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The Folate Branch of the Methionine Biosynthesis Pathway in *Streptomyces lividans*: Disruption of the 5,10-Methylenetetrahydrofolate Reductase Gene Leads to Methionine Auxotrophy

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In enterobacteria, the methyl group of methionine is donated by 5-methyltetrahydrofolate that is synthesized from $N^{5,10}$-methylenetetrahydrofolate by the 5,10-methylenetetrahydrofolate reductase. The *Streptomyces lividans metF* gene, which encodes 5,10-methylenetetrahydrofolate reductase, has been cloned. It encodes a protein of 307 amino acids with a deduced molecular mass of 33,271 Da. S1 exonuclease mapping of the transcription initiation site showed that the *metF* gene is expressed, forming a leaderless mRNA. A 13-bp tandem repeat located immediately upstream of the promoter region shows homology with the consensus MetR-binding sequence of *Salmonella typhimurium*. Expression of *metF* in multicopy plasmids in *S. lividans* resulted in accumulation of a 32-kDa protein, as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Disruption of the *metF* gene led to methionine auxotrophy. Integration of the disrupting plasmid at the *metF* locus was confirmed by Southern hybridization in three randomly isolated transformants. The methionine auxotrophy was complemented by transformation of the auxotrophs with an undisrupted *metF* gene. These results indicate that the folate branch is essential for methionine biosynthesis in streptomycetes, as occurs in enterobacteria.

Methionine, an important amino acid in bacterial metabolism, acts as the initiator of protein synthesis and in protein elongation. In addition, some methionine derivatives (e.g., *S*-adenosylmethionine) serve as methyl donors for a variety of methylation steps in cells (C-1 metabolism). Very little is known about the genes for methionine biosynthesis in *Streptomyces* species despite the interest in this amino acid as a precursor of many metabolites containing methyl groups produced by actinomycetes (5, 22) and gram-negative bacteria (11). In *Escherichia coli* and *Salmonella typhimurium*, methionine biosynthesis is encoded by 12 scattered genes which form the *met* regulon (25, 27). This regulon consists of 10 biosynthetic genes (*metA*, *metB*, *metC*, *metE*, *metF*, *metH*, *metK*, *metL*, *metQ*, and *metX*), 2 regulatory genes (*metJ* and *metR*), and the methionyl-tRNA synthetase gene (*metG*).

In enterobacteria, the last step in methionine biosynthesis is the methylation of homocysteine, which is catalyzed by either of two transmethylase enzymes, the *metE* and *metH* gene products (27) (Fig. 1). The methyl group transferred by these enzymes to homocysteine is donated by 5-methyltetrahydrofolate. This compound is synthesized from $N^{5,10}$-methylenetetrahydrofolate by the 5,10-methylenetetrahydrofolate reductase (the *metF* gene product).

In *E. coli* and *S. typhimurium*, the methyl group of N^5-methyltetrahydrofolate derives necessarily from $N^{5,10}$-methylenetetrahydrofolate, an intermediate of the so-called folate branch of the methionine pathway (27). It is unclear, however, whether the same pathway occurs in *Streptomyces* species or other gram-positive bacteria. In this paper, we report the cloning and characterization of the *Streptomyces lividans metF* homolog and its involvement in methionine biosynthesis.

Total DNA of *S. lividans* 1326 was used for genomic library construction; the same strain was used as the host in gene disruption experiments. *E. coli* DH5α (13) was used for plasmid isolation and subcloning of DNA fragments, and *E. coli* WK6 was used for the isolation of single-strand DNA. All plasmid constructions used (Table 1) derive from pIJ2921 (17), pBluescript KS$^+$ (Stratagene), pIJ699 (20), pGM7 (24), and pULVK99 (4).

Streptomyces strains were grown on solid MEY (16) or R2YE (32) medium or in YEME with 34% sucrose for dispersed growth in liquid cultures (16) and were supplemented with thiostrepton (25 μg/ml for solid media and 5 μg/ml for liquid media) when required. For overexpression of the *metF* gene, *S. lividans*(pMETF150) was cultured in minimal NMMP medium without Casamino Acids and with 0.5% glucose as a carbon source (16) and supplemented with thiostrepton (5 μg/ml). *E. coli* strains were grown in Luria broth or Luria agar medium supplemented with ampicillin (100 μg/ml) when required.

S. lividans total DNA, total RNA, and plasmids were isolated as described by Hopwood et al. (16). *E. coli* plasmids were isolated as reported by Kieser (19) or by the boiling method of Holmes and Quigley (15). All restriction endonuclease digestions, ligations, and DNA manipulations were performed by standard protocols (28) under conditions recommended by the manufacturers (Boehringer, Mannheim, Germany; Promega, Madison, Wis.; and Fermentas AB, Vilnius, Lithuania).

Cloning and sequencing of the *S. lividans metF* gene. *S. lividans* total DNA was digested with *Pst*I, and the resulting fragments were analyzed by Southern hybridization with a de-

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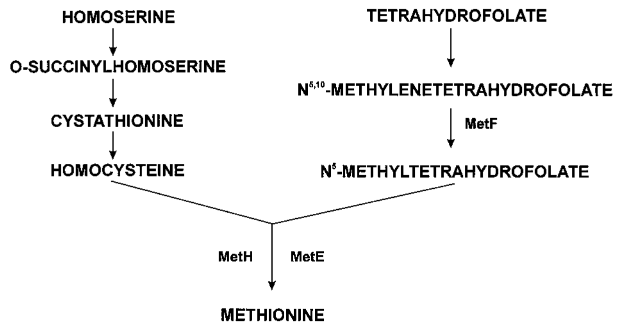

FIG. 1. Biosynthetic pathway of methionine showing the formation of the homocysteine moiety from homoserine (left branch) and the origin of the methyl group from the folate branch (right branch). *metF* encodes the 5,10-methylenetetrahydrofolate reductase (MetF); MetE and MetH are alternative methyltransferases.

generate 36-mer oligonucleotide, 5′-AAGCCHAAGTTCGTHTCHGTHACHTACGGHGCHAAC-3′ (where H is G or C), as a probe designed according to a conserved amino acid motif present in the homologous proteins from *E. coli* and other enterobacteria (see Fig. 3). A 10-kb *Pst*I DNA band that hybridized with the ^{32}P-labelled probe was detected. *Pst*I DNA fragments with similar sizes (9.5 to 10.5 kb) were isolated from an agarose gel run under the same conditions. These fragments were ligated to dephosphorylated *Pst*I-digested plasmid pIJ2921, and the ligation mixture was used to transform *E. coli* DH5α. The transformants were analyzed by colony hybridization with the same ^{32}P-labelled 36-mer oligonucleotide. A strongly hybridizing clone that harbored a recombinant pIJ2921-derived plasmid with a 10-kb *Pst*I insert of DNA from *S. lividans* was isolated. By restriction analysis and Southern hybridization experiments, the oligonucleotide hybridizing sequence was located to a 1.7-kb *Pvu*II-*Pst*I fragment (Fig. 2A).

TABLE 1. Plasmids used in this study

Plasmid	Relevant features	Source or reference
pIJ2921	2.7-kb *E. coli* plasmid; *lacZ* complementation; *bla*	17
pBluescript	2.9-kb *E. coli* phagemid; *lacZ* complementation; *bla* (sequencing vector)	Stratagene
pIJ699	9.6-kb bifunctional *E. coli-Streptomyces* positive selection vector; *tsr aph vph*	20
pGM7	4.95-kb *Streptomyces* temperature-sensitive replication plasmid; *tsr* (gene disruption vector)	24
pULVK99	7.8-kb bifunctional *E. coli-Streptomyces* positive selection vector; *tsr kan*	4
pMETF1	pIJ2921 carrying a 10-kb *Pst*I DNA fragment of *S. lividans* containing *metF* (original clone)	This work
pMETF50	pIJ2921 carrying a 1.4-kb *Kpn*I DNA fragment containing *metF*	This work
pMETF100	pIJ2921 carrying a 255-bp *Sal*I DNA fragment internal to *metF*	This work
pMETF150	pIJ699 carrying a 1.4-kb *Bgl*II DNA fragment derived from pMETF50 and containing *metF*	This work
pMETF200	pGM7 carrying a 0.3-kb *Bgl*II DNA fragment derived from pMETF100 and containing the 255-bp *Sal*I DNA fragment internal to *metF*	This work
pVKK-*metF*	pULVK99 carrying a 1.4-kb *Bgl*II DNA fragment derived from pMETF50 and containing *metF*	This work

The nucleotide sequence of this DNA fragment revealed the presence of three open reading frames (ORF), two of which (ORF1 and ORF3) were incomplete. Compared with proteins in the SWISS-PROT database, the product of ORF1 displayed high homology with the *Bacillus subtilis thiC* gene product, which is involved in thiamine biosynthesis (36), and the product of ORF3 showed homology with a hypothetical 10.2-kDa *B. subtilis* membrane protein. The protein encoded by ORF2 showed strong homology with MetF proteins of *E. coli* (34.5% identical amino acids), *S. typhimurium* (33.8% identity), and *Haemophilus influenzae* (34.6% identity) (Fig. 3). The *metF* gene product (5,10-methylenetetrahydrofolate reductase) is involved in the folate branch of the methionine biosynthesis pathway. The gene encoded by ORF2 was tentatively designated *metF*.

Close analysis of the known bacterial 5,10-methylenetetrahydrofolate reductases revealed seven conserved motifs (Fig. 3) that may be involved in the catalytic activity of these enzymes.

Promoter region of the *metF* gene. The *S. lividans metF* gene has a G+C content of 69% and codes for a putative 307-amino-acid protein. Sequence analysis of the *metF* upstream region revealed the presence of a putative promoter, showing −10 and −35 boxes (Fig. 2A) similar to the consensus sequences reported for *Streptomyces* promoters (31). To confirm the presence of the promoter and to identify the transcription initiation site, high-resolution S1 mapping was carried out with a 590-bp *Kpn*I-*Sal*I DNA fragment labelled with ^{32}P at the 5′ end of *Sal*I (Fig. 2B), as described by Fernández-Abalos et al. (9). A 283- or 284-bp protected DNA fragment was observed, which gives a transcription start site for the *metF* gene located at an adenine or thymine coinciding with the first nucleotide of the first in-frame ATG codon of ORF2; this result is in good agreement with the expected site based on the putative −10 region and indicates that this gene has a leaderless promoter (Fig. 2C) (18). No obvious ribosome binding site sequence was detected upstream from the translation initiation codon. This is also the case for other *Streptomyces* genes in which the transcription start point is at or near the translation initiation point (1).

The intergenic region, between the *thiC* and *metF* genes, contains a 13-bp tandem repeat, TGGACAACAACTC, located immediately upstream from the −35 box of the *metF* promoter that shows homology with the MetR-binding consensus sequence of *S. typhimurium* (5′-TGAANN[T/A]NNTTCA-3′ (33, 34). MetR belongs to the LysR family of bacterial activator proteins (14) and takes part in the positive regulation of *metE*, *metF*, and *metH* in *S. typhimurium* (6, 35). This sequence may be the equivalent MetR-binding motif in gram-positive bacteria to that reported in *Salmonella* species (see "Regulation of *metF* expression," below).

Expression of *metF* in *S. lividans* results in accumulation of a 32-kDa protein. The *S. lividans metF* gene codes for a protein with a deduced molecular mass of 33,271 Da. To confirm that *metF* was expressed in *S. lividans*, a 1.4-kb *Kpn*I fragment containing the *metF* gene and its promoter was subcloned in pIJ699 (a multicopy *Streptomyces* plasmid), creating plasmid pMETF150. *S. lividans*(pMETF150) transformants were cultured in liquid minimal medium containing ammonium sulfate as the only nitrogen source, since the *metF* gene is known to be

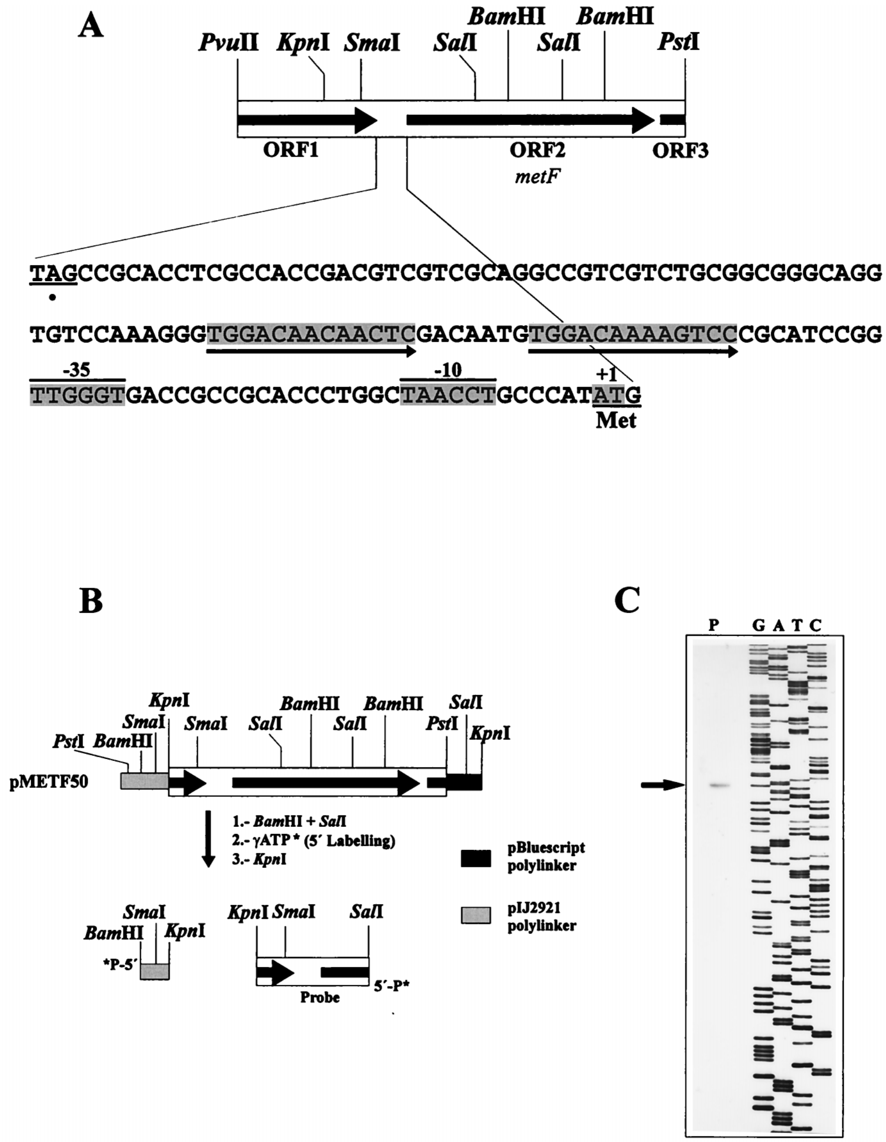

FIG. 2. (A) Physical map of the 1.7-kb *Pvu*II-*Pst*I DNA region of *S. lividans* containing the *metF* gene (ORF2). The first in-frame ATG codon of ORF2 is underlined and labelled "Met." The transcription start point is shaded and labelled +1, and the −10 and −35 boxes of the promoter region are indicated. The 13-bp direct repeat (putative MetR-binding site) is underlined with arrows. (B) Strategy for high-resolution S1 nuclease protection studies of the transcription start point. (C) Protected band (arrow) on S1 mapping experiments.

regulated negatively in *E. coli* and *S. typhimurium* by the presence of methionine and vitamin B_{12} in the medium (21, 23). As shown in Fig. 4, the *S. lividans*(pMETF150) crude extract contains a protein band revealed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of increased intensity, with a molecular mass of 32 kDa, which agrees with the mass estimated for the deduced *metF* gene product. The intensity of the protein band is not very high despite the increased copy number (50 to 300 copies per cell, since pMETF150 contains the pIJ101 replicon) (16), suggesting that expression of the *metF* promoter is strictly regulated.

Disruption of *metF* leads to methionine auxotrophy. In order to study the role of *metF* gene product in methionine biosynthesis, we inactivated *metF* by gene disruption. To achieve this, a 255-bp *Sal*I fragment, internal to the *metF* gene (previously converted to *Bgl*II ends), was subcloned in vector pGM7, a *Streptomyces* plasmid with a temperature-sensitive replicon (24). The resulting integrative plasmid (pMETF200) was transformed into *S. lividans* protoplasts, and the transformants were incubated at 39°C (a nonpermissive temperature) to eliminate autonomously replicating plasmids. After 3 days of incubation, 10^6 cells were plated on MEY medium supplemented with thiostrepton. About 0.01% of the original *S. lividans*(pMETF200) cells were able to grow on this medium. These colonies were replicated to thiostrepton minimal medium. About 10% of them did not grow on this medium, suggesting that they were putative methionine auxotrophs in which homologous recombination had disrupted the coding region of the chromosomal *metF* gene. The rest of the colonies were able to grow and represented either spontaneous thiostrepton-resistant mutants or incompletely cured colonies.

Homologous integration was verified by Southern hybridization experiments using the 255-bp *Sal*I fragment internal to *metF* as a probe. Total DNA from three randomly isolated

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                                       a                                                  b
Sl  MALGTASTRTDRARTVRDILATGKTTYSFEFSAPKTPKGEKNLWSALRRVEAVAPDFVSVTYGAGGSTRAGTVRETQQIVADTTLTPVAHLTAVDHSVAE 100
Ec  MSFFHASQRDALNQSLAEV--QGQINVSFEFFPPRTSEMEQTLWNSIDRLSSLKPKFVSVTYGANSGERDRTHSIIKGIKDRTGLEAAPHLTCIDATPDE  98
St  MSFFHANQREALNQSLAEV--QGQINVSFEFFPPRTSEMEQTLWNSIDRLSSLKPKFVSVTYGANSGERDRTHSVIKGIKERTGLEAAPHLTCIDPTRDE  98
Hi  MSY--AKEIDTLNQHIADF--NKKINVSFEFFPPKNEKMETLLWDSIHRLKVLKPKFVSVTYGANSGERDRTHGIVKAIKQETGLEAAPHLTGIDATPEE  96
    *.   *          . . .    .   **** .*.    *  ** .. *.  . * ******** .. *  *    . *   * * . .*** .*  .  *

               c                                        d                             e
Sl  LRNIIGQYADAGIRNMLAVRGD-PPGDPNADWIAHPEGLTYAAELVRLIKESGDFCVGVAAFPEMHPRSADWDTDVTNFVDKCRAGADYAITQMFFQPDS 199
Ec  LRTIARDYWNNGIRHIVALRGDLPPGSGKPE--------MYASDLVTLLKEVADFDISVAAYPEVHPEAKSAQADLLNLKRKVDAGANRAITQFFFDVES 190
St  LRTIARDYWNNGIRHIVALRGDLPPGSGKPE--------MYAADLVGLLKEVADFDISVAAYPEVHPEAKSAQADLLNLKRKVDAGANRAITQFFFDVES 190
Hi  LKQIARDYWDSGIRRIVALRGDEPKGYAKKP--------FYASDLVELLRSVADFDISVAAYPEVHPEAKSAQADLINLKRKIDAGANHVITQFFFDIEN 188
    *. *  .* . *** ..*.*** * *  .                  **..** *..  .** ..***.**.** .   ..*. *.  *  ***.  *** **. ..

      f                                                                           g
Sl  YLRLRDRVAAAGCATPVIPEVMPVTSVKMLERLPKLSNASFPAELKERILTAKDDPAAVRSIGIEFATEFCARLLAEGVPGLHFITLNNSTATLEIYENL 299
Ec  YLRFRDRCVSAGIDVEIIPGILPVSNFKQAKKFADMTNVRIPAWMAQMFDGLDDDAETRKLVGANIAMDMVKILSREGVKDFHFYTLNRAEMSYAICHTL 290
St  YLRFRDRCVSAGIDVEIIPGILPVSNFKQAKKFADMTNVRIPSWMSLMFEGLDNDAETRKLVGANIAMDMVKILSREGVKDFHFYTLNRAEMSYAICHTL 290
Hi  YLRFRDRCASIGIDTEIVPGILPVTNFKQLQKMASFTNVKIPAWLVKAYDGLDNDPTTRNLVAASVAMDMVKILSREGVNDFHFYTLNRSELTYAICHML 288
    ***.***  . *     ..* ..**.. *   .  .  .* .*. .          .*.  ..   *.    * *** ..** ***  .   .  * . *

Sl  GLHHPPRA 307   S.lividans
Ec  GVR--PGL 296   E.coli
St  GVR--PGL 296   S.typhimurium
Hi  GVR--P-- 292   H.influenzae
    *..  *
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FIG. 3. Alignment of the amino acid sequences of the methylenetetrahydrofolate reductases of *S. lividans* (AJ001630), *E. coli* (P00394), *S. typhimurium* (P11003), and *H. influenzae* (P45208) by using the CLUSTAL program. Conserved amino acids are in white-on-black type. Motifs a to g are sequences conserved in all tetrahydrofolate reductases. The amino acid sequence used for constructing the degenerate probe is underlined.

putative methionine auxotrophs (MD1 to MD3) was digested with *Bgl*II and *Pst*I. Results of the Southern hybridization experiment are shown in Fig. 5A. The three putative auxotrophs gave the same DNA hybridization pattern, which was clearly different from the control. The integration mechanism is shown in Fig. 5B. Further confirmation of the homologous integration was achieved by plasmid rescue. Total DNA from one of the mutant strains (MD2) was isolated and digested with *Xba*I and *Eco*RI (two enzymes which do not cut the *metF* gene). The DNA fragments obtained were religated and transformed into *S. lividans*. Restriction analysis of the plasmids derived from the thiostrepton-resistant colonies revealed the presence of the pGM7 plasmid plus part of the *metF* gene and a DNA fragment located downstream from *metF* in the chromosome.

Spores from one of the mutants, *S. lividans* MD1 *metF*, were plated on minimal medium with or without methionine. The mutant was able to grow only in the medium supplemented with methionine (Fig. 6). Introduction of the pVKK-*metF* plasmid, which contains the undisrupted *metF* gene, restored the ability of the MD1 mutant to grow on minimal medium without methionine.

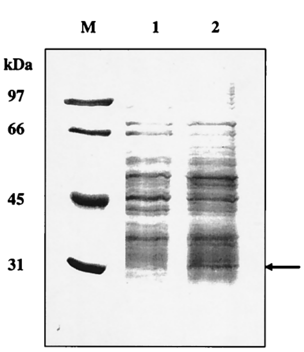


FIG. 4. SDS-PAGE (12% polyacrylamide) of crude extracts of *S. lividans* (pIJ699) (lane 1) and *S. lividans*(pMETF150) (lane 2). Cultures were grown in NMMP medium (16) supplemented with thiostrepton (5 μg/ml). Lane M, SDS-PAGE molecular weight standards (low range; Bio-Rad). Sizes are indicated on the left. The overexpressed protein is indicated with an arrow.

Role of MetF in the methionine pathway. The *E. coli metF* gene product is involved in the folate branch of the methionine biosynthetic pathway (27). It catalyzes the reduction of $N^{5,10}$-methylenetetrahydrofolate to N^5-methyltetrahydrofolate, which in turn gives its methyl group to homocysteine in order to form methionine, in a reaction catalyzed by either a vitamin B_{12}-dependent methyltransferase (the *metH* gene product) or a vitamin B_{12}-independent methyltransferase (the *metE* gene product).

Genes homologous to *metF* have been found in other gram-negative bacteria, such as *S. typhimurium* (30) and *H. influenzae* (10). However, this is the first time that a *metF* homolog has been found in a gram-positive bacterium. Its involvement in methionine biosynthesis has been proved by gene disruption that resulted in methionine auxotrophy. Growth of the disrupted mutant was restored by transformation with an undisrupted *metF*-containing plasmid (pVKK-*metF*). These results indicate that the folate branch is essential to provide the methyl group of methionine in actinomycetes. Synthesis of the folic acid moiety by the formyl tetrahydrofolate synthetase has been reported recently in *Streptococcus mutans*, another gram-positive bacterium (7).

Regulation of *metF* expression. Expression of the *E. coli* and *S. typhimurium metF* genes is negatively controlled by two mechanisms (2, 26, 27). One of these mechanisms uses the *metJ* gene product as a repressor and *S*-adenosylmethionine as a corepressor (8, 29). The other uses the *metH* gene product as a repressor and vitamin B_{12} as a corepressor (12, 23).

In addition, a positive regulatory mechanism in *S. typhimurium* in which the *metR* gene product modulates expression of *metE*, *metF*, and *metH* has been described (6, 35). In *S. typhimurium*, three putative MetR-binding sites are required for MetR-mediated regulation of *metF* (6). The consensus MetR-binding sequence has been identified as 5′-TGAANN (T/A)NNTTCA-3′ (33, 34). The *S. lividans metF* gene has a

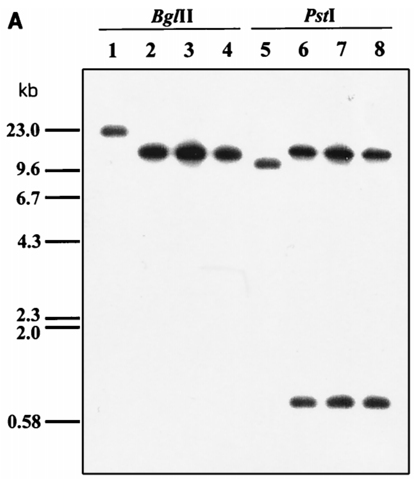
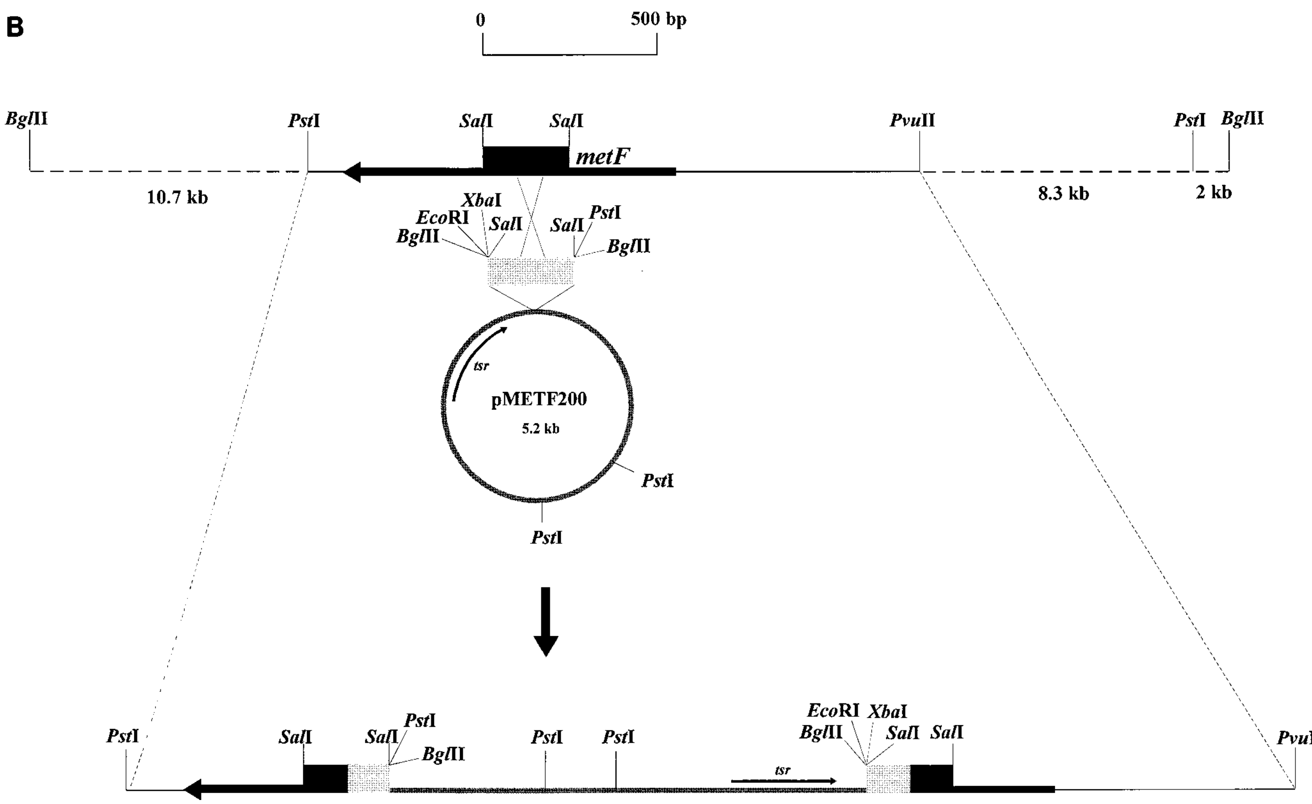


FIG. 5. (A) Disruption of the *S. lividans metF* gene, as shown by hybridization with a 255-bp *Sal*I probe internal to *metF*. Lanes: 1 and 5, undisrupted *S. lividans* 1326 (control); 2 and 6, *S. lividans* MD1 (*met*); 3 and 7, *S. lividans* MD2 (*met*); 4 and 8, *S. lividans* MD3 (*met*). Total DNA of each strain was digested with *Bgl*II (lanes 1 to 4) or *Pst*I (lanes 5 to 8). (B) Disruption of *metF* by integration of pMETF200; *tsr*, thiostrepton resistance gene (used as a marker).



13-bp tandem repeat upstream from its coding region that shows homology with this consensus sequence, suggesting that a similar positive regulation of *metF* occurs in *S. lividans.*

metF belongs to the group of *Streptomyces* genes containing a leaderless mRNA (1, 18). These promoters lack a standard Shine-Dalgarno sequence. Shine-Dalgarno sequences (typically located 5 to 13 nucleotides upstream of translational start codons) are complementary to the anti-Shine–Dalgarno sequences found near the 3′ termini of 16S rRNAs (3). Translation of leaderless mRNAs in the absence of conventional Shine-Dalgarno sequences starts by interaction of the 30S ribosome subunit with the AUG codon as it emerges from the RNA polymerase, thus coupling transcription and translation (18). The efficiency of translation of *metF* and other leaderless promoters is a subject of great interest.

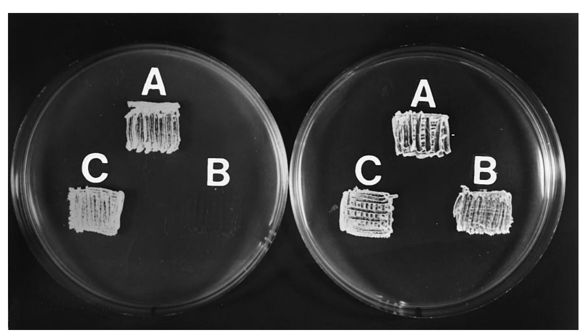


FIG. 6. Growth in *Streptomyces* minimal medium of the parental strain *S. lividans* 1326 (A), the disrupted *S. lividans* MD1 (B), and a transformant of *S. lividans* MD1 with plasmid pVKK-*metF* (C). The righthand plate was supplemented with L-methionine (50 μg/ml).

Nucleotide sequence accession number. The nucleotide sequence of the *S. lividans metF* gene has been deposited in GenBank under accession no. AJ001630.

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