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# Construction and complementation of the first auxotrophic mutant in the spirochaete *Leptospira meyeri*

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In bacteria, the first reaction of the tryptophan biosynthetic pathway involves the conversion of chorismate and glutamine to anthranilate by the action of anthranilate synthase, which is composed of the $\alpha$ (*trpE* gene product) and $\beta$ (*trpG* gene product) subunits. In this study, the tryptophan biosynthetic gene *trpE* of the spirochaete *Leptospira meyeri* was interrupted by a kanamycin-resistance cassette by homologous recombination. The *trpE* double cross-over mutant was not able to grow on solid or in liquid EMJH medium. In contrast, the *trpE* mutant showed a wild-type phenotype when tryptophan or anthranilate was added to the media, therefore showing that disruption of the *L. meyeri trpE* gene resulted in tryptophan auxotrophy. The authors have also characterized a second selectable marker that allows the construction of a spectinomycin-resistant *L. meyeri–E. coli* shuttle vector and the functional complementation of the *L. meyeri trpE* mutant.

## INTRODUCTION

The advance of our knowledge with regard to the biology of the *Spirochaetales* order, which includes the pathogens *Borrelia burgdorferi*, *Treponema pallidum* and *Leptospira interrogans*, has been hampered by the absence of suitable genetic tools (Tilly *et al.*, 2001). Recently, the development of new genetic tools has facilitated the construction of targeted mutants in the saprophytic species *Leptospira biflexa*. Thus, we have previously disrupted the chromosomal *flaB* and *recA* genes of *L. biflexa* with a kanamycin-resistance marker (Picardeau *et al.*, 2001; Tchamedeu Kameni *et al.*, 2002). However, no targeted mutants have been obtained in other *Leptospira* species containing saprophytic and pathogenic members.

Charon *et al.* (1974) demonstrated that *Leptospira* spp. have the potential to synthesize the essential amino acids and that most of them were synthesized by biosynthetic pathways similar to those used in *Escherichia coli*. In previous genetic studies, several leptospiral genes have been isolated by functional complementation of *E. coli* mutants. This methodology has allowed the identification of some amino acid biosynthetic genes such as *asd*, *aroD*, *dapD*, *metX*, *metY*, *trpE*, *proA* and *leuB* (Baril *et al.*, 1992; Belfaiza *et al.*, 1998; Richaud *et al.*, 1990; Yelton & Cohen, 1986). In order to test our genetic exchange system in the saprophytic species *L. meyeri* and to study the tryptophan biosynthetic pathway (Fig. 1), we constructed a *L. meyeri trpE* mutant. This study also describes the use of a second selectable marker, the spectinomycin-resistance cassette, that allows complementation studies in *Leptospira* spp.

## METHODS

**Bacterial strains and growth conditions.** *L. meyeri* serovar semaranga strain Veldrat (Leptospira National Reference Centre, Institut Pasteur, Paris, France) was grown at 30 °C in EMJH

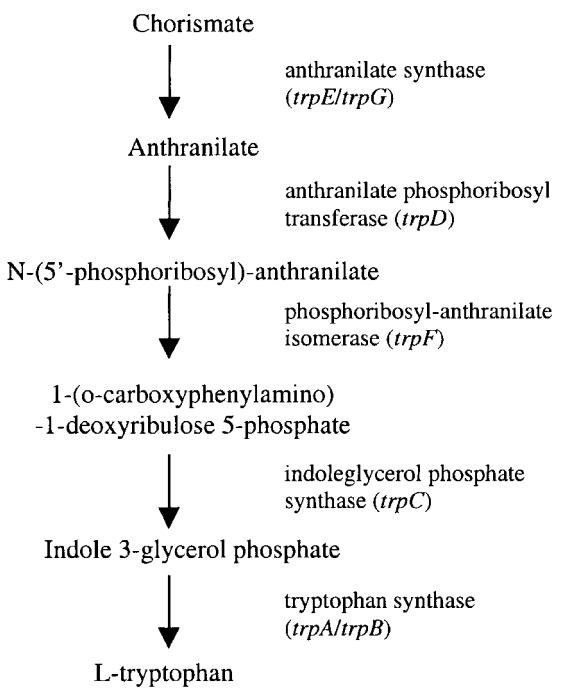



**Fig. 1.** The tryptophan biosynthetic pathway in *E. coli*, a model for *Leptospira* spp.

**Table 1.** Primers used in this study

| Primer | Sequence (5′–3′) |
|---|---|
| TE1 | AGGATCGCACTTAGAAAGGC |
| TE2 | TTTTATCATAGTTAGCTGAC |
| TE4 | GGAGCTGTTGGAAGTTTTGG |
| TK1 | ACCTTTCAATGCCAAATTGG |
| TK2 | TTACGAGCAAGGAGAGTGTC |
| TG1 | ACCCTGCTTATTTTGGTGTG |
| TG2 | AGGATTTGTGGCGAAGACCC |
| FLG5* | TAATACCCGAGCTTCAAGGAA |
| FLG3† | TCTGCAGTTGGTTCATATGGAAACCT |
| SN† | ATTCCATATGAGCAATTTGATTAA |
| SP | TGGTTCTGCAGGTTTTAAAAGTAA |

*See Bono *et al.* (2000).

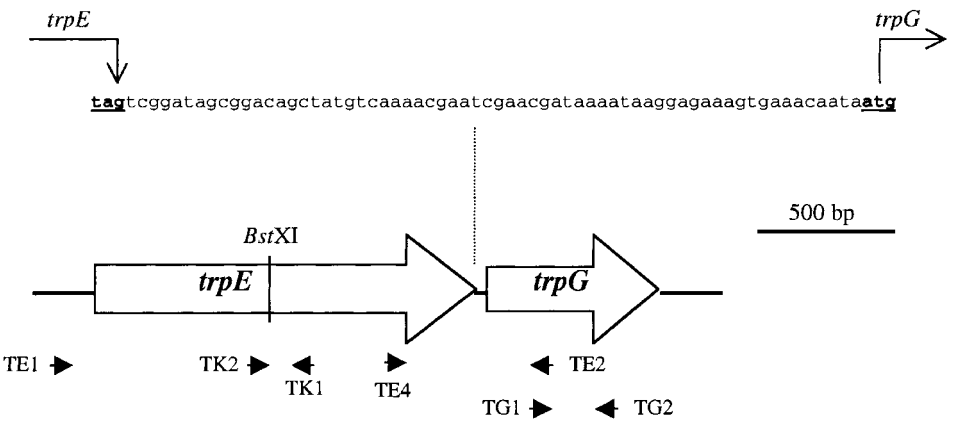
†The *Nde*I restriction site used for the fusion of $P_{lac}$ and *spc* is underlined.

(Ellinghausen & McCullough, 1965; Johnson & Harris, 1967) liquid medium or on 1 % agar plates. When necessary, L-tryptophan and anthranilate were used at 1 mM. Kanamycin and spectinomycin were used at 50 μg $ml^{-1}$.

**DNA manipulations.** Plasmids from *E. coli* were recovered using a Qiaprep Spin miniprep kit (Qiagen). Genomic DNA of *L. meyeri* was extracted as previously described (Picardeau *et al.*, 2001). For Southern blot analysis, DNA of representative clones of *L. meyeri* was digested with *Eco*RI, subjected to electrophoresis overnight, and transferred onto a nylon membrane. The *L. meyeri trpE* gene was amplified with primers TE1–TE2 and radiolabelled with [α-$^{33}$P]dATP (Megaprime, Amersham). The membrane was then hybridized with the labelled probe under stringent conditions as previously described (Picardeau *et al.*, 2001). Amplification with appropriate primers (Table 1) 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.

**RNA isolation and RT-PCR.** Total RNA was extracted from exponentially growing cells of *L. meyeri* ($OD_{420}$ 0·2). After centrifugation (4000 ***g*** for 10 min at 4 °C), cells were washed in water, resuspended in 10 ml Trizol reagent (Gibco) and incubated for 5 min at room temperature. Two millilitres of isoamyl chloroform was added. Cells were then shaken and centrifuged. The supernatant was recovered and 5 ml 2-propanol was added. To precipitate RNA, tubes were incubated for 10 min at room temperature. After centrifugation, the RNA was washed with 75 % ethanol. The pellet was dissolved in 1 × Dnase I buffer (Pharmacia). To remove DNA contamination, the sample was treated with RNase-free DNase I (Pharmacia). RNA was then purified by using the RNeasy mini kit (Qiagen). Reverse transcription of RNA was carried out as described by the manufacturer (SuperScript; Gibco-BRL) by using primer pairs TK1–TK2, TE4–TE2 and TG1–TG2 (Fig. 2, Table 1) at a final concentration of 0·2 μM. After 30 min at 45 °C, reverse transcriptase (RT) was inactivated by incubation at 94 °C for 2 min. PCR conditions were similar to those described above. RNA samples were tested in the presence and absence of RT to test for amplification of contaminant genomic DNA.

**Construction of a *L. meyeri trpE* mutant.** The *trpE* (1714 bp) locus of *L. meyeri* was amplified by PCR with primer pairs TE1–TE2 (Fig. 2, Table 1). The amplified product was then inserted into the *Sma*I site of pUC19, resulting in plasmid pTE. The *Enterococcus faecalis* kanamycin-resistance cassette was amplified as previously described (Picardeau *et al.*, 2001) and inserted into the blunt-ended site *Bst*XI of *trpE* to generate plasmid pTEK (*trpE*::Km). The




| RT-PCR | TK1-TK2 | TE4-TE2 | TG1-TG2 |
|---|---|---|---|
| wild-type strain | + | - | + |
| *trpE* mutant | - | - | + |

**Fig. 2.** Genetic analysis of the *trpE*/*trpG* locus of *L. meyeri*. For the inactivation of the *L. meyeri trpE* gene, the TE1–TE2 amplified product was cloned into a suicide plasmid. The kanamycin-resistance cassette was then inserted into the *Bst*XI restriction site, generating plasmid pTEK, which was used to deliver the mutated allele into *L. meyeri*. Arrows indicate primers (see Table 1) used for the transcriptional analysis of the *L. meyeri trpE* and *trpG* genes (for complete sequence, see Yelton & Peng, 1989). Results of RT-PCR assays with RNA from *L. meyeri* wild-type and *L. meyeri trpE* mutant strains are indicated.

plasmid was then subjected to UV irradiation and used to deliver the inactivated allele into *L. meyeri* as previously described (Picardeau *et al.*, 2001). Optimal conditions (maximum number of transformants) were obtained for 1 μg of circular plasmid DNA treated with low UVB or UVC irradiation (1 mJ $cm^{-2}$) using cells from an early exponential-phase culture ($OD_{420}$ 0·2, corresponding to $4 \times 10^8$ bacteria $ml^{-1}$). Kanamycin-resistant colonies were picked and tested for the insertion of the kanamycin-resistance cassette into the target gene by PCR with primers TK1 and TK2 (Table 1) and Southern blot analysis as previously described (Picardeau *et al.*, 2001).

**Complementation of the *L. meyeri trpE* mutant.** To complement the *L. meyeri* tryptophan (*trpE*::Km) auxotroph, we tested the spectinomycin-resistance cassette of *Staphylococcus aureus* (Chary *et al.*, 1997; Murphy *et al.*, 1985) as a second selectable marker. As previously described (Bono *et al.*, 2000), the promoter of the *B. burgdorferi flgB* gene was amplified with primers FLG5 and FLG3 (Table 1) (introducing *Nde*I and *Pst*I restriction sites at the 3′ end of the promoter) and cloned into the *Sma*I site of pGEM7Zf(+) (Promega), resulting in plasmid pGFB. The promoterless *spc* gene amplified from pVK61 (Chary *et al.*, 1997) using primers SN and SP (Table 1) was cloned into pCR2.1-TOPO (Invitrogen), resulting in plasmid pCRS. The *spc* gene was released from pCRS with *Nde*I and *Nsi*I restriction enzymes and inserted into the *Nde*I–*Pst*I sites of pGFB, to make pGFBS. The $P_{flgB}$–*spc* was amplified from pGFBS with primers FLG5 and SP (Table 1), then inserted into the *Sma*I site of pGKLep4, resulting in plasmid pGKLS (Fig. 3). Spectinomycin-resistant colonies were obtained after electroporation of pGKLS into *L. meyeri*. We then cloned the *trpE* gene (amplified product of TE1–TE2 starting at position −102 upstream of the start codon and ending at position +225 downstream of the stop codon, Fig. 2) into the *Fsp*I sites of pGKLS, resulting in plasmid pGLStrpE (Fig. 3). Plasmid pGSLtrpE was used to transform the *L. meyeri trpE* mutant, by electroporation.

## RESULTS AND DISCUSSION

### Generation of a tryptophan-auxotrophic mutant in *L. meyeri*

In *E. coli*, the synthesis of the aromatic amino acids phenylalanine, tyrosine and tryptophan shares a common pathway leading to the synthesis of the branch point compound chorismate (Pittard, 1996). The first reaction of the tryptophan pathway involves the conversion of chorismate and glutamine to anthranilate by the action of anthranilate synthase (encoded by the *trpE* and *trpG* genes) (Fig. 1). The sequential actions of the products of the *trpD*, *trpF* and *trpC* genes lead to the penultimate compound in the pathway, indoleglycerol phosphate. The last step is catalysed by tryptophan synthase (encoded by the *trpA* and *trpB* genes) (Pittard, 1996) (Fig. 1). The *trpE* and *trpG* genes of *L. meyeri* were previously isolated by complementation of an *E. coli trpE* mutant (Yelton & Cohen, 1986). The suicide plasmid carrying the *L. meyeri* inactivated *trpE* gene, pTEK, was treated by UV irradiation prior to electroporation. UV irradiation of the plasmids yielded up to 100-fold more transformants compared to non-treated DNA. However, no double cross-over event was detected for the *trpE* gene (0/40 tested colonies, all had undergone a single cross-over event), suggesting that auxotrophs were not able to grow on EMJH medium, which is a complex medium and includes undefined components. Therefore, further transformation experiments were done in the presence of tryptophan. This resulted in the identification of double cross-over events for the *trpE* gene of *L. meyeri* (10/50 tested colonies). Confirmation of allelic exchange was obtained by PCR and Southern hybridization (Fig. 4a); an increase in size of either the PCR product or the hybridizing fragment by 1·1 kb is due to the insertion of the kanamycin-resistant cassette into the chromosomal *trpE* locus. The *trpE* mutants resulting from a double cross-over event were unable to grow on solid or in liquid medium (Fig. 4b) lacking supplemental amino acids. Addition of tryptophan restored growth of the *L. meyeri trpE* mutant at the wild-type rate (Fig. 4b). This result shows that *L. meyeri* is able to acquire amino acids from its environment by taking up free amino acids. A wild-type phenotype was also obtained by the addition of anthranilate (data not shown), further confirming that the *L. meyeri* TrpE protein is involved in the first step of the tryptophan biosynthetic pathway, leading to anthranilate (Fig. 1).

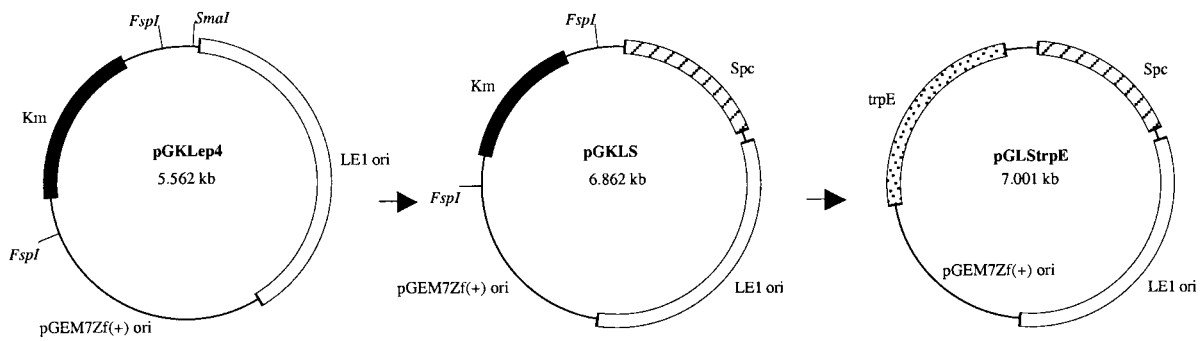



**Fig. 3.** Construction of shuttle vectors pGKLS and pGLStrpE. Relevant restriction sites are indicated. The spectinomycin-resistant cassette (Spc) was amplified and cloned into *Sma*I-cut pGKLep4, to generate pGKLS. We then cloned the *trpE* gene (trpE) into the *Fsp*I sites of pGKLS (then releasing the kanamycin-resistance cassette, Km), resulting in plasmid pGLStrpE.

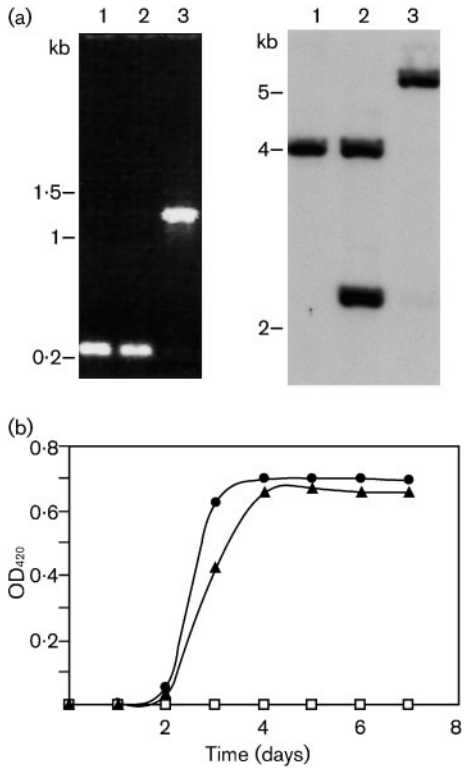



**Fig. 4.** (a) Evidence for homologous recombination with the *L. meyeri trpE* gene. PCR (with primers TK1 and TK2, left panel) and Southern blot analysis (hybridized with the *trpE* probe, right panel) of representative clones. Lanes: 1, *L. meyeri* wild-type strain; 2, single cross-over recombinant strain; 3, double cross-over recombinant strain. Sizes of the fragments of the molecular mass marker are indicated on the left. (b) Growth curve of *L. meyeri*: ●, parental strain; □, *trpE* mutant; ▲, *trpE* mutant supplemented with tryptophan.

The *trpE* and *trpG* genes are separated by only 68 bp and could form an operon (Yelton & Peng, 1989). However, no amplification between *trpE* and *trpG* was obtained by RT-PCR with RNA from either *trpE* mutant or wild-type strains (Fig. 2). Interestingly, inactivation of *trpE* does not affect the transcription of *trpG* in the *L. meyeri trpE* mutant (Fig. 2). Taken together these results suggest that *trpE* and *trpG* do not form an operon and that *trpG* possesses its own promoter.

## Use of a second selectable marker and complementation of the *L. meyeri trpE* mutant

To date, *Leptospira* spp. have only one reliable marker, the *E. faecalis* kanamycin-resistance cassette (Saint Girons *et al.*, 2000). To confirm that the auxotrophy of *L. meyeri* was due to the inactivation of the *trpE* gene, a second selectable marker was tested for complementation experiments. We used the spectinomycin-resistance gene (*spc*) from *S. aureus* (Chary *et al.*, 1997). This cassette was chosen because its sequence shows a G+C content (35 mol%) similar to that of *Leptospira* spp. (34–39 mol%). In addition, similarly to most of the markers used in spirochaetes (Hardham & Rosey, 2000), the *spc* gene is an antibiotic-resistance cassette from a Gram-positive bacterium. Finally, this cassette appears to be expressed in *B. burgdorferi* (Sartakova *et al.*, 2001a). To enhance its transcription in spirochaetes, the promoterless *spc* gene was linked to the promoter region of the *B. burgdorferi flgB* gene as previously described (Bono *et al.*, 2000) and cloned into the *L. meyeri–E. coli* shuttle vector pGKLep4 (Fig. 3). Electroporation of pGKLS into *L. meyeri* resulted in spectinomycin-resistant (Spc$^r$) colonies. The MIC of transformants was $>128$ μg ml$^{-1}$ (versus $<2$ μg ml$^{-1}$ for the wild-type strain). This suggests that the borrelial promoter of *flgB* (Bono *et al.*, 2000) is recognized in *L. meyeri*. We then cloned the *L. meyeri trpE* gene into pGKLS (Fig. 3) and the constructs were transferred into *L. meyeri trpE* mutant by electrotransformation. While transformants containing the vector alone were not able to grow on EMJH medium without tryptophan, the Spc$^r$ transformants containing the *trpE* gene recovered the ability to grow on EMJH medium (data not shown). Plasmid DNAs extracted from several Spc$^r$ *L. meyeri* transformants were analysed after amplification in *E. coli* and showed restriction profiles similar to those from the original plasmid constructs (data not shown). These results show that the Kan$^r$ auxotroph was complemented by the wild-type genes present on the shuttle vector containing the Spc$^r$ marker. Only two other spirochaetal genes have been inactivated and complemented so far: the *T. denticola flgE* (Chi *et al.*, 2002) and the *B. burgdorferi flaB* (Sartakova *et al.*, 2001b) genes.

## Concluding remarks

We report here the characterization of the first auxotroph in *Leptospira* spp. (as well as the first mutant in *L. meyeri*) and the identification of a second selectable marker, the spectinomycin-resistance cassette, for genetic manipulations of *Leptospira* spp. We also demonstrate that tryptophan prototrophy is essential for survival of *Leptospira* spp. in an environment lacking amino acids, such as EMJH medium. During the completion of this work, the genomic sequence of *L. interrogans* serogroup icterohaemorrhagiae pathogenic strain Lai (Chinese Human Genome Center, Shanghai, China; see http://www.chgc.sh.cn/gn/ and http://www.ncbi.nlm.nih.gov/PMGifs/Genomes/micr.html) was released (S. Ren, G. Fu, X. Jiang & 18 other authors, unpublished). Sequence analysis of the genome of the pathogen *L. interrogans* shows a tryptophan pathway similar to that of *E. coli*, as shown in Fig. 1. Interestingly, analyses of the genomic sequences of the evolutionarily related spirochaetes *T. pallidum* and *B. burgdorferi* have shown that genes encoding enzymes of the tryptophan pathway are absent (Fraser *et al.*, 1997, 1998). In fact, these spirochaetes have a minimal genome that lacks genes encoding the enzymes for most biosynthetic pathways, and are naturally auxotrophs for all amino acids. The prospect of soon possessing genetic tools for pathogenic strains of *Leptospira* could allow the construction of a *L. interrogans trpE* mutant as a putative

candidate for vaccines. Indeed, auxotrophs could be vaccine candidates as previously demonstrated in many pathogens such as *Corynebacterium pseudotuberculosis*, *Mycobacterium tuberculosis* and *Legionella pneumophila* (Mintz *et al.*, 1988; Simmons *et al.*, 1997; Smith *et al.*, 2001).

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