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Functions Required for Vitamin B_{12}-Dependent Ethanolamine Utilization in *Salmonella typhimurium*

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When B_{12} is available, *Salmonella typhimurium* can degrade ethanolamine to provide a source of carbon and nitrogen. B_{12} is essential since it is a cofactor for ethanolamine ammonia-lyase, the first enzyme in ethanolamine breakdown. *S. typhimurium* makes B_{12} only under anaerobic conditions; in the presence of oxygen, exogenous B_{12} must be provided to permit ethanolamine utilization. Genes required for ethanolamine utilization are encoded in the *eut* operon. For complementation testing, an F′ plasmid containing the *eut* genes was constructed by transposition of the *eut* operon (flanked by two Tn*10* elements) to an existing F plasmid. Complementation tests defined six genes in the *eut* operon. Three of these genes encode enzymes known to be involved in degradation of ethanolamine: ethanolamine ammonia-lyase (*eutB* and *eutC*) and acetaldehyde dehydrogenase (*eutE*). One gene (*eutR*) seems to encode a positive regulatory protein required for induction of transcription of *eut*. The function of one of the remaining two genes (*eutA*) was shown to be required for ethanolamine utilization only when cyano-B_{12} or hydroxy-B_{12} were the precursors of the adenosyl-B_{12} cofactor of ethanolamine ammonia-lyase; *eutA* mutants could use ethanolamine as the nitrogen source only when adenosyl-B_{12} was provided. No function has been assigned to the *eutD* gene, which is required for use of ethanolamine as a carbon source. Ethanolamine uptake assays of *eut* mutants suggest that no ethanolamine permease is encoded in the *eut* operon.

Ethanolamine can be cleaved to acetaldehyde and ammonia by the enzyme ethanolamine ammonia-lyase when adenosylcobalamin (Ado-B_{12}) is available to serve as a cofactor (4, 28). This enzyme permits *Salmonella typhimurium* to use ethanolamine as the sole source of carbon and nitrogen (9). *S. typhimurium* can synthesize B_{12} de novo but only under anaerobic growth conditions (Fig. 1) (23). The ability to synthesize B_{12} requires an extensive array of genes, but the metabolic importance of B_{12} and the reason B_{12} synthesis occurs only during anaerobic growth are not understood (15, 22). In addition to ethanolamine ammonia-lyase, three other *S. typhimurium* enzymes are known to require a B_{12} cofactor. Propanediol dehydratase (DL-1,2-propanediol hydrolyase; EC 4.2.1.28) enables propanediol to be used as the sole carbon source (30) and has only recently been detected in *S. typhimurium* (R. Jeter, personal communication). A B_{12}-dependent methyl transferase (encoded by the *metH* gene) methylates homocysteine to form methionine (35). The *metH* enzyme is not essential for methionine biosynthesis because a B_{12}-independent enzyme (encoded by the *metE* gene) can catalyze the same reaction (16). Vitamin B_{12} is also required for formation of the modified base queuosine present in the anticodon loop of some tRNAs, but this modification is not essential under standard growth conditions (18). The genetics of ethanolamine utilization is being studied as a tool for analysis of the B_{12} biosynthetic pathway, to determine how genes are regulated in response to B_{12} availability, and to learn the physiological importance of B_{12} to *S. typhimurium*.

During aerobic growth, B_{12} must be obtained from the growth medium to permit ethanolamine utilization. The form of B_{12} encountered in nature is probably hydroxy-B_{12} (HO-B_{12}). While cyano-B_{12} (CN-B_{12}; commercial vitamin B_{12}) is generally provided in laboratory media, neither form of B_{12} can be used directly as the cofactor for ethanolamine ammonia-lyase. To form Ado-B_{12} from exogenous B_{12}, the cell must transport the B_{12}, reduce the central cobalt atom, and add an adenosyl group (Fig. 1) (20). A high-affinity B_{12} transport system has been described for *Escherichia coli* (13); a similar system appears to exist in *S. typhimurium*. The enzymes responsible for reduction and adenosylation have been studied in several species of bacteria (5, 31, 33). Mutants of *E. coli* defective in the *btuR* gene have a decreased pool of Ado-B_{12}, but still contain enough Ado-B_{12} to permit growth on ethanolamine (25). J. Escalante-Semerena, S.-J. Suh, and J. Roth (unpublished results) have isolated mutants (*cobA*) in the same region of the *S. typhimurium* chromosome as *btuR* of *E. coli*. The *cobA* mutants are completely defective in ethanolamine utilization (using CN-B_{12}) when grown in glucose medium with heavy aeration; under these growth conditions, *cobA* mutants can use ethanolamine only if Ado-B_{12} is provided. Anaerobically, the *cobA* mutants express a gene in the CobI operon that can perform the missing (*cobA*) function, permitting adenosylation of assimilated CN-B_{12} and HO-B_{12}. Apparently, at least one of the steps in Ado-B_{12} formation can be catalyzed by either one of two separately encoded enzymes. Since ethanolamine ammonia-lyase requires Ado-B_{12} and the B_{12}-dependent methyl transferase does not, one would expect mutants defective in adenosylation to be defective in ethanolamine degradation but to be capable of using B_{12} for methionine synthesis. One might expect that functions involved in adenosylation of B_{12} would be encoded in the same operon as the other functions required for ethanolamine utilization.

Mutants defective in ethanolamine utilization define the *eut* operon, located at about 50 min on the *S. typhimurium* chromosome (27). Induction of transcription of the *eut* operon requires the simultaneous presence of ethanolamine and B_{12}, but the mechanism by which this regulation pattern

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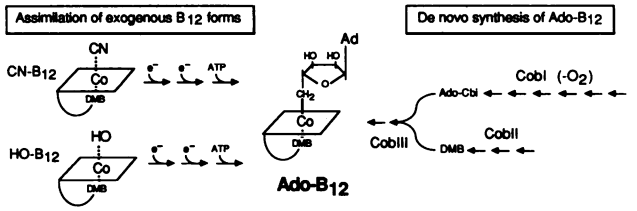

FIG. 1. Sources of Ado-B_{12}, cofactor of ethanolamine ammonia-lyase. The tetrapyrrole ring of B_{12} is indicated by a square with the cobalt atom at the center. The upper ligand (an HO, CN, or adenosyl group) is shown above the plane of the tetrapyrrole ring. HO-B_{12} or CN-B_{12} can be obtained from the growth medium. Under anaerobic growth conditions, the CobI pathway is functional, permitting de novo B_{12} biosynthesis. Ado-Cbi, Adenosylcobinamide; DMB, 5,6-dimethylbenzimidazole; Ad, adenine.

is achieved is not fully understood. A genetic map of *eut* includes mutations that prevent induction of transcription, mutations that eliminate ethanolamine ammonia-lyase, and mutations that eliminate the second enzyme in the degradation pathway, acetaldehyde dehydrogenase (27). The genetic map also includes mutations that do not affect either of these enzyme activities but nevertheless cause an inability to use ethanolamine. The existence of these mutants suggests that additional functions necessary for ethanolamine utilization are encoded in the *eut* region.

We describe here results of complementation tests performed to determine the number of genes encoded in the *eut* operon. Six genes were defined, including two for ethanolamine ammonia-lyase (*eutB* and *eutC*), one for acetaldehyde dehydrogenase (*eutE*), and one whose activity is required for induction of transcription of the *eut* operon (*eutR*). One of the newly defined genes (*eutA*) was shown to be required for ethanolamine utilization only when CN-B_{12} or HO-B_{12} were the precursors of the Ado-B_{12} cofactor of ethanolamine ammonia-lyase. Ethanolamine uptake experiments suggest that no ethanolamine permease is encoded in the *eut* operon. The F′ plasmid used in the complementation tests was constructed by a transpositional method that allows one to place any region of the chromosome on an F plasmid.

MATERIALS AND METHODS

Bacterial strains and transposons. All strains used in this study are derivatives of *S. typhimurium* LT2 (Table 1). The *eut* mutations used in the complementation tests are listed only by their allele numbers (see below); isolation and genetic mapping of these mutations was described previously (27). Two transposition-defective derivatives of the specialized transducing bacteriophage Mu dl(Amp^r *lac* cts) of Casadaban and Cohen (6) were used. The derivatives, MudI1734 Kan^r (7) and Mu dl-8 Amp^r (21) are referred to as MudJ and MudA, respectively; each can form *lac* operon fusions upon insertion. A transposition-defective derivative of Tn*10*, Tn*10d*Cam (14), was used.

Media and growth conditions. The complex medium was nutrient broth (0.8%; Difco Laboratories) with NaCl (0.5%). The minimal medium was the E medium of Vogel and Bonner (32) with glucose (0.2%). The carbon-free minimal medium was NCE (12), and the carbon- and nitrogen-free minimal medium was NCN (26). Ethanolamine hydrochloride (0.2%; Aldrich Chemical Co.) was used as the carbon source in NCE medium, as the nitrogen source in NCN medium with glycerol (0.2%), or as both the carbon and nitrogen sources in NCN medium. 1,2-Propanediol (0.4%; Aldrich) was used as the carbon source in NCE medium or in NCN medium with ethanolamine as the nitrogen source. CN-B_{12}, HO-B_{12}, and Ado-B_{12} (all from Sigma Chemical Co.) were used as exogenous B_{12} sources (0.1 μg/ml). Medium containing Ado-B_{12} was protected from exposure to light during preparation and use. Amino acids were added to minimal media as required at the concentrations previously recommended (12). Cystine (0.058 mM) was added to nutrient broth used for growth of *cys* auxotrophs. Antibiotics were used at the following concentrations (micrograms per milliliter) in mineral and complex media, respectively: ampicillin, 15 and 30; chloramphenicol, 5 and 20; kanamycin, 125 and 50; and tetracycline, 10 and 20. Solid medium contained agar (1.5%; Difco) or, when it was necessary to score growth on medium containing ethanolamine as the sole nitrogen source, Noble agar (1.5%; Difco). Cells were grown aerobically at 37°C.

TABLE 1. Bacterial strains used[a]

Strain	Genotype LT2
LT2	Wildtype
TR35	*his-712 ser-821 arg-501*/F′ T80
TR6583	*metE205 ara-9*
TR7020	*his-2236*
TT10286	*hisD9953*::MudJ
TT11289	*recA1 srl-203*::Tn*10d*Cam
TT11472	*eutE55 zfa-3646*::Tn*10*
TT11514	*eutR100 zfa-3648*::Tn*10*
TT11515	*eutD101 zfa-3648*::Tn*10*
TT11521	*eutB107 zfa-3648*::Tn*10*
TT11524	*eutC110 zfa-3648*::Tn*10*
TT11528	*eutA114 zfa-3648*::Tn*10*
TT11701	DEL730(*cysA1585**MudA**eut-2*)
TT13439	*zfa-3647*::Tn*10*
TT13441	*zfa-3649*::Tn*10*
TT14066	DEL730(*cysA1585**MudA**eut-2*) *zfa-3649*::Tn*10*
TT14068	*zfa-3649*::Tn*10 zfa-3647*::Tn*10*
TT14071	*zfa-3649*::Tn*10 zfa-3647*::Tn*10 hisD9953*::MudJ
TT14074	*zfa-3649*::Tn*10 zfa-3647*::Tn*10 hisD9953*::MudJ/F′ T80
TT14076	*eut-240 hisC9955*::MudA
TT14078	*eut-240 hisC9955*::MudA/F′ 606
TT14082	*eut-240 hisG10082*::Tn*10d*Cam/F′ 606
TT14771	*metE205 ara-9 eutA114 zfa-3648*::Tn*10*

[a] All strains were constructed for this study or were obtained from the laboratory collection.

Genetic techniques. All transductional crosses were performed with the high-frequency, generalized transducing phage mutant P22 HT105/1 *int-201* (29) as previously described (27). Transductants were purified and made phage-free by streaking for single colonies on nonselective green indicator plates (8).

All conjugational crosses were performed by mixing liquid cultures (0.05 ml each) of the donor and recipient strains on one side of a petri dish containing selective medium. The plate was incubated at 37°C for 3 to 6 h, and the cell mixture was then streaked to obtain single exconjugant colonies after further incubation.

Placement of the *eut* region on an F plasmid. A composite transposon, consisting of the *eut* operon and two flanking Tn*10* insertions, was used to introduce the *eut* genes onto the F′ T80 *his*$^+$ plasmid by transposase-mediated transposition. The composite transposon was constructed on the chromosome in two steps. A Tn*10* insertion located adjacent to the promoter-proximal end of the *eut* operon (*zfa-3649*::Tn*10*) was transduced into the *eut-cys* deletion mutant TT11701 by selecting for Tet^r recombinants. A transductant containing

both the Tn*10* insertion and the deletion mutation (Eut$^-$ Cys$^-$ Tet^r) was kept (TT14066). A second Tn*10* insertion (*zfa-3647*::Tn*10*) is located adjacent to the promoter-distal end of the *eut* operon and within the region removed by the *eut-cys* deletion; this insertion was transduced into strain TT14066 by selecting for Cys$^+$ recombinants. Both Eut$^+$ and Eut$^-$ transductants were recovered. The Eut$^-$ transductants contain a deletion of *eut* generated when the donor and recipient Tn*10* elements recombine. The Eut$^+$ transductants that carry two copies of Tn*10* flanking *eut* were identified by instability of the Eut$^+$ phenotype due to loss of *eut* by recombination between the chromosomal Tn*10* elements. The unstable Eut$^+$ transductants carry the composite transposon shown in Fig. 2. The strain containing the composite transposon (TT14068) was maintained on medium containing ethanolamine as the sole nitrogen source to select against loss of *eut* by recombination between the flanking Tn*10* elements. Strain TT14071 is a His$^-$ Kan^r derivative of strain TT14068 made by transduction of the *hisD9953*::MudJ insertion from strain TT10286. The F′ T80 *his*$^+$ plasmid from strain TR35 was introduced into strain TT14071 by conjugation, selecting for a His$^+$ exconjugant (TT14074).

Transposition of the composite transposon into the plasmid F′ T80 *his*$^+$ was detected by conjugational mating of strain TT14074 with a Eut$^-$ Cys$^-$ His$^-$ Amp^r recipient (TT14076). Selection was made for exconjugants that were His$^+$ (specified by plasmid F′ T80), Eut$^+$ (conferred by an insertion of the composite transposon within plasmid F′ T80), and Amp^r (to counter-select the donor). The cross was done by spreading 0.5 ml of an overnight culture of donor cells with 0.5 ml of recipient cells on NCE medium containing ethanolamine, CN-B_{12}, cystine, and ampicillin. Control crosses with the same donor-recipient pair were done without selection for Eut$^+$ on E medium containing glucose, tetracycline, ampicillin, and cystine to select for simple insertions of Tn*10* in the F′ T80 plasmid. Selection for Tet^r yielded about 1,000-fold more exconjugants than selection for Eut$^+$, suggesting that the individual Tn*10* elements transpose substantially more frequently than the composite unit including the *eut* operon. One F′ His$^+$ Eut$^+$ Tet^s plasmid (described in Results and designated F′ 606) was used for complementation tests.

Complementation tests. The *eut* point mutations to be used in complementation tests were placed on F′ 606 by cotransduction with a Tn*10* insertion near the *eut* region (*zfa-3646*::Tn*10* for *eut-1* through *eut-55* or *zfa-3648*::Tn*10* for *eut-56* through *eut-138*). The *eut*::MudJ and *eut*::Tn*10* insertions were placed on F′ 606 in transductional crosses by selecting for Kan^r or Tet^r recombinants. The recipient used was strain TT14078 or strain TT14082. In these crosses, the *eut* mutations and adjacent Tn*10* insertion are inherited only by recombination with the plasmid *eut* region because the recipient chromosome carries a deletion of *eut* and *cysA* that is rarely repaired by P22 transduction. Retention of the chromosomal deletion by all Eut$^-$ recombinants was confirmed by verifying their requirements for cystine. The isolation of the *eut* mutants used in this study and the construction of a genetic map of the *eut* region that includes the mutations have been described previously (27).

To construct recipient strains for the complementation tests, *eut* point mutations were introduced into strain TR7020 (*his-2236*) by transduction using one of the adjacent Tn*10* insertions as the selective marker. The *eut*::MudJ and *eut*::Tn*10* insertions were introduced into strain TR7020 in transductional crosses selecting Kan^r or Tet^r recombinants. To make these strains recombination deficient, P22 grown on a strain containing a Tn*10d*Cam insertion near the *recA1* mutation (TT11289) was used to transduce the Eut$^-$ His$^-$ strains to Cam^r. The *recA* recombinants were identified by UV light sensitivity.

To construct diploids for the complementation tests, each *eut*$^-$ F′ plasmid was introduced into the Eut$^-$ His$^-$ RecA$^-$ recipients by conjugational crosses selecting inheritance of the plasmid *his*$^+$ marker. Donor strains were grown overnight in minimal glucose medium containing cystine, and recipient strains were grown overnight in minimal glucose medium with histidine. The donor and recipient strains were mated on minimal glucose plates, and after growth, four isolated colonies from each cross were picked and patched on minimal glucose plates. These plates were incubated 6 to 12 h and used as masters to make replica prints to medium containing ethanolamine as the sole carbon and nitrogen source. Complementation was scored as positive if significant growth of the four patches occurred after 3 to 5 days of incubation.

Ethanolamine uptake assays. Cells to be used in the uptake assays were grown with vigorous agitation to an A_{650} of 0.4 to 0.6 in NCE medium containing glycerol as the carbon source. The medium was supplemented with ethanolamine and CN-B_{12} (to induce *eut* operon expression) unless indicated otherwise. The cells were washed twice by centrifugation and suspension in NCE medium; following the third centrifugation, cells were suspended in NCE medium containing 0.1% glucose to obtain an A_{650} of 0.66 (about 10^9 cells per ml) and kept on ice. To measure ethanolamine uptake, a 0.5-ml sample of the cell suspension was preincubated for 3 min at 25°C, and [^{14}C]ethanolamine (53 μCi/mmol; Amersham Corp.) was then added to a final concentration of 10 μM. Samples of the reaction mixture were removed after 15, 30, 45, 60, and 120 s of incubation at 25°C; cells from each sample were immediately collected on membrane filters (HA, 0.45-μm pore size; Millipore Corp.) and washed twice with 4 ml of NCE medium containing 0.2% unlabeled ethanolamine. The filters were dried with a heat lamp, and the amount of labeled ethanolamine taken up was determined by scintillation counting.

RESULTS

Construction of an F′ plasmid containing the *eut* operon. F′ plasmids that include the *eut* operon were generated by transposition of the chromosomal *eut* region of *S. typhimurium* to an existing F′ plasmid. Transposition of the *eut* region was achieved by constructing a composite transposon consisting of the *eut*$^+$ genes flanked by Tn*10* elements (Fig. 2). The transposase encoded by the IS*10* elements of Tn*10* can catalyze transposition of the composite element (Tn*10* *eut*$^+$ Tn*10*) to a new site. The behavior of a similar composite transposon consisting of the *malK* gene from *E. coli* and flanking Tn*5* elements has been previously described (19). Figure 2 presents the various *eut*-containing subsets of the composite unit that should be able to transpose; one subset includes only the *eut*$^+$ operon and an IS*10* element from each of the flanking Tn*10* transposons. The plasmid F′ T80 *his*$^+$ was provided as a target for insertion by the composite transposon. This plasmid was chosen because of its ability to transfer and its low copy number. Plasmids that acquired an insertion of the composite transposon were identified by conjugational crosses with a Eut$^-$ His$^-$ recipient by selecting for Eut$^+$ His$^+$ exconjugants. Both Tet^s Eut$^+$ His$^+$ and Tet^r Eut$^+$ His$^+$ plasmids were isolated as expected from the transposable subsets of the composite element (Fig. 2).

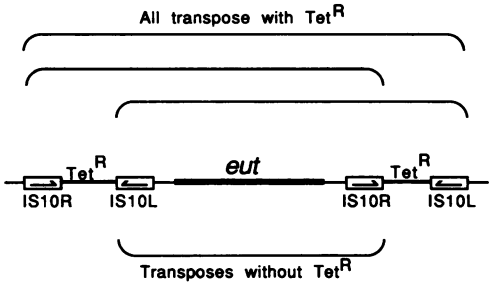

FIG. 2. The composite transposon consisting of the *eut* operon and two flanking Tn*10* insertions. New insertions of the composite transposon which include *eut* also include either Tn*10* or IS*10* at each end. The four possible transposable units are shown. Both Tn*10* elements are in the B orientation as defined relative to other Tn*10* insertions in reference 10. The orientation of an A Tn*10* insertion (*hisG9424*::Tn*10*) relative to the chromosome is known (17), making it possible to define the chromosomal orientation of *zfa-3647*::Tn*10* and *zfa-3649*::Tn*10*. The *zfa-3647*::Tn*10* insertion (shown on the left) is located between *eut* and *purC*, while the *zfa-3649*::Tn*10* insertion (shown on the right) is located at the opposite end of the *eut* operon near *cysA*. Transcription of *eut* is from left to right.

One of the Tet^s plasmids (F′ 606) was used in all subsequent experiments. The *eut*$^+$ and *his*$^+$ markers of F′ 606 were inherited simultaneously in all subsequent conjugational crosses. This plasmid is quite stable; about 10 generations of nonselective growth yielded Eut$^-$ His$^-$ segregants at a frequency of 10^{-3} and no Eut$^-$ His$^+$ segregants were found (frequency $<10^{-3}$). The stability of the *eut* region is probably due to the fact that the repeated flanking IS*10* elements are in inverse orientation and thus do not recombine to eliminate the *eut* operon.

Six complementation groups included in the *eut* locus. The complementation behavior of *eut* mutations was tested in strains made diploid for *eut* by introduction of F′ 606. The *eut* mutations were placed on F′ 606 by transductional crosses, and the resulting *eut* plasmids were introduced into various *eut his recA* recipients by selection for inheritance of the *his*$^+$ marker of the plasmid (see Materials and Methods). Complementation was scored by testing the exconjugants for growth on medium containing ethanolamine as the sole source of carbon and nitrogen.

The results of the complementation tests are shown in Fig. 3. Each mutation was tested in both the chromosomal and plasmid positions. The 40 mutations tested defined six complementation groups. Each gene is defined by several point mutations which do not complement each other but which do complement point mutations in the other genes. In Fig. 3, mutations are presented in order (horizontally and vertically) according to their positions in the genetic map described previously (27). It can be seen that mutations inferred to affect the same complementation group map in a contiguous cluster. Point mutations and Tn*10* insertion mutations that seem to have a polar effect on expression of downstream genes are also included (see below). Occasionally, complementation behavior was affected by whether the alleles being tested were on the plasmid or on the chromosome. Possible explanations for this are presented in the discussion.

Some of the mutations used in the complementation tests were previously tested for their effects on the in vitro activity of the two enzymes required for ethanolamine utilization, ethanolamine ammonia-lyase and acetaldehyde dehydrogenase (27). The gene designation inferred from the complementation tests and a summary of enzyme activities

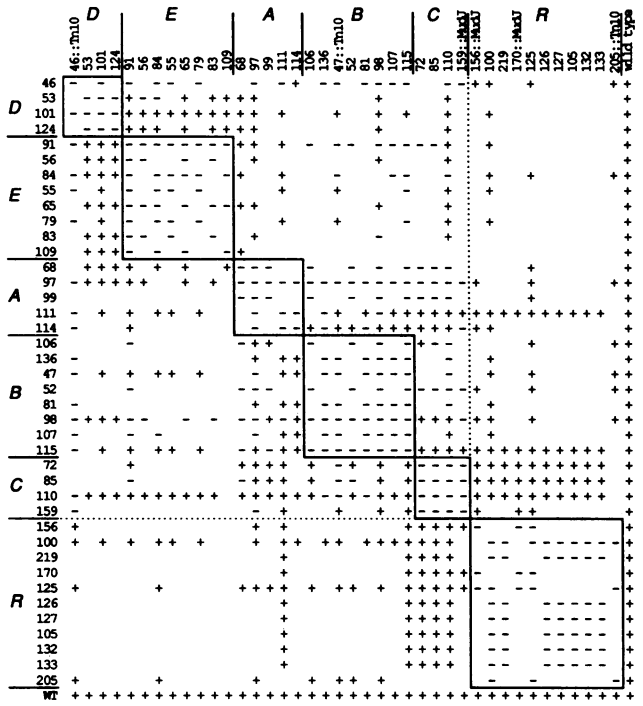

FIG. 3. Complementation behavior of *eut* mutations. The donor strains are listed by *eut* allele number in their map order across the top, and the recipient strains are listed by *eut* allele number in their map order on the left. The map order is from the genetic map of the *eut* operon (27). Transcription of the operon is from left to right. The phenotype of each merodiploid is given as + when complementation occurred to give the Eut$^+$ phenotype and as − when complementation was not observed. A blank indicates that the diploid was not constructed. Solid lines enclose the blocks of negative results resulting from the lack of complementation between mutations inferred to affect a single complementation group. The complementation groups have been named (from left to right) *eutD*, *eutE*, *eutA*, *eutB*, *eutC*, and *eutR*. The dashed line indicates the inferred position of a promoter for the distal *eutR* gene.

is shown in Fig. 4. These results indicate that the *eutB* and *eutC* genes specify ethanolamine ammonia-lyase and that the *eutE* gene specifies acetaldehyde dehydrogenase. All of the mutations previously shown to cause inability to express the entire *eut* operon (27) fell into a single complementation group, *eutR*; we presume that this gene encodes a positive regulatory protein. Results suggesting the role of the *eutA* gene product are presented later. The role of the *eutD* gene product remains undefined, except that it is required only for use of ethanolamine as a carbon source.

Transcriptional polarity and independent expression of *eutR*. Several point mutations and Tn*10* insertion mutations failed to complement mutations inferred to be in downstream genes (examples are *eutD46*::Tn*10* and *eutA68* in Fig. 3). The orientation of the polar effects is consistent with the direction of transcription of the *eut* operon (from left to right in Fig. 3 and 4) which was determined previously by using *eut-lac* operon fusions (27). Nevertheless, none of the mutations in the *eutD*, *eutE*, *eutA*, *eutB*, or *eutC* genes, including the polar mutations mentioned above and the *eutC159*::MudJ insertion, exerted a polar effect sufficient to prevent complementation with *eutR* mutations. This suggests that the *eutR* gene can be transcribed from a promoter located downstream of the *eutC159*::MudJ insertion site. In fact, transcription of the *eutR* gene occurs from both the

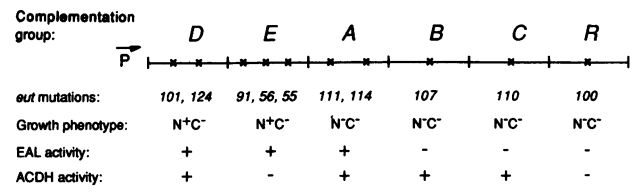

FIG. 4. Gene-function relationships. The name of each complementation group and representative mutations in each group are shown at the top. The mutations are listed in their map order and transcription is from left to right. Below each complementation group is a summary of the phenotype and in vitro enzyme activities of strains containing the listed mutations. C^+ indicates an ability to use ethanolamine as a carbon source (aerobically with CN-B_{12}); N^+ indicates an ability to use ethanolamine as a nitrogen source (aerobically with CN-B_{12}). For in vitro ethanolamine ammonia-lyase activity (EAL) and in vitro acetaldehyde dehydrogenase activity (ACDH), a + indicates an in vitro enzyme activity of 30 to 100% of that present in wild-type strains and a − indicates an in vitro enzyme activity of less than 1% of that present in wild-type strains. The enzyme assay data has been previously published (27). An exceptional mutant, *eutE91*, has in vitro ethanolamine ammonia-lyase activity of only 7% of that present in wild-type strains. This mutation may have a slight polar effect on transcription of the downstream genes for ethanolamine ammonia-lyase (*eutB* and *eutC*).

regulated promoter for the operon and from the promoter located downstream of the *eutC159*::MudJ insertion site (unpublished results).

Utilization of Ado-B_{12} precursors. The first enzyme in ethanolamine utilization, ethanolamine ammonia-lyase, requires the cofactor Ado-B_{12} for activity (4, 28). This cofactor is synthesized de novo during anaerobic growth. The B_{12} biosynthetic pathway is outlined in Fig. 1. To permit ethanolamine utilization during aerobic growth, B_{12} must be obtained from the growth medium. Exogenous B_{12} is usually supplied as CN-B_{12} or HO-B_{12}, neither of which is active as a cofactor until converted to Ado-B_{12} in several enzymatic steps. In fact, the inactive precursor forms of B_{12} are potent inhibitors of ethanolamine ammonia-lyase in vitro (3). *S. typhimurium* can convert HO-B_{12} or CN-B_{12} to the Ado-B_{12} required by ethanolamine ammonia-lyase (Fig. 1).

Mutants defective in the *eutA* gene can use ethanolamine as the nitrogen source when Ado-B_{12} is provided but not when HO-B_{12} or CN-B_{12} is provided. This was determined by testing the abilities of various *eut* mutants to use ethanolamine as a carbon or nitrogen source when Ado-B_{12} was provided (Table 2). These results suggest a role for the *eutA* gene product in the use of HO-B_{12} and CN-B_{12} as precursors of Ado-B_{12}. It should be noted that *eutA* mutants could not use ethanolamine as a carbon source even when Ado-B_{12} was provided (Table 2). This may be due to insufficient transport of intact Ado-B_{12} (see Discussion for alternative explanations).

Strains containing mutations in the *eutB*, *eutC*, and *eutR* genes were unable to degrade ethanolamine for use as either a carbon or a nitrogen source even when Ado-B_{12} was provided (Table 2). Strains containing mutations in the *eutD* and *eutE* genes fail to use ethanolamine as a carbon source but are able to use it as a nitrogen source (with any form of B_{12} provided). Since *eutD* and *eutE* mutants are able to derive ammonia (but not carbon) from ethanolamine, they must retain the ability to transport ethanolamine and synthesize the Ado-B_{12} required as a cofactor by ethanolamine ammonia-lyase; as expected, the growth defect of these mutants was not corrected by providing Ado-B_{12} (Table 2).

The *eutA* mutants were able to use HO-B_{12} or CN-B_{12} for two other B_{12}-dependent enzymes in *S. typhimurium*. One of these enzymes, a B_{12}-dependent methyltransferase, is one of the two enzymes that can methylate homocysteine to form methionine (16). The function of the B_{12}-dependent enzyme was tested in a *metE* mutant which lacks the alternative B_{12}-independent methyltransferase enzyme (Table 2). The *metE eutA* double mutant was unable to grow on minimal medium but did grow on medium supplemented only with HO-B_{12} or CN-B_{12}, indicating that *eutA* mutants continued to transport B_{12} and synthesize the methyl-B_{12} cofactor needed by the B_{12}-dependent methyltransferase. The possibility remains that HO-B_{12} and CN-B_{12} transport is partially impaired in *eutA* mutants.

The *eutA* mutants retain the ability to synthesize Ado-B_{12} from HO-B_{12} or CN-B_{12} precursors. This was evident because the Ado-B_{12} requirement of another Ado-B_{12}-dependent enzyme, propanediol dehydratase, was satisfied by

TABLE 2. Abilities of *eut* mutants to use Ado-B_{12} precursors

Relevant genotype	Carbon source[a]	Nitrogen source[a]	B_{12}-dependent function tested[a]	Growth of strain aerobically on medium containing the indicated form of B_{12}[b]			
				None	CN-B_{12}	HO-B_{12}	Ado-B_{12}
Wild type	Glycerol	EA	EAL (nitrogen)[c]	−	+	+	+
Wild type	EA	NH_4	EAL (carbon)	−	+	+	+
eutA111 or *eutA114*	Glycerol	EA	EAL (nitrogen)	−	−	−	+
eutA111 or *eutA114*	EA	NH_4	EAL (carbon)	−	−	−	−
eutB107, *eutC110*, or *eutR100*	Glycerol	EA	EAL (nitrogen)	−	−	−	−
eutB107, *eutC110*, or *eutR100*	EA	NH_4	EAL (carbon)	−	−	−	−
eutD101 or *eutE55*	Glycerol	EA	EAL (nitrogen)	−	+	+	+
eutD101 or *eutE55*	EA	NH_4	EAL (carbon)	−	−	−	−
metE205	Glucose	NH_4	MT (methionine)	−	+	+	+
metE205 eutA114	Glucose	NH_4	MT (methionine)	−	+	+	+
Wild type	PD	NH_4	PDD (carbon)	−	+	+	+
eutA114	PD	NH_4	PDD (carbon)	−	+	+	+
Wild type	PD	EA	EAL (nitrogen) and PDD (carbon)	−	+	+	+
eutA114	PD	EA	EAL (nitrogen) and PDD (carbon)	−	−	−	−

[a] EA, ethanolamine; EAL, ethanolamine ammonia-lyase; MT, 5-methyltetrahydrofolate-homocysteine transmethylase; PD, propanediol; PDD, propanediol dehydratase.

[b] All forms of B_{12} were supplied at 0.1 μg/ml.

[c] The growth requirement satisfied by the B_{12}-dependent enzyme is indicated in parentheses.

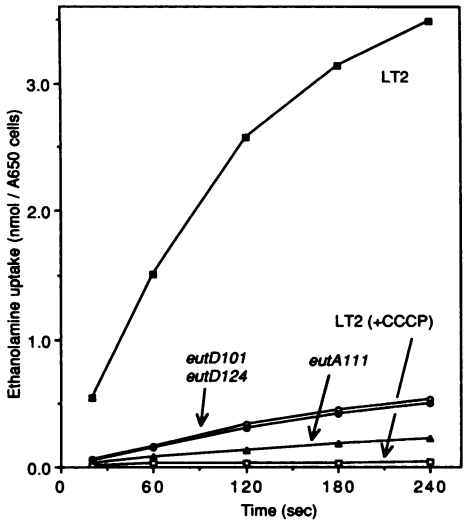

FIG. 5. Effect of ability to degrade ethanolamine on the rate of ethanolamine uptake. Strains containing a *eutE55*, *eutB107*, *eutC110*, or *eutR100* mutation accumulated ethanolamine at a rate less than that shown for *eutA111* but greater than that shown for LT2 pretreated with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (data not shown).

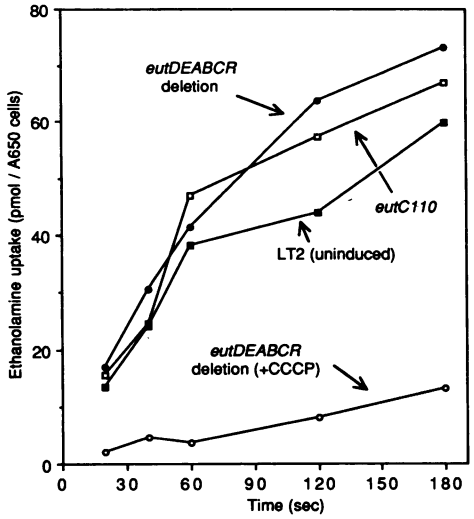

FIG. 6. Ethanolamine uptake by strains which cannot degrade ethanolamine. Strain LT2 was grown without ethanolamine or B_{12} present. CCCP, Carbonyl cyanide *m*-chlorophenylhydrazone.

HO-B_{12} or CN-B_{12} in *eutA* mutants. The Ado-B_{12}-dependent propanediol dehydratase has only recently been identified in *S. typhimurium* (R. Jeter, personal communication). The propanediol utilization pathway enabled both wild-type and *eutA* mutant strains to grow on medium containing propanediol as the sole carbon source and ammonia as the sole nitrogen source when HO-B_{12} or CN-B_{12} was provided (Table 2). It is possible that alternative enzymes which convert HO-B_{12} or CN-B_{12} to Ado-B_{12} are induced only during growth on propanediol. To determine if a function induced during growth on propanediol can substitute for the *eutA* gene product, the *eutA* mutants were tested for the ability to grow on medium containing propanediol as the sole carbon source (to induce this pathway) and ethanolamine as the sole nitrogen source (to test whether sufficient Ado-B_{12} was made) (Table 2). The *eutA* mutants were unable to grow on this medium, indicating that no substitute for the *eutA* gene product appeared during growth on propanediol.

The above observations may be explained by the fact that ethanolamine ammonia-lyase is acutely sensitive to inhibition by CN-B_{12} and HO-B_{12}. The *eutA* gene product may act to prevent this inhibition in vivo. To test this possibility, the ability of a *eutA* mutant to use a mixture of CN-B_{12} and Ado-B_{12} for growth on ethanolamine as the nitrogen source was checked. Ado-B_{12}-dependent growth was completely inhibited by an equal concentration of either HO-B_{12} or CN-B_{12} in the medium; this inhibition was not seen for *eutA*$^+$ cells, which use ethanolamine with either or both of these B_{12} forms at the same concentration. This suggests that the function of the *eutA* gene product may be to remove inhibitory forms of B_{12} or to allow ethanolamine ammonia-lyase to discriminate between these B_{12} forms.

The *eut* operon does not appear to include an ethanolamine permease. The rate of ethanolamine uptake by *eut* mutants was examined to determine whether an ethanolamine permease is encoded in the *eut* operon. The uptake assays were performed on cells that were grown in glycerol-ammonia medium containing ethanolamine and CN-B_{12} to induce expression of the *eut* operon. The data in Fig. 5 show that strain LT2 accumulated ethanolamine at a high and nearly constant rate throughout the 4-min assay. Accumulation of ethanolamine in point mutants defective in any of the individual *eut* genes occurred at lower rates. It appears that uptake of ethanolamine at a high rate requires concurrent degradation of ethanolamine by both ethanolamine ammonia-lyase and acetaldehyde dehydrogenase. Ethanolamine uptake did not occur when the proton gradient was abolished by addition of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone to the cells before the assay began (Fig. 5).

Although transport of ethanolamine was reduced in all of the individual *eut* mutants, a significant residual level of transport remained. This residual level of transport was blocked by carbonyl cyanide *m*-chlorophenylhydrazone, suggesting that the proton gradient may contribute to transport. Since all mutations reduced transport to a similar extent, we presume that all exert their effect by impairing degradation and none of the six known genes in the operon encodes a component of the transport mechanism.

To test more directly for the presence of a permease gene in the *eut* operon, we compared two strains, both blocked in ethanolamine degradation. One carried a deletion of the entire operon (*eut-237*); the other carried a point mutation in one of the genes for ethanolamine ammonia-lyase (*eutC110*). If an ethanolamine permease is encoded in the *eut* region, it should contribute to the lyase-independent ethanolamine uptake activity in the *eutC* point mutant but not in the deletion mutant. The uptake rates observed for the two strains were identical (Fig. 6), suggesting that the deletion mutant lacks only the ability to degrade ethanolamine. The ethanolamine uptake occurred only when the proton gradient was left intact; this level was present in wild-type cells grown in the absence of ethanolamine and B_{12} (Fig. 6). For the assays shown in Fig. 6, 20 μM ethanolamine was present. When the concentration of ethanolamine was increased to 100 μM, the uptake rates increased for both strains, but the two strains could still not be distinguished (data not shown).

DISCUSSION

An F′ plasmid containing the *eut* region was constructed and used to perform complementation tests that defined six complementation groups in the *eut* operon. A summary of representative mutations in each complementation group and the enzymatic or growth defect caused by the mutations is shown in Fig. 4. Mutations in the *eutR* gene prevent expression of the entire *eut* operon; strains containing a *eutR* mutation lack all of the enzymatic activities encoded in *eut* and fail to express *eut-lac* operon fusions located promoter proximal to the *eutR* gene (27). Mutations in the *eutB* and *eutC* complementation groups affect in vitro ethanolamine ammonia-lyase activity. Two complementation groups for ethanolamine ammonia-lyase were expected because this enzyme isolated from *Clostridium* sp. and *E. coli* is composed of two subunit types (3, 34). Mutations in the *eutE* complementation group affect in vitro acetaldehyde dehydrogenase activity; this enzyme is composed of a single subunit type in *E. coli* (24). The function of the *eutD* gene product is not yet known; these mutants are able to use ethanolamine as a nitrogen but not a carbon source.

In the complementation tests, several point and insertion mutations exhibited a polar effect on the expression of downstream genes. Nevertheless, the function of the most promoter-distal gene in the operon (*eutR*) was not eliminated by upstream mutations (including insertion mutations), indicating that *eutR* can be expressed from its own promoter. In a subsequent paper, we will show that *eutR* can also be expressed from the main promoter for the *eutDEABC* operon and that the independent promoter for *eutR* is a constitutive promoter within the operon.

Many mutations that appeared to be nonpolar since they complemented many downstream mutations still failed to complement particular alleles of downstream genes. In these cases, complementation was often restored when the chromosomal and the F′ plasmid locations of the two mutations were switched. Perhaps the relative concentration of each *eut* gene product is important for efficient utilization of ethanolamine. This might reflect the existence of a multienzyme complex of the enzymes for ethanolamine degradation or a toxic effect of accumulated acetaldehyde.

The *eutA* gene product is required for use of ethanolamine in the presence of exogenous HO-B_{12} or CN-B_{12}. The *eutA* mutants could use exogenously supplied Ado-B_{12} to fulfill the cofactor requirement for growth on ethanolamine as the sole nitrogen source (but not as the sole carbon source) (Table 2). Several results suggest that the growth defect of *eutA* mutants is not caused by a complete lack of Ado-B_{12} synthesis but by inhibition of ethanolamine ammonia-lyase by accumulated HO-B_{12} or CN-B_{12}. First, the *eutA* mutants could still convert HO-B_{12} or CN-B_{12} to Ado-B_{12} to fulfill the cofactor requirement of the Ado-B_{12}-dependent enzyme DL-1,2-propanediol dehydratase. The Ado-B_{12} inferred to be available in *eutA* mutants during growth on propanediol as the carbon source when HO-B_{12} or CN-B_{12} was provided exogenously did not permit the simultaneous use of ethanolamine as the nitrogen source. A more direct indication that accumulated HO-B_{12} or CN-B_{12} might inhibit ethanolamine ammonia-lyase is the fact that *eutA* mutants failed to use Ado-B_{12} if HO-B_{12} or CN-B_{12} was provided simultaneously. The *eutA* gene product may serve to protect ethanolamine ammonia-lyase from inactivation by preventing accumulation of a high concentration of inhibitory B_{12} forms in the cytoplasm. This could be accomplished by increasing the rate of adenosylation, by slowing entry of HO-B_{12} or CN-B_{12} into the cytoplasm, or by inactivating internal forms of B_{12} other than Ado-B_{12}. Alternatively, the *eutA* gene product could interact with ethanolamine ammonia-lyase directly to protect it from inactivation by the toxic B_{12} forms. The ethanolamine ammonia-lyase enzyme itself seems to be functional in *eutA* mutants because cell extracts of *eutA* mutants retain normal ethanolamine ammonia-lyase activity in an assay dependent on added Ado-B_{12} (27).

Exogenously supplied Ado-B_{12} allowed the *eutA* mutants to use ethanolamine as the sole nitrogen source but not as the sole carbon source (Table 2). During the course of catalysis by ethanolamine ammonia-lyase, the carbon-cobalt bond of Ado-B_{12} undergoes reversible cleavage and reformation (1). It is thought that the carbon-cobalt bond occasionally fails to reform correctly, generating inactive (inhibitory) forms of B_{12} as a by-product of catalysis (2, 20). We suggest that these inactive forms of B_{12} accumulate in a *eutA* mutant during the large number of reaction cycles required to use ethanolamine as the sole carbon and energy source. Thus, the role for the *eutA* function may be to regenerate Ado-B_{12} and thereby protect ethanolamine ammonia-lyase from inhibition by its own accumulated by-products. Such an activity might also protect the enzyme from inhibition by cytoplasmic HO-B_{12} or CN-B_{12}.

An ethanolamine permease does not seem to be encoded in the *eut* operon since a deletion mutant that lacks the entire *eut* operon still transported ethanolamine at the same rate as a strain that contains only a point mutation in one of the genes for ethanolamine ammonia-lyase. It is possible that the *eut* operon encodes an ethanolamine permease whose activity is not detectable under our assay conditions or whose activity requires the presence of active ethanolamine ammonia-lyase enzyme. All uptake measured was inhibited by pretreatment of cells with a drug that abolishes the proton gradient, suggesting that ethanolamine uptake is mediated by an active transport system. So far, no Eut$^-$ mutant unlinked to the *eut* operon has been isolated that lacks ethanolamine uptake activity (D. Roof, unpublished). If several permeases act on ethanolamine (as is the case for some other growth substrates [11]) or if ethanolamine diffuses through the membrane, our failure to recover permease-deficient Eut$^-$ mutants would be explained.

The method used here to construct an F′ plasmid containing the *eut* operon can easily be applied to other bacterial genes. The main advantage of the method is that a large (40 kilobases or more) genetically predetermined region can be placed on a low-copy-number F plasmid. Plasmids such as these are difficult to construct by other methods.

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