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A Rationale for Autoinduction of a Transcriptional Activator: Ethanolamine Ammonia-Lyase (EutBC) and the Operon Activator (EutR) Compete for Adenosyl-Cobalamin in *Salmonella typhimurium*

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The ethanolamine utilization (*eut*) operon of *Salmonella typhimurium* is controlled by a positive regulatory protein (EutR) which stimulates *eut* operon expression in response to the simultaneous presence of two effectors, ethanolamine and adenosyl-cobalamin (Ado-B_{12}). Ado-B_{12} is a cofactor for ethanolamine ammonia-lyase (lyase), the first enzyme in the ethanolamine-degradative pathway. The dependence of this pathway on the use of Ado-B_{12} as an effector in *eut* operon induction may be explained by its role in the degradation of ethanolamine and the fact that this cofactor is not always made by *S. typhimurium*. The *eutR* gene lies within the *eut* operon, and its autoinduction is required for maximum operon expression. Evidence is presented that the placement of the *eutR* regulatory gene within the operon provides a means of balancing the competition between lyase and the regulatory protein for a very small pool of Ado-B_{12}. Since both lyase and the regulatory protein are induced, they can compete more equally for a small pool of Ado-B_{12}. This permits both continued *eut* operon induction and lyase activity. Two general observations support this model. First, mutations that inactivate lyase allow the operon to be fully induced by a lower level of exogenous cobalamin (CN-B_{12}) than required by a wild-type operon. This increase in sensitivity is measured as a reduction in the apparent K_m for operon induction by exogenous CN-B_{12}. Second, the maximum level of operon induction by excess CN-B_{12} is dictated by the level of EutR regulatory protein, regardless of the level of lyase.

The *eut* operon of *Salmonella typhimurium* encodes enzymes necessary for utilization of ethanolamine as a carbon, nitrogen, and energy source (Fig. 1) (21–23). The first enzyme in the ethanolamine-degradative pathway, ethanolamine ammonia-lyase (lyase) (EC 4.3.1.7) (4–6, 11, 24), catalyzes conversion of ethanolamine to acetaldehyde and ammonia. The two protein subunits of lyase are encoded by the *eutB* and *eutC* genes (22). A second enzyme in the pathway, acetaldehyde dehydrogenase (EC 1.2.1.10) (17, 18) catalyzes conversion of acetaldehyde to acetyl coenzyme A and is encoded by the *eutE* gene. Lyase activity alone allows utilization of ethanolamine as a nitrogen source, whereas dehydrogenase is also required to initiate utilization of ethanolamine as a carbon source (7). Adenosyl-cobalamin (Ado-B_{12}) performs two distinct functions in ethanolamine utilization; it is a cofactor for lyase activity and is required, with ethanolamine, as an effector for *eut* operon induction (21–23). Induction of the *eut* operon by these two effectors requires the action of a positive regulatory protein, EutR. The *eutR* gene is located at the promoter distal end of the *eut* operon (Fig. 1). In the absence of *eut* operon induction, the *eutR* gene is expressed by one ($eutP_{II}$) or more weak constitutive promoters located within the operon. This low constitutive level of EutR, with the effectors ethanolamine and Ado-B_{12}, initiates induction of the *eut* operon. Induced operon expression from the main promoter ($eutP_I$) (Fig. 1) can result in as much as a 200-fold increase in transcription of the *eutR* gene. This increase in EutR is required for full induction of the *eut* operon (23). The autoinduction of the EutR regulatory protein is the object of this study.

Autoinduction of EutR seems to help the operon manage its regulation in the face of low pools of Ado-B_{12} and competition from lyase for binding of Ado-B_{12}. Ado-B_{12} can be synthesized de novo by *S. typhimurium* anaerobically but not aerobically (15, 16). Under anaerobic growth conditions, cells contain approximately 100 molecules of cobalamin (1, 2). Under both aerobic and anaerobic conditions, exogenously supplied Ado-B_{12} or cyanobalamin (CN-B_{12}) can be imported (3, 22), increasing the cobalamin level to several thousand molecules per cell (1). Imported CN-B_{12} can be converted to Ado-B_{12} by the *cobA* gene product, thereby providing this effector and cofactor under aerobic conditions (13). Regardless of whether these corrinoids are synthesized or imported, a small pool of not more than a few thousand molecules of Ado-B_{12} must serve as inducer of the *eut* operon (by EutR) and as cofactor for the pool of any newly synthesized lyase enzyme. One might expect that the new lyase molecules, produced following induction, would compete with the EutR regulatory protein for limiting levels of Ado-B_{12}.

Two consequences of the autoinduction of EutR regulatory protein may be important for achieving and maintaining levels of *eut* operon induction that are sufficient for ethanolamine utilization. First, the increase in EutR regulatory protein levels insures that the major *eut* promoter ($eutP_I$) approaches saturation with the EutR–ethanolamine–Ado-B_{12}-activating complex. An increased level of EutR protein would allow significant levels of the activating complex to form, even at relatively low intracellular concentrations of Ado-B_{12}. Second, under inducing conditions, a parallel increase in EutR and lyase could prevent newly synthesized lyase from sequestering a

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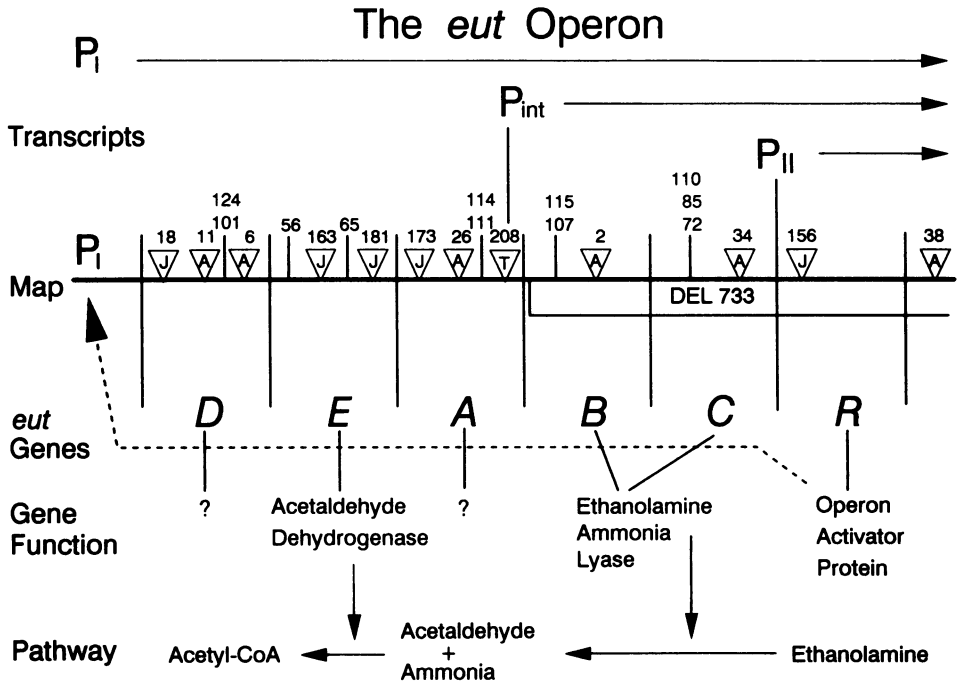

FIG. 1. *eut* operon. The pathway for ethanolamine breakdown to acetyl coenzyme A (acetyl-CoA) is shown at the bottom of the figure. The horizontal line in the middle is the genetic map, with inferred transcription starts indicated above. Inverted triangles indicate insertion mutations; J, Mu dJ insertion; A, Mu dA insertion; T, Tn*10d*(Tc) insertion; allele numbers are given above the triangles. Other numbers above the map indicate point mutations. Mutation DEL733 is a constructed deletion that extends from the insertion *eut-5*::Mu dA (located near the border between the *eutA* and *eutB* genes) rightward to the *cysA* gene. P_{int} is a promoter [(probably within *eutA208*::Tn*10d*(Tc)] which was created or activated by mutation.

disproportionate amount of Ado-B_{12}. Such a mechanism could partition Ado-B_{12} more equitably between the regulatory protein and lyase, thereby permitting both continued degradation of ethanolamine (by lyase) and *eut* operon induction (by EutR).

The proposed sequence of events in *eut* operon induction can be summarized as follows. Initial induction is achieved with the effectors ethanolamine and Ado-B_{12} and the basal levels of EutR regulatory protein. EutR levels increase by autoinduction, and this allows the maximum level of expression typical of the *eut* operon to be achieved. In addition, the coordinated induction of both EutR and lyase permits limiting Ado-B_{12} to continue to play a significant role as both cofactor for lyase and effector for operon induction.

In this study, strains that produce altered relative levels of lyase and activator protein have been constructed. The effect of these alterations on apparent K_m values for induction by exogenous CN-B_{12} and on maximum operon expression have been used to infer the distribution of Ado-B_{12} between lyase and EutR protein. The results confirm some of the predictions inherent in the above model for *eut* operon induction. (i) Increased levels of EutR protein result in a higher maximum level of operon induction; (ii) reduction or loss of lyase allows maximum *eut* operon expression to occur at reduced levels of Ado-B_{12} effector, suggesting that the normal binding of the Ado-B_{12} cofactor to lyase represents a competition for available Ado-B_{12}.

MATERIALS AND METHODS

Bacterial strains and transposons. All strains are derivatives of *S. typhimurium* LT2 (Table 1). The *eut* mutations have been previously described previously (21–23). The Mu dJ(Kn) (9) and Mu dA(Ap) (8) elements are transposition-defective derivatives of the specialized transducing phage Mu d1(Ap^r *lac cts*) (14). A transposition-defective Tn*10* derivative, Tn*10d*(Tc) (27), was used by Roof and Roth to produce the *eutA208*::Tn*10d*(Tc) mutation described here (21).

Media. Complex medium was nutrient broth (NB) (0.8%; Difco Laboratories) supplemented with NaCl (0.5%). The carbon-free minimal medium was NCE or Vogel and Bonner E medium (12), and minimal medium free of both carbon and nitrogen sources was NCN (20). Ethanolamine hydrochloride (0.2%; Aldrich) was used as a carbon source in NCE medium, as a nitrogen source in NCN medium with glycerol (0.2%), or both as the carbon and nitrogen source in NCN medium. CN-B_{12} (Sigma Chemical Co.) was used as the exogenous vitamin B_{12} source at the concentrations specified. Amino acids and purines were added to minimal medium as described previously (12). Complex medium contained the following antibiotics at the concentrations indicated: ampicillin, 30 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 20 μg/ml. Agar (1.5% Difco) was added to E, NCE, NCN, and NB media to prepare solid media.

Genetic techniques. Transductional crosses were performed with the high-frequency generalized transducing phage mutant P22 HT105/1 *int-201* as previously described (25). Transductant clones were purified and made phage free by streaking for single colonies on nonselective green indicator plates (10).

Enzyme assays. Strains to be assayed for β-galactosidase activity were inoculated from a 1/150 dilution of an overnight NB culture into 2.5 ml of NCE-glycerol medium containing 20.5 mM ethanolamine and CN-B_{12} concentrations ranging from 0 to 0.08 μM. All strains employed were able to transport

TABLE 1. Bacterial strains used

Strain	Genotype LT2
TT10271	*eutD18*::Mu dJ
TT10638	*eutB2*::Mu dA
TT10642	*eutD6*::Mu dA
TT10647	*eutD11*::Mu dA
TT10662	*eutA26*::Mu dA
TT10670	*eutC34*::Mu dA
TT10674	*eut-38*::Mu dA
TT11704	*eut* DEL733 (*cysA1585* Mu dA *eut-5*)
TT13738	*eutR156*::Mu dJ
TT13745	*eutE163*::Mu dJ
TT13754	*eutA173*::Mu dJ
TT13762	*eutE181*::Mu dJ
TT14492	*eutA208*::Tn*10d*(Tc) *eut-38*::Mu dA
TT17296	*eutD18*::Mu dJ *eutA208*::Tn*10d*(Tc) P_{int} *eutB115*
TT17297	*eutD18*::Mu dJ *eutA208*::Tn*10d*(Tc) P_{int} *eutB107*
TT17298	*eutD18*::Mu dJ *eutA208*::Tn*10d*(Tc) P_{int} *eutC110*
TT17299	*eutA208*::Tn*10d*(Tc) *eutR156*::Mu dJ
TT17300	*eutA208*::Tn*10d*(Tc) P_{int} *eutR156*::Mu dJ
TT17301	*eutA208*::Tn*10d*(Tc) *eut-38*::Mu dA
TT17302	*eutA208*::Tn*10d*(Tc) P_{int} *eut-38*::Mu dA
TT17303	*eutA208*::Tn*10d*(Tc) P_{int} *eutB115 eut-38*::Mu dA
TT17304	*eutA208*::Tn*10d*(Tc) P_{int} *eutC110 eut-38*::Mu dA
TT17305	*eutA208*::Tn*10d*(Tc) P_{int} *eutB107 eut-38*::Mu dA
TT17306	*eutA208*::Tn*10d*(Tc) P_{int}
TT17307	*eutA208*::Tn*10d*(Tc) P_{int} DEL733
TT17308	*eutA208*::Tn*10d*(Tc) P_{int} DEL733
TT17309	*eutD124 eut-38*::Mu dA
TT17310	*eutE56 eut-38*::Mu dA
TT17311	*eutE65 eut-38*::Mu dA
TT17312	*eutA114 eut-38*::Mu dA *zfa-3648*::Tn*10 metE205 ara-9*
TT17313	*eutA111 eut-38*::Mu dA
TT17314	*eutB115 eut-38*::Mu dA
TT17315	*eutB107 eut-38*::Mu dA
TT17316	*eutC85 eut-38*::Mu dA
TT17317	*eutC110 eut-38*::Mu dA
TT17318	*eutC72 eut-38*::Mu dA
TT17319	*eutD101 eut-38*::Mu dA
TT17320	*eutD18*::Mu dJ *eutA208*::Tn*10d*(Tc) P_{int}
TT17321	*eutD18*::Mu dJ *eutA208*::Tn*10d*(Tc)

$CN\text{-}B_{12}$ and to catalyze its conversion to $Ado\text{-}B_{12}$. Following growth to mid-exponential phase (approximately 8 h) at 37°C on a New Brunswick shaker, model 50, cells were held at 4°C until assayed. β-Galactosidase enzyme activity was determined as previously described by Miller (19), with toluene-sodium dodecyl sulfate-permeabilized cells. Enzyme activity was expressed in units defined as nanomoles of nitrophenol produced per minute per optical density at 650 nm of cell culture turbidity.

Determination of apparent K_m values for *eut* operon induction by $CN\text{-}B_{12}$. Strains were grown in NCE-ethanolamine-glycerol medium over a range of $CN\text{-}B_{12}$ concentrations. Induction of the *eut* operon was determined by measuring β-galactosidase expression from a Mu d *lac* operon fusion element inserted in the *eut* operon. When K_m determinations were made for strains containing specific *eut* gene insertion mutations, β-galactosidase expression was measured directly from the Mu d *lac* insertion fusion in that particular *eut* gene. When K_m determinations were made for strains containing point mutations in specific genes, *eut* operon expression was measured from the *eut-38*::Mu dA *lac* fusion insertion located distal to all known point mutations. This insertion mutation does not result in any known *eut* phenotype (21–23) and is used routinely in this study either to measure the wild-type rate of *eutR* expression or to measure the effect of a *eut* point mutation on downstream *eut* operon expression. Apparent K_m values for *eut* operon induction by $CN\text{-}B_{12}$ were determined from the *x*-axis intercept of double-reciprocal plots of the following type: (1/percent maximal β-galactosidase) versus $(1/CN\text{-}B_{12})$.

Isolation of mutants with a new internal promoter. A saturated NB culture of strain TT17299 (*eutA208*::Tn*10d*(Tc) *eutR156*::Mu dJ *lac*) (0.1 ml) was plated onto NCE-lactose agar medium. After several days, spontaneous Lac^+ mutant colonies arose with a frequency of about 10 to 20 per plate. One isolate (TT17300) was characterized further. The mutation in this strain results in constitutive expression of the *eutR156*::Mu dJ *lac* fusion and is referred to as P_{int}.

RESULTS

Experimental design. In the experiments presented below, the general procedure is to construct a series of strains, each with a Mu d *lac* operon fusion at some point in the *eut* operon. In these strains, the inducibility of β-galactosidase is dictated by the position of the fusion and the presence of additional mutations. These strains vary in their levels of EutR and lyase, and these differences affect regulation of the main promoter. These strains were grown with an excess of ethanolamine (20.5 mM) and various concentrations of $CN\text{-}B_{12}$. Each culture was assayed for β-galactosidase to determine the level of induction of the *eut* operon achieved at each concentration of $CN\text{-}B_{12}$. The internal concentration of the effector $Ado\text{-}B_{12}$ was increased following the addition of exogenous $CN\text{-}B_{12}$ (1). Although the internal levels of $Ado\text{-}B_{12}$ have not been measured directly, the linear increase in *eut* operon induction over a limiting range of exogenous $CN\text{-}B_{12}$ concentrations (Fig. 2A) suggests a proportional relationship between exogenous concentrations of $CN\text{-}B_{12}$ and internal concentrations of $Ado\text{-}B_{12}$. The apparent K_m value for induction by exogenous $CN\text{-}B_{12}$ was determined. The percent maximum β-galactosidase was used to determine K_m values because strains containing different Mu d::*lac* insertion mutations exhibit different maximum levels of β-galactosidase expression. The maximum achievable level of operon induction at excess $CN\text{-}B_{12}$ was also determined.

Some strains tested carry the *eut-38*::Mu dA insertion; this is an insertion within the operon, distal to all known *eut* genes. This insertion creates a *eut*::*lac* operon fusion but causes no known Eut phenotype. We presume that expression of the *lacZ* gene in this strain reflects the behavior of a wild-type *eut* operon. Additional mutations were added to this strain to assess their effect on operon induction. Although the *eut-38*::*lac* fusion detects the combined effect of the $eutP_I$ and $eutP_{II}$ promoters, the $eutP_{II}$ promoter is weak and only the $eutP_I$ promoter is stimulated by the EutR–ethanolamine–$Ado\text{-}B_{12}$ complex (21–23). Thus the variation in *eut* operon induction in response to the $CN\text{-}B_{12}$ concentration reflects the response of the $eutP_I$ promoter to $Ado\text{-}B_{12}$ (via the EutR-ethanolamine-$Ado\text{-}B_{12}$ complex.). The locations of fusions, point mutations, and the promoters used can be seen in Fig. 1.

Point mutations affect sensitivity of the operon to induction by exogenous $CN\text{-}B_{12}$. When strains were surveyed for inducibility of the *eut* operon by low levels of $CN\text{-}B_{12}$, two patterns of induction were seen. Sensitive strains were induced by a significantly lower level of $CN\text{-}B_{12}$ than insensitive strains. This is illustrated in Fig. 2A. A strain with a simple *eut-38*::*lac* fusion (TT10674) was insensitive to induction. This sensitivity was greatly increased by the addition of a mutation eliminating lysase production (*eutC110*). A mutation in the dehydrogenase (*eutE56*) had only a slight effect on induction sensitivity. The sensitive and insensitive strains differed by about 10-fold in

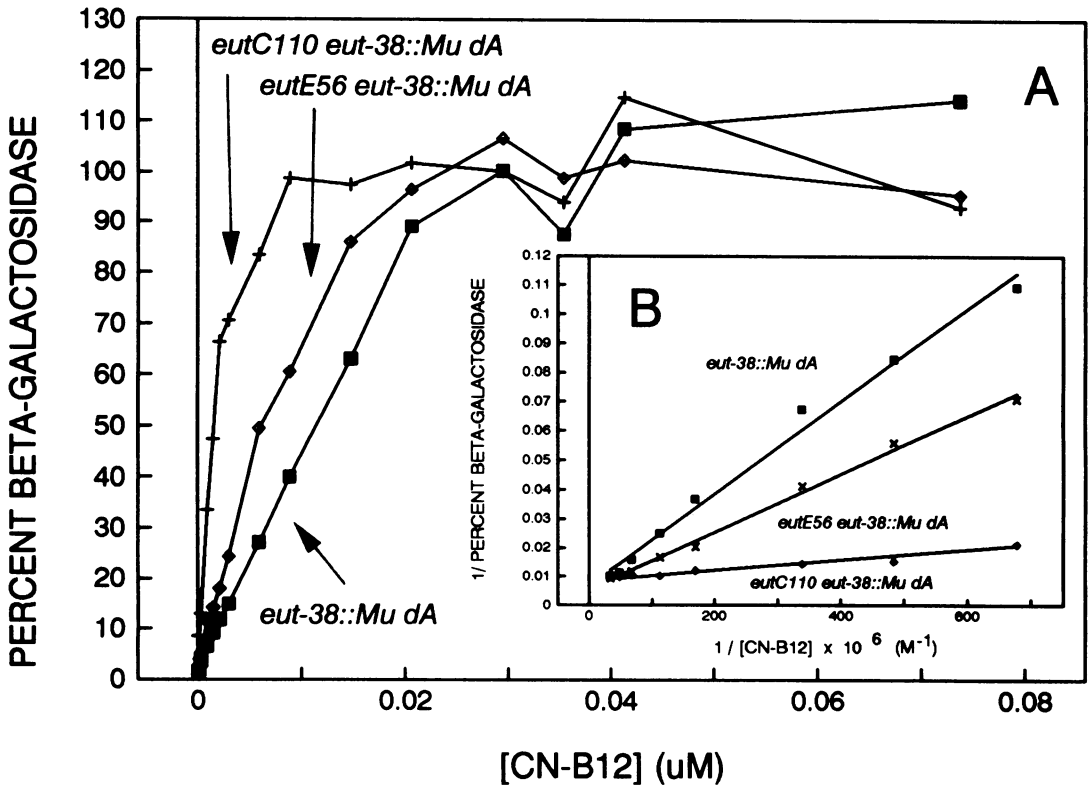

FIG. 2. Induction of the *eut* operon by exogenous CN-B_{12}: effect of *eut* point mutations on induction sensitivity. Cultures were grown aerobically on NCE-glycerol-ethanolamine as described in Materials and Methods over a range of CN-B_{12} concentrations. β-Galactosidase activities for each strain are presented as a fraction of the maximum induced level achieved. This was done to allow easy and direct comparison of the sensitivities of operon induction in these strains by exogenously supplied CN-B_{12}, some of which show very different maximum induced levels of operon induciton. These differences in maximum induced levels in strains with point mutations and insertion mutations (Tables 2 to 4) will be described later. (A) Operon expression was measured from a Mu dA *lac* fusion located in a noncritical downstream region of the *eut* transcription unit. The strains assayed were *eut-38*::Mu dA (TT10674), *eutE56 eut-38*::Mu dA (TT17310), and *eutC110 eut-38*::Mu dA (TT17317). (B) Double-reciprocal plots were used to demonstrate the differences in induction sensitivity of the various strains and to calculate the apparent K_m values for induction by CN-B_{12} (Ado-B_{12}). Linear regression analysis was used to fit the data to straight lines.

their apparent K_m values for induction by exogenous CN-B_{12} (Fig. 2B and Table 2).

When this survey was extended to a series of point mutations, the results (Table 2) provided the initial evidence suggesting that lyase levels might affect induction sensitivity. The most striking increase in sensitivity was caused by mutations in genes for lyase (*eutB* and *eutC*). Mutations in genes other than lyase had only small effects on the apparent K_m of the operon for induction by exogenous CN-B_{12}.

While these results were suggestive, they included some exceptions. Several mutations outside of *eutB* and *eutC* (e.g., *eutE65* [Table 2]) caused a significant increase in sensitivity. We suggest that these mutations cause a slight reduction in lyase levels by exerting a polar effect on expression of the *eutB* and *eutC* genes. Furthermore, two mutations in the *eutC* gene failed to exhibit the increase in induction sensitivity. One of these mutations (*eutC72*) is "leaky" and likely to allow production of a lyase with ability to bind Ado-B_{12}; the other mutation is not phenotypically leaky but may produce a protein able to bind Ado-B_{12}. It is possible that only the *eutB* subunit of lyase binds Ado-B_{12} directly; if this were the case, then many *eutC* mutations might allow production of nonfunctional lyase in which the wild-type *eutB* subunit is capable of binding Ado-B_{12}. Despite the exceptions, the general pattern of mutations affecting sensitivity suggested that lyase influences sensitivity to induction by Ado-B_{12}.

These data suggested that lyase might sequester Ado-B_{12}, making it more difficult for limiting levels of Ado-B_{12} to induce the operon in the face of an increasing level of lyase (Fig. 3). The data above could be explained if mutations that eliminate lyase (or its Ado-B_{12}-binding ability) allowed all Ado-B_{12} to serve as inducer and thus caused the sensitive induction phenotype seen. Stated as a model for function of the normal operon, we suggest that the operon is very sensitive to initial induction (no lyase is present), but full induction occurs only if Ado-B_{12} levels are sufficient to populate both the lyase and the regulatory protein. Since both lyase and the activator protein are induced together, they can compete equally for available Ado-B_{12} throughout the course of induction. If a sufficient level of Ado-B_{12} is present, the cell can populate both ligase and the activator protein and maintain induction even when lyase levels are very high. Maintenance of maximal induction may require sufficient Ado-B_{12} to saturate the high levels of both lyase and activator protein.

Fusions early in the operon show increased sensitivity to induction. To test the above-described model, we assayed strains with insertion mutations (Mu dJ and Mu dA) which form fusions of the *lac* operon to various points in the *eut* operon. The fusion points are within structural genes, either within or promoter-proximal to the *eutB* and *eutC* genes (Fig. 1). All of the upstream insertion mutations are strongly polar on expression of distal genes in the operon and eliminate

TABLE 2. Apparent K_m values and maximum *eut* operon induction by Ado-B_{12}: effect of *eut* point mutations on induction of the *eut-38*::*lac* fusion

Strain	*eut* point mutation	K_m (10^9)	Km relative to *eut-38*::Mu dA	*eut* operon induction (β-galactosidase)[a]		
				Uninduced	Fully induced	Fully induced relative to *eut-38*::Mu dA
TT10674		23.3	1.00	4.9	305.8	1.0
TT17309	*eutD124*	9.7	0.42	4.2	165.3	0.5
TT17319	*eutD101*	9.6	0.41	5.0	192.6	0.6
TT17310	*eutE56*	18.2	0.78	4.4	177.8	0.6
TT17311	*eutE65*	5.4	0.23	4.5	105.5	0.3
TT17313	*eutA111*	7.9	0.34	4.6	208.2	0.7
TT17312	*eutA114*	20.7	0.89	4.4	213.5	0.7
TT17314	*eutB115*	1.3	0.06	4.6	316.4	1.0
TT17315	*eutB107*	1.4	0.06	3.8	239.5	0.8
TT17317	*eutC110*	1.2	0.05	3.5	39.9	0.1
TT17316	*eutC85*	12.4	0.53	4.6	293.0	1.0
TT17318	*eutC72*	38.5	1.65	4.4	327.7	1.1

[a] Cultures were grown for analysis as described in Materials and Methods. Induction of the *eut* operon was measured from the *eut38*::*lac* fusion. Apparent K_m values for CN-B_{12} induction were determined from the value of the *x*-axis intercept of reciprocal plots: (1/percent maximal β-galactosidase) versus (1/CN-B_{12}) (Fig. 2).

expression of both the *eutB* and *eutC* genes. These fusion strains retain inducibility by Ado-B_{12} because the *eutR* gene is intact and can be expressed from the weak internal promoter (P_{II}). The behavior of these fusion strains (all of which lack lyase) was compared with behavior of the distal fusion (*eut-38*), which leaves the entire operon intact. Typical results are presented in Fig. 4. When operon induction is monitored from internal fusion points, the sensitivity to induction is much greater than that seen for the fusion outside of the coding sequences, *eut-38*::*lac*. The apparent K_m values for induction of these fusions by exogenous CN-B_{12} are presented in Table 3; all the fusions show a strongly increased sensitivity (reduced apparent K_m values) for induction compared with that of the *eut-38*::*lac* fusion.

P_{int}, a mutationally altered internal promoter that causes high constitutive expression of lyase and EutR regulatory protein. To test further the hypothesis that lyase and EutR regulatory protein compete for Ado-B_{12}, we isolated a mutant that expresses both the lyase and regulatory protein genes at high constitutive levels. This mutant was selected as a derivative of a strain (TT17299) which carries a Tn*10d*(Tc) insertion in the *eutA* gene, just promoter-proximal to the lyase genes (Fig. 1). This strain also carries insertion *eutR156*::Mu dJ *lac*, which fuses the *lac* operon to the *eutR* gene. In this strain, the *eutA208*::Tn*10d*(Tc) polar insertion mutation prevents expression of all distal genes by the major *eut* promoter (*eut*P_1). The low level of expression of *eutR*::*lac* from the internal weak constitutive *eutR* promoter *eut*P_{II} is not sufficient to permit this strain to use lactose as a sole carbon and energy source. By selecting for Lac$^+$ derivatives of this strain, one can obtain mutants in which the transcription of the *eutR*::*lac* fusion is increased. One Lac$^+$ derivative, TT17300, showed an constitutive level of β-galactosidase fivefold higher than that of a fully induced wild-type *eut* operon measured with a fusion distal to all structural genes (Table 4, lines 1 to 4). When the *eutR156*::*lac* fusion was replaced with the *eut-38*::*lac* fusion (to form strain TT17302), the new promoter, P_{int}, caused constitutive expression of the distal *lac* fusion at levels slightly higher than that seen during full induction of P_{int}^+ strains with the same fusion (Table 4, lines 4 to 6).

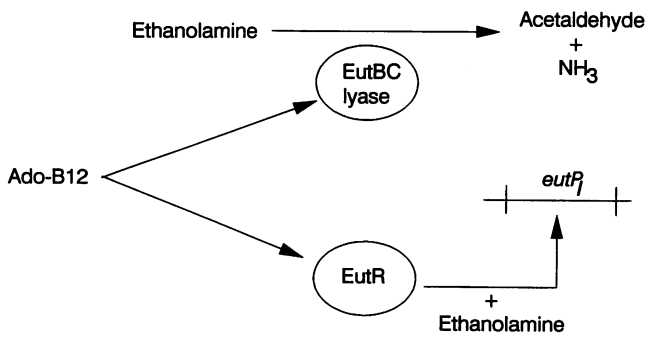

FIG. 3. Model for the distribution of Ado-B_{12} between EutBC lyase and EutR regulatory protein.

Map position of P_{int}. Several pieces of evidence suggest that the mutation which creates or activates P_{int} is upstream of the *eutB* gene and probably lies within the *eutA208*::Tn*10d*(Tc) element in strain TT17300. In the transductional crosses used in mapping and in construction of several strains, the P_{int} mutation has never been separated by recombination from the Tc^r determinant. To test linkage of P_{int} to *eutA208*::Tn*10d*(Tc), a P22 transducing phage lysate grown on TT17306 [*eutA208*::Tn*10d*(Tc) P_{int}] was used to transduce *eut* mutation DEL733 (in strain TT11704) to Tc^r. DEL733 is a constructed deletion mutation extending from the *cysA* gene into the promoter-distal end of the *eut* operon; the deletion ends near the border between the *eutA* and *eutB* genes (Fig. 1) and has a Mu dA (Ap^r Lac) element marking the site of the deleted material (21). In this cross, 10 of 96 Tc^r transductants were Ap^r, indicating that recombination occurred between *eutA208*::Tn*10d*(Tc) and the endpoint of the Ap^r-marked deletion. All 10 Ap^r recombinants are Lac$^+$, indicating that none of the 10 exchanges within the portion of the *eutA* gene distal to the Tn*10d*(Tc) insertion separated Tc^r from P_{int}. (These exchanges could also occur within the *lac* sequences shared by both the donor and recipient.)

P22 transducing phage lysates were prepared on two double mutants that carried both the *eutA*::Tn*10d*(Tc) P_{int} insertion and the Ap^r-marked DEL733 mutation (TT17307 and TT17308). This phage lysate was used to transduce a recipient strain containing fusion *eutR156*::*lac* (TT13738) to Tc^r. If the P_{int} mutation is located within the Tn*10d*(Tc) element or the region between this element and the region defined by the

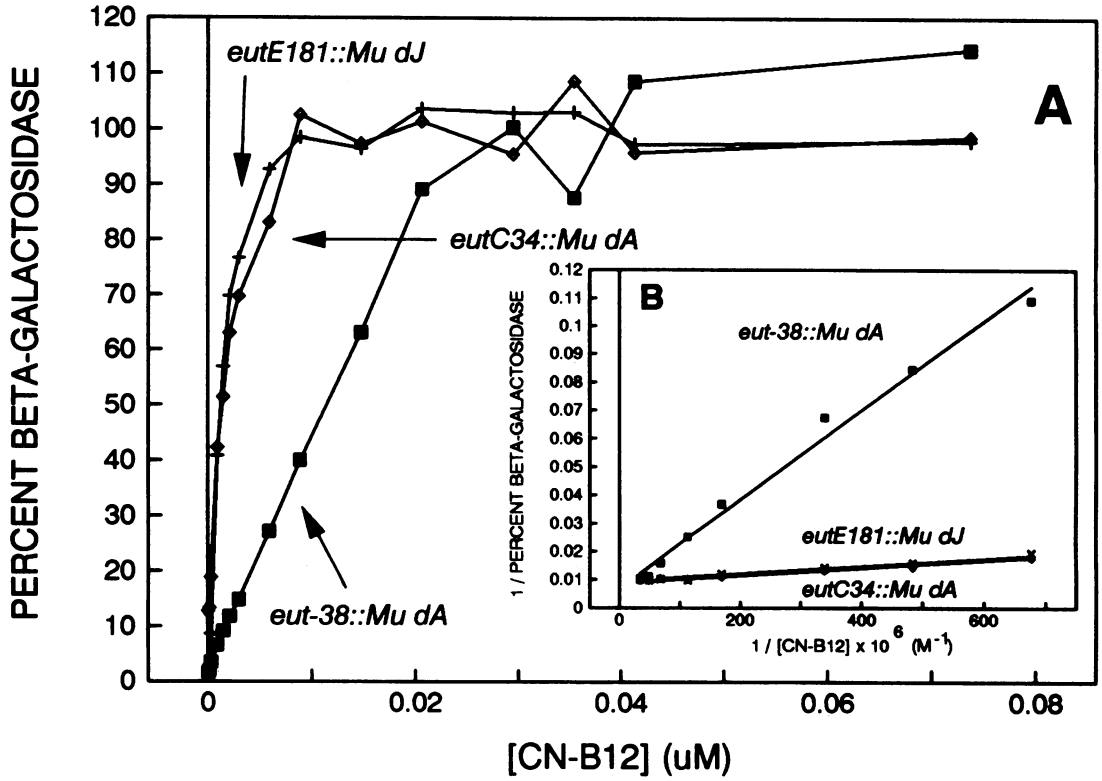

FIG. 4. Induction of the *eut* operon by exogenous CN-B_{12}: effect of *eut* insertion mutations on induction sensitivity. Cultures were grown and data were analyzed as described in the legend to Fig. 2. Induction of the *eut* operon was measured directly from the Mu dA *lac* or Mu dJ *lac* insertion mutation. The strains assayed were *eut-38*::Mu dA (TT10674), *eutC34*::Mu dA (TT10670), and *eutE181*::Mu dJ (TT13762).

beginning of DEL733, two Tc^r Ap^s $cysA^+$ recombinant types are possible; recombinants that contain the P_{int} mutation will be Lac^+, whereas those in which the P_{int} mutation has been separated from Tn*10d*(Tc) will be Lac^-. None of 192 recombinant events occurring between *eutA208*::Tn*10d*(Tc) and the Mu dA element of DEL733 separated the P_{int} mutation from the Tn*10d*(Tc) element. This indicates that the P_{int} mutation is not located within DEL733 and is not easily separated from the Tn*10d*(Tc) element by recombination.

The Tn*10d*(Tc) element includes promoters for divergent genes that confer the Tc^r phenotype. These promoters direct tetracycline-inducible transcripts that extend out of both sides of the transposable material into the chromosomal region adjoining the insertion site (26). The P_{int} mutation described here may cause constitutive expression of a promoter within the Tn*10d*(Tc) element. Further support for the positioning of the P_{int} mutation within Tn*10d*(Tc) will require sequence analysis and an analysis of P_{int} transcripts.

Effect of P_{int} on the phenotype of *eutA208*::Tn*10d*(Tc)-containing strains. *S. typhimurium* can use ethanolamine as both a carbon and nitrogen source when grown aerobically and provided with cobalamin. Strains defective in the *eut* operon

TABLE 3. Apparent K_m values and maximum *eut* operon induction by Ado-B_{12}: effect of *eut* insertion mutations

Strain	*lac* fusion	K_m (10^9)	K_m relative to *eut-38*::Mu dA	*eut* operon induction (β-galactosidase)[a]		
				Uninduced	Fully induced	Fully induced relative to *eut-38*::Mu dA
TT10674	*eut-38*::Mu dA	23.3	1.00	4.9	305.8	1.0
TT10271	*eutD18*::Mu dJ	1.1	0.05	1.9	184.1	0.6
TT10642	*eutD6*::Mu dA	2.8	0.12	1.2	61.1	0.2
TT10647	*eutD11*::Mu dA	6.8	0.29	1.3	111.0	0.4
TT13762	*eutE181*::Mu dJ	1.6	0.07	1.6	145.1	0.5
TT13745	*eutE163*::Mu dJ	3.4	0.15	2.0	181.6	0.6
TT13754	*eutA173*::Mu dJ	1.7	0.07	1.7	118.0	0.4
TT10662	*eutA26*::Mu dA	1.7	0.07	2.9	151.4	0.5
TT10638	*eutB2*::Mu dA	2.1	0.09	4.2	44.2	0.1
TT10670	*eutC34*::Mu dA	1.4	0.06	4.2	32.9	0.1

[a] Cultures were grown for analysis as described in Materials and Methods. Induction of the *eut* operon was measured directly from the *eut*::*lac* insertion mutation. Apparent K_m values for Ado-B_{12} induction were determined from the value of the *x*-axis intercept of reciprocal plots: (1/percent maximal β-galactosidase) versus (1/CN-B_{12}) (Fig. 4). The fully induced levels of *eut* operon expression from different *eut*::*lac* insertion mutations varied considerably. This is a common characteristic of different Mu d *lac* insertion mutants and does not appear to have regulatory significance for the *eut* operon.

have been placed into two physiologically distinct groups. Strains lacking lyase activity (*eutBC*) are unable to use ethanolamine as either nitrogen or carbon source (N^- C^-). Strains that have lyase but are unable to catabolize sufficient acetaldehyde (*eutD* and *eutE*) can use ethanolamine as a nitrogen but not as a carbon source (N^+ C^-). Nonpolar *eutA* mutants exhibit a N^- C^- phenotype when given CN-B_{12} as a cobalamin source, even though 40% of wild-type levels of lyase and 75% of wild-type levels of dehydrogenase activity can be detected in these strains in cell-free assays (22). These nonpolar *eutA* mutants are N^+ C^- when Ado-B_{12} is provided. It has been suggested that the *eutA* function may protect lyase from inhibition by excess CN-B_{12} (22) or a toxic breakdown product of Ado-B_{12} produced during the lyase enzymatic reaction.

Strains containing the polar *eutA208*::Tn*10d*(Tc) mutation exhibit the N^- C^- phenotype (even with Ado-B_{12}) since the insertion prevents expression of the *eutB* and *eutC* (lyase) genes. The P_{int} mutation changes the phenotype of the *eutA* insertion mutant from N^- C^- to N^+ C^- in the presence of CN-B_{12}, suggesting that it restores lyase expression (*eutBC*). The P_{int} mutation causes an increase in the expression of both lyase and EutR regulatory protein and must lie upstream of both the *eutB* and *eutC* genes. This location of P_{int} is supported by the observation that strains TT17307 and TT17308 [*eutA*::Tn*10d*(Tc) P_{int} DEL733] show high constitutive expression of *lacZ* from the Mu dA fusion present at the deletion endpoint near the junction of the *eutA* and *eutB* genes (Fig. 1). This places P_{int} either within the Tn*10d*(Tc) element or within the *eutA* gene or immediately upstream of the *eutB* gene.

Effect of high levels of lyase and activator protein on *eut* operon induction. Three isogenic strains that produced different relative levels of lyase and regulatory protein were constructed. In each case, expression of the operon was monitored by the promoter-proximal *eutD18*::*lac* fusion. Strain TT17321 carries the original *eutA208*::Tn*10d*(Tc) polar insertion and produces no lyase but a low level of EutR regulatory protein. Strain TT17320 carries the *eutA208*::Tn*10d*(Tc) polar insertion with P_{int} and produces high levels of both lyase and EutR. Strain TT17298 carries the *eutA208*::Tn*10d*(Tc) polar insertion with P_{int} and the nonpolar lyase mutation *eutC110*; it produces no lyase but makes a high level of EutR. These three strains, TT17321, TT17320, and TT17298, were tested for their sensitivity to induction by CN-B_{12}. The results are shown in Fig. 5; K_m values are presented in the last six lines of Table 4.

Strain TT17298 (high EutR and no lyase) exhibits a higher absolute level of *eut* operon expression than does strain TT17321 (low EutR and no lyase) over the entire range of CN-B_{12} concentrations tested (Fig. 5A). This demonstrates that in the absence of lyase increased EutR synthesis allows *eut* operon induction to be maintained at low levels of exogenous CN-B_{12} (and presumably correspondingly low intracellular concentrations of Ado-B_{12}). The strain with a high EutR level also shows a higher maximal level of operon expression. This is expected, since under these conditions a greater degree of saturation of the main *eut* promoter ($eutP_I$) by the EutR–ethanolamine–Ado-B_{12} complex should occur.

The competition between lyase and the EutR regulatory protein for Ado-B_{12} is demonstrated by comparing the sensitivity of *eut* operon induction in strain TT17320 (high EutR and high lyase) with strain TT17298 (high EutR and no lyase). With high lyase production, more Ado-B_{12} is required to induce $eutP_I$. When synthesis of functional lyase is blocked by the mutation *eutC110* (Fig. 5A) or by two different *eutB* point mutations (Table 4), the apparent K_m values for operon induction are decreased five- to 10-fold relative to those of the *eutBC*$^+$ strain TT17320 (see strains TT17296, TT17297, and TT17298 in Table 4).

The maximum level of operon expression is dictated by EutR protein level and is not affected by lyase levels. Isogenic strains harboring various *eut* mutations differed not only in their sensitivity to induction by low levels of exogenous CN-B_{12} but also exhibited differences in the maximal induction levels achieved in the presence of excess CN-B_{12}. These differences correlate with the expression level of the *eutR* gene and not with the expression of *eutBC* lyase. For example, strains containing fusions located promoter-proximal to the *eutR* gene, such as *eutD18*::*lac*, exhibit a low level of *eutR* gene expression because of the weak internal promoter ($eutP_{II}$) and thus exhibit a low level of maximal *eut* operon expression (Table 4, strain TT10271). Presumably the main $eutP_I$ promoter is only partially saturated by the EutR–ethanolamine–Ado-B_{12} complex. When the P_{int} mutation is added to such a strain, the *eutD18*::*lac* fusion attains a much higher maximal level of induction (Table 4, strain TT17320). Presumably the higher level of expression of the *eutR* gene results in a greater saturation of $eutP_I$ by the EutR–ethanolamine–Ado-B_{12} complex. These high maximal levels of *eut* operon induction (seen with excess Ado-B_{12}) are not altered by the introduction of nonpolar lyase point mutations in *eutB* and *eutC* (Table 4, strains TT17296, TT17297, and TT17298). Similar results are obtained when *eut* operon induction is measured from the *eut-38*::*lac* fusion at the distal end of the operon (Table 4, strains TT14492, TT17302, TT17303, TT17305, and TT17304).

DISCUSSION

The *eut* operon in *S. typhimurium* requires ethanolamine, the substrate of this degradative pathway, and Ado-B_{12}, the cofactor for the first enzyme in the pathway (lyase) as effectors for induction. Induction is mediated by the EutR activator protein (21–23). The *eutR* gene is positioned within the operon at the distal end of the cotransduced region (Fig. 1). Transcription initiated at the regulated main promoter ($eutP_I$) extends through the *eutR* gene. Thus synthesis of EutR protein increases with the expression of other genes in the operon. This autoinduction of EutR protein is necessary to provide levels of the EutR–ethanolamine–Ado-B_{12} complex sufficient to achieve maximal induction of the operon from the $eutP_I$ promoter and to successfully compete with newly synthesized EutBC lyase for available Ado-B_{12} (Fig. 3).

To understand the physiological significance of the autoinduction of EutR protein, we examined the sensitivity of the *eut* operon to induction by the effector CN-B_{12}, a precursor of the Ado-B_{12} effector (presented with excess ethanolamine). Strains that contained different combinations of *eut*::*lac* fusions and point mutations were tested. The apparent K_m values for *eut* operon induction by exogenous CN-B_{12} varied over a 10-fold range. Low apparent K_m values were exhibited in strains with *eut*::*lac* fusions within or upstream of the *eutBC* lyase genes (sensitive strains). Fusions that did not disrupt any *eut* gene exhibited a relatively high apparent K_m value for operon induction (insensitive strains). This high K_m value was reduced by point mutations that eliminate synthesis of lyase. Point mutations in genes other than the *eutBC* lyase genes had little effect on the sensitivity of induction of a distal operon fusion, and the slight effects seen probably result from polarity effects of those point mutations on expression of the *eutBC* gene. The strongly polar Mu d *lac* fusion mutations in these same *eut* genes are uniform in their reduction of the apparent K_m value for operon induction; we attribute this to low expression of the lyase. Two exceptional *eutC* lyase mutations,

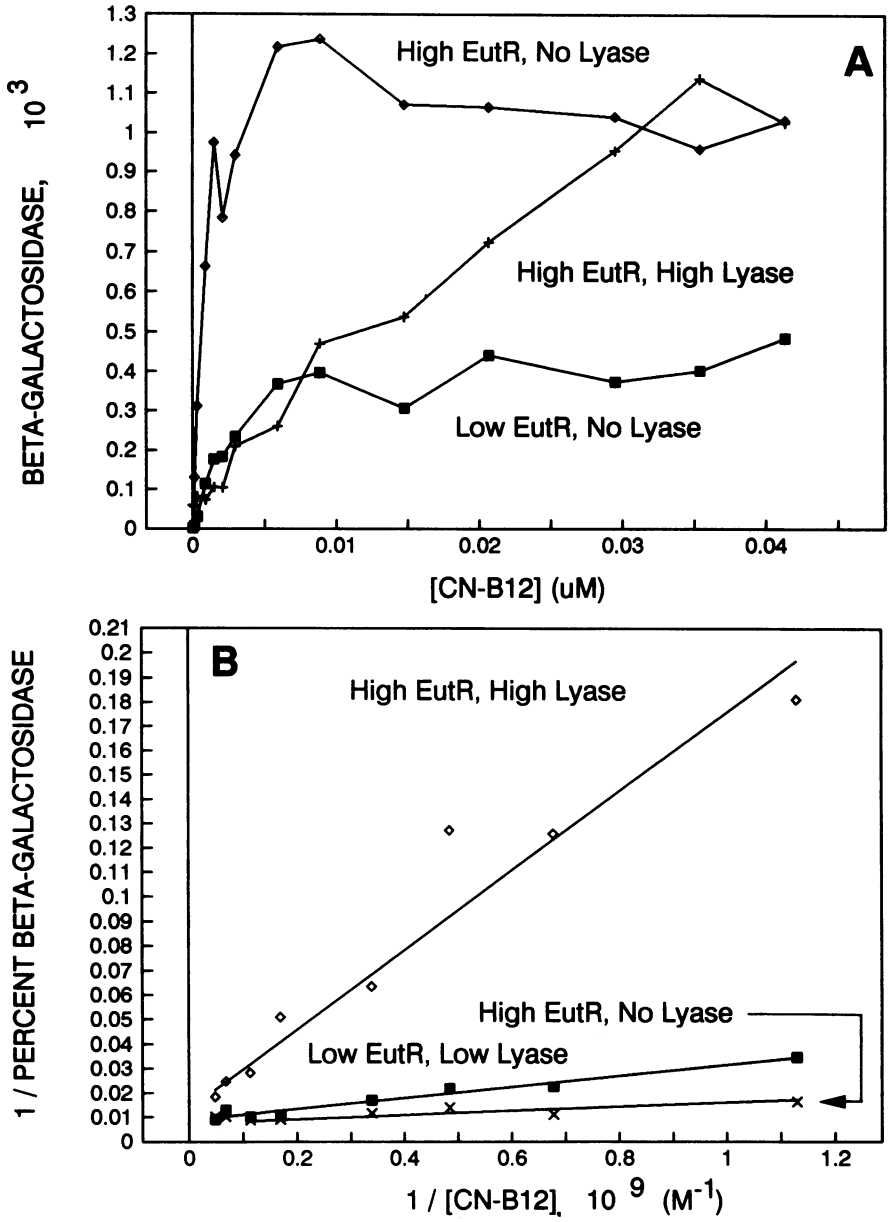

FIG. 5. Induction of the *eut* operon by exogenous CN-B_{12}: effect of high levels of lyase (*eutB* and *eutC*) and activator protein (*eutR*) on induction sensitivity. Cultures were grown and data were analyzed as described in the legend to Fig. 2, except that for panel A β-galactosidase activity was expressed in absolute units rather than as a percentage of the maximum expressed levels so that absolute levels of *eut* operon induction can be compared. The strains assayed were TT17298 (High EutR, No Lyase), TT17320 (High EutR, High Lyase), and TT17321 (Low EutR, No Lyase). (A) actual data; (B) double reciprocal plots of the data.

both nonpolar point mutations, failed to improve the sensitivity of induction. One of these is a leaky mutation. We think such mutations are expected and can be explained as missense mutants which produce lyase with impaired catalytic activity but normal ability to bind to Ado-B_{12}. If the mutations do not eliminate protein capable of binding Ado-B_{12}, they are not expected to improve induction sensitivity. Since impaired catalytic activity alone does not increase sensitivity to induction, it seems likely that this regulatory effect is due to binding of Ado-B_{12} by lyase protein rather than depletion of the inducer ethanolamine by lyase enzymatic activity. In summary, strains that are sensitive to induction have reduced levels of lyase synthesis. We interpret this to mean that lyase competes with the EutR regulatory protein for Ado-B_{12}. Eliminating lyase allows all available Ado-B_{12} to serve as an effector for *eut* operon induction.

The autoinduction model for EutR synthesis predicts that the absolute level of *eut* operon induction should be controlled by the level of EutR protein available to the main promoter ($eutP_I$). To test this prediction, a mutant was selected with high constitutive expression of both the *eutBC* lyase and *eutR* regulatory protein genes. An internal promoter mutation located just upstream of the *eutB* gene is responsible for this expression. This made it possible to measure the absolute expression from $eutP_I$ at both a low and high constitutive rate of expression of *eutR*, with and without competition by lyase.

TABLE 4. Apparent K_m and maximum *eut* operon induction by Ado-B12: effect of high and low rates of expression of EutB,C (lyase) and EutR (operon activator protein)

Strain	Genotype[a]							K_m (10^9)[b]	K_m relative to *eut-38*::Mu dA	*eut* operon induction (β-galactosidase)[c]		
	eutD	*eutA*	P_{int}	*eutB*	*eut*	*eutR*	*eut-38*			Uninduced	Fully induced	Fully induced relative to *eut-38*::Mu dA
TT13738						*eutR156*::Mu dJ				1.6	2.1	0.0
TT17299		*eutA208*::Tn*10d*Tet				*eutR156*::Mu dJ				4.3	<1	—
TT17300		*eutA208*::Tn*10d*Tet	P_{int}			*eutR156*::Mu dJ				1107.7	904.2	0.6
TT10674							*eut-38*::Mu dA			4.5	196.8	1.0
TT14492		*eutA208*::Tn*10d*Tet					*eut-38*::Mu dA			9.9	9.0	0.0
TT17302		*eutA208*::Tn*10d*Tet	P_{int}				*eut-38*::Mu dA			361.3	318.0	1.6
TT17303		*eutA208*::Tn*10d*Tet	P_{int}	*eutB115*			*eut-38*::Mu dA			386.5	291.6	1.5
TT17305		*eutA208*::Tn*10d*Tet	P_{int}	*eutB107*			*eut-38*::Mu dA			354.8	277.2	1.4
TT17304		*eutA208*::Tn*10d*Tet	P_{int}		*eutC110*		*eut-38*::Mu dA			203.7	163.9	0.8
TT10674							*eut-38*::Mu dA	23.3	1.00	1.9	305.8[d]	1.0
TT10271	*eutD18*::Mu dJ							1.1	0.05	1.9	184.1[d]	0.6
TT17321	*eutD18*::Mu dJ	*eutA208*::Tn*10d*Tet						2.6	0.11	1.9	398.4[d]	1.3
TT17320	*eutD18*::Mu dJ	*eutA208*::Tn*10d*Tet	P_{int}					12.2	0.52	59.0	1325.7[d]	4.3
TT17296	*eutD18*::Mu dJ	*eutA208*::Tn*10d*Tet	P_{int}	*eutB115*				2.3	0.10	32.2	1424.9[d]	4.7
TT17297	*eutD18*::Mu dJ	*eutA208*::Tn*10d*Tet	P_{int}	*eutB107*				1.0	0.04	29.3	1413.8[d]	4.6
TT17298	*eutD18*::Mu dJ	*eutA208*::Tn*10d*Tet	P_{int}		*eutC110*			1.2	0.05	10.7	1083.7[d]	3.5

[a] Mutations present in each strain are presented in map order (Fig. 1) and are aligned vertically for each of comparison. No entry indicates that the particular genetic region is wild type.
[b] Apparent K_m values were determined as described in Materials and Methods.
[c] All cultures were grown for analysis as described in Materials and Methods. Operon induction was measured from *eut*::*lac* fusions located at different positions within the operon.
[d] These are the average of several values obtained from cultures grown with excess CN-B_{12}.

Measurement of $eutP_I$ expression in otherwise isogenic strains demonstrates that an increase in *eutR* expression causes an increase in the maximal level of $eutP_I$ induction.

An autoinduced regulatory gene, such as seen for the *eut* operon, has several properties that appear to be valuable. Uninduced cells need only produce a low basal level of activator regulatory protein sufficient to start the process of induction. Since little or no lyase is present before induction, the initial phase of induction should be extremely sensitive to Ado-B_{12}. Once operon induction starts and the level of lyase increases, a competition will develop between the EutR protein, which requires Ado-B_{12} as an effector for induction, and lyase, which requires Ado-B_{12} as a cofactor for enzymatic activity. At low levels of Ado-B_{12}, full induction will fail since lyase will sequester the inducer. By increasing the level of EutR regulatory protein in parallel with lyase, the cell can partition available Ado-B_{12} between the enzyme and the available protein. This permits the EutR protein to continue to sense the Ado-B_{12} level even when a high level of lyase competes for Ado-B_{12} binding. While autoinduction of *eutR* is important in maintaining an effective distribution of Ado-B_{12} between EutR regulatory protein and lyase, differences in the binding constants of these two proteins may also contribute to the balance between *eut* operon induction and ethanolamine degradation. In the absence of separate regulatable promoters for *eutBC* and *eutR*, it is not possible to determine whether the condition of high levels of EutBC lyase with low levels of EutR regulatory protein results in less-efficient induction. This premise will be tested when these constructs become available.

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