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Role of the MetR Regulatory System in Vitamin B_{12}-Mediated Repression of the *Salmonella typhimurium metE* Gene

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The vitamin B_{12} (B_{12})-mediated repression of the *metE* gene in *Escherichia coli* and *Salmonella typhimurium* requires the B_{12}-dependent transmethylase, the *metH* gene product. It has been proposed that the MetH-B_{12} holoenzyme complex is involved directly in the repression mechanism. Using *Escherichia coli* strains lysogenized with a λ phage carrying a *metE-lacZ* gene fusion, we examined B_{12}-mediated repression of the *metE-lacZ* gene fusion. Although B_{12} supplementation results in a 10-fold repression of *metE-lacZ* expression, homocysteine addition to the growth medium overrides the B_{12}-mediated repression. In addition, B_{12}-mediated repression of the *metE-lacZ* fusion is dependent on a functional MetR protein. When a *metB* mutant was transformed with a high-copy-number plasmid carrying the *metE* gene, which would be expected to reduce intracellular levels of homocysteine, *metE-lacZ* expression was reduced and B_{12} supplementation had no further effect. In a *metJ* mutant, B_{12} represses *metE-lacZ* expression less than twofold. When the *metJ* mutant was transformed with a high-copy-number plasmid carrying the *metH* gene, which would be expected to reduce intracellular levels of homocysteine, B_{12} repression of the *metE-lacZ* fusion was partially restored. The results indicate that B_{12}-mediated repression of the *metE* gene is primarily a loss of MetR-mediated activation due to depletion of the coactivator homocysteine, rather than a direct repression by the MetH-B_{12} holoenzyme.

In *Escherichia coli* and *Salmonella typhimurium*, the final reaction in the synthesis of methionine is the methylation of homocysteine (14, 15) (Fig. 1). Two alternate transmethylases can catalyze this reaction. One is a vitamin B_{12} (B_{12})-independent enzyme, the *metE* gene product; the other is a B_{12}-dependent enzyme, the *metH* gene product.

The *metE* gene, as well as all of the other methionine biosynthetic genes except *metH*, is negatively controlled by the *metJ* gene product, with *S*-adenosylmethionine (SAM) acting as the corepressor (14, 15). In addition, the *metE* gene is repressed by the addition of B_{12} to the growth medium (7, 8). This B_{12}-mediated repression requires the *metJ* gene product and the *metF* gene product (the *metF* gene product provides the methyl donor for homocysteine methylation) (3, 12), as well as the MetH-B_{12} holoenzyme. Although the mechanism of repression by the MetH-B_{12} holoenzyme requires the MetJ protein, this repression system is distinct from the repression system mediated by the MetJ protein and SAM (11).

Recently it was demonstrated that transcription of the *metE* gene (and the *metH* gene) is positively regulated by the *metR* gene product (9, 21–23), a DNA binding protein (1, 22). Homocysteine, an intermediate in methionine biosynthesis, was shown to be required for activation of the *metE* gene both in vivo (24) and in vitro (2).

Here we provide evidence that a large part of the B_{12}-mediated repression of the *metE* gene is a result of reduced MetR-homocysteine activation. The MetH-B_{12} holoenzyme, along with the *metF* and *metJ* gene products, probably plays an indirect role in B_{12}-mediated repression by changing the intracellular levels of both the MetR activator protein and its coactivator homocysteine.

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MATERIALS AND METHODS

Bacteria, bacteriophage, and plasmids. All strains used are from the laboratory collection. GS243 is Δ*metE*::Mu, GS244 is Δ*metR*::Mu, GS597 is *metJ97*, and GS718 is *metB1*. In addition, all strains carry the *pheA905*, *thi*, *araD129*, *rpsL*, *and* Δ*lacU169* mutations. The Δ*lacU169* mutation is a complete deletion of the *lac* operon from the chromosome. The λElac phage carries a fusion of the *S. typhimurium metE* control region and the first 22 amino acid codons fused to the 8th amino acid codon of *lacZ* (13). λElac1, a derivative of λElac, expresses high levels of β-galactosidase activity because of the presence of a *metE* operator-constitutive mutation (13). Plasmid pGS192 carries the *S. typhimurium metH* gene (21). Plasmid pGS69 carries the *S. typhimurium metE* gene (16). Plasmid pGS69::Tn*5*B is a derivative of pGS69 carrying transposon Tn*5* within the *metE* gene (16).

Media and growth conditions. Glucose minimal medium (GM) has been described previously (18). Lactose minimal medium (LM) is identical to GM except that 0.2% lactose replaces glucose. Phenylalanine and B_{12} were added to GM since all strains carry the *pheA905* and *thi* mutations. Supplements were added at the following concentrations (in micrograms per milliliter): amino acids, 50; B_1 and B_{12}, 1; ampicillin (Ap), 100; D,L-homocysteine; 100.

Construction of λElac and λElac1 lysogens. Appropriate strains were lysogenized with λElac or λElac1 fusion phage by the procedure described previously (19). After purification, the lysogens were tested for a single copy of the λ phage by infection with phage λ *c*I90 *c*17 (17). All λ lysogens contain the temperature-sensitive mutation *c*I857 and were grown at 30°C.

β-Galactosidase assay. β-Galactosidase levels were measured as described by Miller (10) by using the chloroform-sodium dodecyl sulfate lysis procedure. The results are the averages of two or more assays, and each sample was run in triplicate.

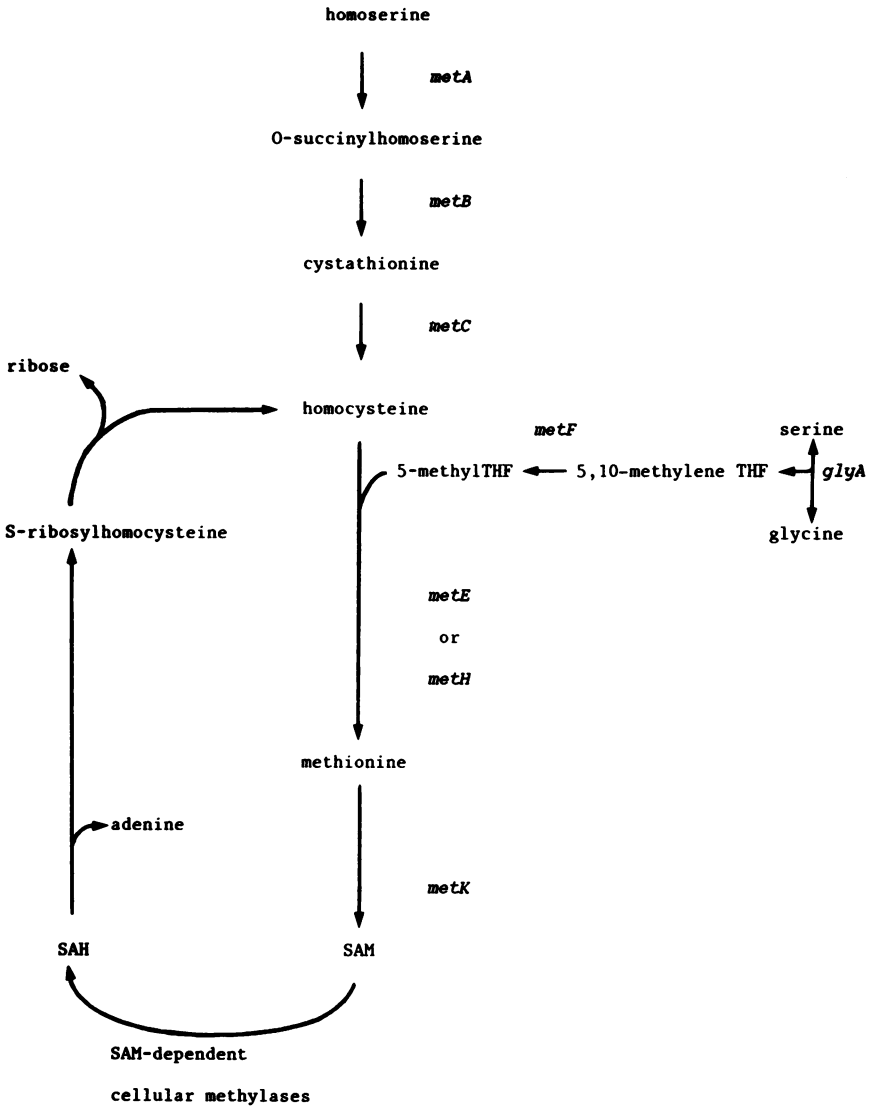

FIG. 1. Methionine biosynthetic pathway in *E. coli* and *S. typhimurium* (4, 14). The *metA*, *metB*, and *metC* genes encode enzymes in the nonfolate branch of the methionine pathway. The *glyA* and *metF* genes encode enzymes in the folate branch of the methionine pathway. The transmethylation of homocysteine to methionine is catalyzed by either the *metE* or the *metH* gene product. The *S*-adenosylhomocysteine (SAH) formed as a by-product of SAM-dependent methylation reactions is salvaged as the adenine base and homocysteine by a cyclical regenerative branch of the pathway (5), which has not been characterized genetically. THF, tetrahydrofolate.

RESULTS

Effect of homocysteine on *metE-lacZ* expression in B_{12}-supplemented media. In early experiments investigating B_{12}-mediated repression of *metE*, it was suggested that the MetH-B_{12} holoenzyme might function directly as a repressor of *metE* gene expression (7, 8). However, at that time the role of MetR-homocysteine-mediated activation of the *metE* gene was unknown. An initial experiment was designed to determine if repression of the *metE* gene by the addition of B_{12} to the growth medium occurs directly via the formation of a MetH-B_{12} repressor complex or indirectly by the MetH-B_{12} holoenzyme lowering the levels of the coactivator homocysteine by methylation to methionine (Fig. 1). The *metE* mutant strain GS243 was lysogenized with λElac. This strain, designated 243λElac, cannot grow on LM plates supplemented with B_{12} because of a deletion of the *lac* operon on the chromosome and insufficient β-galactosidase production from the *metE-lacZ* gene fusion because of B_{12}-mediated repression. If the MetH-B_{12} holoenzyme represses *metE* expression by lowering homocysteine levels below what is required for activation of the *metE-lacZ* fusion, then exogenous homocysteine would be expected to overcome growth inhibition of 243λElac on LM plates supplemented with B_{12}. Lysogen 243λElac grew well on LM plates supplemented with both B_{12} and homocysteine.

To test more directly the effect of homocysteine on the B_{12}-mediated repression of a *metE-lacZ* gene fusion, the *metB* mutant strain GS718 was lysogenized with λElac1. The λElac1 phage carries a point mutation (O^c-*1*) in the *metE* control region that interferes with MetJ repression of the *metE-lacZ* fusion but allows normal regulation by B_{12} (13). The elevated *metE-lacZ* expression caused by this O^c-*1*

TABLE 1. Effects of homocysteine and the *metR* gene product on MetH-B_{12} repression of a *metE-lacZ* gene fusion

Lysogen[a]	GM supplement	β-Galactosidase (U)[b]
718λElac1[pGS192]	L-Methionine	662
	L-Methionine + B_{12}	59
	L-Methionine + homocysteine	866
	L-Methionine + B_{12} + homocysteine	920
244λElac1[pGS192]	L-Methionine	35
	L-Methionine + B_{12}	35

[a] Strain GS718 is a *metB* mutant and strain GS244 is a *metR* mutant. Both strains were lysogenized with the *metE-lacZ* fusion phage λElac1 and transformed with the multicopy *metH* plasmid pGS192.
[b] Units of activity are Miller units (10).

TABLE 2. Effects of multicopy *metE* on expression of the *metE-lacZ* gene fusion

Lysogen[a]	GM supplement	β-Galactosidase (U)[b]
718λElac1[pGS69::Tn*5*B]	D-Methionine	379
	D-Methionine + B_{12}	133
718λElac1[pGS69]	D-Methionine	126
	D-Methionine + B_{12}	122

[a] The *metB* mutant strain GS718 was lysogenized with the *metE-lacZ* fusion phage λElac1 and then transformed with either multicopy *metE* plasmid pGS69 or pGS69::Tn*5*, a pGS69 derivative with transposon Tn*5* inserted in and inactivating the *metE* gene (16).
[b] Units of activity are Miller units (10).

mutation enabled us to supplement all lysogen cultures with L-methionine, minimizing any effects of changes in internal methionine levels and corresponding changes in MetJ-mediated repression. Because of the *metB* mutation in lysogen 718λElac1, this strain does not produce homocysteine via the nonfolate branch of the methionine pathway (Fig. 1). When supplemented with methionine, however, lysogen 718λElac1 produces low levels of homocysteine from the utilization of SAM via a regenerative pathway that exists in *E. coli* (Fig. 1). The lysogen was transformed with a multicopy plasmid carrying the *metH* gene (pGS192) to increase the levels of the MetH protein. Lysogen 718λElac[pGS192] was grown in GM supplemented with L-methionine, and B_{12} or homocysteine was added as indicated in Table 1. β-Galactosidase levels were then measured for each growth condition. Expression of the *metE-lacZ* gene fusion was repressed more than 10-fold when the lysogen was grown on GM supplemented with B_{12} (Table 1, compare lines 1 and 2). The addition of homocysteine, however, overrides the B_{12}-mediated repression, resulting in a high β-galactosidase level (Table 1, compare lines 2 and 4).

Repression of the *metE-lacZ* fusion by B_{12} is dependent on the MetR protein. Since homocysteine acts as a coactivator for the MetR protein to activate *metE* expression (23), we tested whether the MetR protein is involved with homocysteine in overriding the B_{12}-mediated repression of the *metE-lacZ* fusion. The *metR* mutant strain GS244 was lysogenized with λElac1, and the lysogen was subsequently transformed with the *metH* plasmid pGS192. This strain, designated 244λElac1[pGS192], was then tested for B_{12}-mediated repression of the *metE-lacZ* gene fusion. Cells were grown in GM supplemented with L-methionine or L-methionine plus B_{12}, and β-galactosidase levels were measured. As expected, β-galactosidase levels in lysogen 244λElac1[pGS192] were greatly reduced compared with those in lysogen 718λElac1[pGS192] because of the *metR* mutation (Table 1). However, even in the presence of excess levels of MetH protein, there is no further repression of the *metE-lacZ* fusion in lysogen 244λElac1[pGS192] when cells are grown in the presence of B_{12}. Thus, B_{12}-mediated regulation of the *metE-lacZ* fusion is dependent on a functional MetR protein. These data, along with the above results, suggest that B_{12}-mediated repression is due to depletion of homocysteine rather than to a direct repression by the MetH-B_{12} holoenzyme.

***metE-lacZ* expression in the presence of multiple copies of the *metE* gene.** Both the *metE* and *metH* gene products transmethylate homocysteine to form methionine. Thus, the presence of a multicopy plasmid carrying the *metE* gene should utilize excess homocysteine, having an effect similar to that of B_{12} supplementation. To test this hypothesis, lysogen 718λElac1 was transformed with either pGS69 or pGS69::Tn*5*B, designated 718λElac1[pGS69] and 718λElac1 [pGS69::Tn*5*B], respectively. Plasmid pGS69 carries an intact *metE* gene. Plasmid pGS69::Tn*5*B is identical to pGS69 except that it has a Tn*5* element inserted in the *metE* structural gene and serves as a control for any decrease in *metE-lacZ* expression due to the titration of the MetR protein by the multicopy *metE* plasmid. Lysogens 718λElac1[pGS69::Tn*5*B] and 718λElac1[pGS69] were grown in GM supplemented with either D-methionine or D-methionine plus B_{12}, and β-galactosidase levels were measured. D-Methionine is converted to L-methionine by the cell and can be used as a methionine-limiting source (6). D-Methionine instead of L-methionine was used to keep the basal level of homocysteine generated by the cyclic branch of the methionine pathway low (Fig. 1), as well as to prevent *metJ*-mediated repression of *metE* expression from pGS69. The level of β-galactosidase in the control lysogen 718λElac1[pGS69::Tn*5*B] was decreased threefold when growth was in the presence of D-methionine plus B_{12} compared with growth in the presence of D-methionine (Table 2, compare lines 1 and 2). However, the level of β-galactosidase in 718λElac1[pGS69] was reduced in the absence or presence of B_{12} (Table 2, lines 3 and 4). These results suggest that the high MetE levels from the multicopy *metE* plasmid pGS69 have an effect similar to that of B_{12} supplementation, depleting homocysteine and decreasing *metE-lacZ* expression.

***metE-lacZ* expression in a *metJ* mutant.** Previous studies (8) have shown that in *metJ* mutants, the repression of *metE* by B_{12} is less than twofold. We tested the effect of *metJ* mutations on B_{12}-mediated repression of the *metE-lacZ* gene fusion. The *metJ* mutant strain GS597 was lysogenized with λElac (designated 597λElac), and the lysogen was grown in GM supplemented with either D-methionine or D-methionine plus B_{12}. The addition of B_{12} to the growth medium resulted in about twofold repression of the *metE-lacZ* fusion (Table 3).

It was shown previously that the MetJ protein and SAM negatively regulate *metR* gene expression and that *metR* expression is increased in a *metJ* mutant strain (20). The inability of B_{12} to repress the *metE-lacZ* fusion in the 597λElac lysogen could be due either to a requirement for a functional MetJ protein in the B_{12}-mediated repression mechanism or to higher homocysteine levels and MetR

TABLE 3. Effects of the *metJ* gene product on B_{12}-mediated repression of a *metE-lacZ* gene fusion

Lysogen[a]	GM supplement	β-Galactosidase (U)[b]
597λElac	D-Methionine	4,184
	D-Methionine + B_{12}	2,067
597λElac[pGS192]	D-Methionine	2,049
	D-Methionine + B_{12}	516

[a] The *metJ* mutant strain GS597 was lysogenized with the *metE-lacZ* fusion phage λElac and was either nontransformed or transformed with the multicopy *metH* plasmid pGS192.
[b] Units of activity are Miller units (10).

protein in the *metJ* mutant strain. To distinguish between these two possibilities, 597λElac was transformed with the *metH* plasmid pGS192. This strain, designated 597λElac[pGS192], was then grown in GM supplemented with D-methionine or D-methionine plus B_{12}, and the β-galactosidase levels were determined. If the inability of B_{12} to repress *metE-lacZ* expression in the *metJ* mutant is due to higher levels of homocysteine and MetR protein, resulting in activation of the *metE-lacZ* fusion, then the multicopy *metH* plasmid should result in the utilization of excess homocysteine because of high levels of the MetH-B_{12} transmethylase, thus lowering levels of expression of the *metE-lacZ* fusion. The β-galactosidase levels were significantly reduced in the transformed cells (Table 3).

DISCUSSION

Previous work suggested that the MetH-B_{12} holoenzyme has both an enzymatic and a regulatory role (11), serving as a homocysteine transmethylase and, in an unknown way, repressing *metE* gene expression. The results presented here suggest that a large part of the B_{12}-mediated repression of the *metE* gene is a result of utilization of homocysteine (the coactivator for *metE* expression) by the MetH-B_{12} holoenzyme. We have not been successful in measuring accurately the intracellular levels of homocysteine; however, by manipulating both the growth medium composition and the genetic status of the lysogens, we have been able to examine *metE-lacZ* expression under various conditions that would be expected to drive the levels of homocysteine either higher or lower. We have shown that homocysteine supplementation overrides B_{12}-mediated repression of a *metE-lacZ* gene fusion, resulting in increased β-galactosidase levels in cells grown in the presence of B_{12} (Table 1). Furthermore, repression of the *metE-lacZ* fusion by B_{12} is dependent on a functional MetR protein (Table 1). Although the level of β-galactosidase is low in lysogen 244λElac1[pGS192] because of the absence of MetR, it would have been possible to detect additional repression by B_{12} in this lysogen if B_{12} regulates *metE* by a MetR-independent mechanism. Although the low β-galactosidase levels in the 244λElac1 [pGS192] could be explained by a secondary promoter in the *metE-metR* intergenic region, independent from the MetH-B_{12} regulatory mechanism, this is unlikely since previous S1 nuclease mapping experiments showed that the low levels of *metE-lacZ* expression in a *metR* mutant initiate from the normal *metE* start site (23).

If the MetH-B_{12} holoenzyme acts indirectly to reduce *metE* expression by depleting the homocysteine pool, it should be possible to reproduce this effect by any mechanism that reduces the homocysteine pool. Thus, we transformed the 718λElac1 lysogen with the multicopy plasmid pGS69, which carries the *S. typhimurium metE* gene. We assumed that a high level of MetE protein, the B_{12}-independent homocysteine transmethylase, would reduce the homocysteine pool. Although plasmid pGS69 carries the MetR binding site and could titrate the MetR activator protein, resulting in a low level of expression of *metE-lacZ* fusion, we controlled for this titration effect by comparison to plasmid pGS69::Tn*5*B, which also has an intact MetR binding site. Thus, any influence on *metE-lacZ* expression due to the insufficiency of the MetR protein would be equally reflected in both lysogens. The level of β-galactosidase in lysogen 718λElac1[pGS69] grown in GM supplemented with D-methionine was threefold lower than that in lysogen 718λElac1[pGS69::Tn*5*B], whose *metE* gene was inactivated by a Tn*5* insertion (Table 2). Furthermore, the addition of B_{12} to the growth medium reduced β-galactosidase levels in lysogen 718λElac1[pGS69::Tn*5*B] to those of lysogen 718λElac1[pGS69] grown on either D-methionine or D-methionine plus B_{12}. These results show that a second mechanism for depleting the homocysteine pool has an effect similar to that of B_{12} supplementation.

The results of our work support a model in which the MetH-B_{12} complex acts indirectly to regulate *metE* expression by reducing the level of homocysteine. Alternative models in which the MetH-B_{12} holoenzyme might be proposed to inhibit the activity of the MetR protein (e.g., by direct interaction or by methylation of MetR) are not supported by our data. No further repression of the *metE-lacZ* fusion was observed in the 718λElac1[pGS69] lysogen when B_{12} was added (Table 2, compare lines 3 and 4). If the MetH-B_{12} holoenzyme interferes directly with MetR activity, an additional decrease in *metE-lacZ* expression would have been expected.

Kung et al. (8) have shown that in a *metJ* strain, B_{12} represses *metE* expression less than twofold. We also found that B_{12} represses a *metE-lacZ* fusion only twofold in a *metJ* mutant (Table 3) as well as a *metJ metB* double mutant (data not shown). We believe that the failure to see the full 10-fold B_{12}-mediated repression that is observed in a *metJ*$^+$ strain (Table 1) is due to overproduction of homocysteine by the regenerative branch of the methionine pathway (Fig. 1). Consistent with this hypothesis, transformation of the *metJ* lysogen 597λElac with the *metH* plasmid pGS192 partially restores B_{12}-mediated repression of the *metE-lacZ* gene fusion (Table 3). The high-copy-number *metH* plasmid results in increased levels of the B_{12}-dependent homocysteine transmethylase and would be expected to reduce homocysteine levels in the presence of B_{12}. It is not clear why overproduction of the MetH protein does not result in full repression of the *metE-lacZ* fusion in the presence of B_{12} (Table 3). Since *metJ* mutants also overexpress the *metR* gene (20), it is possible that the elevated levels of MetR protein are sufficient to maintain a higher basal level of expression of the *metE-lacZ* fusion. It should be noted that even in the absence of B_{12}, lysogen 597λElac transformed with pGS192 has twofold-lower β-galactosidase levels than the untransformed lysogen (Table 3). The MetR binding site is overrepresented in the transformed lysogen because of multiple copies of the *metH* gene. It is possible that enough MetR protein is sequestered at these sites to deplete the MetR pool to a level insufficient to fully activate the *metE-lacZ* fusion.

Mulligan et al. (12) reported that in a *metF*::Tn*5* mutant, *metE-lacZ* expression is derepressed in the presence of B_{12}, indicating that a functional *metF* gene product is necessary

for repression of *metE* by B_{12}. A nonfunctional MetF protein would result in the absence of 5-methyltetrahydrofolate, the methyl donor for methionine synthesis (Fig. 1), leading to the accumulation of homocysteine. Our results indicate that accumulated homocysteine is probably responsible for the increased expression of the *metE* gene in *metF* mutants.

The *metF* gene is also repressed by the *metH* gene product and B_{12} (3, 25). Whitehouse and Smith (25) found that in some *S. typhimurium metE metH* double mutants which lack B_{12}-dependent homocysteine transmethylase activity, B_{12} supplementation still represses *metF* gene expression. Since homocysteine would be expected to accumulate in these mutants, it is difficult to reconcile these results with our model. We have tested for B_{12} repression of a *metF-lacZ* fusion in one of their strains [DS952 (*metH465 metE205*)] and found no repression (data not shown). Additional studies will be necessary to determine the role of B_{12} in *metF* gene expression.

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