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Mutations Affecting the Regulation of the *metB* Gene of *Salmonella typhimurium* LT2

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We isolated and characterized *cis*-acting mutations that affect the regulation of the *metB* gene of *Salmonella typhimurium* LT2. The mutations were isolated in an *Escherichia coli lac* deletion strain lysogenized with λ bacteriophage carrying a *metB-lacZ* gene fusion (λJBlac) in which β-galactosidase production is dependent upon *metB* gene expression. The mutant lysogens show elevated, poorly regulated β-galactosidase production. The altered regulation is a result of disruption of the methionine control system mediated by the *metJ* repressor. The mutations are located in a region of dyad symmetry centered near the −35 sequence of the *metB* promoter. We propose that these mutations alter the repressor binding site and define the *metB* operator sequence. In addition, we discuss a highly conserved, nonsymmetric DNA sequence of unknown function which occurs in the control regions of the *metA*, *metC*, *metE*, *metF*, *metG*, and *metJB* genes of both *S. typhimurium* and *E. coli*.

The methionine biosynthesis genes in *Salmonella typhimurium* and *Escherichia coli* are scattered around the chromosome (1, 19). The cell coordinates the expression of these genes for the efficient synthesis of methionine by means of a negative control system (for a review, see reference 17). The current model for methionine regulation suggests that the *met* repressor (encoded by the *metJ* gene) binds to the control regions of the *met* genes and negatively regulates the amount of transcription from these genes. The extent to which each *met* gene is repressed is thought to result from the different affinity of the repressor for each individual operator, the position of the operator with respect to the promoter, or a combination of the two. Attempts have been made to discern the sites of action of the *met* repressor by analyzing and comparing DNA sequences of various *E. coli met* genes (3, 13) and by studying in vitro repressor DNA binding (21). In these investigations, sequences were found that possess dyad symmetry and show homology among the *met* genes, suggesting that the sequences could be repressor binding sites. We report here the isolation and characterization of *cis*-acting mutations that genetically identify the operator site for the *S. typhimurium metB* gene.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. JL781 (wild type) and JB672 (*metJ*) are *S. typhimurium* LT2 strains. GS243 (*metE ΔlacU169 pheA905 araD129 rpsL thi*) and GS597 (*metJ97 ΔlacU169 pheA905 araD129 rpsL thi*) are *E. coli* K-12 strains. Plasmids pGS107 (23) and pBR322 (10) were described previously. Plasmid pMC1403 (5) was from M. Casadaban. Phage λgt2 (15) was from R. Davis. Phage λ*c*I90 *c*17 (20) was from M. Feiss. Other plasmids and phages were isolated during this investigation.

Media. L agar and glucose minimal (GM) medium have been described previously (22). Lactose minimal (LM) medium is identical to GM medium except that glucose is replaced by 0.4% lactose. Supplements were added at the following concentrations (micrograms per milliliter): phenylalanine, 50; vitamin B_1, 1; D-methionine, 150; L-methionine, 50; ampicillin, 100; 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal), 40.

DNA manipulations. The general procedures used for restriction enzyme cleavage, ligation, plasmid isolation, and isolation of DNA fragments from polyacrylamide and agarose gels have been described before (10). Phage DNA was isolated by using a modification (27) of the rapid method of Benson and Taylor (4). DNA sequence analysis was done by the method of Maxam and Gilbert (12).

Construction of λJBlac phage. The λJBlac phage construction was similar to that previously reported for the λBlac phage (25), except that λJBlac carries a functional *metJ* gene. Plasmid pGS107, which carries the *S. typhimurium metB* and *metJ* genes, was partially digested with restriction enzyme *Rsa*I, and a 732-base-pair (bp) fragment was isolated. This fragment contains a functional *metJ* gene and a truncated *metB* gene. The *Rsa*I cleavage site within the *metB* gene occurs between codons 31 and 32. The fragment was ligated into the *Sma*I site of the *lacZYA* fusion vector pMC1403, and the ligation mixture was used to transform the *lac* deletion strain GS243. Transformants were plated onto L agar plates containing ampicillin and X-gal, and blue colonies were isolated. Plasmid DNA isolated from several blue transformants was analyzed by restriction enzyme digestion to determine the orientation of the 732-bp fragment. One plasmid was chosen in which the truncated *metB* gene was fused to the *lacZ* gene in pMC1403. To construct the λJBlac phage from this plasmid, the $metJ^+$ gene, the *metB-lacZ* fusion, and the *lacY* and *lacA* genes carried on this plasmid were cloned into the single *Eco*RI site in phage λgt2 by the method described previously (25).

Selection of λJBlac mutant phage. Strain GS243 was lysogenized with the λJBlac phage by putting 1 drop of the phage suspension onto a soft agar overlay of GS243 and incubating the plate at 30°C overnight. Phage-resistant cells were picked from the zone of lysis, and the presumed lysogens were single colony purified on L agar plates containing X-gal. One lysogen, designated 243λJBlac, was again streaked for isolated colonies on an L agar plate containing X-gal. Individual colonies were grown overnight in GM medium supplemented with phenylalanine, vitamin B_1, and methionine. Cells were then washed, centrifuged, and concentrated fivefold. A 0.1-ml sample (2×10^9 cells) of each

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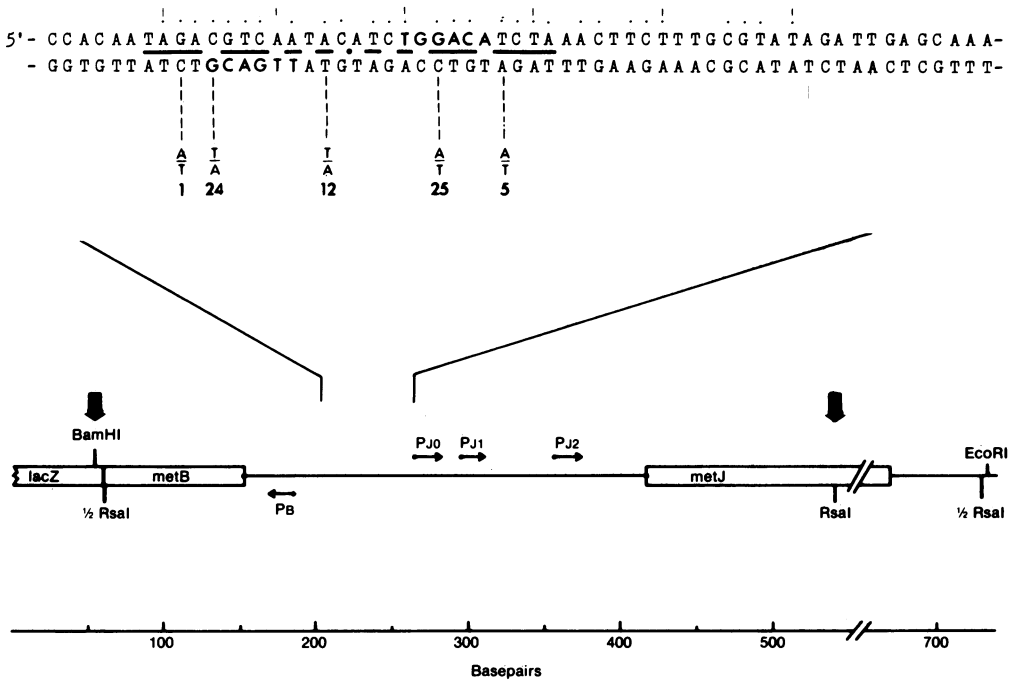

FIG. 1. Essential elements of the λJBlac phage used in the mutant selection. The phage carries a functional *metJ*$^+$ gene, a *metB-lacZ* fusion and the *lacY* and *lacA* genes. Details are given in the text. Horizontal arrows indicate the start site and direction of transcription of the *metB* promoter, p_B, and the three metJ promoters, p_{J0}, p_{J1}, and p_{J2} (24; also see the text). The upper expanded part of the figure shows the nucleotide sequence of the region that contains the *metB* operator site for the *metJ* repressor. Base changes for each mutant are indicated below the sequence. The proposed −35 region for promoters p_B and p_{J0} is shown in bold letters. The dyad symmetry of the proposed *metB* operator, which is disrupted in mutations Blac1, Blac12, Blac25, and Blac5, is shown as a broken line between the two strands. Bases that are homologous to the 8-bp repeat unit of Belfaiza et al. (3) are indicated by dots above the sequence. The two heavy arrows indicate the *Bam*HI-*Rsa*I fragment used to construct the λBlac derivatives of the mutant phage.

culture was plated onto LM plates supplemented with phenylalanine, vitamin B_1, and methionine and incubated at 30°C. Two colonies from each plate which arose within 24 h (colonies numbered between 50 to 200 per plate) were streaked for purity onto L agar plates containing X-gal and again tested for the Lac$^+$ phenotype with LM plates supplemented with phenylalanine, vitamin B_1, and methionine. A phage stock was prepared from each mutant by temperature induction, and these phage were again used to lysogenize GS243. The new lysogens were single colony purified and then scored for the Lac$^+$ phenotype as described above. Lac$^+$ lysogens were assumed to have a phage-associated mutation and were retained for further study. Phage DNA was prepared from each Lac$^+$ lysogen, and the *metJB* control region DNA was sequenced to locate the mutation.

Construction of λBlac mutant phage. Phage DNA from selected λJBlac mutants was digested with restriction enzyme *Eco*RI, and from each mutant a 6,900-bp fragment that carries the *metB-lacZ* fusion, the *metJ* gene, and the *lacY* and *lacA* genes was isolated from low-melting-temperature agarose gels and ligated into the *Eco*RI site of plasmid pBR322. Each recombinant plasmid was then digested with restriction enzymes *Bam*HI and *Rsa*I, and a 483-bp fragment that carries the *metB* control region and a truncated *metJ* gene was isolated (Fig. 1). This fragment was then ligated into the pMC1403 vector at the *Bam*HI and *Sma*I sites. The orientation of the fragment was confirmed by restriction enzyme analysis. Each plasmid was then digested with *Eco*RI, and a 6,700-bp *Eco*RI fragment that carries the mutant *metB-lacZ* fusion (now *metJ*) was ligated into λgt2 as described before (25). A purified stock of each λBlac mutant phage was prepared and used to lysogenize GS243 and GS597 as described above.

β-Galactosidase assay. β-Galactosidase activity was assayed as described by Miller (14), using the chloroform-sodium dodecyl sulfate lysis procedure.

Chemicals and enzymes. All chemicals and enzymes used are commercially available. The lambda packaging system was from Amersham Corp. (Arlington Heights, Ill.).

RESULTS

Selection of mutations in the *metJB* control region. Our primary interest in this study was to genetically define the *metJ* repressor binding site within the *metJB* shared control region. To select *cis*-acting up mutations in this region, we used a *metB-lacZ* fusion phage (λJBlac). This phage encodes a MetB-LacZ fusion protein identical to that encoded by the λBlac phage reported earlier (25), and also encodes a functional *metJ* repressor protein (Fig. 1). Thus, in the 243λJBlac lysogen used in the mutant selection, there are two copies of the *metJ* gene, one at the normal chromosomal locus and the other at the site of the λJBlac insertion. Although it is possible that hybrid *E. coli-S. typhimurium* repressors will be formed, two experimental results indicate that such repressors should function normally. (i) The amino acid sequences show ≥98% homology. (ii) A single copy of either the *E. coli* or *S. typhimurium metJ* gene produces similar levels of repression of the *metB-lacZ* fusion (unpublished data). Strain 243λJBlac does not grow on LM plates supple-

mented with phenylalanine, vitamin B_1, and methionine because of repression of the *metB-lacZ* fusion by the *metJ* repressor and thus insufficient β-galactosidase production. Mutations in this lysogen which lead to the ability to grow on the selection plates are likely to alter the *metB* operator or promoter site, rather than inactivate the repressor. Although the operator is presumably a smaller target than the *metJ* gene, inactivation of both *metJ* genes would require two independent mutation events. Another class of mutations that could lead to derepression of the *metB-lacZ* fusion are *metK* mutations. The *metK* gene encodes *S*-adenosylmethionine synthetase, which is responsible for the synthesis of the corepressor *S*-adenosylmethionine (17). However, *metK* mutants normally grow very slowly even in GM media (data not shown), and such mutants would not be evident on the selection plates within 24 h. With this selection procedure, 19 out of 20 Lac^+ mutants isolated have mutations located near *metB*.

Locations of mutations in λJBlac mutant phage. The location of the mutation in each of 13 Lac^+ mutants was determined by DNA sequence analysis of the JBlac control region. The base changes found are shown in Fig. 1. Three types of mutations were found: transitions, transversions, and one deletion (not shown). Five different base changes were identified, designated Blac1 (isolated seven times independently), Blac5, Blac12, Blac24, and Blac25 (isolated three times independently). All five changes are clustered in the region around the −35 sequence for the *metB* promoter (p_B). In addition, this region overlaps the −35 sequence for the furthest upstream *metJ* promoter (designated p_{J0}). Promoter p_{J0} was recently discovered in the *E. coli metJ* gene (9) and subsequently found in *S. typhimurium* (manuscript in preparation).

Analysis of the DNA sequence around the mutations revealed a region of dyad symmetry (Fig. 1) that is disrupted by mutations Blac1, Blac12, Blac25, and Blac5. Mutation Blac24 increases the symmetry beyond the sequence underlined, and also alters the −35 sequence of promoter p_B, making this sequence a perfect match with the −35 consensus sequence for *E. coli* promoters (16).

β-Galactosidase activity in wild-type λJBlac and mutant λJBlac lysogens. β-Galactosidase levels were measured in GS243 lysogenized with wild-type λJBlac and λJBlac mutants grown under conditions which normally either repress (L-methionine) or derepress (D-methionine) the *metB* gene (Table 1). The five mutants isolated all have β-galactosidase levels substantially higher than the level in the wild-type lysogen, especially when grown with L-methionine. These elevated values are sufficient to explain the Lac^+ phenotype shown on the LM selection plates. In addition, regulation of β-galactosidase synthesis is altered in the mutant lysogens. A comparison of the derepressed and repressed levels in the wild-type λJBlac lysogen shows that regulation varies over a 38-fold range, whereas in the mutant lysogens, regulation varies over only a 1.6- to 10.7-fold range.

TABLE 1. Comparison of β-galactosidase levels in GS243 lysogenized with λJBlac wild type and λJBlac mutants

Lysogen[a]	β-Galactosidase activity[b]		D-Methionine/ L-methionine ratio
	D-Methionine	L-Methionine	
243λJBlac	1,434	37	38.7
243λJBlac1	4,163	1,082	3.8
243λJBlac5	5,590	3,503	1.6
243λJBlac12	2,212	1,022	2.2
243λJBlac24	4,541	424	10.7
243λJBlac25	3,490	424	8.2

[a] Each lysogen used was shown to carry a single copy of the appropriate λ phage by infection with λ *c*I90 *c*17 (20).

[b] Units of specific activity are nanomoles of *O*-nitrophenol produced per minute per milligram of protein at 28°C. Growth medium was GM medium supplemented with phenylalanine, vitamin B_1, and either D-methionine (methionine limitation) or L-methionine (methionine excess).

TABLE 2. Comparison of β-galactosidase levels in GS243 and GS597 lysogenized with λBlac wild type and λBlac mutants

Lysogen[a]	*metJ* genotype	β-Galactosidase activity[b]		D-Methionine/ L-methionine ratio
		D-Methionine	L-Methionine	
243λBlac	+	3,101	221	14.0
243λBlac1	+	4,494	1,500	3.0
243λBlac12	+	1,893	1,127	1.7
243λBlac25	+	4,123	751	5.5
597λBlac	−	4,794	4,510	1.0
597λBlac1	−	5,553	5,276	1.0
597λBlac12	−	1,928	1,796	1.1
597λBlac25	−	5,470	4,771	1.1

[a] Each lysogen used was shown to carry a single copy of the appropriate λ phage by infection with λ *c*I90 *c*17 (20).

[b] Units of specific activity are nanomoles of *O*-nitrophenol produced per minute per milligram of protein at 28°C. Growth medium was GM medium supplemented with phenylalanine, vitamin B_1, and either D-methionine (methionine limitation) or L-methionine (methionine excess).

Evidence for *metJ* repressor involvement. The altered regulation of β-galactosidase levels in the λJBlac mutants (Table 1) and the disruption of a region of dyad symmetry in the control regions of these mutants (Fig. 1) suggest that the mutations have altered the *metJ* repressor binding site for the *metB* gene. To show that the altered regulation was due to an altered *metJ* repressor binding site and not some other independent system, we measured the level and the regulation of β-galactosidase production in cells in which the *metJ* repressor was inactivated.

To construct such a test system, it was necessary to inactivate the *metJ* gene carried on each of the mutant λJBlac phage and lysogenize a *metJ* mutant with each reconstructed mutant phage. For this reconstruction, only the wild-type λJBlac and mutant λJBlac1, λJBlac12, and λJBlac25 phage were used (see above).

β-Galactosidase levels were measured in strain GS597 (*metJ*) lysogenized with wild-type λBlac phage and the λBlac1, λBlac12, and λBlac25 mutant phage. As a control, β-galactosidase levels were also measured in strain GS243 ($metJ^+$) lysogenized with each phage (Table 2). In the GS243 lysogens, the regulation of β-galactosidase production follows the same pattern seen with the λJBlac lysogens except that the enzyme levels are somewhat higher and the range of regulation is not as great (cf. Tables 1 and 2). This difference most likely reflects the presence of two copies of the *metJ* repressor gene in the λJBlac lysogens. In contrast, the GS597 lysogens, which do not produce an active repressor, show elevated, nonrepressible levels of β-galactosidase, which are not significantly higher in the mutants when compared with levels in the wild type. This result suggests that the altered regulation of β-galactosidase synthesis seen in 243λBlac1, 243λBlac12, and 243λBlac25 is due to disruption of the *metJ* repressor system, rather than a mutation in some other independent system that increases *metB* expression. Mutations in an independent *metB* regulatory system would have shown an additive effect in the GS597 lysogens.

DISCUSSION

We isolated mutations in vivo which affect the expression of a *metB-lacZ* gene fusion. β-Galactosidase assays of these mutants showed elevated, partially regulated enzyme synthesis, typical of repressor binding site mutations (Table 1). A DNA sequence analysis located the mutations within or near the −35 region of the *metB* promoter p_B, and four out of five of the mutations disrupt a region of dyad symmetry (Fig. 1). The fifth mutation alters the −35 sequence of promoter p_B so that it perfectly matches the consensus sequence 5′-TTGACA-3′ (16). For three of the mutants, we showed that the altered regulation of promoter p_B is due to disruption of the *metJ* repressor system, most likely by alterations in the repressor binding site (Table 2).

At least two DNA regions have been proposed as *metJ* repressor binding sites in *E. coli*. The first, suggested by Duchange et al. (6) and analyzed for homologies by Michaeli et al. (13), is a short palindromic sequence, 5′-ATCTA-AC------GT-TAGAT-3′. For the *S. typhimurium metB* gene, this sequence begins 1 base to the left of the Blac5 mutation (Fig. 1), and includes the Blac5 mutation. However, three other mutations affecting *metB* regulation, Blac1, Blac12, and Blac25, map well outside this symmetry region. A second possibility, suggested by Belfaiza et al. (3), is that the repressor binding region may be composed of tandemly repeating 8-bp palindromes which vary in their frequency of repetition and degree of homology to the consensus palindrome 5′-AGACGTCT-3′. The region of repetition proposed by these investigators for the *E. coli metB* gene encompasses most of the sequence given in Fig. 1 and includes all of our mutations. Interestingly, most of the sequence forming the dyad symmetry defined by our mutations Blac1, Blac12, Blac25 and Blac5 (Fig. 1) can be generated by three repetitions of the above consensus sequence.

The Blac12 mutation requires further comment. It was isolated as an up mutation, which is reflected in the 27-fold increase of β-galactosidase activity in the 243λJBlac12 lysogen over that of the wild-type 243λJBlac lysogen under the repressing growth conditions used on the original selection plate (Table 1). However, when the Blac12 mutation is compared with wild-type Blac in a system which does not have a functional *metJ* repressor, it appears as a down mutation (cf. the 597λBlac12 and 597λBlac lysogens [Table 2]). In the GS597 lysogens, RNA polymerase does not have to compete with repressor for promoter binding, and thus the β-galactosidase activity in these strains probably reflects the intrinsic efficiency of the promoters involved. Hawley and McClure (7) have shown that bases near the −35 consensus sequence 5′-TTGACA-3′ are also conserved to some extent, including the T residue 2 bases upstream of the conserved sequence. The Blac12 mutation changes this T residue of the consensus sequence to an A residue. A similar change (T→G) at this position in the *ant* gene of bacteriophage P22 results in a mild down mutation (26).

The Blac24 mutation changes the −35 sequence of promoter p_B to a perfect match of the −35 consensus sequence. This change also increases the dyad symmetry region (Fig. 1), as well as changes a base in the repressor consensus sequence of Belfaiza et al. (3). It will be interesting to see if this mutation alters expression of *metB* solely by increasing the homology to the −35 promoter consensus, generating a promoter up mutation, or whether it also alters the affinity of this sequence for the repressor. It will be necessary to measure the affinity in vitro of the Blac24 DNA with purified repressor and RNA polymerase to resolve this question.

Binding of purified *E. coli metJ* repressor to the DNA of the *E. coli metJB* control region has been demonstrated in vitro by Smith et al. (21). Using a DNase protection assay, they found that at low concentrations of repressor protein in the presence of the corepressor *S*-adenosylmethionine, the repressor preferentially protects a region of DNA encompassing the −35 sequences of promoter p_B and the nearest *metJ* promoter (our p_{J0}). This is the same region in which our mutations are located, suggesting that these mutations are affecting the *metJ* repressor binding site for the *metB* gene. Although this region is very highly homologous between *S. typhimurium* and *E. coli*, an important exception is the A · T base pair immediately to the left of the Blac1 mutation (Fig. 1), which in *E. coli* has been reported as a G · C base pair (6, 9). This substitution in *E. coli* would disrupt both the symmetry defined by our mutation and the consensus sequence proposed by Belfaiza et al. discussed above (3).

The *metJB* control region contains not only promoters p_B and p_{J0}, but also two other *metJ* promoters, p_{J1} and p_{J2} (Fig. 1). We have shown previously that in a *metJ*$^+$ host, promoter p_{J1} is subject to repression by methionine (25). Since the RNA polymerase binding sites for promoters p_{J0} (defined by inspection of the sequence) and p_B (24) both overlap the region of dyad symmetry defined by the mutations as an operator site, it is of interest to question whether the *metJ* repressor binding site involved in *metB* regulation also plays a role in the autoregulation of the *metJ* gene. To answer this question, we fused the mutant *metJB* control fragments to the *lacZ* gene in which β-galactosidase production is directed by the three *metJ* promoters. Our results indicate that the mutations do affect *metJ* gene expression, but that the mechanism is complex and involves interactions between the overlapping promoters and the shared operator region (manuscript in preparation).

GENE	ORGANISM		REFERENCE
metJB	S.T.	5-GACGTCAATACATC TGGA CATC TAAAC TTCTTTGCGT^{-42}	(23)
metJB	E.C.	5-GACGTCAATACATC TGGA CATC TAAAC TTCTTTGCGT^{-179}	(9)
metC	S.T.	5-TGATTTTAACCGTC TGGA TGTC TAAAC TAGCATGAAT	THIS LAB
metC	E.C.	5-TGTTTTTATACGTC TGGA TGTC TAAAC TAGCATGAAT^{331}	(3)
metE	S.T.	5-GTACCTTTACATTA TGGA TGTG TAAAC ATCCAGACGT	THIS LAB
metF	S.T.	5-TTCATTTTTACATC TGGA CGTC TAAAC GGATAGATGT	THIS LAB
metF	E.C.	5-TTCATCTTTACATC TGGA CGTC TAAAC GGATAGATGT^{65}	(18)
metA	E.C.	5-TATCTTCAGCTATC TGGA TGTC TAAAC GTATAAGCGT^{246}	(13)
metG	E.C.	5-CCCGACTCCTTTTT TGTA TAGA TAAAC CATCAGCTGA^{-95}	(2)
metK	E.C.	5-ACCTAAAGAGAATT TGGT TAGC TAAAC TGTTGTGTGG^{-48}	(11)
CONSENSUS SEQUENCE:		Tc TGGA yrTc TAAAC	

FIG. 2. DNA sequences of *S. typhimurium* and *E. coli met* genes aligned to show a highly conserved 15-bp sequence within the various promoters. The most highly conserved bases, except for the first T residue, are boxed for clarity, and a consensus sequence is given at the bottom of the figure. The numbers above the rightmost bases correspond to the positions of those bases in the DNA sequences published in the respective references. Asterisks indicate the start sites of transcription. The proposed −35 (---) and −10 (—) regions, if known, are overlined for genes transcribed to the right (*metJ*, *metE*, *metF*, and *metA*) or underlined for genes transcribed to the left (*metB* and *metC*). The *metK* and *metG* genes show less homology to the consensus sequence, but are included for completeness. S.t., *S. typhimurium* LT2; E.c., *E. coli* K-12; y, pyrimidine; r, purine.

The dyad symmetry found in the *S. typhimurium metJB* operator region shows only partial homology with the control regions of other *S. typhimurium met* genes (unpublished data). This is similar to the results found in *E. coli*, as discussed above (3, 12). The imperfect homologies may reflect the various responses of the different *met* genes to repression by methionine.

In Fig. 2 the DNA sequences of various *met* gene control regions from both *S. typhimurium* and *E. coli* are aligned to emphasize a highly conserved, nonsymmetric sequence, 5′T-TGGA----TAAAC-3′. This sequence is located near the point of transcription initiation, except for the *metJB* control region. Note that the orientation of the sequence in the *metB* and *metC* genes to the direction of transcription is opposite that of the other genes. The occurrence of this sequence at a position between the *metB* and *metJ* genes, instead of near the transcription initiation points, may indicate that this sequence, like that of the repressor binding site, is shared by both genes. We have not determined a role for this sequence, but it is probably not the operator site for the *metJ* repressor. The mutations reported here which affect the regulation of *metB* expression by the repressor fall outside this sequence, except for the Blac25 mutation. The effect of the Blac25 mutation on the function of this highly conserved sequence is difficult to discern, since the particular G · C base pair changed by the Blac25 mutation is also part of the dyad symmetry and the −35 sequence of promoter p_{J0}. We examined the DNA sequences of the Genbank library for the occurrence of the conserved sequence. Except for the *E. coli lexA* gene (8), this sequence has not been found in any other genes. Whether or not this highly conserved sequence is involved in another level of control of the *met* regulon is currently being investigated.

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