Regulation of the *Salmonella typhimurium metF* Gene by the MetR Protein

JOHN M. COWAN, MARK L. URBANOWSKI, MICHAL TALMI,
AND GEORGE V. STAUFFER*

Department of Microbiology, The University of Iowa, Iowa City, Iowa 52242

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The *metF* gene in *Escherichia coli* and *Salmonella typhimurium* is under negative transcriptional control by the MetJ repressor. Expression of an *S. typhimurium metF-lacZ* gene fusion is repressed up to 10-fold by methionine addition to the growth medium in *E. coli* hosts encoding wild-type MetJ repressor; this repression is not seen in *metJ* mutants. *metR* mutations which eliminate the MetR activator protein result in two- to threefold-more-severe repression by the MetJ repressor. In a *metJ metR* double mutant, however, the level of *metF-lacZ* expression is the same as in a *metJ* mutant, suggesting that MetR antagonizes MetJ-mediated methionine repression of the *metF* promoter. A DNA footprint analysis showed that MetR binds to a DNA fragment carrying the *metF* promoter and protects two separate regions from DNase I digestion: a 46-bp region from position –50 to –95 upstream of the transcription initiation site and a 24-bp region from about position +62 to +85 downstream of the transcription initiation site and within the *metF* structural gene. Nucleotide changes in each of the MetR-binding sites away from the consensus sequence disrupt MetR-mediated regulation of the *metF-lacZ* fusion.

The folate branch of the methionine pathway in *Salmonella typhimurium* includes the *metE*, *metH*, and *metF* genes. The *metF* gene product (5,10-methylenetetrahydrofolate reductase) is involved in the biosynthesis of 5-methyltetrahydrofolate from 5,10-methylenetetrahydrofolate (20). 5-Methyltetrahydrofolate serves as a substrate for the methylation of homocysteine to form methionine by either the *metE* or the *metH* gene product (20).

The MetJ repressor negatively regulates the *metF* gene, as well as all other *met* biosynthetic genes except *metH* (20). The MetJ repressor is believed to function as a dimer, binding to the 8-bp recognition sequence 5'-AGACGTCT-3' (1, 19). Five degenerate forms of this 8-bp sequence occur as tandem repeats in the *metF* promoter region (24, 25). In addition to MetJ-mediated repression, two genes in the folate branch of the methionine pathway, *metE* and *metH*, are positively regulated by the *metR* gene product (29). The MetR protein belongs to the *lysR* family of bacterial activator proteins (9). DNase I protection experiments have shown that MetR binds to the *metE metR* intergenic promoter region (3, 28) and also to the *metA* (15) and *metH* (2) control regions.

The DNA sequence of the *S. typhimurium metF* gene has been determined (24). A computer search of the DNA sequence identified two possible MetR-binding sites in the *metF* promoter region and a third possible binding site in the structural gene (Fig. 1). We show here that the MetR protein is involved in *metF* regulation and that all three putative MetR-binding sites are required for MetR-mediated regulation of the *metF* gene.

MATERIALS AND METHODS

Strains, plasmids, and phages. All bacterial strains used in this study were derived from *Escherichia coli* K-12 and are described in Table 1. Plasmid pMC1403 (4), a *lac* fusion

vector, has been described previously, as have bacteriophages M13mp18 (30) and λ gt2 (18). Plasmid pGS174 carries the *S. typhimurium metF* gene (24), and pFlac carries a fusion of the *S. typhimurium metF* control region and the first 12 amino acid codons of the structural gene to the eighth codon of *lacZ* (25).

Media and growth conditions. Luria agar, Luria broth, and glucose minimal medium (GM) have been described previously (23). Supplements were added at the following concentrations: amino acids, 50 μ g/ml; vitamin B₁, 1 μ g/ml; ampicillin, 150 μ g/ml; and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal), 40 μ g/ml.

β -Galactosidase enzyme assay. β -Galactosidase activity was assayed as described by Miller (17), using the chloroform-sodium dodecyl sulfate lysis procedure. Results are the averages of two or more assays in which each sample was measured in triplicate.

DNA manipulation. Restriction enzyme digestions, ligations, plasmid and phage DNA isolations, and DNA fragment isolations were done as described by Maniatis et al. (14). Transformations were done by using polyethylene glycol- and dimethyl sulfoxide-prepared competent cells (5).

Site-directed mutagenesis. A 746-bp *EcoRI-BamHI* fragment carrying the *metF* control region was isolated from plasmid pFlac (25) and was cloned into the *EcoRI* and *BamHI* sites of phage M13mp18, creating phage M13mp18-Flac. Point mutations were introduced into the *metF* control region by the oligonucleotide-directed mutagenesis procedure of Kunkle (12) as modified by Byerly et al. (2). Changes were made that were shown previously to prevent MetR binding to the *metH* control region (2). The mutations were verified by DNA sequence analysis, and then the *EcoRI-BamHI* fragments carrying the desired mutations were re-cloned into plasmid pMC1403, creating mutant derivatives of the original pFlac fusion plasmid. Each mutant *metF-lacZ* fusion and the *lacY* and *lacA* genes were cloned onto bacteriophage λ gt2, and appropriate strains were lysogenized with the *metF-lacZ* fusion phage λ Flac or mutant derivatives, as previously described (26). Lysogens were

* Corresponding author.

FIG. 1. Nucleotide sequence of the *metF* control region. The transcription start site (+1) and the -10 and -35 promoter sequences are underlined (24). The ATG translation initiation codon for *metF* begins at base +70, within MetR-binding site 3. The sequence ends at codon 12 of the structural gene, the fusion point with *lacZ* in plasmid pFlac. The solid bars indicate MetR-binding sites, with consensus bases indicated in boldface type (18). The five degenerate forms of the 8-bp MetJ-binding sequence are indicated as J1 through J5. Dots above the sequence indicated bases homologous to the MetJ consensus binding sequence (1). Brackets above the MetR-binding sites indicate sequences protected by MetR from DNase I digestion (see Fig. 3). ----- indicates that DNase I cleavage in the unprotected lane was nonrandom and the precise endpoints of protection are not discernible. The nucleotide changes constructed by site-directed mutagenesis (see Materials and Methods) in the MetR-binding sites away from consensus are indicated below the sequence. The allele designations indicate both the base changes and their positions in the DNA sequence relative to the transcription initiation site for *metF* (+1).

tested for a single copy of the λ phage by infection with phage λ C190c17 (22) and then tested for the effects of the mutations on *metF-lacZ* expression.

Mobility shift assay. The mobility shift assay was based on the methods developed by Fried and Crothers (6) and by Garner and Revzin (7). A double-stranded 467-bp *Bst*EII-*Hinf*I fragment carrying the *metF* control region was isolated from plasmid pGS174 (24) and was 32 P labeled at the *Hinf*I site. The labeled DNA fragment was added to 20- μ l reaction mixtures containing 1 $\times$ DNA-binding buffer (10 mM Tris hydrochloride [pH 7.5], 50 mM KCl, 0.5 mM EDTA, 2.5% glycerol, 1 mM dithiothreitol) plus 125 μ g of bovine serum albumin (BSA) per ml. The reaction mixtures were incubated at 37°C for 5 min, and then 2 μ l of purified MetR protein (2) was added to the reaction mixtures as indicated in Fig. 2 and incubation was continued for an additional 15 min. One microliter of loading-dye mixture (0.1% xylene cyanol and 50% glycerol in H₂O) was added to each reaction mixture, and the samples were immediately loaded onto a 5% polyacrylamide-glycine gel and run at 12 V/cm for 3 h.

DNase I protection assay. The DNase I protection assay was based on the method of Schmitz and Galas (21). A 746-bp *Eco*RI-*Bam*HI fragment carrying the *metF* control region from plasmid pFlac (25) was 32 P labeled at the *Bam*HI site of the template strand. The labeled DNA fragment was

added to 160- μ l reaction mixtures containing 1 $\times$ DNA-binding buffer plus 125 μ g of BSA per ml. The reaction mixtures were preincubated at 37°C for 5 min, and then, 16 μ l of MetR protein was added to the reaction mixtures as indicated in Fig. 3 and incubation was continued for an additional 15 min. An 8- μ l portion of a solution of DNase I (2.5 μ g/ml dissolved in 20 mM sodium acetate [pH 7] plus 32 mM CaCl₂) was added, and incubation was continued at 37°C for 30 s. Reactions were stopped by adding 33 μ l of stop solution (3 M ammonium acetate, 15 μ g of sonicated calf thymus DNA per ml, 0.25 M EDTA). Samples were precipitated, resuspended in loading buffer, and loaded onto a 5% polyacrylamide-urea sequencing gel alongside the A+G and C+T sequencing reactions (16) of the same DNA fragment.

RESULTS

MetR-binding site analysis. A computer search of the *S. typhimurium metF* sequence using the MetR-binding consensus sequence 5'-TGAANNT/ANNTTCA-3' (27, 28) identified three possible MetR-binding sites (Fig. 1). Two are centered 80 and 60 bp upstream from the +1 transcription initiation site, and the third is centered 77 bp downstream of the +1 transcription initiation site.

To determine whether the MetR protein binds to the *metF* control region, we used a gel mobility shift assay. A 467-bp *Bst*EII-*Hinf*I DNA fragment carrying the *metF* control region was isolated from plasmid pGS174, 32 P labeled at the *Hinf*I end, and used in the gel mobility shift assay (see Materials and Methods). The labeled DNA fragment was shifted to three positions of slower mobility as the MetR concentration was increased from 0.25 to 1 μ g/ml (Fig. 2). The same concentrations of MetR did not shift a control fragment that did not contain a MetR-binding site (data not shown). These results suggest that all three MetR-binding sites identified by the computer search might bind the MetR protein.

A DNase I protection assay was used to show where the MetR protein specifically binds in the *metF* control region (see Materials and Methods). At a low concentration (0.25

TABLE 1. *E. coli* strains used in this investigation

Strain ^a	Genotype
GS243.....	Δ metE::Mu pheA905 thi Δ lacU169 araD129 rspL
GS244.....	Δ metR::Mu pheA905 thi Δ lacU169 araD129 rspL
GS597.....	metJ97 pheA905 thi Δ lacU169 araD129 rspL
GS748.....	Δ metR::Mu metJ97 pheA905 thi Δ lacU169 araD129 rspL
BW313.....	HfrKL16 PO/45 lysA(61-62) dut-1 ung-1 thi-1 relA1
JM107.....	endA1 gyrA96 thi hisR17 supE44 relA1 λ^- Δ (lac-proAB) (F' traD36 proAB lacI ^q lacZ Δ M15)

^a Strains GS243, GS244, GS597, and GS748 were constructed in this laboratory. Strains BW313 and JM107 were obtained from M. Feiss.

FIG. 2. Mobility shift assay of the 467-bp *BstEII-HinI* fragment containing the *metF* control region. Lane 1, no MetR protein; lanes 2 through 4, 0.25, 0.5, and 1 µg of MetR protein per ml, respectively. The arrow indicates the position of the unbound DNA fragment.

µg/ml), the MetR protein protected a region from approximately position -68 to -95 upstream of the transcription initiation site (site 1 in Fig. 3). At a higher concentration (1 µg/ml), MetR protein also protected two additional regions, from approximately position -50 to -67 upstream of +1 (site 2 in Fig. 3) and from about +62 to +85 downstream of

FIG. 3. DNase I protection assay of the 746-bp *BamHI-EcoRI* fragment containing the *metF* control region. The DNA fragment was ^{32}P labeled at the *BamHI* site of the template strand. The A+G (lanes 1) and C+T (lanes 2) DNA-sequencing reactions were done according to the protocol of Maxam and Gilbert (16). Lanes 3, no MetR protein; lanes 4 through 7, 0.25, 0.5, 1, and 2 µg of MetR protein per ml, respectively. The two panels show different running times of the samples to optimize the positions of the DNase I-protected regions in the gel. The bars labeled 1, 2, and 3 indicate the three regions protected by MetR from DNase I digestion and are shown in Fig. 1.

TABLE 2. β -Galactosidase levels in *E. coli* strains carrying a wild-type *metF-lacZ* fusion

Lysogen	Relevant genotype	β -Galactosidase activity ^a with:	
		D-Methionine	L-Methionine
243λFlac	<i>metE</i>	1,129	178
244λFlac	<i>metR</i>	565	66
597λFlac	<i>metJ</i>	2,366	2,322
748λFlac	<i>metJ metR</i>	2,137	2,177

^a Units of activity are nanomoles of *o*-nitrophenol produced per minute per optical density unit at 600 nm and 28°C. The growth medium was GM plus phenylalanine, vitamin B₁, and either D-methionine or L-methionine.

+1 (site 3 in Fig. 3). It should be noted that the DNase I control lane (lane 3) in Fig. 3 is overloaded. This is especially apparent for the shorter run to optimize site 3 in the autoradiogram. However, since lanes 4 and 5 (0.25 and 0.5 µg/ml, respectively) look identical for both sites 2 and 3, the results show that MetR protects these sites only at higher concentrations of MetR (1 and 2 µg/ml).

Regulation of the *metF-lacZ* fusion. A *metF-lacZ* gene fusion constructed previously (25) and carried on phage λgt2 (designated λFlac) was used to reexamine the effect of MetR on the regulation of the *metF* gene. This phage was used to lysogenize appropriate strains, the lysogens were grown in GM supplemented with either D-methionine (derepressing condition) or L-methionine (repressing condition), and β -galactosidase levels were measured. D-Methionine is converted to L-methionine by the cell and serves as a growth rate-limiting source of methionine (8). In the *metR* mutant strain GS244, expression of the *metF-lacZ* fusion with D-methionine was reduced twofold compared with that in the *metE* control strain GS243 (Table 2). With L-methionine, however, *metF-lacZ* expression in the *metR* mutant was reduced 2.7-fold compared with that in the *metE* control strain. These results suggest that the role of MetR becomes more important as MetJ-mediated repression is increased. This hypothesis would predict that in a *metJ* mutant, in which all MetJ-mediated repression is abolished, MetR would have no function in *metF-lacZ* expression. To test this hypothesis, strains GS597 (*metJ*) and GS748 (*metJ metR*) were lysogenized with λFlac, the lysogens were grown in GM supplemented with D-methionine or L-methionine, and β -galactosidase levels were measured. In lysogen 748λFlac, the level of *metF-lacZ* expression was essentially the same as in lysogen 597λFlac under both growth conditions (Table 2).

Effects of MetR-binding site mutations on *metF-lacZ* expression. Mutations that changed each of the three MetR-binding sites individually away from the MetR consensus binding sequence were introduced into the *metF* control region (see Materials and Methods and Fig. 1). Two base pairs were changed in site 1, since it has a higher affinity for MetR than sites 2 and 3 (Fig. 3). The mutated *metF-lacZ* fusions were subcloned into phage λgt2, and the phage was used to lysogenize strains GS748 (*metJ metR*), GS243 (*metE*), and GS244 (*metR*). The lysogens were grown in GM supplemented with D-methionine or L-methionine, and β -galactosidase levels were determined. In strain GS243 (*metE*), the mutant λFlac lysogens had about twofold-lower β -galactosidase levels than the wild-type λFlac lysogen when grown in the presence of D-methionine (derepressing condition) and about threefold-lower β -galactosidase levels than the wild-type lysogen when grown in the presence of L-methionine

TABLE 3. β -Galactosidase levels in *E. coli* strains carrying mutant *metF-lacZ* fusions

Lysogen	Relevant genotype	β -Galactosidase activity ^a with:	
		D-Methionine	L-Methionine
243 λ Flac	<i>metE</i>	1,129	178
243 λ Flac-85A-74G	<i>metE</i>	460	48
243 λ Flac-55T	<i>metE</i>	471	69
243 λ Flac+83G	<i>metE</i>	571	56
244 λ Flac	<i>metR</i>	565	66
244 λ Flac-85A-74G	<i>metR</i>	583	44
244 λ Flac-55T	<i>metR</i>	470	60
244 λ Flac+83G	<i>metR</i>	724	82
748 λ Flac	<i>metJ metR</i>	2,137	2,177
748 λ Flac-85A-74G	<i>metJ metR</i>	1,957	2,113
748 λ Flac-55T	<i>metJ metR</i>	2,130	2,198
748 λ Flac+83G	<i>metJ metR</i>	3,026	2,318

^a Units of activity are nanomoles of *o*-nitrophenol produced per minute per optical density unit at 600 nm and 28°C. The growth medium was GM plus phenylalanine, vitamin B₁, and either D-methionine or L-methionine.

(repressing condition) (Table 3). In strain GS244 (*metR*), the mutant and wild-type λ Flac lysogens had essentially the same β -galactosidase levels when grown under the derepressing condition. The addition of L-methionine further repressed *metF-lacZ* expression, and the β -galactosidase levels were essentially the same in the wild-type and mutant λ Flac lysogens. In strain GS748 (*metJ metR*), the mutant and wild-type λ Flac phage had essentially the same β -galactosidase levels under both growth conditions (Table 3). These results suggest that the mutations are specifically affecting a MetR-dependent mechanism and that all three MetR-binding sites identified by DNase I footprinting are necessary for normal *metF-lacZ* expression.

DISCUSSION

A computer search of the *S. typhimurium metF* sequence identified three possible MetR-binding sites (Fig. 1). In vitro binding assays and DNase I protection experiments verified that MetR binds to *metF* promoter DNA and protects two separate regions from DNase I attack (Fig. 1): (i) a ca. 46-bp region from position -50 to -95 upstream from the transcription initiation site and encompassing two of the predicted MetR-binding sites (sites 1 and 2) in the *metF* promoter region and (ii) a ca. 24-bp region from approximately position +62 to +85 downstream from the transcriptional initiation site and encompassing the third predicted MetR-binding site located in the *metF* structural gene (site 3). It should be noted that sites 2 and 3 required higher concentrations of the MetR protein than did site 1 for protection from DNase I digestion, suggesting that they have a lower affinity for MetR protein.

Results from previous work suggested that MetR has only a minor effect on *metF-lacZ* expression. β -Galactosidase levels from a *metF-lacZ* gene fusion were only two- to threefold lower in a *metR* mutant than in the wild-type strain (29). In addition, a *metR* mutation could be complemented by a multicopy plasmid carrying either a *metE* or *metH* gene; presumably the residual expression of either gene from multicopy plasmids in the *metR* mutant is sufficient for growth. Since the transformants did not require a multicopy plasmid carrying *metF*, the results suggest that *metF* expression is sufficient in the *metR* mutant to support growth.

Subsequent studies to determine whether the smaller effect on *metF* expression is due to a direct or indirect effect of the MetR protein were not done.

We reexamined the effect of MetR protein on *metF-lacZ* expression in light of the in vitro binding assays and the DNase I protection experiment. In the *metR* mutant strain GS244 grown under methionine-repressing conditions, *metF-lacZ* expression was reduced 2.7-fold compared with that in the *metR*⁺ control strain GS243 (Table 2). However, at intermediate levels of methionine repression (D-methionine), *metF-lacZ* expression in the *metR* mutant was reduced only twofold compared with that in the *metR*⁺ control strain. Under completely nonrepressing conditions (*metJ* mutants), there was essentially no difference between the *metR* strain GS748 and the *metR*⁺ control strain GS597. These results suggest that MetR is required for activation of *metF-lacZ* only under MetJ-mediated repressing conditions. Superrepression by MetJ in a *metR* mutant is also seen for *metE-lacZ* expression (29). However, in the absence of MetJ-mediated repression, *metE-lacZ* is still regulated over a 100-fold range by MetR (28). These results and those described above suggest that MetR could function by two different mechanisms for *metE-lacZ* and *metF-lacZ* expression: as an activator and as an antirepressor, respectively. The mechanism of MetJ-mediated repression of the *metF* gene is not known. If repression involves steric hindrance of RNA polymerase, then MetR alone or a MetR-RNA polymerase complex could act as an antirepressor to destabilize MetJ binding. As shown in Fig. 1, the MetR- and MetJ-binding sites do not overlap. However, MetR bound at MetR-binding sites 1 and 2 in the promoter region could interact directly with MetR bound at site 3 in the structural gene, possibly forming a DNA loop (10, 11, 13) that could antagonize repressor function. Mutations introduced by site-directed mutagenesis into each of the MetR-binding sites away from the consensus MetR-binding sequence completely disrupted MetR-mediated regulation (Fig. 1 and Table 3).

Alternatively, MetR could function as an activator for *metF*. The repressor may interfere with RNA polymerase binding, open complex formation, or promoter clearance at the *metF* promoter; MetR could partially compensate by affecting any of these parameters. In this model, then, differences in the architectures of the nonrepressed promoters may account for the observation that the *metE* promoter is intrinsically weak and thus always requires activation for full expression (28), whereas *metF* requires activation only under repressing conditions. This model also presumes that the step blocked by MetJ is not rate limiting in the absence of the repressor, since *metF-lacZ* expression in the *metJ metR* double mutant is only slightly reduced compared with that in the *metJ* mutant (Table 2).

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