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Direct Sulfhydrylation for Methionine Biosynthesis in *Leptospira meyeri*

J. BELFAIZA,[1] A. MARTEL,[2] D. MARGARITA,[3] AND I. SAINT GIRONS[3]*

Faculté des Sciences d'El-Jadida, Université Chouaib Doukkali, El-Jadida, Morocco,[1] and Laboratoire de Bioorganique et Biotechnologies, Ecole Nationale Supérieure de Chimie de Paris, 75005 Paris,[2] and Unité de Bactériologie Moléculaire et Médicale, Institut Pasteur, 75724 Paris Cedex 15,[3] France

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A gene library of the *Leptospira meyeri* serovar semaranga strain Veldrat S.173 DNA has been constructed in a mobilizable cosmid with inserts of up to 40 kb. It was demonstrated that a *Leptospira* DNA fragment carrying *metY* complemented *Escherichia coli* strains carrying mutations in *metB*. The latter gene encodes cystathionine γ-synthase, an enzyme which catalyzes the second step of the methionine biosynthetic pathway. The *metY* gene is 1,304 bp long and encodes a 443-amino-acid protein with a molecular mass of 45 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The deduced amino acid sequence of the *Leptospira metY* product has a high degree of similarity to those of *O*-acetylhomoserine sulfhydrylases from *Aspergillus nidulans* and *Saccharomyces cerevisiae*. A lower degree of sequence similarity was also found with bacterial cystathionine γ-synthase. The *L. meyeri metY* gene was overexpressed under the control of the T7 promoter. MetY exhibits an *O*-acetylhomoserine sulfhydrylase activity. Genetic, enzymatic, and physiological studies reveal that the transsulfuration pathway via cystathionine does not exist in *L. meyeri*, in contrast to the situation found for fungi and some bacteria. Our results indicate, therefore, that the *L. meyeri* MetY enzyme is able to perform direct sulfhydrylation for methionine biosynthesis by using *O*-acetylhomoserine as a substrate.

The biosynthetic pathways of sulfur amino acids are well documented. Two alternative methionine biosynthetic pathways exist in microorganisms (Fig. 1). One, called the transsulfuration pathway, involves cystathionine formation, and the other bypasses cystathionine via direct sulfhydrylation of *O*-acylhomoserine to homocysteine (29).

In enteric bacteria, the sulfur atom is incorporated first into a serine ester (*O*-acetylserine) to yield cysteine (16). Sulfur is then transferred from cysteine to homocysteine via transsulfuration. In *Escherichia coli*, it requires the sequential action of cystathionine γ-synthase (EC 4.2.99.9), the product of the *metB* gene (7), and cystathionine β-lyase (EC 4.4.1.8), the *metC* gene product (1), with the intermediary formation of cystathionine (Fig. 1A, steps 2 and 3).

The direct sulfhydrylation pathway has been reported to be the main pathway for homocysteine biosynthesis in *Saccharomyces cerevisiae* (5) and bacteria such as *Brevibacterium flavum* and *Pseudomonas aeruginosa* (10, 23). In *S. cerevisiae*, which is the best-studied example, the direct synthesis of homocysteine is catalyzed by an *O*-acetylhomoserine sulfhydrylase, the Met25 (or Met17) product (Fig. 1B, step 4) (5, 34). The resulting homocysteine is used as a direct precursor for methionine and is converted to cysteine via the reverse transsulfuration pathway (Fig. 1B, steps 5 and 6).

In addition, it should be kept in mind that the ester of homoserine used for homocysteine biosynthesis differs depending on the organisms: enteric bacteria use *O*-succinylhomoserine, while fungi and most gram-positive bacteria use *O*-acetylhomoserine (Fig. 1, steps 1 and 1′) (for a review, see reference 33).

* Corresponding author. Mailing address: Unité de Bactériologie Moléculaire et Médicale, 25, rue du docteur Roux, Institut Pasteur, 75724 Paris Cedex 15, France. Phone: 33 (0)1 45 68 83 66. Fax: 33 (0)1 40 61 30 01. E-mail: isgirons@pasteur.fr.

Little is presently known about the regulation of the metabolite flux of the methionine pathway. However, it has been reported that the control at the enzymatic level in bacteria and *S. cerevisiae* occurred at an early step of the methionine biosynthetic pathway. The first enzyme of the methionine biosynthetic pathway in *E. coli*, *O*-succinylhomoserine transferase, is feedback inhibited by methionine and *S*-adenosylmethionine (20), while the activity of *O*-acetylhomoserine transferase from *S. cerevisiae* is inhibited only by *S*-adenosylmethionine (6). In previous work, we demonstrated that *O*-acetylhomoserine transferase activity in *Leptospira meyeri* is not regulated by methionine and/or *S*-adenosylmethionine (2).

Our goal was to investigate the evolution of sulfur metabolism in *L. meyeri*. We report here the construction of a representative cosmid *L. meyeri* DNA library and the cloning of a biosynthetic gene, *metY*, which complements *E. coli metB* mutants. Analysis of the inferred *L. meyeri* MetY amino acid sequence, growth impairment of *E. coli* mutants carrying *metY*, and results of enzymatic assays allow us to propose a direct sulfhydrylation pathway catalyzed by an *O*-acetylhomoserine sulfhydrylase.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *L. meyeri* serovar semaranga strain Veldrat S.173 (National Reference Center, Paris, France), isolated from a rat, was grown in EMJH medium at 30°C (8, 14).

E. coli strains (Table 1) were grown in LB broth or on L agar plates at 37°C except when indicated (27). The antibiotics used and their concentrations were as follows: kanamycin, 25 μg/ml; tetracycline, 8 μg/ml; chloramphenicol, 30 μg/ml; and ampicillin, 100 μg/ml. Minimal medium M9 supplemented with 0.04% glucose and 1 μg of thiamine per ml, plus appropriate amino acids (1 mM), was used to characterize *E. coli* transformants at 30°C (27).

$pill_{200}$ (Km^r Mob^+ Tra^-) (6.9 kb long), derived from pILL575 by deletion of a 3.2-kb *Hin*dIII-*Pst*I fragment (18), was used as a cosmid vector to clone *L. meyeri* DNA. pUC18 (Amp^r), pBR322 (Tet^r Amp^r), and pSU18 (Cm^r) (22, 30, 35) were used for subcloning experiments.

Construction of an *L. meyeri* genomic DNA library in a cosmid. Total DNA from *L. meyeri* was prepared as described previously (11) from 500 ml of culture

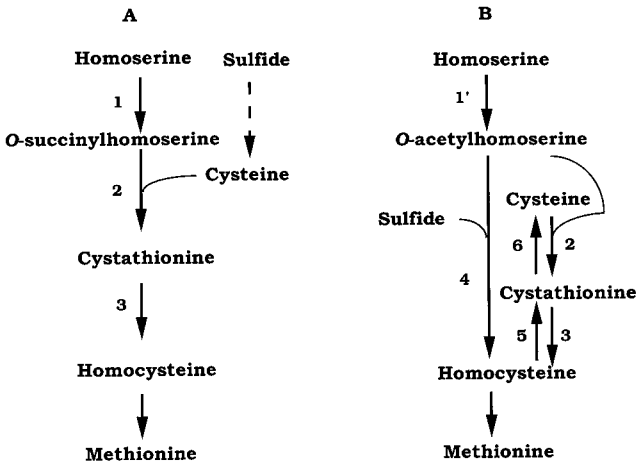

FIG. 1. Biosynthetic pathways of sulfur amino acids in *E. coli* (A) and *S. cerevisiae* (B). Enzyme steps: 1, *O*-succinylhomoserine transferase (*metA*); 1′, *O*-acetylhomoserine transferase; 2, cystathionine γ-synthase (*metB*); 3, cystathionine β-lyase (*metC*); 4, *O*-acetylhomoserine sulfhydrylase (Met17 [or Met25]); 5, cystathionine β-synthase; 6, cystathionine γ-lyase. Genes shown in parentheses in this legend are the corresponding *E. coli* genes; for step 4 the *S. cerevisiae* gene is indicated.

at 5×10^8 bacteria/ml. Genomic DNA partially cleaved with restriction endonuclease *Sau*3A and sized on a 10 to 40% sucrose density gradient was ligated into the *Bam*HI-digested and alkaline-phosphatase-treated cosmid vector $pill_{200}$ (1 μg). The ligated mix was packaged into phage lambda particles as described by the supplier (Gigapack III Gold 11 kit; Stratagene) and used to infect *E. coli* HB101 harboring helper plasmid pRK212.1 (9).

Complementation of *E. coli* methionine auxotrophs. Two different strategies were used for complementation of *E. coli* methionine auxotrophs: (i) direct infection of *E. coli* methionine auxotrophs with lambda phage particles from the cosmidic library at 30°C and (ii) mobilization of the recombinant cosmids as follows. Individual clones of the *L. meyeri* gene library (HB101 harboring the IncP helper plasmid plus the hybrid plasmid to be mobilized) were grown in LB broth at 30°C with kanamycin. Aliquots (2 μl) of each clone were transferred to L agar plates spotted with the recipient cells (2 μl of *E. coli* spontaneous Rif^r AB1932 and WA802 methionine auxotrophs).

In each case, the plates were incubated at 30°C overnight and cells were replica plated on minimal medium containing tetracycline, kanamycin, rifampin, and the appropriate amino acids.

DNA analysis, DNA sequencing, and computer analysis. Restriction endonucleases purchased from Boehringer Mannheim were used as described in the manufacturer's instructions. Calf intestinal alkaline phosphatase, T4 DNA ligase (high concentration), and the Wizard DNA cleanup system were purchased from Promega, Madison, Wis. *Taq*I DNA polymerase was from Cetus. Ligations, agarose gel electrophoresis, and electroporation were performed by standard procedures (27).

Double-stranded plasmid DNA was sequenced by using the Pharmacia T7 sequencing kit, [α-^{33}P]dATP (111 TBq/mmol; ICN), and synthetic oligonucleotides. Comparisons to protein databases were done by using the BLAST e-mail server.

Overexpression of *metY*. A plasmid allowing expression of the *metY* structural gene under the control of the T7 promoter was constructed by oligonucleotide mutagenesis. The final construct (pETmetY), verified by nucleotide sequencing, contained the whole *metY* gene (starting at the ATG and continuing to the *Bam*HI site located 44 bp beyond the *metY* stop codon) inserted into $pET20b^+$ (Novagen, Madison, Wis.). The pETmetY plasmid was transformed into *E. coli* BL21 (DE3) (Novagen), which carries the T7 RNA polymerase gene on λDE3 integrated on the chromosome of BL21. The conditions of overexpression of *metY* under the control of the T7 promoter were as described previously for overexpression of *L. meyeri metX* (2).

Enzymatic assays. *O*-Acetylhomoserine sulfhydrylase and *O*-acetylserine sulfhydrylase activities were assayed as described by Ravanel et al. (25). The amount of homocysteine or cysteine formed in a 0.1-ml reaction mixture was determined by the nitroprusside reaction (32) or by the procedure described by Kredich and Becker (17). Since MetY does not contain cysteine, dithiothreitol was omitted from the reaction mixture. The reaction was started by addition of 2 mM sodium sulfide (Na_2S). The reaction mixtures overlaid with 50 μl of paraffin oil were incubated at 30°C for 10 and 30 min. When aliphatic thiols were determined with the nitroprusside test, the incubation was stopped by 3 min of heating at 100°C instead of acid precipitation (which can cause formation of thiolactone from homocysteine at a lower pH). The assays were found to be reproducible.

Cystathionine γ-synthase was assayed in the same reaction mixture as that described above by using a 1 mM final concentration of L-cysteine. Disappearance of L-cysteine was measured by using either DTNB (13) or the ninhydrin reaction (25). Proteins were estimated by the method of Bradford (4).

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

E. coli strain	Genotype	Origin or reference
HB101	*hsdR hsdM recA supE44 lacZ4 leuB6 proA2 thi-1* Sm^r	3
WA802	*metB1 lac-3* (or *lacY1*) *galK2 galT22 supE44 hsdR* Rif^r	31
AB1932	*metA28 argH1 thi-1 lacY1 lacZ4 galZ2 xyl4* (or *-5*) *tsx6 F* Rif^r	E. A. Adelberg
EC972	*metB185 araD139* Δ(*argF-lac*)*205 trpB202 flB350 ptsF25 relA1 rpsL150 deoG* λ−	B. Bachmann
β254	*metC*::Tet^r	26
GT1107	*metC*::Tet^r *metB1 lac-3* (or *lacY1*) *galK2 galT22 supE44 hsdR* Rif^r	Transduction of WA802 by a P1 lysate grown on β254
NK3	*cysK cysM*	N. M. Kredich
β180	*metA*::Cm^r	26
GT1140	*metA*::Cm^r *metB1 lac-3* (or *lacY1*) *galK2 galT22 supE44 hsdR* Rif^r	Transduction of WA802 by a P1 lysate grown on β180

RESULTS

Cloning of an *L. meyeri* DNA fragment able to complement *E. coli metB* and *metA* mutants. A cosmid library with 25- to 40-kbp inserts of *L. meyeri* DNA was obtained. Km^r clones were screened for complementation of the *metB1 E. coli* mutant (WA802) at 30°C. Seven recombinant cosmids were found in two separate experiments using either mobilization (1,152 clones) or direct infection of WA802 with transducing phage particles (5,760 clones). Several restriction fragments were common to the seven cosmids. Cosmid pb10 containing a 25-kbp insert was kept for further study and was shown to also complement *E. coli* EC972 carrying the *metB185* allele. No major rearrangement had occurred during the cloning experiment since *Bam*HI restriction fragments from cosmid pb10 were the same size as fragments of the genomic DNA of *L. meyeri* as determined by Southern blotting (data not shown).

The pb10 cosmid carrying *L. meyeri metY* (the *metB* complementing activity) was shown to also carry the *metX* gene able to complement *E. coli metA* mutants (2). Further subcloning allowed us to locate *metY* more precisely within the 25-kbp insert of pb10 (Fig. 2A). Plasmid pb12 carrying a 6.8-kbp *Pst*I fragment in pBR322 still complemented *metB* and *metA* mutants. Plasmids pb13s8, pb13c8, and pb13c9, generated by cloning an *Xba*I-*Pst*I insert (2.9 kb) from pb12 into pSU21, pUC18, and pUC19, respectively, complemented only the *metB* mutant. Such expression of *metY* in both orientations (pUC18 and pUC19) is evidence for transcription of the *metY* gene from its own promoter. When a *Bam*HI-*Xba*I fragment was cloned into pUC18 to yield pcn (Fig. 2A), no complementation was found, indicating that a *Bam*HI site is located within the *L. meyeri metY* gene.

High similarity of MetY to *O*-acetylhomoserine sulfhydrylases. The determination of the sequence of a 2.8-kb *Pst*I-*Xba*I insert of pb13s8 (Fig. 2A) demonstrated an open reading frame of 1,304 nucleotides encoding a 443-amino-acid protein. Two putative start codons, separated by 21 nucleotides, were found, and the first one was chosen conservatively. The amino

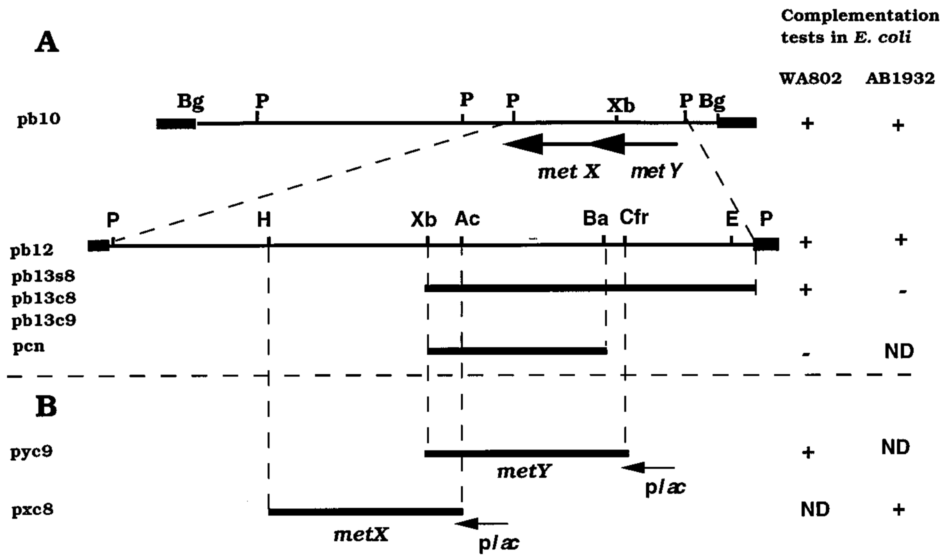

FIG. 2. (A) Subcloning of *L. meyeri* DNA able to complement an *E. coli metB* mutant (WA802) and/or an *E. coli metA* mutant (AB1932). Large arrows indicate the orientations of the *metX* and *metY* genes. Names of the recombinant plasmids (or cosmid for pb10) are indicated on the left. Restriction site abbreviations: Ac, *Acc*I; Ba, *Bam*HI; Bg, *Bgl*II; Cfr, *Cfr*101; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Xb, *Xba*I. c8, c9, and s8 indicate the vectors pUC18, pUC19, and pSU21, respectively. ND, not done. (B) Cloning of the *L. meyeri metX* and *metY* genes under the control of the p*lac* promoter (small arrows).

acid sequence deduced from *metY* and analyzed by the BLAST program showed the following sequence identities: 55% with *Aspergillus nidulans O*-acetylhomoserine sulfhydrylase (28), 50% with yeast *O*-acetylhomoserine sulfhydrylase (Met17 or Met25), and 40.5% with *P. aeruginosa O*-succinylhomoserine sulfhydrylase (MetZ) (10, 19). Amino acid sequence alignments of these proteins indicated a high overall similarity (Fig. 3). *E. coli* cystathionine γ-synthase (MetB) (7) was less similar to MetY (30% identity). In this respect, the deduced MetY amino acid sequence showed a stronger similarity to the sequences of fungal transsulfuration enzymes than to those of the corresponding bacterial enzymes, MetB and MetZ, which, as already mentioned (10, 12), had a gap of about 40 amino acids in their middle portion, unlike the sequences of the yeast enzymes (see Fig. 3 for MetZ).

All transsulfuration enzymes and enzymes catalyzing the incorporation of reduced sulfur in carbon chains utilize pyridoxal phosphate as a cofactor. From the sequence alignment depicted in Fig. 3, Lys-216 from *L. meyeri O*-acetylhomoserine sulfhydrylase appears to be strictly conserved in all other enzymes. It suggests that this lysine is the pyridoxal phosphate binding residue, in agreement with the location of the pyri-

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MetYLm    MVGPSGESMPRNFKPETIALHGGQEP--DPTTTSRAVPLYQTTSYVFKDTDHAARLFGLQEFGNIYTRLMNPTTDVL 75
OAHSAn          MSDPSPKRFETLQLHAGQEP--DPATNSRAVPIYATTSYTFNDSAHGARLFGLKEFGNIYSRIMNPTVDVF 69
MT17Sc               PSHFDTVQLHAGQENPGDNAHRSRAVPIYATTSYVFENSKHGSQLFGLEVPGYVYSRFQNPTSNVL 66
MetZPa   MTQDWDAGRLDSDLEGAAFDTLAVRAGQRRTPEGEHGEALFTTSSYVFRTAADAAARFAGEVPGNVYSRYTNPTVRTF 78

MetYLm   EKRVAALEGGVAALATASGQSAEMLALLNIVEAGQEIVASSSLYGGTYNLLHYTFPKLGIKVHFVDQSDPENFRKASN 153
OAHSAn   EKRIAALEGGVAAVAASSGQAAQFMAISALAHAGDNIVSTSNLYGGTYNQFKVLFPRLGITTKFVQGDKAEDIAAAID 147
MT17Sc   EERIAALEGGAAALAVSSGQAAQTLAIQGLAHTGDNIVSTSYLYGGTYNQFKISFKRFGIEARFVEGDNPEEFEKVFD 144
MetZPa   EERIAALEGAEQAVATASGMSAILALVMSLCSSGDHVLVSRSVFGSTISLFDKYFKRFGIQVDYPPLSDLAAWEAACK 156

                                                                  #
MetYLm   DKTRAFYAETLGNPKLDTLDIAAVSKVAKEVGVPLVIDNTM-PSPYLVNPLKHGADIVVHSLTKFLGGHGTSIGGIII 230
OAHSAn   DRTKAVYVETIGNPRYNVPDFEAIAKVAHEKGIPLVVDNTFGAGGYFVRPIEHGADIVVHSATKWIGGHGTTIGGVVV 225
MT17Sc   ERTKAVYLETIGNPKYNVPDFEKIVAIAHKHGIPVVVDNTFGAGGYFCQPIKYGADIVTHSATKWIGGHGTTIGGIIV 222
MetZPa   PNTKLFFVESPSNPLAELVDIAALAEIAHAKGALLAVDNCFCTPA-LQQPLKLGADVVIHSATKYIDGQGRGMGGVVA 233

MetYLm   DGGSFNWGN--GKFKNFTEPDPSYHGLKFWEVFGKFEPFGGVNIAFILKARVQGLRDLGPAISPFNAWQILQGVETLP 306
OAHSAn   DSGKFEWGKNAARFPQFTQPSEGYHGLNFWETFG--------PIAFAIRVRVEILRDLGSALNPFAAQQLILGLETLS 295
MT17Sc   DSGKFPWKDYPEKFPQFSQPAEGYHGTIYNEAYG--------NLAYIVHVRTELLRDLGPLMNPFASFLLLQGVETLS 292
MetZPa   GRGE------------------------------------------QMKEVVGFLRTAGPTLSPFNAWLFLKGLETLR 269

MetYLm   LRMERHSGNALKVAEFLQKHPKIEWVNYPGLSTDKNYATAKKYHERGLFGAIVGFQIKGGVEKAK----------KFI 374
OAHSAn   LRAERHASNALALANWLKKNDHVSWVSYVGLEEHPSHGVAKKYLKRG-FGGVLSFGVKGEAAV----------GSQVV 362
MT17Sc   LRAERHGENALKLAKWLEQSPYVSWVSYPGLASHSHHENAKKYLSNG-FGGVLSFGVKDLPNADKETDPFKLSGAQVV 369
MetZPa   IRMQAHSASALALAEWLERQPGIERVYYAGLPSHPQHELARR--QQSGFGAVVSFDVKGGRDAAW----------RFI 335

MetYLm   DGLELFSLLANIGDAKSLAIHPASTTHQQLTGPEQISAGVTPGFVRLSVGLENIDDILVDLEEALKNIX         443
OAHSAn   DNFKLISNLANVGDSKTLAIHPWSTTHEQLTDQERIDSGVTEDAIRISVGTEHIDDIIADFEQSFAATFKVVRGA   437
MT17Sc   DNLKLASNLANVGDAKTLVIAPYFTTHKQLNDKEKLASGVTKDLIRVSVGIEFIDDIIADFQQSFETVFAGQKP    443
MetZPa   DATRMVSITTNLGDTKTTIAHPATTSHGRLSPEDRARAGIGDSLIRVAVGLEDLDDLKADMARGLAAL          403
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FIG. 3. Alignment of *L. meyeri* serovar semaranga *metY* (MetYLm) deduced amino acid sequence with the *O*-acetylhomoserine sulfhydrylases of *A. nidulans* (OAHSAn; accession no. U19394) and *S. cerevisiae* (MT17Sc; accession no. P06106) and the *O*-succinyl homoserine sulfhydrylase of *P. aeruginosa* (MetZPa; accession no. U10904). Identical residues are represented by bold characters, and similar residues are indicated by dots. A hyphen indicates a gap. The alignment was performed by use of Clustal V. The proposed lysine (K) of the pyridoxal phosphate binding site is indicated by # above the sequences.

TABLE 2. Growth of *E. coli* mutants bearing *Leptospira metX* and *metY*

Plasmid[a]	*Leptospira* gene(s)	Relevant *E. coli* chromosomal mutation(s)	OD_{600}[b]
pb12	*metX metY*	*metB1*	0.25
pxc8	*metX*	*metA*	ND[c]
pyc9	*metY*	*metB1*	0.2
pb13s8	*metY*	*metB1*	0.3
pxc8 and pb13s8	*metX metY*	*metB1*	0.18
pb13c9	*metY*	*metB1 metC*	0.83
pb13c9	*metY*	*metB1*	0.73
pb12	*metX metY*	*metA metB1*	1

[a] c8, c9, and s8 indicate the vectors pUC18, pUC19, and pSU18, respectively.
[b] Growth in minimal medium without methionine was evaluated after 24 h.
[c] ND, not done.

doxal phosphate binding site found experimentally for β-cystathionase and cystathionine γ-synthase (21).

Evidence for direct sulfhydrylation in *L. meyeri*. *E. coli metB* mutants carrying the pb12 plasmid (*metX metY*) grew very slowly (generation time, about 24 h) and stopped growing at an optical density at 600 nm (OD_{600}) of 0.2 (Table 2). This slow growth could be due to a weak expression of the *L. meyeri* methionine genes in *E. coli*. To improve expression, *L. meyeri metX* and *metY* genes were cloned under the control of the *lac* promoter. pxc8 (*metX*) was obtained by cloning the *Acc*I-*Hin*dIII fragment from pb12 into pUC18, and pyc9 (*metY*) was obtained by deletion of the *Pst*I-*Cfr*101 insert of pb13c9 (Fig. 2B). However, the growth of *metA* and *metB E. coli* strains (AB1932 and WA802) harboring pxc8 and pyc9, respectively, was not improved.

Based on the knowledge that *E. coli* cystathionine γ-synthase preferentially uses *O*-succinylhomoserine as a substrate (15) and that the product of the *L. meyeri metX* gene (*O*-acetyhomoserine transferase) uses only acetyl coenzyme A (2), two other hypotheses were tested. (i) Overexpression of the *L. meyeri metX* gene leading to high synthesis of *O*-acetylhomoserine might compensate for the deficit in *E. coli* of the substrate required for MetY activity. However, the growth of the *E. coli metB1* mutant was not improved after transformation by both plasmids pxc8 and pb13s8. (ii) The cause of impaired growth could be found at the level of homocysteine biosynthesis, which could be either direct or via cystathionine (Fig. 1). The comparison of growth rates of *E. coli* mutants carrying the *L. meyeri metY* gene in a *metB1 metC* double mutant (indication of direct pathway) or in a *metB1 metC*$^+$ mutant (indication of homocysteine synthesis via cystathionine) revealed that the growth rates were the same. This is in keeping with the fact that *E. coli* cystathionine β-lyase (Fig. 1A, step 3), the second enzyme of the transsulfuration pathway, is not needed within the recombinant *E. coli* carrying *metY*.

It is therefore suggested that MetY uses acetylhomoserine much better than it uses succinylhomoserine. Along these lines, much improved growth (an OD_{600} of 1 was reached with a generation time of 8 h) was obtained with *E. coli metA metB1* double mutants carrying the pb12 plasmid (*metX metY*) compared to that from the *E. coli metB1* single mutant carrying the pb12 plasmid (*metX metY*) (generation time, 24 h; growth stopped at an OD_{600} of 0.2) (Table 2). This suggested the proposed roles for reaction steps 1′ and 4 (Fig. 1B) for homocysteine biosynthesis in vivo.

MetY, an enzyme with *O*-acetylhomoserine sulfhydrylase activity. MetY was overproduced by cloning the *metY* gene under the control of a very strong and inducible T7 promoter. The molecular mass of the overexpressed MetY protein as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown) was 45 kDa, in agreement with that calculated from the sequence. Crude cellular extracts of *E. coli* BL21 (D23) transformed with pET20b$^+$ bearing *metY* exhibited, after induction, two very high activities, *O*-acetylhomoserine sulfhydrylase (15 μmol of homocysteine/min/mg of protein) and *O*-acetylserine sulfhydrylase (13 μmol of cysteine/min/mg of protein). A control was made with crude extracts of the same strain transformed with the pET20b$^+$ vector alone. No acetylhomoserine sulfhydrylase activity was found, whereas *O*-acetylserine sulfhydrylase activity was observed at a level similar to that found in the strain bearing *metY*. However, no cystathionine γ-synthase activity was found. The *O*-acetylhomoserine sulfhydrylase activity was specific for the acetyl substrate; succinylhomoserine was not used as a substrate.

A question arises as to the regulation of the enzymatic activity by the end products of the pathway. The results indicate that *L. meyeri* MetY exhibited an *O*-acetylhomoserine sulfhydrylase activity which is feedback inhibited (33 or 25% residual activity) at very high concentrations (10 mM) of methionine or *S*-adenosylmethionine.

DISCUSSION

The *L. meyeri metY* gene was cloned by functional complementation of an *E. coli metB* mutant. However, the results suggest that cystathionine is not an intermediary metabolite of methionine synthesis in *L. meyeri*. The data are consistent with the enzymatic activity exhibited in vitro by MetY. Indeed, MetY protein can utilize sulfide for reaction with *O*-acetylhomoserine to yield homocysteine (Fig. 1B, step 4). In contrast to *E. coli* MetB protein (Fig. 1A, step 2), MetY protein does not use cysteine as a substrate, indicating that MetY protein is devoid of cystathionine γ-synthase activity. It is clear that complementation of *E. coli metB* mutants by the *L. meyeri metY* gene, however poor, became possible by marginal use of *E. coli* substrates. In fact, two substrates used by MetY protein differ from those used in *E. coli*: the homoserine derivative is an *O*-acetyl derivative, and the source of sulfur is not cysteine. A previous study from our laboratory has also shown that the *L. meyeri metX* product, *O*-acetylhomoserine transferase, the enzyme catalyzing the step upstream of MetY (2), does not transfer the acetyl group of acetyl coenzyme A to serine, showing its exclusive specificity for homoserine.

In *S. cerevisiae*, the Met17 (or Met25) enzyme exhibits both *O*-acetylserine sulfhydrylase and *O*-acetylhomoserine sulfhydrylase activities in vitro (32). Unfortunately, it was not possible to determine if *L. meyeri* MetY had an *O*-acetylserine sulfhydrylase activity in vitro since the latter activity was also present in *E. coli* extracts (see Results). The synthesis of cysteine by MetY was thus experimentally tested in vivo by functional complementation of the double *E. coli cysK cysM* mutant (these two genes specify two isoenzymes with *O*-acetylserine sulfhydrylase activity) (16). However, no complementation was found (data not shown). This could suggest that *L. meyeri* MetY is indeed similar to *S. cerevisiae* Met17 (or Met25), which was found ultimately to behave in vivo only as an *O*-acetylhomoserine sulfhydrylase (5), and that MetY may also have evolved from the same common ancestor of the γ-family of the transsulfuration enzymes (24).

At this point, the key goal is to determine the physiological role of the *O*-acetylhomoserine sulfhydrylase activity of *L. meyeri* MetY and whether it represents a major or alternative pathway. The organization of the two genes *metX* and *metY* in

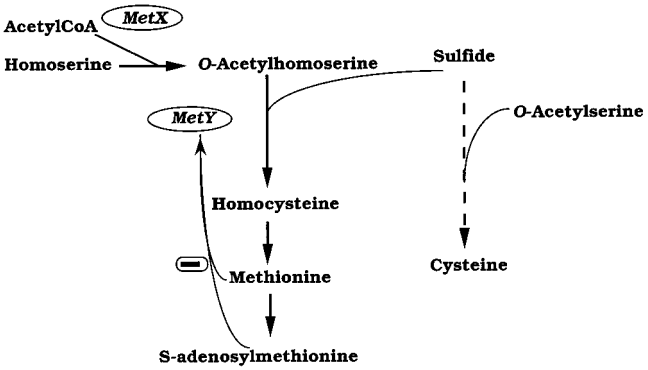

FIG. 4. Proposed pathway for methionine biosynthesis in *L. meyeri*. MetX, *O*-acetylhomoserine transferase; MetY, *O*-acetylhomoserine sulfhydrylase.

an operon (2) suggests the participation of both genes in the methionine pathway. Since the complementation of an *E. coli metB* mutant by the *L. meyeri metY* gene was effective, we rather expected to isolate an *L. meyeri* gene encoding cystathionine γ-synthase. However, the *L. meyeri* inserts from the seven recombinant cosmids able to complement both *metA* and *metB E. coli* mutants overlapped, indicating that they originated from the same region of the chromosome. We thus propose that the transsulfuration pathway via cystathionine does not exist in *L. meyeri*; this is in contrast to the situation found for fungi, which have both operating pathways for methionine biosynthesis (transsulfuration and sulfhydrylation). The proposed pathway for methionine biosynthesis for *L. meyeri* is shown in Fig. 4. With regard to metabolic regulation, we have reported that MetX, the first enzyme of the *L. meyeri* pathway, is not feedback inhibited (2). The concentration of methionine and *S*-adenosylmethionine giving 67 and 75% inhibition of *O*-acetylhomoserine sulfhydrylase (MetY), respectively, was 10 mM. The methionine or *S*-adenosylmethionine inhibition of *O*-acetylhomoserine sulfhydrylase seems not to be physiologically significant. Further studies are needed to examine regulation at the level of repression by methionine or its metabolites.

It was of interest to compare the methionine biosynthetic pathway in a pathogenic species of *Leptospira* to that in the saprophytic *L. meyeri* species. A 525-bp DNA fragment from *Leptospira interrogans* serovar icterohaemorrhagiae strain Verdun was amplified by PCR assay with appropriate oligonucleotides chosen within *metY* (data not shown). The deduced amino acid sequence of the amplified product (Fig. 3, amino acids 110 to 285) was 86% identical to the amino acid sequence of MetY. Interestingly, the large insertion of 40 amino acids characteristic of MetY protein (Fig. 3, amino acids 234 to 276) and of yeast enzymes was found in the corresponding amino acid sequence of this pathogenic species. These results could suggest that the direct sulfhydrylation pathway for methionine is also operating in a pathogenic species of *Leptospira*.

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