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Genetic evidence for the existence of two pathways for the biosynthesis of methionine in the *Leptospira* spp

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Abstract

There are two major pathways for methionine biosynthesis: the enterobacterial type transsulfuration pathway and the sulfhydrylation pathway as previously identified in the spirochete *Leptospira meyeri*. Sequence analysis of the *L. meyeri metYX* locus allows the identification of a third gene, called *metW*, which encodes a protein exhibiting similarities with homologs in many organisms belonging to the α-, β-, and γ-subdivisions of proteobacteria. The *metW*, *metX* and *metY* genes of *L. meyeri* were disrupted by a resistance cassette by homologous recombination. While the *L. meyeri metX* mutant shows methionine auxotrophy, the *metY* mutant (as well as the *metW* and *metYmetW* mutants) conserves methionine prototrophy, suggesting the presence of additional route(s) which may bypass the direct sulfhydrylation pathway. In addition, a *L. interrogans* gene, called *metZ*, was found to complement an *Escherichia coli metB* mutant, further suggesting that the transsulfuration pathway is also present in *Leptospira* spp.

Keywords: *Leptospira*; Methionine; Transsulfuration; Direct sulfhydrylation

1. Introduction

In this paper, we studied the methionine biosynthetic pathway in *Leptospira* spp., the only genus described so far in the phylum of spirochetes as containing genes involved in the biosynthesis of methionine. Methionine biosynthesis is a central pathway, as it controls a large number of cellular processes such as translation of mRNA into proteins and transmethylation reactions via the formation of *S*-adenosylmethionine. Since spirochetes differ considerably from well-studied Gram-negative and Gram-positive bacteria, some of the steps of the methionine pathway may be unique to these organisms and potentially useful as therapeutic target. Methionine biosynthesis is absent in mammals but is a general metabolic capability of prokaryotes, eukaryotic microorganisms and higher plants. There are two major pathways for methionine biosynthesis in microorganisms. The transsulfuration pathway found in enterobacteria allows the conversion of cysteine and *O*-succinylhomoserine to homocysteine via the intermediary formation of cystathionine (Fig. 1). In *Escherichia coli*, this pathway requires the sequential action of cystathionine γ-synthase (EC 4.2.99.9) and cystathionine β-lyase (EC 4.4.1.8) [1]. The sulfhydrylation pathway directly incorporates sulfide into *O*-acetylhomoserine to produce homocysteine (Fig. 1). This pathway is present in bacteria such as *Pseudomonas aeruginosa* [2] and *Leptospira meyeri* [3]. In *L. meyeri*, this step is catalyzed by an *O*-acetylhomoserine sulfhydrylase (EC 4.2.99.10), encoded by *metY*, which is organized in operon with *metX* [3] (Fig. 1). Although yeast, fungi and higher plants have both transsulfuration and direct sulfhydrylation pathways, only the bacteria *Corynebacterium glutamicum* [4], *Bacillus subtilis* [5], *P. aeruginosa*, and *Pseudomonas putida* [6,7] have been shown to have both pathways.

2. Materials and methods

2.1. Bacterial strains and growth conditions

L. meyeri serovar semaranga strain Veldrat and *L. interrogans* serogroup icterohemorrhagiae strain Lai (Leptospira National Reference Center, Institut Pasteur, Paris, France) were grown at 30°C in EMJH [8,9] liquid medium

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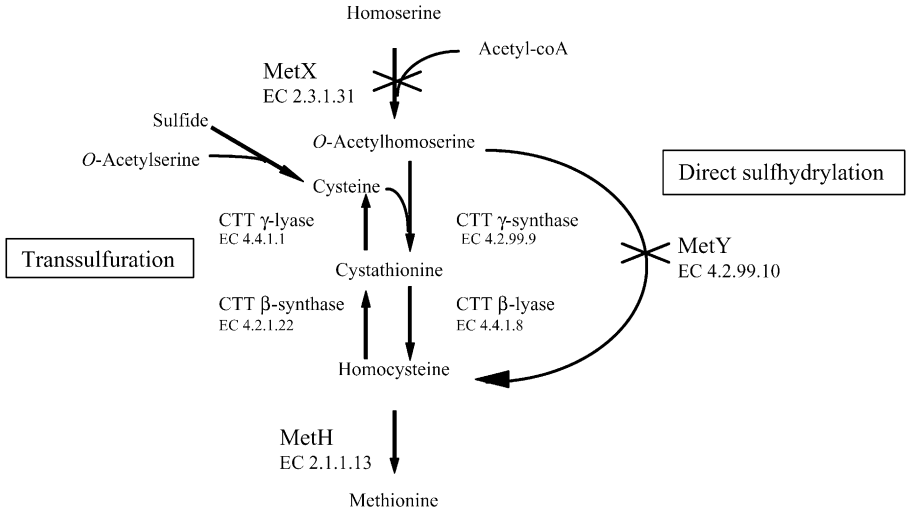

Fig. 1. Model for the methionine biosynthetic pathway in *Leptospira* spp. *Leptospira* spp. may utilize both transsulfuration and direct sulfhydrylation pathways. While inactivation of *L. meyeri metX* results in methionine auxotrophy, the inactivation of *metY* results in prototrophy. In enterobacteria, *O*-succinylhomoserine is used as the substrate instead of *O*-acetylhomoserine. CTT: Cystathionine, used as an intermediary substrate in transsulfuration.

or on 1% agar plates. *E. coli metB* (strain WA802) and *metC* (strain CAG18475) were grown at 37°C in Luria–Bertani medium, or at 30°C in M9 minimal medium. When necessary, L-methionine, was used at 1 mM. Kanamycin and spectinomycin were used at 50 μg ml^{-1}.

2.2. DNA and RNA manipulations

Plasmids from *E. coli* were recovered using a Qiaprep Spin miniprep kit (Qiagen). Genomic DNA of *L. meyeri* was extracted as previously described [10]. For Southern blot analysis, digested DNA was subjected to electrophoresis overnight, transferred onto a nylon membrane, and hybridized with (α-^{33}P) dATP labelled probes under stringent conditions as previously described [10]. Amplification was achieved using one cycle of denaturation (94°C, 5 min), followed by 35 cycles of amplification consisting of denaturation (94°C, 30 s), annealing (55°C, 30 s), and primer extension (72°C, 1 min 30 s), and a final cycle of extension of 10 min at 72°C. RT-PCR was performed on total RNA extracted from exponentially growing cells of *L. meyeri* strains as previously described [11].

2.3. Construction of L. meyeri insertional mutants

The *L. meyeri metX*, *metY*, and *metW* genes were disrupted by allelic exchange as previously described [10–12]. The *L. meyeri metY*, *metX*, and *metW* DNA fragments were amplified with primer pairs MTA–MTB, MXA–MXB, and MX1–MWC, respectively (Table 1), cloned into a suicide plasmid and alleles were interrupted by either a kanamycin (*metY* and *metX*) or a spectinomycin (*metW*) resistance cassette. Plasmids were then subjected to UV irradiation and used to deliver the inactivated allele into the *L. meyeri* chromosome by homologous recombination. Kanamycin- or spectinomycin resistant colonies were picked and tested for the insertion of the kanamycin resistance cassette in the target gene by PCR and Southern blot analysis.

2.4. Complementation studies of E. coli mutant strains

To complement *E. coli* methionine auxotrophs, the *L. interrogans metZ* and *metW* genes were expressed under the control of the *lac* promoter in pCR2.1-TOPO (Invitrogen). The complete coding sequences (from the putative start codon to the downstream region of the stop codon) of *metZ* and *metW* of *L. interrogans* were amplified by PCR with primer pairs MZ1-MZ2 and MW1-MW2, respectively (Table 1), and inserted into pCR2.1-TOPO by

Table 1
Primers used in this study

Primer	Sequence (5′–3′)
MK1	GATAACACAATGCCTTCTCC
MK2	CATGGTATGACGGATCTGGC
MTA	AACTAGACCCACCGGCATCG
MTB	AGAGTCAAAGACTCAAATCG
MY1	TTACCCAGGCCTTTCAACTG
MY2	AAGTCTACCAGAATGTCATC
MXA	AGGATTTGAAATCAAAGGTG
MXB	CATACCCACAACCAAGATCC
MX1	TCTTCTTCCGAACATTCTGC
MX2	TGGTAGATTAGATAACTTCC
MX3	AGATATGGTGAATGCTCAAG
MW1	ATGACTCCTCTTGAAAAAAA
MW2	TATCGTAAACGGAGAACCTC
MWA	AGTTTATATGTACATCATGG
MWB	TACCAGTGGAAAGGCATAAG
MWC	TGTGTCTCACGTAAACTTGG
MXT	TGAGCAACAAGACTCGATCC
MWD	CAATCCCTTGGACACGAACC
MZ1	ATGTTAAAAGAAATCAACGAACC
MZ2	ATAATGTCCATAAAACGACG

using the TOPO TA cloning kit (Invitrogen). Recombinant plasmids pCRmetZ and pCRmetW were then used to transform *E. coli metB* and *metC* mutants.

3. Results and discussion

3.1. Genetic characterization of the metYXW operon in L. meyeri

The *metX* (encoding an homoserine *O*-acetyltransferase, EC 2.3.1.31), and *metY* (encoding an *O*-acetylhomoserine sulfhydrylase, EC 4.2.99.10) genes of *L. meyeri* were previously isolated by complementation of an *E. coli metB* mutant [3]. MetX and MetY are involved in the first two steps of the methionine biosynthesis pathway (Fig. 1). In order to find other genes involved in methionine biosynthesis, we cloned and sequenced the upstream and downstream region of the *metYX* operon of *L. meyeri* by LM-PCR [13]. Comparison of sequences in databanks revealed that the downstream coding sequence of *L. meyeri metYX* exhibits more than 40% sequence identity (over 195 residues) with the MetW proteins of *P. aeruginosa*, *P. putida* and *P. syringae*. In *P. syringae* and *P. putida*, *metW* is organized in an operon with *metX* [7,14], which encodes a protein exhibiting 54% identity with *L. meyeri* MetX. We named this *L. meyeri* gene *metW*, based on its deduced amino acid sequence similarity (Fig. 2). When described by Andersen et al. [14], the *P. aeruginosa* MetW protein showed no significant homology to any known protein. We searched for putative *metW* homologs in the genomes of other prokaryotes (at the time of this writing 205 prokaryotic genomes have been sequenced, including archae). The genomes of 27 microorganisms (including *L. meyeri* and *L. interrogans*) contain a *metW* homolog (193–231 residues). They all are hypothetical

Table 2
Microbial genomes containing both putative *metX* and *metW* genes

Proteobacteria	Bacterial species	MetX (aa)	MetW (aa)	Intergenic sequence (bp)[a]
α-subdivision	**Caulobacter group**			
	Caulobacter crescentus CB15	382	221	+128
	Rhizobiaceae group			
	Mesorhizobium loti[b]	389	205	+59
	Rhodopseudomonas palustris[b]	400	231	+3
	Rhodospirillaceae			
	Magnetospirillum magnetotacticum[b]	394	208	−3
	Rhodospirillum rubrum[b]	391	211	−3
β subdivision	**Bordetella**			
	Bordetella parapertussis[b]	415	204	−3
	Bordetella pertussis[b]	415	204	−3
	Bordetella bronchiseptica[b]	415	204	−3
	Burkholderia/Oxalobacter/Ralstonia group			
	Burkholderia fungorum[b]	381	202	−3
	Burkholderia mallei[b]	381	198	−22
	Burkholderia pseudomallei[b]	381	198	−22
	Ralstonia metallidurans[b]	390	203	−3
	Ralstonia solanacearum	403	215	−3
	Neisseriaceae			
	Neisseria gonorrhoeae[b]	379	193	−3
	Neisseria meningitidis MC58	379	193	−3
	Neisseria meningitidis Z2491	379	193	−3
	Others			
	Nitrosomonas europaea[b]	377	205	+26
γ-subdivision	**Pseudomonadaceae**			
	P. aeruginosa PA01	379	206	+8
	Pseudomonas fluorescens[b]	379	206	+208
	P. putida KT2440[b]	379	206	+8
	Pseudomonas syringae pv. *syringae* B728a[b]	379	206	+8
	Others			
	Microbulbifer degradans 2–40[b]	381	198	+2
	Azotobacter vinelandii[b]	363	199	+2
	Acidithiobacillus ferrooxidans[b]	416	201	−3
Spirochaetales	*L. interrogans* serovar lai str. 56601	366	205	+4
	L. meyeri serovar semaranga str. Veldrat[c]	366	195	+29
Others	*Magnetococcus* sp. MC-1[b]	394	210	−3

[a]Length of intergenic sequence between putative *metX* and *metW* genes; overlapping open reading frames are indicated by a negative value.
[b]Unfinished genomic sequences.
[c]Partial sequences.

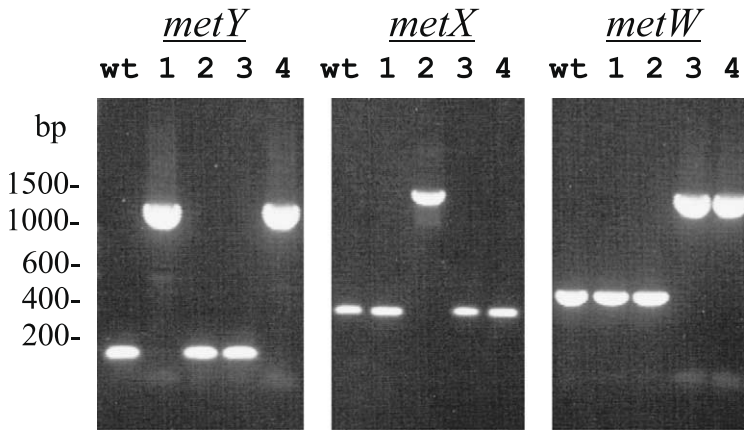

Fig. 2. Evidence for homologous recombination with the *metYXW* locus of *L. meyeri*. PCR with primer pairs MK1-MK2 (*metY*), MX3-MX2 (*metX*), and MXT-MWB (*metW*) (Table 1). Lanes: wt, *L. meyeri* wild-type strain; 1, *metY*::Km mutant; 2, *metX*::Km mutant; 3, *metW*::Spc mutant; 4, *metY*::Km *metW*::Spc mutant. Sizes of the fragments of the molecular mass markers are indicated on the left.

proteins of unknown function and they share more than 50% similarity at the amino acid level. In addition, *metW* homologs are found adjacent to a *metX* homolog (363–416 residues). Interestingly, the *metXW* locus is only found in proteobacteria (at the time of this writing, 93 genomes of proteobacteria have been sequenced/205 microbial genomic sequences): five genera within the α-proteobacteria, all the 13 sequenced genomes (five genera) within the β-proteobacteria, four genera (including four *Pseudomonas* species) within the γ-proteobacteria, the spirochetes of the *Leptospira* genus (but not found in *Borrelia* and *Treponema* genera), and in *Magnetococcus* sp. (Table 2). The putative genes encoding MetX and MetW proteins have the same direction of transcription and their coding sequences either overlap (in 15/27 *metX-metW* loci) or are separated by only 2 to 208 bp (Table 2), suggesting that they could form operons. Transcriptional analysis by RT-PCR revealed that the *L. meyeri metY*, *metX* and *metW* genes are part of an operon (data not shown).

3.2. *Construction and analysis of* metX, metY, metW, *and* metYmetW *mutants of* L. meyeri

Recently, the development of new genetic tools has facilitated the construction of targeted mutants in saprophytic *Leptospira* spp. [10–12]. In order to clarify the methionine biosynthetic pathway in *Leptospira* spp., the *metX*, *metY*, and *metW* genes were disrupted by allelic exchange as previously described [10–12]. Since the EMJH medium does not contain methionine, transformation experiments were done in the presence of 50 μg ml^{-1} L-methionine. This resulted in the identification of double cross-over events for the *metY*, *metX*, and *metW* genes of *L. meyeri* (Fig. 2). To obtain a *metYmetW* double mutant, the cloned *metY* was interrupted by a kanamycin resistance marker, and the mutated allele was then introduced into the chromosome of the *L. meyeri metW*::Spc mutant strain by homologous recombination. RT-PCR assays on the *L. meyeri met* mutants revealed that inactivation of each gene of the operon does not exert a polar effect on the other methionine biosynthetic genes (Table 3). The *metY*, *metX*, *metW*, and *metY metW* mutants were further analyzed. The *L. meyeri metX* mutant does not show any visible growth unless methionine was added to the EMJH solid medium (Table 3). Growth of the *metX* mutant was also restored by transformation with *L. meyeri metX* gene-containing replicative plasmid (data not shown). In contrast, despite the absence of methionine in the EMJH medium, the *L. meyeri metY* mutant grows as well as the wild-type strain (Table 3). Similarly, the *metW* and the *metYmetW* mutants are prototrophs for methionine (Table 3). However, it has to be noted that the EMJH medium used for the growth of leptospira is extremely complex [8,9]. It includes undefined components and it contains

Table 3
Genotypic and phenotypic analysis of *L. meyeri met* mutants

Strain	Methionine prototrophy	RT-PCR		
		metY	*metX*	*metW*
Wild-type	yes	+	+	+
metY::Km	yes	−	+	+
metX::Km	no	+	−	+
metW::Spc	yes	+	+	−
metY::Km *metW*::Spc	yes	−	+	−

Transcriptional analysis of the *metX*, *metY* and *metW* genes in *L. meyeri* was performed by RT-PCR. Primer pairs MY1–MY2 (*metY*), MX1–MX2 (*metX*), and MWA–MWB (*metW*) were used for RT-PCR assays. (Table 1). Strains were grown in EMJH medium lacking supplemental methionine.

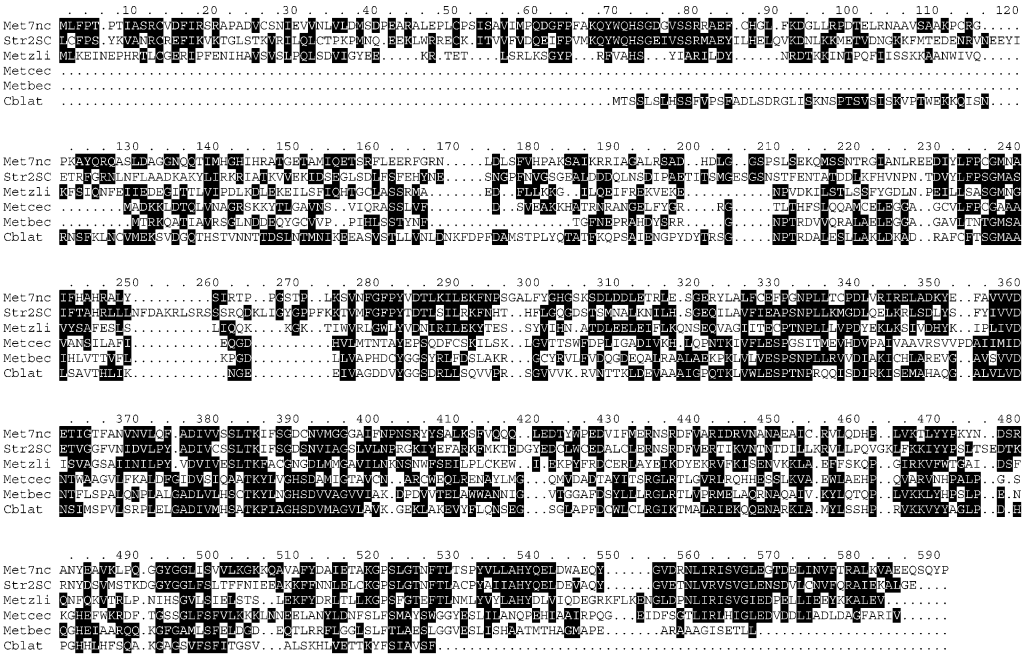

Fig. 3. Alignment of amino acid sequences of *L. interrogans* MetZ (Metzli) and the cystathionine γ-synthases of *E. coli* (Metbec), *N. crassa* (Met7nc), and *S. cerevisiae* (Str2sc), the cystathionine β-lyases of *A. thaliana* (Cblat), and *E. coli* (Metcec) by using the Clustal program. Amino acids similar in at least two sequences are shown on a black background.

high levels of sulfate, which may serve as a sulfur source for the synthesis of methionine (formation of cysteine from sulfate and conversion of cysteine to methionine by transsulfuration, Fig. 1). Development of a minimal medium is therefore necessary to test for growth of *met* mutants with a variety of different sulfur sources.

3.3. Evidence for two distinct pathways for methionine biosynthesis

The *metY* mutant is prototroph for methionine. This suggests that *L. meyeri* could utilize a second pathway distinct from direct sulfhydrylation (Fig. 1). *L. meyeri* and *L. interrogans* are phylogenetically related and, therefore, metabolic genes found in *L. interrogans* should be also present in *L. meyeri*. Since complete genomic information is now available for *L. interrogans* [15], we searched for similarities with proteins involved in the methionine biosynthesis pathway. Sequence analysis of the genome of *L. interrogans* suggests that the last step of the methionine pathway is catalyzed by a cobalamin (vitamin B12)-dependent homocysteine methyltransferase (EC 2.1.1.13) (Fig. 1). Further genome analysis reveals a 1500-bp coding sequence, referred as *metZ*, that encodes a putative protein with 27 to 31% identity (over the 500 residues) with cystathionine γ-synthases of the fungus *Neurospora crassa*, and the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* (Fig. 3). The C-terminal region (from residue 200 to 500) of this *L. interrogans* putative protein also shows similarities with *E. coli* MetB (cystathionine γ-synthase) and the cystathionine β-lyases of *E. coli* and *Arabidopsis thaliana* (Fig. 3). The ability to convert cysteine to homocysteine (direct transsulfuration) or vice versa (reverse transsulfuration) is due to the specificity of the cystathionine synthases present in the organisms (Fig. 1). These cystathionine synthases are evolutionary related, constituting a protein family [16]. Therefore, the function of the newly identified gene *metZ* cannot be assigned based on sequence similarity, making it impossible to determine whether *metZ* is involved in the direct transsulfuration (cystathionine γ-synthase or cystathionine β-lyase) or reverse transsulfuration (cystathionine γ-lyase) (Fig. 1). To test for the function of the *L. interrogans metW* and *metZ* genes, we studied their ability to complement the *E. coli metB* and *metC* mutants (lacking cystathionine γ-synthase and cystathionine β-lyase activities, respectively) in M9 minimal medium. No growth was observed on minimal liquid and solid media for the *E. coli* mutants carrying the *L. interrogans metW* gene. Growth on minimal medium was observed for the *E. coli metB* mutant carrying the *L. interrogans metZ* gene, but not for the *E. coli metC* mutant carrying *metZ*. The *metB* complemented strain exhibits a lag period longer than that of the *metB* mutant growing in cultures supplemented with methionine (data not shown). This suggests a different substrate specificity (*O*-succinylhomoserine/*O*-acetylhomoserine), as previously found for the *L. meyeri* MetY [3].

In conclusion, our mutational and sequence analysis shows that the methionine biosynthesis pathway of *Leptospira* is far more intertwined than suspected. Evidence of both transsulfuration (from cysteine to homocysteine) and sulfhydrylation pathways in *Leptospira* spp. was shown by

(i) methionine prototrophy of the *L. meyeri metY* mutant, characterization of the *L. interrogans metZ* gene, which (ii) encodes a protein exhibiting similarities with cystathionine synthases (closely related to cystathionine γ-synthases) and (iii) complements an *E. coli metB* mutant, and (iv) identification of *metW* homologs. The organization of the two genes *metX* and *metW* in an operon suggests the participation of both genes in the methionine pathway. The *metW* methionine auxotroph mutant of *P. syringae* exhibited growth on cystathionine, suggesting that this gene is involved in either of the two first steps of the transsulfuration pathway [14]. Since MetX (homoserine *O*-acetyltransferase, EC 2.3.1.31) has been shown to be the first enzyme in the methionine biosynthesis pathway in several organisms, MetW may be a cystathionine synthase, despite of its lack of similarity to any protein of known function.

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References

[1] Greene, R.C. (1996) Biosynthesis of Methionine. *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, 2nd edn., pp. 542–560.

[2] Foglino, M., Borne, F., Bally, M. and Patte, J.C. (1995) A direct sulhydrylation pathway is used for methionine biosynthesis in *Pseudomonas aeruginosa*. Microbiology 141, 431–439.

[3] Belfaiza, J., Martel, A., Margarita, D. and Saint Girons, I. (1998) Direct sulfhydrylation for methionine biosynthesis in *Leptospira meyeri*. J. Bacteriol. 180, 250–255.

[4] Hwang, B.J., Yeom, H.J., Kim, Y. and Lee, H.S. (2002) *Corynebacterium glutamicum* utilizes both transsulfuration and direct sulfhydrylation pathways for methionine biosynthesis. J. Bacteriol. 184, 1277–1286.

[5] Auger, S., Yuen, W.H., Danchin, A. and Martin-Vertraete, I. (2002) The *metIC* operon involved in methionine biosynthesis in *Bacillus subtilis* is controlled by transcription antitermination. Microbiology 148, 507–518.

[6] Vermeij, P. and Kertesz, M.A. (1999) Pathways of assimilative sulfur metabolism in *Pseudomonas putida*. J. Bacteriol. 181, 5833–5837.

[7] Alaminos, M. and Ramos, J.L. (2001) The methionine biosynthetic pathway from homoserine in *Pseudomonas putida* involves the *metW*, *metX*, *metZ*, *metH* and *metE* gene products. Arch. Microbiol. 176, 151–154.

[8] Ellinghausen, H.C. and McCullough, W.G. (1965) Nutrition of *Leptospira pomona* and growth of 13 other serotypes: fractionation of oleic albumin complex and a medium of bovine albumin and polysorbate 80. Am. J. Vet. Res. 26, 45–51.

[9] Johnson, R.C. and Harris, V.G. (1967) Differentiation of pathogenic and saprophytic leptospires. J. Bacteriol. 94, 27–31.

[10] Picardeau, M., Brenot, A. and Saint Girons, I. (2001) First evidence for gene replacement in *Leptospira* spp. Inactivation of *L. biflexa flaB* results in non-motile mutants deficient in endoflagella. Mol. Microbiol. 40, 189–199.

[11] Bauby, H., Saint Girons, I. and Picardeau, M. (2003) Construction and complementation of the first auxotrophic mutant in the spirochaete *Leptospira meyeri*. Microbiology 149, 689–693.

[12] Tchamedeu Kameni, A.P., Couture-Tosi, E., Saint-Girons, I. and Picardeau, M. (2002) Inactivation of the spirochete *recA* gene results in a mutant with low viability and irregular nucleoid morphology. J. Bacteriol. 184, 452–458.

[13] Prod'hom, G., Lagier, B., Pelicic, V., Hance, A.J., Gicquel, B. and Guilhot, C. (1998) A reliable amplification technique for the characterization of genomic DNA sequences flanking insertion sequences. FEMS Microbiol. Lett. 158, 75–81.

[14] Andersen, G.L., Beattie, G.A. and Lindow, S.E. (1998) Molecular characterization and sequence of a methionine biosynthetic locus from *Pseudomonas syringae*. J. Bacteriol. 180, 4497–4507.

[15] Ren etal., S. (2003) Unique and physiological and pathogenic features of *Leptospira interrogans* revealed by whole genome sequencing. Nature 422, 888–893.

[16] Alexander, F.W., Sandmeier, E., Mehta, P.K. and Christen, P. (1994) Evolutionary relationships among pyridoxal-5′-phosphate-dependant enzymes, region-specific alpha, beta and gamma families. Eur. J. Biochem. 219, 953–960.