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Localization of the *Leptospira interrogans metF* gene on the CII secondary chromosome

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Abstract

An open reading frame of 885 nucleotides was identified as the *Leptospira interrogans metF* gene. The deduced amino acid sequence (294 amino acids) showed similarities with *Escherichia coli* methylene tetrahydrofolate reductase (MetF or MTHFR) (33% identity) and with the N-terminal part of human MTHFR (33% identity). The *L. interrogans metF* gene complements an *E. coli metF* mutant to prototrophy, suggesting the functionality of the folate branch converging to form methionine. In addition, the *L. interrogans* MetF was found to be thermolabile. The *metF* gene belonged to the CII secondary chromosome, in contrast to the previously isolated *metY* and *metX* genes, which have been localized to the CI chromosome of *Leptospira* sp.

Keywords: *Leptospira interrogans*; *metF*; Methylene tetrahydrofolate reductase; Thermolabile enzyme; Secondary chromosome

1. Introduction

Folic acid derivatives are coenzymes for reactions in the biosynthesis of methionine. In Enterobacteriaceae, a methylene tetrahydrofolate reductase (MetF or MTHFR) catalyzes the flavin adenine nucleotide (FAD) reduction of $N^{5,10}$-methylenetetrahydrofolate to N^{5}-methyltetrahydrofolate, a cofactor for methylation of homocysteine to methionine [1]. It should be noted that *Escherichia coli* MetF is similar to the N-terminal domain of yeast and other eukaryotic MTHFRs [2,3]. The C-terminal domain of the porcine enzyme contains the regulatory center while its N-terminal domain contains the catalytic center [4]. This is consistent with the known regulation of the eukaryotic MTHFR by *S*-adenosylmethionine [5], regulation which is absent from bacterial MTHFRs.

Molecular and biochemical data have shown that the first part of the methionine pathway for *Leptospira*, a bacterium belonging to the spirochete phylum, is similar to that from yeast [6]. The first step, esterification of homoserine, is catalyzed by a homoserine *O*-acetyltransferase (MetX) [7]. Then direct sulfhydrylation of *O*-acetylhomoserine to homocysteine involves MetY, an *O*-acetylhomoserine sulfhydrylase [6]. The *metX* and *metY* genes, organized in an operon, belong to the large CI chromosome of *Leptospira*. We have cloned the *metF* gene and determined its nucleotide sequence and functionality. The thermolability of *Leptospira interrogans* MetF was investigated. The *metF* gene has been localized to the CII secondary chromosome. The dispersion of the *met* biosynthetic genes on the two chromosomes of *Leptospira* is discussed.

2. Materials and methods

2.1. Bacterial strains and media

L. interrogans serovar icterohaemorraghiae strain Verdun (National Reference Center, Paris, France) was grown in EMJH medium at 30°C [8,9]. *E. coli* strain JM109 was used for cloning experiments whereas strain JM110 (deficient in Dam methylase) was used for the preparation of a recombinant plasmid with a methylated *Cla*I site (Promega, France). Growth in LB broth or LB agar plates was performed at 37°C [10]. *E. coli* RC 709 cells (*metF63*, *pro-22*) (kindly given by R. Clowes) were made competent by the $CaCl_2$ method [10]. This strain, grown on minimal

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agar plates [10], was used for complementation experiments at 30°C and 42°C. Isopropylthio-β-D-galactoside (IPTG) was used at a concentration of 0.5 mM, 3-indolyl-β-D-galactoside (X-gal) at 80 μg ml^{-1}, and ampicillin at 100 μg ml^{-1}.

2.2. Cloning of the L. interrogans metF gene

A cosmid library of *L. interrogans* serovar icterohaemorraghiae strain Verdun was made as described for another *Leptospira* species [6]. A recombinant cosmid (XG10) harboring an insert of around 40 kb in size was cleaved with the *Sau*3A restriction endonuclease according to the manufacturer's specifications. The obtained fragments measuring between 0.5 and 3 kb were ligated into the plasmid vector pGEM-7Zf(+) (Promega).

2.3. DNA sequencing and sequence analysis

The recombinant plasmid DNAs were sequenced on both strands with the ALFexpress™ AutoRead™ Sequencing kit (Pharmacia Biotech) and Cy5-labeled primers. Nucleotide and deduced amino acid sequences were analyzed with the BLAST program (NCBI) to find similarities, CLUSTAL software [11] for multiple alignment, PHI-Blast (NCBI) for motif research. The *metF* nucleotide sequence has been assigned the GenBank accession number AF253051.

2.4. Pulsed-field gel electrophoresis and Southern blot

Pulsed-field gel electrophoresis (PFGE) was performed with the CHEF-DRII apparatus (Bio-Rad Laboratories, Richmond, CA, USA), in 0.5×TBE. Chromosomes CI and CII were separated in a 1% agarose gel for 26 h with a pulse ramp of 3–110 s at 200 V. Southern blots of PFGE agarose gels were transferred on Hybond-N nylon membrane (Amersham, UK) by passive transfer [10]. The [α-^{33}P]dATP labeling of the probes was performed with the Megaprime kit (Amersham). Several probes originating from *L. interrogans* strain Verdun were used: (i) plasmid pAIL4 was used as a probe for the *asd* gene [12]; (ii) a 530-bp probe specific for the *metY* gene [6] was synthesized with two oligonucleotides 5′-CAAGAAATTGTTGCGTCTTC-3′ and 5′-CTAGCGCTTCTGGAAC-3′ by polymerase chain reaction; (iii) plasmid pGMetF3 was used as a probe for the *metF* gene. Hybridizations were carried out for 18 h at 65°C.

3. Results and discussion

3.1. The L. interrogans metF gene belongs to the CII secondary chromosome

We had demonstrated that *L. interrogans* had a secondary chromosome since it carried the *asd* essential gene, encoding aspartate β-semialdehyde dehydrogenase [13]. The screening of a *L. interrogans* genomic library with *asd* as a probe yielded the XG10 recombinant cosmid. Random subcloning of *Sau*3A partial inserts of XG10 into pGEM-7Zf(+) generated recombinant plasmids which were sequenced from each extremity. Analysis of the nucleotide sequence of the recombinant plasmids and comparison of the deduced amino acid sequences with the content of the databases allowed identification of the 5′-end and 3′-end of the putative *metF* gene each on a separate subclone. Since, for rigorous interpretation of complementation experiments (see below), it was important to obtain the smallest fragment carrying the *L. interrogans* *metF* gene, the next step was a search for restriction sites flanking the gene. Endonuclease restriction sites for *Cla*I and *Nsi*I were found 100 bp downstream and 175 bp upstream of the *metF* gene respectively. Cloning of this 1.2-kb fragment into pGEM-7Zf(+) yielded the expected pGMetF3 clone, carrying the smallest fragment which contained the whole *metF* gene and short flanking regions.

We verified that *metF* belongs to the CII secondary chromosome (370 kb), which carries the *asd* gene [13]. The presence of *metF* on CII (partly linearized at random) and CII linearized with *Not*I is shown in Fig. 1B (lanes 1 and 2 respectively).

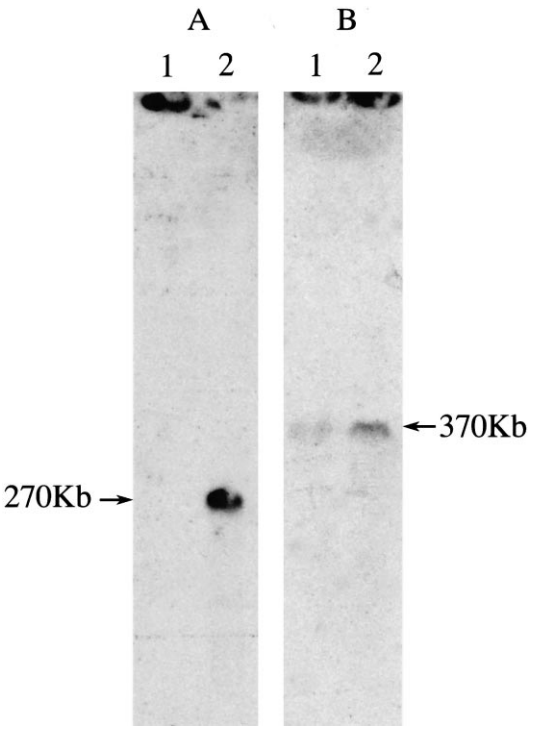

Fig. 1. Localization of the *metY* and *metF* genes to the CI and CII chromosomes respectively. *L. interrogans* strain Verdun DNA uncut (lanes 1) or cut with *Not*I (lanes 2), then separated by PFGE, and blotted to a membrane were hybridized with the *metY* (A) and *metF* probes (B). The size of the bands obtained is indicated by arrows.

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MetF L.i.                                                MKKVSEIYGS--AKGPVY-SFEFFPPKTPEGDSKLMETVKELSLL   42
MetF E.c.                                          MSFFHASQRDALNQSLAE-VQGQINVSFEFFPPRTSEMEQTLWNSIDRLSSL   51
MTHFR H.s. MVNEARGNSSLNPCLEGSASSGSESSKDSSRCSTPGLDPERHERLREKMRRRLESGDKWFSLEFFPPRTAEGAVNLISRFDRMAAG   86
                                                                 *    * ***** * *    *

MetF L.i.  NPDFVTVTY---GAGGSTRDKTVQILSQISKDY-SFPTVSHFTCVGANKNQILETLKGIRSSGILNLMALRGDPPKGEGEFKKVEN  124
MetF E.c.  KPKFVSVTY---GANSGERDRTHSIIKGIKDRT-GLEAAPHLTCIDATPDELRTIARDYWNNGIRHIVALRGDLPPGSGK----PE  129
MTHFR H.s. GPLYIDVTWHPAGDPGSDKETSSMMIASTAVNYCGLETILHMTCCRQRLEEITGHLHKAKQLGLKNIMALRGDPI--GDQWEEEEG  170
            *    **    *                          * **                 *     *****

MetF L.i.  GFGNATELVSFIRSEKLD-FCIGGGCYPEKHPNAKTLEEDVENLKLKVDAGTDFLVSQLFFVNSIFENFLNLVRKVGIQVPVIPGI  209
MetF E.c.  MY--ASDLVTLLK-EVAD-FDISVAAYPEVHPEAKSAQADLLNLKRKVDAGANRAITQFFFDVESYLRFRDRCVSAGIDVEIIPGI  211
MTHFR H.s. GFNYAVDLVKHIRSEFGDYFDICVAGYPKGHPEAGSFEADLKHLKEKVSAGADFIITQLFFEADTFFRFVKACTDMGITCPIVPGI  256
               *  **     *  * * *    **  ** *     *   ** ** **      * **       *       **     ***

MetF L.i.  MPITSFSQIDRFRSMAGCEFPSSLIQDLQEVEHRPEEFYRRSLNFSVKQCRELLAMG-VPGIHLYTLNQSHASYDIVRELKGESAZ  294
MetF E.c.  LPVSNFKQAKKFADMTNVRIPAWMAQMFDGLDDDAETRKLVGANIAMDMVK-ILREG-VKDFHFYTLNRAEMSYAICHTLGVRPGL  295
MTHFR H.s. FPIQGYHSLRQLVKLSKLEVPQEIKDVIEPIKDNDAAIRNYGIELAVSLCQELLASGLVPGLHFYTLNREMATTEVLKRLGWALSA  342
           *                  *                                 *  * *   * ****           *
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Fig. 2. Alignment of MetF from *L. interrogans* (L.i.) with MetF from *E. coli* (E.c.) and MTHFR from *Homo sapiens* (H.s.). Only the N-terminal part of the 656 amino acids long *H. sapiens* MTHFR is presented. Identical amino acids in the three proteins are indicated by asterisks. A motif belonging to the FAD binding site [15] is shown in bold characters. The Thr175 in *L. interrogans*, corresponding to Ala177 in *E. coli* and Ala222 in *H. sapiens*, is underlined. The *L. interrogans metF* nucleotide sequence has been assigned the GenBank accession number AF253051.

3.2. The L. interrogans MetF is homologous to other bacterial MetFs and to the N-terminal domain of yeast and mammalian MTHFRs

The whole 885-bp *L. interrogans metF* gene was sequenced. Similarities were high with bacterial MetFs and the N-terminal part of eukaryotic MTHFRs. Fig. 2 shows a comparison with *E. coli* MetF (33% identity) and *Homo sapiens* MTHFR (33% identity) [2,14]. Functional analysis had been performed with *E. coli* MetF and mammalian MTHFR [1,5]. This validates the assumption that *L. interrogans* MetF does indeed correspond to MTHFR. The X-ray analysis of *E. coli* MetF provided a model for the catalytic domain, which should be shared by all MTHFRs [15]. This domain is a β8α8 barrel, which binds FAD in a novel fashion [15]. Close analysis of the MTHFRs revealed several conserved motifs (see grouped asterisks of Fig. 2). Among them, the pattern A-[L/V]-R-G-D-(0/X)-[P/I/V], part of the FAD binding site [15], was found to be characteristic of all MTHFRs. When programs of motif research were used with this pattern, only MTHFRs were selected. We therefore searched for the functionality of the *L. interrogans* MetF.

3.3. The L. interrogans MetF is functional in E. coli at 30°C

The recombinant plasmid pGMetF3 (carrying the whole *L. interrogans metF* gene) and pGEM-7Zf(+) as a control were transformed into an *E. coli metF* mutant. The transformants were tested for their growth on minimal medium plates with or without methionine at 30°C and 42°C. Two independent experiments gave an identical outcome. The results showed that all transformants grew on the control plate with added methionine (Fig. 3, top panels). However, only the transformants containing plasmid pGMetF3 grew on the plate without methionine at 30°C (Fig. 3, bottom left panel, quadrants B, C and D). The control transformants with the pGEM-7Zf(+) vector did not grow (Fig. 3, bottom left panel, quadrant A). Complementation of the *E. coli metF* mutant by the *L. interrogans metF* gene occurs at 30°C and not at 42°C (Fig. 3, compare bottom panels) indicative of the thermolability of the corresponding *L. interrogans* MetF (see below).

3.4. The L. interrogans MetF is similar to a thermolabile E. coli MetF with an Ala177Val change

In humans, high plasma homocysteine levels are associated with an increased risk of cardiovascular disease [16]. Folate treatment decreases homocysteine levels, most probably by targeting MTHFR. In most cases, the cause of elevated plasma homocysteine is the MTHFR polymorphism Ala222→Val. It should be added that Ala is conserved at this position for all MTHFRs known to date except that from *L. interrogans*.

The bacterial mutation Ala177→Val (which mimics Ala222→Val in human MTHFR) does not affect the kinetic parameters of the *E. coli* enzyme but instead increases the tendency for *E. coli* MetF to lose its essential flavin cofactor associated with its thermolability [15]. It should be stressed that while the Ala177→Val mutation affects FAD binding, it alters a residue that is not part of the FAD binding site [15]. For *L. interrogans*, at the critical 177 position, a threonine is found instead of an alanine (underlined in Fig. 2), thus analogous to the

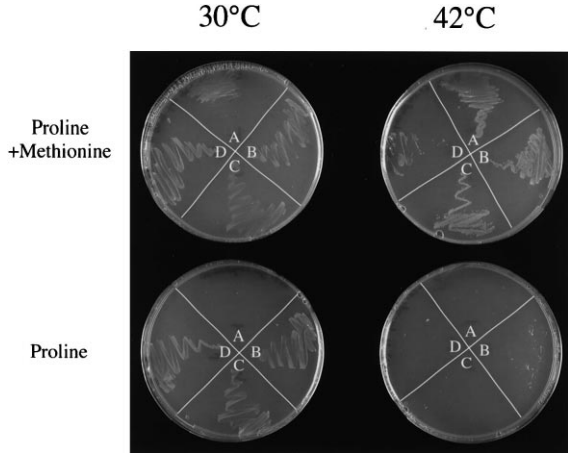

Fig. 3. Complementation results. *E. coli* strain RC709 was transformed either with the pGEM vector (A) or with pGMetF3 (B, C and D). Transformants were streaked onto minimal medium supplemented with proline plus methionine or proline only and grown at 30°C or 42°C.

Ala177→Val change of the *E. coli* mutant mentioned above. This might suggest that the leptospiral MetF is a thermolabile enzyme that loses its flavin cofactor more readily than does the wild-type *E. coli* MetF. Indeed, we have given experimental evidence that *L. interrogans MetF* complements an *E. coli metF* mutant at 30°C and not at 42°C (see Section 3.3). Since it is known that addition of folate derivatives results in protection of MTHFR from thermosensitivity, further studies are needed to establish folate levels within *Leptospira*.

3.5. Dispersion of the methionine genes on the two leptospiral chromosomes

Three genes have been identified for the leptospiral methionine biosynthetic pathway. The *metX* and *metY* genes catalyze the first steps of the pathway [6,7] and the *metF* gene corresponds to the folate branch (this study). The methionine genes are dispersed over the two chromosomes of *Leptospira* since *metX* and *metY* are located on CI [6,7] and *metF* on CII (see Section 3.1). However, the data concerning *metX* and *metY* had been performed with the *L. meyeri* strain Veldrat [7], which differs from the *L. interrogans* Verdun strain used in this study. It was thus important to validate the findings for strain Verdun. Restriction fragments produced following digestion of DNA from *L. interrogans* strain Verdun DNA were separated by PFGE and probed with a labeled *metY* probe. DNA that either had not been digested or had been digested with *Not*I gave a strong signal located in the well and at 270 kb in size respectively (Fig. 1A, lanes 1 and 2). This localized the *metY* gene to the large CI chromosome and precisely to the *Nt*F band from a *Not*I digest of CI [13]. These data of dispersion of the methionine genes on the two chromosomes of *Leptospira* are similar to the results obtained for the genes of the tryptophan pathway in *Rhodococcus sphaeroides* [17]. The latter was the first bacterium to be shown to carry two chromosomes [18]. Transposon mutagenesis of the *R. sphaeroides* genome yielded tryptophan auxotrophs and the mutations were on either chromosome [17]. Our results correspond to a second example of this kind of topological organization of genes involved in a biosynthetic pathway and indicate a functional link between the two chromosomes of the genome.

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