine catabolism remains unclear, and the physiological reasons for its evolution remain an open question. The *eutSMNLK*

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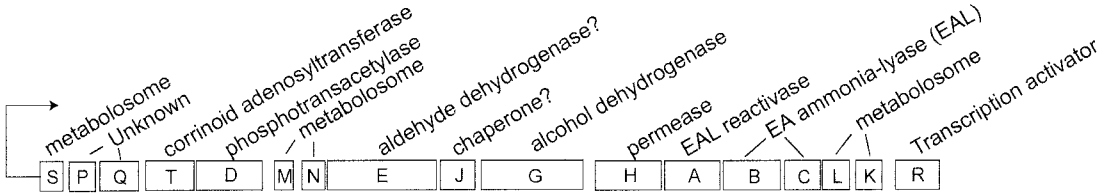

FIG. 1. The *e*thanolamine *ut*ilization (*eut*) operon of *S. enterica*. The *eutR* gene encodes an AraC-type protein that activates *eut* operon transcription in response to AdoCbl and ethanolamine. *eutR* expression is controlled independently from the operon.

genes are inferred by homology to encode the shell proteins of the metabolosome (2, 22, 32, 35). To further investigate the role of the metabolosome shell proteins, in-frame deletion mutants of the genes encoding these proteins were constructed and the abilities of the resulting strains to catabolize ethanolamine were assessed under various growth conditions. We have identified conditions that bypass the need for the metabolosome and for the EAL reactivase enzyme.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All *Salmonella* strains used in this study were derivatives of *S. enterica* serovar Typhimurium LT2. The relevant genotypes of bacterial strains and plasmids used are listed in Table 1. *S. enterica* strains were grown in a minimal medium (4) supplemented with ethanolamine (30 mM), $MgSO_4$ (1 mM), and L-methionine (0.5 mM), trace minerals (3), and vitamin B_{12} (cyanocobalamin) (150 nM). Lysogenic broth (LB) was used to cultivate both *S. enterica* and *Escherichia coli* strains. When added, oxidized L-glutathione (GSSG) was at 2.5 mM. Working concentrations of antibiotics in LB medium were 100 μg/ml for ampicillin and 12 μg/ml for chloramphenicol (Cm). When necessary, genes under the control of the P_{araBAD} promoter were induced for expression by addition of L-(+)-arabinose to a final concentration of 100 μM. Expression of genes cloned into vector pTAC-85 was induced by addition of IPTG (isopropyl-β-D-thiogalactoside) to a final concentration of 100 μM. Growth behavior was analyzed in liquid in 16- by 150-mm Kimax borosilicate tubes. Each tube contained 5 ml of fresh medium, which was inoculated with 75 μl (1.5% [vol/vol]) of a culture of an *S. enterica* strain grown for 24 h in LB medium containing the appropriate antibiotic. Tubes were shaken at 37°C. Growth was monitored as the increase in the absorbance at 650 nm on a Spectronic 20D spectrophotometer (Milton Roy Company, Rochester, NY). Growth behavior was also analyzed by using a microtiter dish (Becton-Dickinson) format using a computer-controlled Ultra Microplate Reader (Bio-Tek Instruments) equipped with the KC4 software package. The temperature of the incubation chamber was set at 37°C. Each well of the plate contained 190 μl of fresh medium supplemented with ethanolamine (30 mM), glycerol (0.5 mM), and ammonium chloride (30 mM), which was inoculated with 10 μl of a 24-h-old inoculum. Growth was monitored as the increase in the absorbance at 630 nm due to fixed-wavelength filters in the microplate reader. Data points were collected every 15 min; cultures were shaken for 870 s between readings.

Phage P22 transductions. All transduction crosses were performed as previously described (12) with phage P22 HT105/1 *int-210* (29, 30). Transductants were freed of phage as previously described (8).

Construction of nonpolar *eut* deletion mutants. In-frame deletion mutants of *eut* genes constructed during this investigation were constructed by using a modification of the method described previously by Datsenko and Wanner (11). Primers were obtained from Integrated DNA Technologies (Coralville, IA) and are listed in Table 2. Briefly, the cat^+ cassette was amplified with these primers by using template plasmid pKD3. Manipulations were performed in the *S. enterica* recipient strain JE6692. The insertion of the cat^+ gene was confirmed by P22 transduction and PCR amplification. Removal of the cat^+ gene was performed as described previously and the resulting deletion was confirmed by sequencing using BigDye (ABI PRISM) protocols (University of Wisconsin—Madison Biotechnology Center).

Construction of a *eutMNLK* deletion mutant. Strain JE8071 (Δ*eutMN*) was transduced to Cm resistance by using phage P22 grown on JE8032 as donor. Cm^r transductants were freed of phage and screened by PCR to confirm the presence of the strain with the *eutMN* allele deleted. Construction of the quadruple deletion mutant from this point was as described above.

Plasmid constructions. (i) Plasmid pEUT31. *S. enterica* $eutBC^+$ cloned into the NdeI and BamHI site of pET22-b. A generous gift from G. Reed.

(ii) Plasmid pEUT33. Allele $eutA^+$ from strain TR6583 was amplified from the chromosome using the forward primer 5′-GAA AGA TGA GCT CGC CCA GGT GAA AA-3′ and the reverse primer 5′-CAA TGT GGT CTC TAG ATT CAT AAG TCG-3′. Bases underlined indicate the SacI and XbaI restriction sites engineered into the primers. The resulting 1.4-kb fragment was A-tailed and gel purified by using the QIAquick gel extraction kit (QIAGEN, Valencia, CA). This product was ligated into plasmid pGEM-T-Easy (Promega, Madison, WI) according to manufacturer's instructions. The resulting plasmid contained the $eutA^+$ gene under the control of the T7 promoter. This $eutA^+$ plasmid was cut with restriction enzymes XbaI and SacI to release a 1.4-kb fragment that was ligated into plasmid pBAD33 (14) cut with the same enzymes. The resulting 6.7-kb plasmid was named pEUT33.

(iii) Plasmid pEUT34. Plasmid pEUT31 was cut with SalI and XbaI to release a 2.3-kb fragment containing allele $eutBC^+$ from *Salmonella enterica*. The $eutBC^+$ fragment was ligated into plasmid pEUT33 cut with the same enzymes. The resulting 9-kb plasmid containing $eutABC^+$ was named pEUT34.

(iv) Plasmid pEUT40. The $eutE^+$ allele from *S. enterica* was amplified from the chromosome using the forward primer 5′-CAT AAA TAG GAT CCA ACA TCA TGA ATC AAC AG-3′ and the reverse primer 5′-GTT CGT CGT GCG TCT AGA GTC ATC-3′. Underlined bases indicate the BamHI and XbaI restriction sites engineered into the primers. The resulting 1.4-kb fragment was A-tailed and gel purified by using the QIAquick gel extraction kit (QIAGEN). The product was ligated into plasmid pGEM-T-Easy (Promega) according to manufacturer's instructions. The resulting plasmid contained the $eutE^+$ allele under the control of the T7 promoter. The $eutE^+$ allele was cut out of this plasmid by using BamHI and SalI and ligated into the same sites of plasmid pTAC-85. The resulting 6.5-kb plasmid was named pEUT40.

(v) Plasmid pEUT42. Plasmid pEUT31 was cut with SalI and XbaI to release a 2.3-kb fragment containing allele $eutBC^+$ from *Salmonella enterica*. The $eutBC^+$ fragment was ligated into pBAD33 cut with the same enzymes. This resulting 7.6-kb plasmid was named pEUT42.

TEM. Strains used for transmission electron microscopy (TEM) were grown in minimal medium supplemented with the appropriate carbon source. When cultures attained an optical density (650 nm) of 1 or greater, 3 ml of culture was pelleted at 18,000 × *g* in a Microfuge 18 microcentrifuge (Beckman-Coulter) for 3 min. Samples were fixed in 2.5% glutaraldehyde–2.0% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h at room temperature. They were then postfixed in 1% osmium tetroxide for 1 h at room temperature in the same buffer. Next, the samples were dehydrated in a graded ethanol series, rinsed in propylene oxide and embedded in Spurr's epoxy resin. Following resin polymerization, the samples were cut into 60- to 90-nm sections for transmission electron microscopy using a Reichert-Jung Ultra-Cut E Ultramicrotome. Prior to transmission microscopic observation, the sectioned samples were stained with routing concentrations of ethanoic uranyl acetate and Reynolds' lead citrate. The specimens were observed by using a Philips CM 120 transmission electron microscope, and images were documented with a SIS Mega-View III digital camera.

RESULTS

Strains lacking EutS, EutMN, or EutLK proteins can grow on ethanolamine as the sole source of carbon and energy; a strain lacking EutMNLK cannot. Although the conversion of ethanolamine to Ac-CoA is straightforward, the genetic information encoded within the *eut* operon suggests that physiological problems arise during ethanolamine catabolism. To inves-

TABLE 1. Strains used in this study

Strain or plasmid	Genotype	Reference/source[a]
Strains		
TR6583	*metE205 ara-9*	K. Sanderson via J. Roth
Derivatives of TR6583		
JE2261	*zfa-3648** Tn*10* zfa-3649* (Δ*eut*)[b]	Laboratory collection
JE4175	pBAD30 *bla*$^+$	Laboratory collection
JE6692	pKD46 *bla*$^+$	Laboratory collection
JE7963	*eutE1151*::*cat*$^+$	
JE7973	Δ*eutE1164*	
JE7979	Δ*eutE1164*/pBAD30	
JE8032	*eutLK1152*::*cat*$^+$	
JE8033	*eutMN1153*::*cat*$^+$	
JE8064	*eutS1154*::*cat*$^+$	
JE8070	Δ*eutLK1158*	
JE8071	Δ*eutMN1159*	
JE8072	Δ*eutS1160*	
JE8087	*eutA1155*::*cat*$^+$	
JE8088	*eutBC1156*::*cat*$^+$	
JE8089	*eutABC1157*::*cat*$^+$	
JE8093	Δ*eutA1161*	
JE8094	Δ*eutBC1162*	
JE8095	Δ*eutABC1163*	
JE8102	*eutLK1152*::*cat*$^+$ Δ*eutMN1159*	
JE8103	Δ*eutLK1158*/pBAD30	
JE8105	Δ*eutMN1159*/pBAD30	
JE8133	Δ*eutLK1158* Δ*eutMN1159*	
JE8135	Δ*eutLK1158* Δ*eutMN1159*/pBAD30	
JE8138	Δ*eutABC1163*/pEUT34	
JE8139	Δ*eutABC1163*/pBAD33	
JE8142	*zfa-3648** Tn*10* zfa-3649* (Δ*eut*)/pBAD33, pTAC-85	
JE8144	*zfa-3648** Tn*10* zfa-3649* (Δ*eut*)/pEUT34, pTAC-85	
JE8191	Δ*eutE1164*/pEUT40	
JE8217	*zfa-3648** Tn*10* zfa-3649* (Δ*eut*)/pEUT34, pEUT40	
JE8298	pBAD33 pTAC-85	
JE8327	*zfa-3648** Tn*10* zfa-3649* (Δ*eut*)/pEUT42, pEUT40	
JE8392	pBAD33 *cat*$^+$	
JE8393	Δ*eutA1161*/pEUT33	
JE8395	Δ*eutA1161*/pBAD33	
JE8411	Δ*eutBC1162*/pEUT42	
JE8412	Δ*eutBC1162*/pBAD33	
JE8424	pTAC-85 *bla*$^+$	
JE8425	Δ*eutE1164*/pTAC-85	
JE8426	Δ*eutS1160*/pBAD30	
E. coli strain DH5α	F$^-$ *f80ΔlacZDM15* Δ*(lacZYA-argF)U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1*	15
Plasmids		
pBAD30	*bla*$^+$	14
pBAD33	*cat*$^+$	14
pET22-b	*bla*$^+$	Novagen
pTAC-85	*bla*$^+$	20
pEUT31	*eutBC*$^+$ in pET22-b	G. Reed
pEUT33	*eutA*$^+$ in pBAD33	
pEUT34	*eutABC*$^+$ in pBAD33	
pEUT40	*eutE*$^+$ in pTAC-85	
pEUT42	*eutBC*$^+$ in pBAD33	

[a] Unless otherwise indicated, all strains were constructed during the course of this work.
[b] Asterisks indicate a Tn*10*-borne deletion.

tigate the role of the proteins exhibiting similarity to the carboxysome shell proteins of CO_2-fixing prokaryotes, in-frame deletion mutants of the genes encoding these proteins were constructed, and the ability of the resulting strains to grow on ethanolamine was assessed. In-frame deletion mutants of the genes *eutS*, *eutMN*, and *eutLK* had little effect on ethanolamine-dependent growth. A 14-h doubling time was measured for cultures of Δ*eutS*, Δ*eutL*, Δ*eutMN*, and wild-type strains. In contrast, a strain with *eutLKMN* deleted failed to grow on ethanolamine (data not shown). Although the Δ*eutS*, Δ*eutL*, and Δ*eutMN* strains lacked one or two of the five proteins that comprise the ethanolamine metabolosome, they grew on ethanolamine suggesting that some type of structure was still formed by the remaining proteins. Recent crystallo-

TABLE 2. Primers used for the construction of in-frame, nonpolar *eut* deletion mutants

Primer	Sequence
*eutA*5′KO	5′-GTGAACACTCGCCAGCTACTGAGCGTCGGTATCGATATCGGCACCACCACCGTGTAGGCTGGAGCTGCTTC-3′
*eutA*3′KO	5′-TCAGGAAGGAAATGCGAGTGATTTCACCGTCACCGGCACAACCGATCCGCCCATATGAATATCCTCCTTAG-3′
*eutB*5′KO	5′-ATGAAACTAAAGACCACATTGTTCGGCAATGTTTATCAGTTTAAGGATGTAGTGTAGGCTGGAGCTGCTTC-3′
*eutC*3′KO	5′-TTAACGGGTCATGTTGATGCCGGACGCTTTCTGCTCCAGCATCCGTTTGGCCATATGAATATCCTCCTTAG-3′
*eutE*5′KO	5′-ATGAATCAACAGGATATTGAACAGGTGGTGAAAGCGGTACTGCTGAAAATGGTGTAGGCTGGAGCTGCTTC-3′
*eutE*3′KO	5′-TTATACAATGCGAAACGCATCCACCAGCACGCATCGACGCAGCCGCACAAACATATGAATATCCTCCTTAG-3′
*eutLK*5′KO	5′-ATGCCTGCATTAGATTTAATTCGACCTTCCGTGACTGCCATGCGCGTGATTGTGTAGGCTGGAGCTGCTTC-3′
*eutLK*3′KO	5′-TTAATTTTTGATGCGATAGCGACTACTGCGTTTACCGAACGCTCCGTCAGACATATGAATATCCTCCTTAG-3′
*eutMN*5′KO	5′-ATGGAAGCATTAGGAATGATTGAAACCCGGGGCCTGGTTGCGCTGATTGAGGTGTAGGCTGGAGCTGCTTC-3′
*eutMN*3′KO	5′-CTATTTATGGAAAACCACTTTCCCGCCAGCCACCACTTCATCGACGATGCCCATATGAATATCCTCCTTAG-3′
*eutS*5′KO	5′-ATGAATAAAGAACGCATTATTCAGGAATTTGTGCCGGGCAAACAGGTCACGGTGTAGGCTGGAGCTGCTTC-3′
*eutS*3′KO	5′-TTAACTTTTTGTTAACTCACACAGGGTAAAGTTTAATAATCGCCCCAGCCCCATATGAATATCCTCCTTAG-3′

graphic studies performed with carboxysome shell proteins CcmK2 and CcmK4 showed that these proteins form hexameric units of the polyhedral shell (16). It appears that the loss of one or two of the metabolosome proteins does not prevent the assembly of a variant of the metabolosome capable of functioning.

The simplest explanation for the lack of growth of the *eutLKMN* strain on ethanolamine was that in the absence of a functional metabolosome, ethanolamine catabolism did not occur because the concentration of the ethanolamine catabolic enzymes was diluted to the point of inefficiency.

TEM. The growth phenotypes of strains lacking *eutS*, *eutMN*, *eutLK*, or *eutMNLK* suggested that a variant of the ethanolamine metabolosome capable of supporting growth on ethanolamine occurred in all of them except the EutMNLK-deficient strain. To address this possibility, we performed TEM studies with strains grown in 30 mM ethanolamine to induce synthesis of the metabolosome or 22 mM glycerol as the negative control. In all cases, cells were harvested from stationary-phase cultures (optical density of ≥1 at 650 nm). When grown in a minimal medium containing ethanolamine as the sole source of carbon and energy, a strain with a fully intact *eut* operon (JE8392 *eut*$^+$) showed electron-dense structures likely to be the metabolosome shell (Fig. 2A). These structures were reminiscent of those observed by Bobik et al. during growth of *S. enterica* on 1,2-propanediol (5). These structures were absent when the same strain was grown on glycerol (Fig. 2B), and in a strain lacking the entire *eut* operon but harboring genes encoding ethanolamine ammonia-lyase and acetaldehyde dehydrogenase (JE8217 Δ*eut*/p*eutABC*$^+$ p*eutE*$^+$). Strain JE8217 did exhibit, however, a large electron-dense region present in almost every cell viewed in the microscope field (Fig. 2C). Strains lacking one or more of the ethanolamine metabolosome shell proteins also exhibited these electron-dense zones. The nature of the latter is unknown.

Complementation studies with strains lacking functions critical for ethanolamine catabolism. If the idea of the metabolosome serving as a concentrator of catabolic enzymes were correct, one would predict that raising the level of the ethanolamine catabolic enzymes would circumvent the need for the metabolosome. To test this possibility, we constructed in-frame deletion mutants of genes encoding the ethanolamine ammonia-lyase, its reactivase and acetaldehyde dehydrogenase, and performed complementation analyses with plasmids carrying the appropriate genes to verify that the deletion mutants generated did not have any negative effect on the expression of downstream genes.

A culture of a strain lacking EAL (EutBC) and the EAL reactivase (EutA) protein (Δ*eutABC*, JE8139) failed to grow on ethanolamine as carbon and energy source (Fig. 3A). Introduction of plasmid pEUT34 (P_{araBAD}-*eutABC*$^+$) into strain JE8095 and inclusion of arabinose (100 μM) in the medium restored growth on ethanolamine with a doubling time of 14 h and the culture reached full density (Fig. 3A). Similarly, a culture of a strain carrying an in-frame deletion mutant of *eutBC* (JE8412) behaved like strain JE8139 (Δ*eutABC*) (Fig. 3B). Introduction of plasmid pEUT42 (P_{araBAD}-*eutBC*$^+$) into strain JE8094 restored growth with a doubling time of 22 h, and the culture reached full density (Fig. 3B).

An in-frame deletion mutant of the gene encoding the pu-

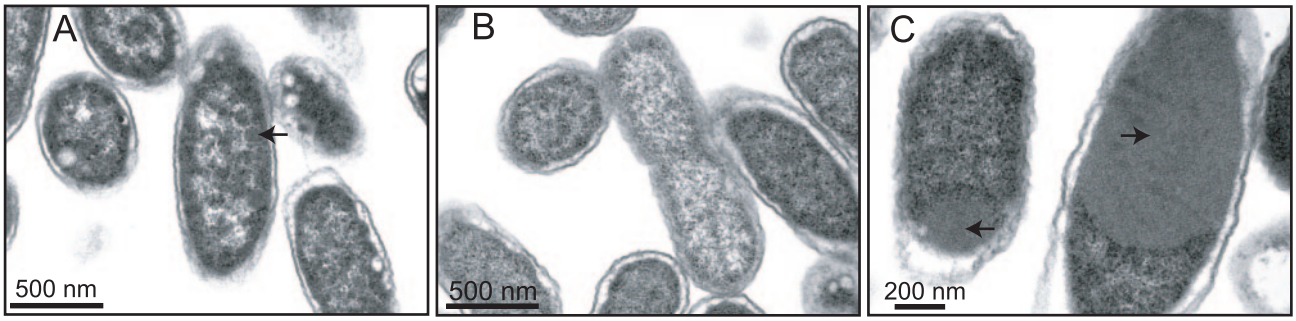

FIG. 2. Transmission electron micrographs of *S. enterica* strains grown on ethanolamine as carbon and energy source. A. JE8392 (*eut*$^+$) grown in 30 mM ethanolamine viewed at 40,000-fold magnification. B. JE8392 grown in 22 mM glycerol viewed at 40,000-fold magnfication. C. Strain JE8217 (Δ*eut* p*eutABC*$^+$ p*eutE*$^+$) grown in 30 mM ethanolamine, viewed at 53,000-fold magnification.

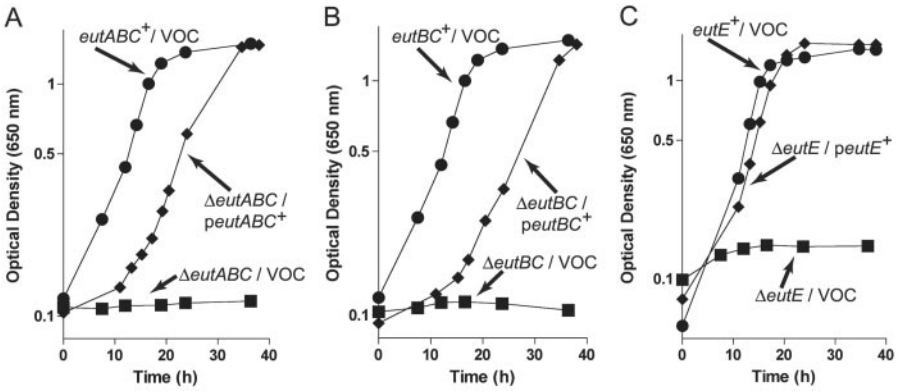

FIG. 3. Complementation studies. In all panels VOC means vector-only control. A. A strain lacking the *eutABC* genes encoding the EAL reactivase EutA and the large and small subunits of EAL, respectively, was tested for the ability to use ethanolamine as sole carbon and energy source. Expression of genes encoded by plasmid pEUT34 (P_{araBAD}-*eutABC*$^+$) was induced with L-(+)-arabinose (JE8138) (100 μM) (solid diamonds); empty cloning vector pBAD33 was introduced into JE8095 (Δ*eutABC1163*) and the growth behavior of the resulting strain (JE8139) was also analyzed in the presence of L-(+)-arabinose (100 μM) (solid squares). Strain JE8392 (*eut*$^+$/pBAD33) (solid circles) was used as a positive control. B. Strain JE8094 (Δ*eutBC*) was tested for its ability to grow on ethanolamine. Strain JE8411 (JE8094/pEUT42 (P_{araBAD}-*eutBC*$^+$) was grown on ethanolamine in the absence of L-(+)-arabinose (solid diamonds). Strain JE8412 (JE8094/pBAD33) (solid squares) was used negative control. Strain JE8392 (*eut*$^+$/pBAD33) (solid circles) was used as positive control. C. Strain JE7973 (Δ*eutE*) was tested for the ability to grow on ethanolamine. Strain JE8191 (JE7973/pEUT40 P_{tac}-*eutE*$^+$) was grown in ethanolamine medium containing 100 μM IPTG (solid diamonds). Strain JE8425 (JE7973/pTAC-85) (solid squares) was used as the negative control, and strain JE8424 (TR6583/pTAC-85) (solid circles) was used as the positive control.

tative aldehyde dehydrogenase *eutE* was also constructed. Strain JE8425 (Δ*eutE*) failed to grow on ethanolamine (Fig. 3C). Introduction of plasmid pEUT40 (P_{tac}-*eutE*$^+$) into strain JE7973 and inclusion of IPTG (100 μM) in the medium restored growth of strain JE7973 on ethanolamine with a doubling time of 10 h, and the culture reached full density (Fig. 3C).

Collectively, the results described above make two points. First, they confirmed that the deletions of *eutABC*, *eutBC*, and *eutE* were in-frame (as suggested by DNA sequencing), since they could be complemented by the missing function(s) on a plasmid. Second, they showed that the *eutABCE* functions were critical to ethanolamine catabolism.

The metabolosome is dispensable when ethanolamine ammonia-lyase (EutBC) and acetaldehyde dehydrogenase (EutE) enzyme levels are elevated. Once reliable mutant strains and complementing plasmids were available, we tested the idea that elevated levels of ethanolamine catabolic enzymes would bypass the need for the metabolosome. For this purpose, two compatible plasmids, pEUT34 (P_{araBAD}-*eutABC*$^+$) and pEUT40 (P_{tac}-*eutE*$^+$), were moved into strain JE2261 carrying a chromosomal deletion of the entire *eut* operon (Δ*eut*), and growth of the resulting strain on ethanolamine was monitored over time.

In the absence of the metabolosome, plasmids pEUT34 (P_{araBAD}-*eutABC*$^+$) and pEUT40 (P_{tac}-*eutE*$^+$) restored growth of strain JE2261 (Δ*eut*) on ethanolamine, reaching full density with a doubling time of 67 h (Fig. 4A). The addition of glutathione to the medium shortened the lag and reduced the doubling time to 56 h (Fig. 4A). When plasmid pEUT42 (P_{araBAD}-*eutBC*$^+$) was substituted for plasmid pEUT34 (P_{araBAD}-*eutABC*$^+$), the resulting strain (JE8327) grew only when the medium was supplemented with glutathione with a doubling time of 100 h (Fig. 4B). In all instances, control experiments showed that the stimulatory effect of glutathione was not due to its use as a carbon and energy source (data not shown).

EutBC reactivase (EutA) is conditionally required, and nonspecific acetaldehyde dehydrogenases partially compensate for the lack of EutE. Under some growth conditions, the absence of EutE protein was partially compensated by nonspecific acetaldehyde dehydrogenase activities. For example, when plasmid pEUT34 (P_{araBAD}-*eutABC*$^+$) was moved into strain JE2261 (Δ*eut*) but plasmid pEUT40 (P_{tac}-*eutE*$^+$) was not, the resulting strain (JE8144) struggled to grow (doubling time, 167 h) and the cell density of the culture was poor (~0.35) (Fig. 4C). The absence of EutE protein may lead to the accumulation of acetaldehyde to toxic levels, hence arresting growth. This idea was consistent with the effect obtained by the effect of the addition of glutathione to the medium, which drastically stimulated growth, allowing strain JE8144 to reach near full density after 96 h of incubation (doubling time, 58 h) (Fig. 4C). These results revealed the existence of alternative nonspecific acetaldehyde dehydrogenases that can compensate for the absence of EutE. Possible explanations for the stimulatory effect of glutathione are discussed below.

We also found conditions under which EutA activity was not required for growth on ethanolamine. Strain JE8395 (Δ*eutA*) displayed a severe growth defect on ethanolamine (Fig. 5A). These results suggested that under the aerobic conditions used, EAL was deactivated and EutA activity was needed to keep EAL functional. Introduction of plasmid pEUT33 (P_{araBAD}-*eutA*$^+$) and inclusion of arabinose (100 μM) in the medium restored wild-type growth of strain JE8093 (Fig. 5A), indicating that the plasmid-encoded EutA was functional, and that the *eutA* mutation in the chromosome did not affect the expression genes downstream of it. It is noteworthy that when strain JE8093 (Δ*eutA*) was inoculated into ethanolamine medium in a microtiter plate, the culture grew to full density with a doubling time of 53 h, compared to 10 h for the strain carrying plasmid pEUT33 (P_{araBAD}-*eutA*$^+$) (Fig. 5B).

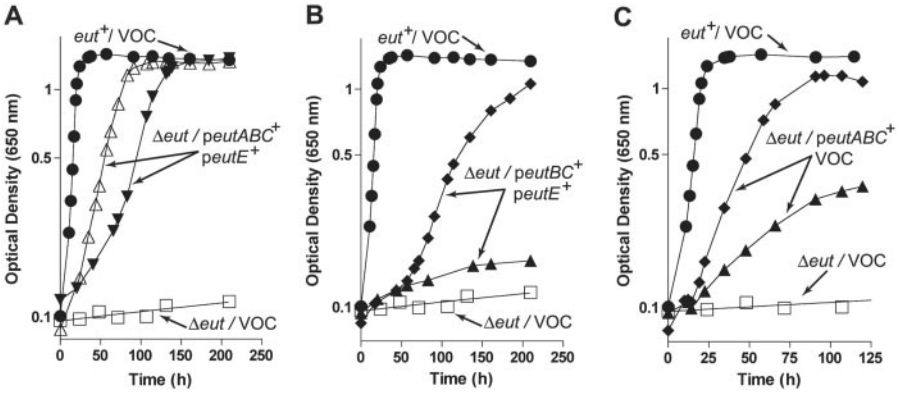

FIG. 4. Minimal functions for the ethanolamine catabolism. For all panels, VOC means vector-only control, strain JE8142 (Δ*eut*/pBAD33 pTAC-85) was used as the negative control (open squares), and strain JE8298 (*eut*$^+$/pBAD33 pTAC-85) (solid circles) was used as the positive control. When added, arabinose or IPTG was at 100 μM. A. Strain JE2261 (Δ*eut*) was transformed with plasmids pEUT34 (P_{araBAD}-*eutABC*$^+$) and pEUT40 (P_{tac}-*eutE*$^+$) and phenotypes assessed. For growth on ethanolamine as the sole source of carbon and energy, 2.5 mM oxidized GSSG (open triangles) or no GSSG (inverted solid triangles) was added to the medium. B. Strain JE2261 (Δ*eut*) was transformed with plasmids pEUT42 (P_{araBAD}-*eutBC*$^+$) and plasmid pEUT40 (P_{tac}-*eutE*$^+$), and phenotypes were assessed. For growth on ethanolamine as the sole source of carbon and energy: plus GSSG (solid diamonds) or without GSSG (solid triangles) addition to the medium. C. Strain JE2261 (Δ*eut*) was transformed with plasmids pEUT34 (P_{araBAD}-*eutABC*$^+$) and pTAC-85 and phenotypes were assessed: plus GSSG (solid diamonds) or without GSSG (solid triangles) addition to the medium.

DISCUSSION

The availability of a large collection of in-frame deletion mutant strains and plasmids carrying functional *eut* alleles allowed us to query the role of the metabolosome multimolecular complex during growth of *Salmonella enterica* on ethanolamine.

Results from experiments reported here clearly show that a strain of *S. enterica* lacking the genes encoding the ethanolamine metabolosome can grow on ethanolamine as the sole source of carbon and energy as long as ethanolamine ammonia-lyase (EutBC) and acetaldehyde dehydrogenase (but not necessarily EutE) enzymes are present in the cell at elevated levels. The metabolosome is not involved in the biochemistry of ethanolamine catabolism and may represent a strategy for reducing the level of enzymes needed to support growth on a poor carbon and energy source while serving as a containment structure for controlling the level of toxic acetaldehyde. By using the ethanolamine metabolosome, *S. enterica* may effectively reduce the level of acetaldehyde by concentrating within the metabolosome two enzymes that would rapidly consume acetaldehyde, i.e., acetaldehyde dehydrogenase (EutE) and alcohol dehydrogenase (presumably EutG).

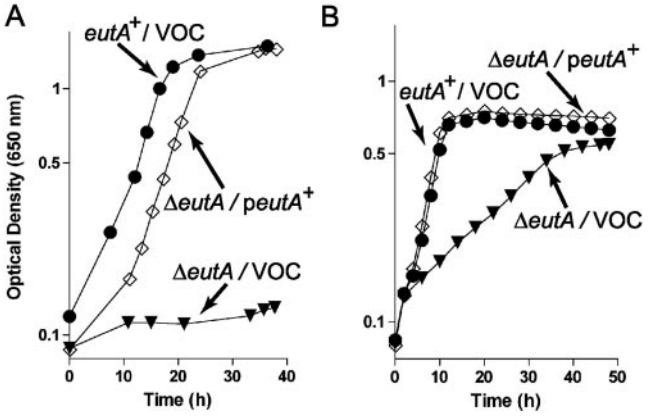

FIG. 5. Conditional requirement of *eutA* function. Strain JE8093 (Δ*eutA*) was transformed with either plasmid pEUT33 [gene expression induced with 100 μM L-(+)-arabinose] (open diamonds) or pBAD33 (solid inverted triangles). Strain JE8392 (solid circles) was used as a positive control. In both panels, VOC means vector-only control. A. The experiment was performed in 5-ml cultures in 16- by 150-mm borosilicate tubes. B. The experiment was performed in a microtiter plate with medium supplemented with ethanolamine (30 mM) and glycerol (0.5 mM). Glycerol was needed to prime cell growth; in its absence the onset of exponential growth was drastically delayed. Strain JE8142 (Δ*eut*/pBAD33 pTAC-85) was used as the negative control.

The need for EutA reactivase function can be bypassed by glutathione and reduced-oxygen conditions. Given the known role of EutA in maintaining EAL activity (21), it was not unexpected to see the lack of growth of an EutA-deficient strain on ethanolamine (Fig. 5A). It was very surprising, however, to see that the effect of the lack of EAL reactivase could be largely circumvented by glutathione (Fig. 4B). It was not surprising to learn that the EAL enzyme was more prone to inactivation under aerobic conditions than under reduced-oxygen (microtiter dish format) growth conditions (Fig. 5A versus B), since adenosylcoblamin (the coenzyme of EAL) can be readily inactivated by oxidation to Co^{2+} cobalamin (18). The meaning of these results may be explained by studies of EAL inactivation under aerobic and microaerophilic conditions as a function of substrate limitation.

The role of glutathione. The requirement for glutathione during growth of *S. enterica* on ethanolamine was reported a decade ago, when the activity of EAL in a glutathione-deficient *gshA* strain was noted to be >90% reduced relative to the activity of the EAL enzyme in the *gshA*$^+$ strain (25). Although it is tempting to speculate that glutathione may be involved in the EutA reaction, a role for this tripeptide in EutA-dependent EAL reactivation was not evident from the results recently reported by Toraya and coworkers (21).

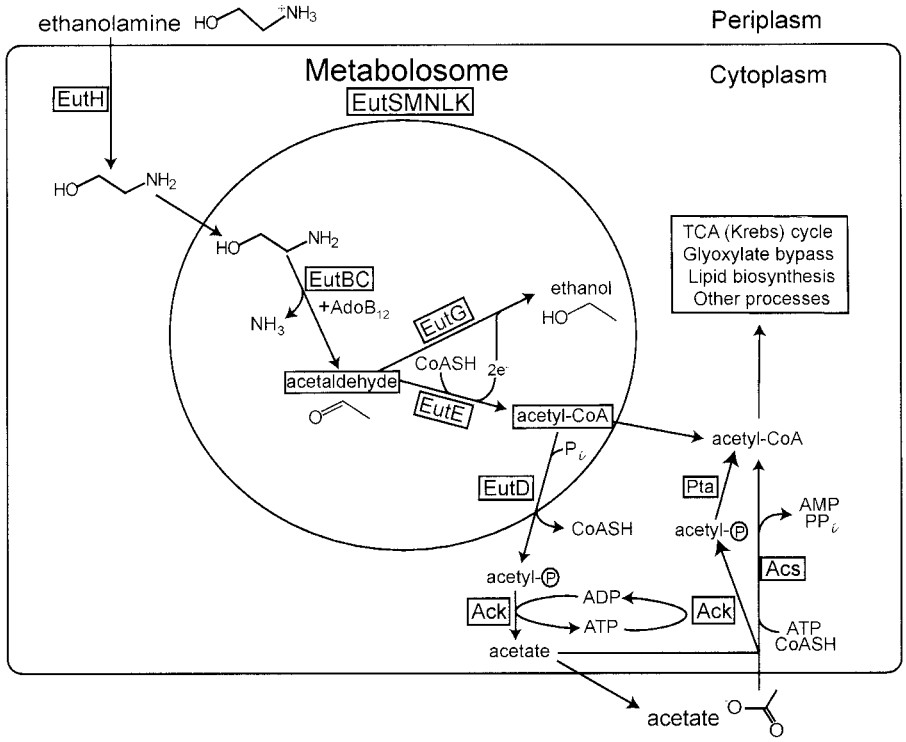

FIG. 6. Model for ethanolamine catabolism in *S. enterica*. The metabolosome representation is not to scale.

The role of glutathione in ethanolamine catabolism may be more complex than previously thought. The striking positive effect of glutathione on the growth of an EutE-deficient strain on ethanolamine was different than the effect on the EutA-deficient strain, since the EutE-deficient strain contained a wild-type *eutA* allele. It is possible that in the case of the EutE-deficient strain, glutathione enhances toxic acetaldehyde quenching (10, 19) or the substrate for the alternative acetaldehyde dehydrogenase is glutathionyl hemithioacetal. Furthermore, GSH may be a required cofactor for the alternative acetaldehyde dehydrogenase. The role of glutathione in ethanolamine catabolism requires further investigation.

A new perspective on ethanolamine catabolism and the role of the metabolosome. Fig. 6 shows our current model for the catabolism of ethanolamine. Ethanolamine enters the cell either by diffusion or via the EutH permease (23), depending on its protonation state. Within the metabolosome multimolecular complex (EutSMNLK), ethanolamine is converted to Ac-CoA via acetaldehyde by the concerted actions of EAL and EutE enzymes.

We hypothesize that the rate of Ac-CoA synthesis in the metabolosome may exceed the rate of Ac-CoA consumption, leading to the accumulation of Ac-CoA and probably acetaldehyde. Two safety valves appear to be built into the system to avoid problems created by flux variations. One is the putative EutG alcohol dehydrogenase, which would detoxify acetaldehyde by reducing it to ethanol. The latter, not redox balance, is likely to be the role of EutG, as *S. enterica* respires ethanolamine aerobically by using the electron transport chain. Although at present the kinetic analysis of EutG is lacking and the protein has not been studied in any detail, we predict that EutG will be a very efficient enzyme, so the level of toxic acetaldehyde can be maintained low.

The second safety valve is EutD, which converts Ac-CoA to Ac-P (6). The latter is used to conserve energy by the substrate level phosphorylation reaction catalyzed by acetate kinase (Ack) with the concomitant excretion of acetate, which is later recaptured by the Ac-CoA synthetase (33). Although the EutD phosphotransacetylase may play additional a role in ATP generation, we see EutD as an efficient strategy to maintain free CoA levels from becoming limiting.

We view the ethanolamine metabolosome as a strategy to concentrate low levels of ethanolamine catabolic enzymes. If the ethanolamine metabolosome were not made, the cell would have to synthesize large amounts of Eut enzymes, increasing the level of acetaldehyde generated by EAL in the absence of a containment structure. We propose that the metabolosome is also an effective means to concentrate acetaldehyde for the EutG and EutE enzymes that use it as a substrate. The same argument applies to the buildup of Ac-CoA. In other words, by confining ethanolamine catabolism to the metabolosome, the cell can swiftly respond to increases in the concentrations of these intermediates of the pathway beyond tolerable levels. We posit that the need to respond to even low levels of these intermediates may have been the selective pressure for the evolution of the EutD phosphotransacetylase, which has much higher affinity for its substrates and a higher catalytic efficiency than the housekeeping phosphotransacetytlase (Pta) enzyme (6), hence EutD is poised to maintain the level of Ac-CoA low. It is unclear why EutD activity is necessary, since the Pta enzyme present in the cell could replace EutD in the conversion of Ac-CoA to Ac-P. We

speculate that Ac-CoA levels during ethanolamine catabolism are kept low, since in the absence of EutD function, acetate excretion is abolished in spite of the presence of the Pta enzyme (34), suggesting that the levels of Ac-CoA are not high enough to serve as the substrate for Pta.

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