Molecular Microbiology (2001) **40**(1), 189–199

First evidence for gene replacement in *Leptospira* spp. Inactivation of *L. biflexa flaB* results in non-motile mutants deficient in endoflagella

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Summary

***Leptospira* spp. offer many advantages as model bacteria for the study of spirochaetes. However, homologous recombination between introduced DNA and the corresponding chromosomal loci has never been demonstrated. A unique feature of spirochaetes is the presence of endoflagella between the outer membrane sheath and the cell cylinder. We chose the *flaB* flagellin gene, constituting the flagellar core, as a target for gene inactivation in the saprophyte *Leptospira biflexa*. The amino acid sequence of the FlaB protein of *L. biflexa* was most similar to those of spirochaetes *Brachyspira hyodysenteriae* (agent of swine dysentery), *Leptospira interrogans* (agent of leptospirosis) and *Treponema pallidum* (agent of syphilis). A suicide vector containing the *L. biflexa flaB* gene disrupted by a kanamycin marker was UV irradiated or alkali denatured before electroporation. This methodology allowed the selection of many kanamycin-resistant colonies resulting from single and double cross-over events at the *flaB* locus. The double recombinant mutants are non-motile, as visualized in both liquid and semi-solid media. In addition, a *flaB* mutant selected for further analysis was shown to be deficient in endoflagella by electron microscopy. However, most of the transformants had resulted from a single homologous recombination event, giving rise to the integration of the suicide vector. We evaluated the effect of the *sacB* and *rpsL* genes in *L. biflexa* as potential counterselectable markers for allelic exchange, and then used the *rpsL* system for the positive selection of *flaB* double recombinants in a streptomycin-resistant strain. Like the *flaB* mutant studied above, the Str^r double cross-over mutant was non-motile and deficient in endoflagella. Our results demonstrate that FlaB is involved in flagella assembly and motility. They also show the feasibility of performing allelic replacement in *Leptospira* spp. by homologous recombination.**

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Introduction

Spirochaetes are a diverse group of bacteria, many of which are medically important pathogens, including *Leptospira interrogans* (leptospirosis), *Borrelia burgdorferi* (Lyme disease), *Treponema pallidum* (syphilis) and *Brachyspira* (formerly *Serpulina*) *hyodysenteriae* (swine dysentery). The genus *Leptospira* belongs to the order Spirochaetales and is composed of both pathogenic and saprophytic species (Faine *et al*., 1999). The study of *Leptospira* spp. and other spirochaetes has been hindered by a lack of genetic tools. This might result from the fact that spirochaetes differ considerably from Gram-negative and Gram-positive bacteria that have well-developed genetic systems; indeed, 16S rRNA analysis indicates that the phylum of spirochaetes has a deep branching lineage (Woese, 1987). Previous attempts to create defined mutants of *Leptospira* spp. using a suicide vector delivering an inactivated gene into the chromosome have been unsuccessful. This lack of success is probably due to the low efficiency of transformation and/or to the low frequency of homologous recombination events. Previous studies have shown that homologous recombination events in mycobacteria and *Streptomyces* spp. can be increased by UV irradiation or alkali denaturation of the delivery vector (Oh and Chater, 1997; Hinds *et al*., 1999). However, the proportion of allelic exchange mutants is low, and single recombination (integration of the entire vector bearing the inactivated gene) is the general rule. A strategy that could be used to promote allelic exchange and to allow identification of double cross-over mutants is to use a plasmid vector containing a counterselectable marker (Reyrat *et al*., 1998). Counterselectable markers are genes that promote bacterial death in the presence of a counterselective compound but have no effect on bacterial growth in the absence of this compound. The most widely used counterselectable markers in bacteria are the genes that confer sucrose (*Bacillus subtilis sacB*) and streptomycin (*rpsL*) sensitivity (Reyrat *et al*., 1998). Construction of a

plasmid vector with a counterselectable marker adjacent to the inactivated gene should eliminate clones harbouring the plasmid vector (when the vector is replicative) and clones that have integrated the vector by a single cross-over event. On a counterselective medium, clones that result from the integration of the inactivated gene into the chromosome by a double cross-over event should be obtained more frequently.

Until recently, no antibiotic resistance gene that could be used as a selection marker was available in *Leptospira* spp. We demonstrated that a *Leptospira biflexa–Escherichia coli* shuttle vector based on the replication region of leptobacteriophage LE1 could express the kanamycin gene from the Gram-positive bacterium *Enteroccocus faecalis* in the saprophyte *L. biflexa* (Saint Girons *et al*., 2000). This kanamycin cassette was chosen for subsequent development of the gene inactivation system in *L. biflexa*. Another important step in the development of reverse genetic methods is the choice of the target gene. We used the *flaB* flagellin gene of *L. biflexa* as a target for gene inactivation. As spirochaetes and other bacteria mutated for flagellin genes were affected in their motility (Rosey *et al*., 1995; Limberger *et al*., 1999; Motaleb *et al*., 2000), the phenotype of the resulting *L. biflexa flaB* mutant should be easy to detect.

In this study, we show that successful gene replacement in the saprophyte *L. biflexa* is possible under conditions (either by UV irradiation or alkali denaturation of the suicide plasmid DNA) in which homologous recombination is enhanced. To identify a valuable counterselectable marker for allelic exchange in *Leptospira*, we evaluated the effect of the *sacB* and *rpsL* genes in *L. biflexa* and then used the *rpsL* system for the positive selection of *flaB* double recombinants. This work represents the first evidence for allelic exchange (as well as the first phenotypic and genetic analyses of mutants deficient in endoflagella) in *Leptospira* spp., and the first efficient use of counterselectable markers in spirochaetes.

Results

Identification of L. biflexa flaB *homologue and construction of plasmid for allelic exchange*

To study homologous recombination in *Leptospira* spp., we chose the saprophyte *L. biflexa* as a model. Strains of this species are non-pathogenic and therefore easy to manipulate as well as fast-growing (colonies formed in a week on solid medium). In addition, we have shown recently that the kanamycin (Km) gene of *E. faecalis* was expressed in and conferred Km resistance to *L. biflexa* (Saint Girons *et al*., 2000). Another important issue was the choice of target gene. Amplification by polymerase chain reaction (PCR) of *L. biflexa* DNA with degenerate primers located in conserved regions of *Leptospira* spp. *flaB* allowed the generation of a single product. Flanking regions of *L. biflexa flaB* were then cloned by LM-PCR (Prod'hom *et al*., 1998). A 751 bp open reading frame (ORF), similar in size to that of spirochaete *flaB* homologues, exhibited a possible ribosome binding site (5′-GAAAAG-3′) upstream of the ATG start codon and was therefore likely to represent the *flaB* coding region. The deduced amino acid sequence of the *L. biflexa* amplified product had significant sequence similarity to flagellar proteins from other bacteria: the *L. biflexa* sequence exhibits 63% and 64% identity with FlaB proteins of *B. hyodysenteriae* and *L. interrogans* respectively. *L. biflexa* FlaB also shows 57% and 43% identity with FlaB from *T. pallidum* and *Bacillus subtilis* respectively. To create a specific mutant of the *L. biflexa flaB*, we constructed a suicide vector (unable to replicate in *Leptospira* spp.) that contains the *E. faecalis* Km cassette inserted within the *L. biflexa flaB* (Fig. 1), thereby generating plasmid pPA2 (Table 1). The extent of homologous *L. biflexa* DNA present on either side of the Km marker of pPA2 was approximately 0.9 kb and 0.8 kb respectively (Fig. 1).

Stimulation of gene replacement in L. biflexa

Electroporation of *L. biflexa* with suicide vector pPA2 containing the inactivated gene (*flaB*::Km) produced only one Km-resistant (Km^r) colony in three independent experiments. This single transformant resulted from a single cross-over event at the *L. biflexa flaB* locus (data not shown). We then used another methodology, previously applied to Actinomycetales (Oh and Chater, 1997; Hinds *et al*., 1999), in which plasmid pPA2 was treated by UV irradiation or alkaline denaturation before electroporation. Figure 2 shows that a significant increase in Km^r

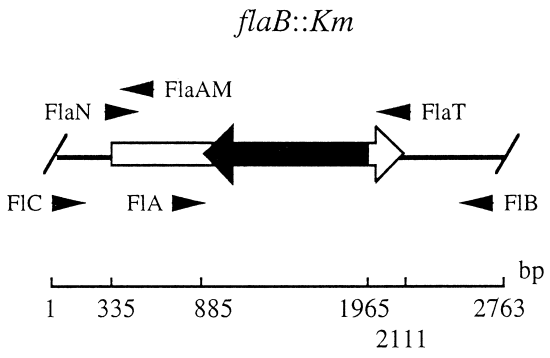


Fig. 1. Diagram of the *flaB*::Km construction cloned into the delivery plasmid pPA2. The kanamycin gene cassette is indicated by solid bar, and the open box represents the *flaB* open reading frame. The location of primers within the *L. biflexa flaB* locus are indicated by arrows. Primers FlA and FlB were used for the detection of double cross-over mutants.

Table 1. Primers and plasmids used in this study.

Primers or plasmids	Description	Source or reference[a]
DNA primers (5′−3′)		
FlA	ATC GGA CTT TGT GAT GAT GC	+583 to +602 downstream the *L. biflexa flaB* start codon
FlB	CAC CAA ACT TAC GTA CCT GG	+637 to +656 downstream the *L. biflexa flaB* stop codon
FlC	TTT TAG AAA AGG GGA AGT TC	−280 to −261 upstream from the *L. biflexa flaB* start codon
Kan3	ATC GGC TCC GTC GAT ACT AT	−171 to −152 upstream the *E. faecalis kanamycin* start codon
Kan5	GTA GTC TCA TAC CTG TCA ACG CCT ACA T	+78 to +97 downstream the *E. faecalis kanamycin* stop codon
FlaN	GTG GAG CTC ATG ATT ATC AA(T/C) CA(C/T) AA(C/T) CT	−9 to +20 from the *Leptospira* spp. *flaB* start codon
FlaT	ACA GGA TCC TCA GAT (A/G)TG CTG CAG AAG (C/T)TT	−20 to +10 from the *Leptospira* spp. *flaB* stop codon
flaAM	AGT CCA GAT GCG TCA TCT CC	+19 to +38 downstream the *L. biflexa flaB* start codon
LK1	AAT TGC TCG TGC	*Eco*RI linker
LK2	TAG AGT ATT CCT CAA GGC ACG AGC	*Eco*RI linker
sacB1	TTG TGC GTA ACT AAC TTG CC	−87 to −68 upstream the *B. subtilis sacB* start codon
sacB2	TCG GTA AAT TGT CAC AAC GC	+32 to +53 downstream the *B. subtilis sacB* stop codon
LpR1	TAC AAA ATG TCG CTA TCT GG	−79 to −60 upstream the *L. biflexa rpsL* start codon
LpR2	TGT ATT TAG GAT CGC CTT CG	+52 to +71 downstream the *L. biflexa rpsL* stop codon
Plasmids		
pGKlep4	*E. coli–L. biflexa* shuttle vector (containing the *E. faecalis* Km^r gene)	Saint Girons *et al.* (2000)
pGKLS	pGKLep4 with a PCR fragment containing *B. subtilis sacB*	This study
pGKLrL	pGKLep4 with a PCR fragment containing *L. biflexa rpsL*$^+$	This study
pPAR1	pGKLrL with *flaB*::Km	This study
pPA2	pUC19 with *flaB*::Km replacing Amp^r	This study

a. Primer locations are indicated in basepairs (bp). Orientation of primers within the *L. biflexa flaB* locus is indicated in Fig. 1.

colonies was then obtained. UV irradiation of plasmid DNA at 25 mJ cm^{-2} or a 30 min alkaline treatment increased the number of recombinants up to 40 colonies per electrotransformation. In contrast, longer exposure of plasmid DNA to UV or to sodium hydroxide was less effective, although still more efficient than the absence of DNA treatment (Fig. 2). Fifty colonies were selected for further analysis by PCR and Southern blot. All colonies analysed resulted from homologous recombination with the *L. biflexa flaB* locus, i.e. no spontaneous Km-resistant mutants or integration of suicide vector into a chromosomal region other than the *flaB* locus were detected (Fig. 3). Three classes of mutants were identified: single cross-over mutants resulting from a single cross-over event with either the left or the right arm of *flaB*::Km and double cross-over mutants (Fig. 3B and C). Among the Km^r colonies, 5–10% (3/30 for 25 mJ cm^{-2} UV irradiation treatment and 1/20 for a 30 min alkaline treatment at 37°C) had undergone a double cross-over event.

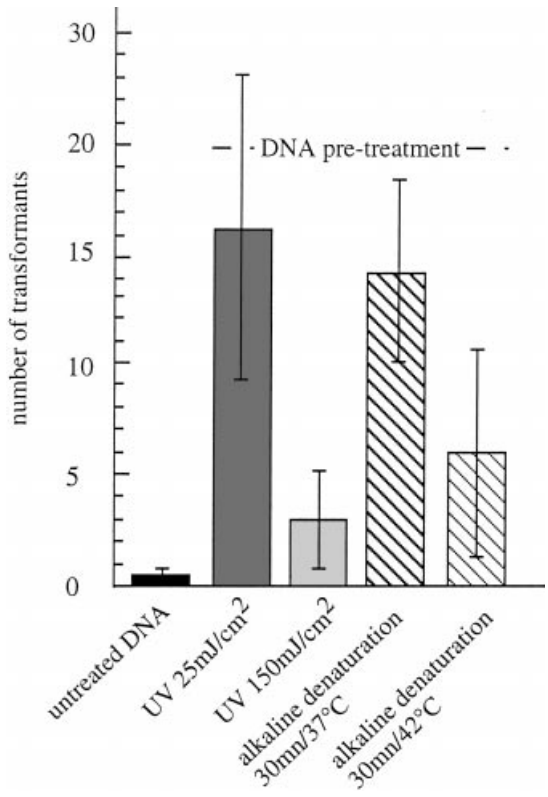
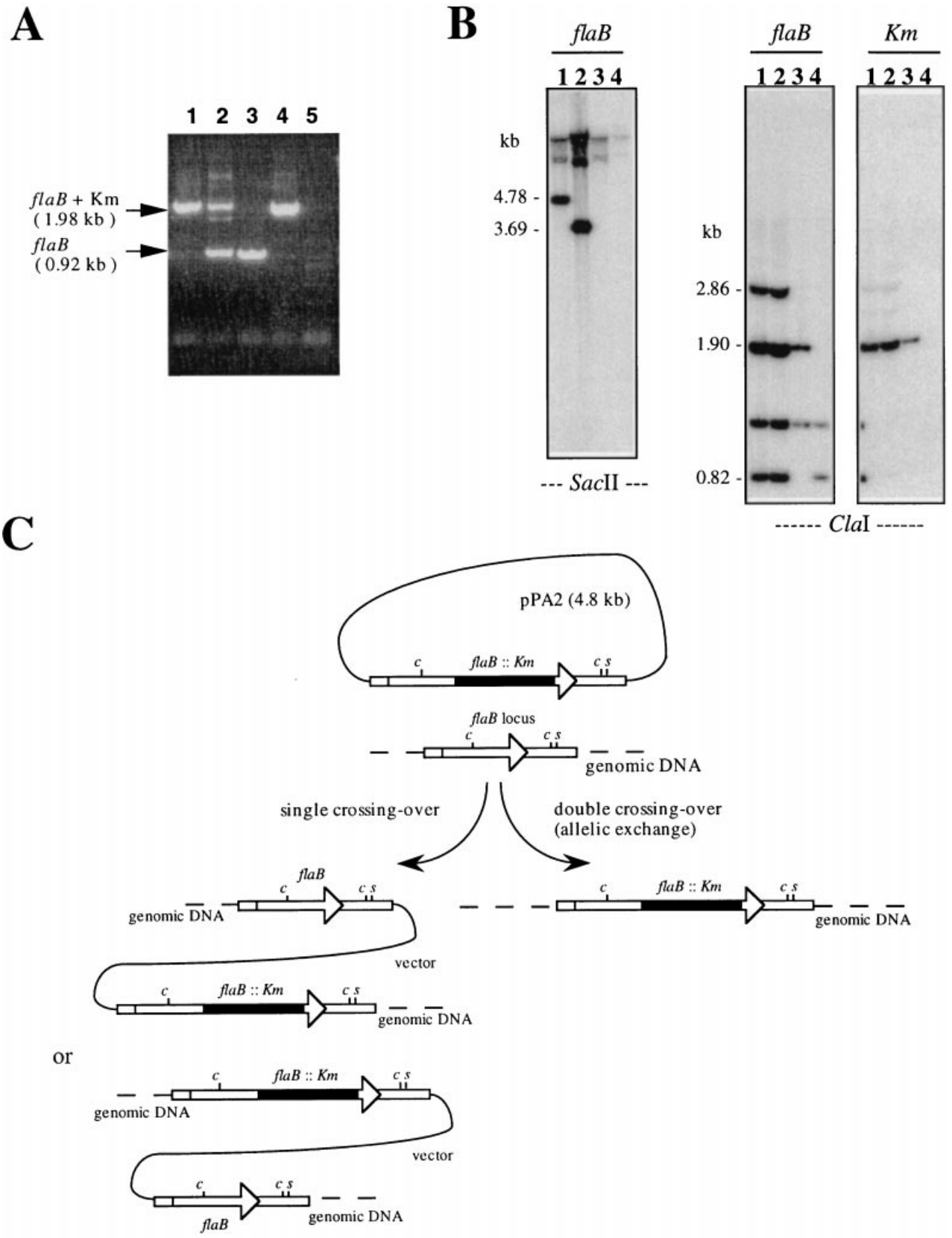


Fig. 2. Effect of pretreatment of plasmid DNA on the number of kanamycin-resistant transformants. The means and the standard deviations of the number of Km^r colonies of three independent experiments are given. DNA was treated as described in *Experimental procedures*.

Assessment of sacB *and* rpsL *as counterselectable markers in* L. biflexa

We used pGKLep4-derived plasmids to transfer putative counterselectable markers via electroporation from *E. coli* into recipient *L. biflexa*. The *L. biflexa–E. coli* shuttle vector pGKLep4 contains the following elements: a 2.2 kb fragment from bacteriophage LE1 responsible for replication of the vector in *L. biflexa*; an *E. coli* replication origin derived from pGEM7Zf$^{(+)}$ (Promega); and a Km resistance


A
1 2 3 4 5
flaB + Km
(1.98 kb)
flaB
(0.92 kb)
B
flaB
1 2 3 4
kb
4.78 -
3.69 -
--- SacII ---
flaB
Km
1 2 3 4
1 2 3 4
kb
2.86 -
1.90 -
0.82 -
------ ClaI ------
C
pPA2 (4.8 kb)
c
flaB :: Km
c s
flaB locus
genomic DNA
single crossing-over
double crossing-over
(allelic exchange)
flaB
genomic DNA
vector
or
flaB

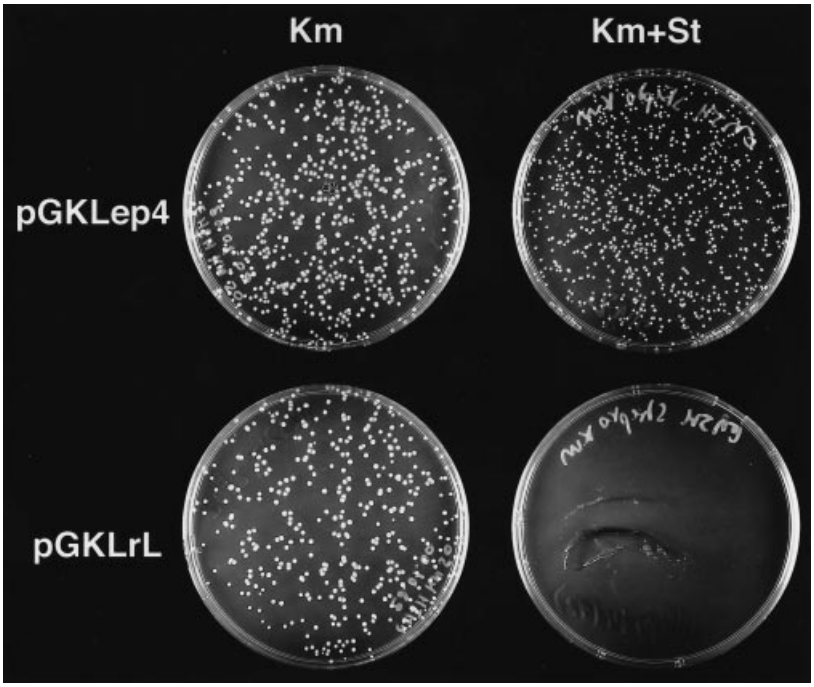


Fig. 4. Assessment of *rpsL* as a counterselectable marker in *L. biflexa*. Transformation of streptomycin-resistant *L. biflexa* with plasmids pGKLep 4 (top) and pGKLrL (bottom) plated onto EMJH supplemented with kanamycin (left) or with kanamycin plus streptomycin (right). Plates were incubated for 3 weeks at 32°C.

gene from the Gram-positive bacterium *E. faecalis* (Saint Girons *et al*., 2000).

The *rpsL* gene encodes the ribosomal protein S12. When provided in *trans*, the *rpsL* gene renders a streptomycin-resistant (Str^r) strain streptomycin sensitive (Str^s) in many bacteria (Reyrat *et al*., 1998). Spontaneous Str^r mutants of *L. biflexa* were selected from EMJH medium containing streptomycin. In Str^r mutants of *E. coli*, the nucleotide sequence of the *rpsL* gene is altered at specific locations (Funatsu and Wittman, 1972). The Str^r *L. biflexa* strain has a mutation in codon 88 (AAA → AGA) of the *rpsL* gene, resulting in an amino acid change of lysine to arginine, formerly identified as being responsible for the resistance to Str in other bacteria (Funatsu and Wittman, 1972). The *rpsL* gene of the *L. biflexa* wild-type strain (Zuerner and Charon, 1990) was amplified and cloned into pGKLep4 to generate pGKLrL. Electrotransformation of the Str^r *L. biflexa* strain with pGKLep4 or pGKLrL was equally efficient (Fig. 4). However, when pGKLrL was introduced into the Str^r *L. biflexa* strain, no colonies were obtained on medium supplemented with Km and Str (Fig. 4). In fact, transformation of pGKLrL into the Str^r *L. biflexa* strain resulted in a 100 000- to 120 000-fold lower number of transformants on Km-containing medium supplemented with Str. This result indicates that spontaneous Str^r colonies occurred at a frequency of around 10^{-5} in pGKLrL-transformed cells. This value was low enough to permit the use of *rpsL* as a counterselectable marker.

The *B. subtilis sacB* gene encodes the secreted enzyme levan sucrase (Steinmetz *et al*., 1983), the expression of which is lethal in a wide range of Gram-negative bacteria in the presence of sucrose (Reyrat *et al*., 1998). The *B. subtilis sacB* gene was cloned into the *L. biflexa–E. coli* shuttle vector pGKLep4, resulting in a vector called pGKLS. Although *E. coli* cells harbouring pGKLS were not able to grow on media that contained 10% sucrose, *L. biflexa* cells containing pGKLS grew as small colonies in the presence of 1%, 2%, 5% and 10% sucrose (data not shown). This indicates that *sacB* cannot be used as a counterselectable marker in *L. biflexa*.

Fig. 3. Evidence for homologous recombination within the *L. biflexa flaB* gene.
A. PCR with FIA and FIB primers of representative clones. Lanes: 1, double cross-over mutant strain; 2, single cross-over mutant strain; 3, *L. biflexa* wild-type strain; 4, plasmid pPA2; 5, negative control. The expected sizes of amplified products are indicated in parentheses.
B. Southern blot analysis of representative clones resulting from homologous recombination. Lanes 1 and 2, Km^r colonies resulting from a single crossing over with the right and left arm of the *flaB* locus respectively; 3, double cross-over mutant strain; 4, *L. biflexa* wild-type strain. Genomic DNA was digested with *Cla*I or *Sac*II and hybridized with the *flaB* or Km probe. The expected sizes are indicated on the left. For *Sac*II-digested DNA, flanking regions of the *flaB* integration site are the two upper bands. For *Cla*I-digested DNA, flanking regions correspond to the restriction fragment between 0.82 kb and 1.90 kb and a second restriction fragment lower than 0.82 kb (not shown here).
C. Schematic representation of clones obtained after homologous recombination within the *L. biflexa flaB* gene. Plasmid pPA2 represents the suicide vector used to inactivate the *L. biflexa flaB* gene. The black rectangle corresponds to the Km gene. *flaB*::Km indicates the mutant allele of the *flaB* gene. Km, gene encoding for kanamycin resistance; *c*, *Cla*I site; *s*, *Sac*II site.

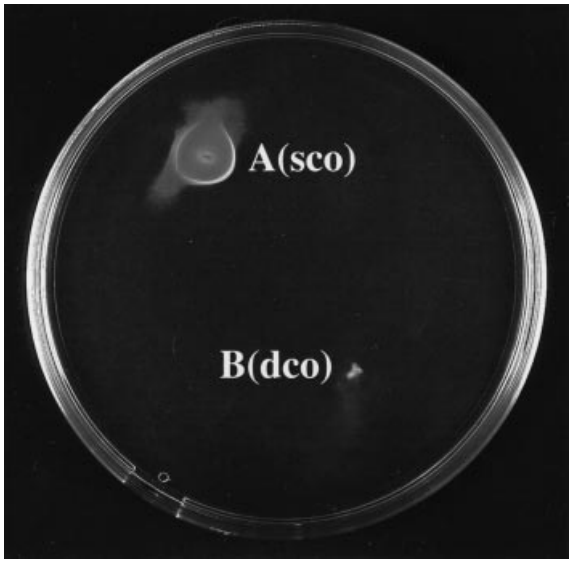


Fig. 5. Motility assay on 0.3% agar in EMJH medium supplemented with 50 μg ml^{-1} kanamycin. Single cross-over (sco) mutant strain (A) and double cross-over (dco) mutant strain (B) were incubated at 32°C for 4 days.

Use of rpsL *for gene replacement in* L. biflexa

Our results indicate that the native *rpsL* gene of *L. biflexa* is a suitable counterselectable marker in Str^r *L. biflexa*. The presence of two *Fsp*I sites in pGKLrL allowed the removal of the Km gene, generating blunt ends where a chosen inactivated target gene can be inserted. The interrupted *flaB*::Km gene was then cloned into a pGKLrL derivative vector, resulting in plasmid pPAR1. The plasmid was treated (or not) before electroporation as described above. After transformation of Str^r *L. biflexa*, a few kanamycin- and streptomycin-resistant (Km^r Str^r) colonies were obtained on counterselective medium only when pPAR1 was pretreated by UV irradiation of DNA at 25 mJ cm^{-2} or alkali denaturation (0.2 M sodium hydroxide) at 37°C for 30 min. The number of Str^r Km^r colonies is in agreement with the frequency of double recombination events obtained with the suicide vector. In all *L. biflexa* Str^r Km^r transformants tested thus far, PCR assays and hybridization patterns are consistent with a double cross-over between sequences flanking the Km resistance gene and the corresponding regions of homology in the *L. biflexa* genome, as shown in Fig. 3. The *rpsL* system can also be used for selective enrichment of allelic exchange by a two-step procedure. Transformants were first selected on Km, then on Km

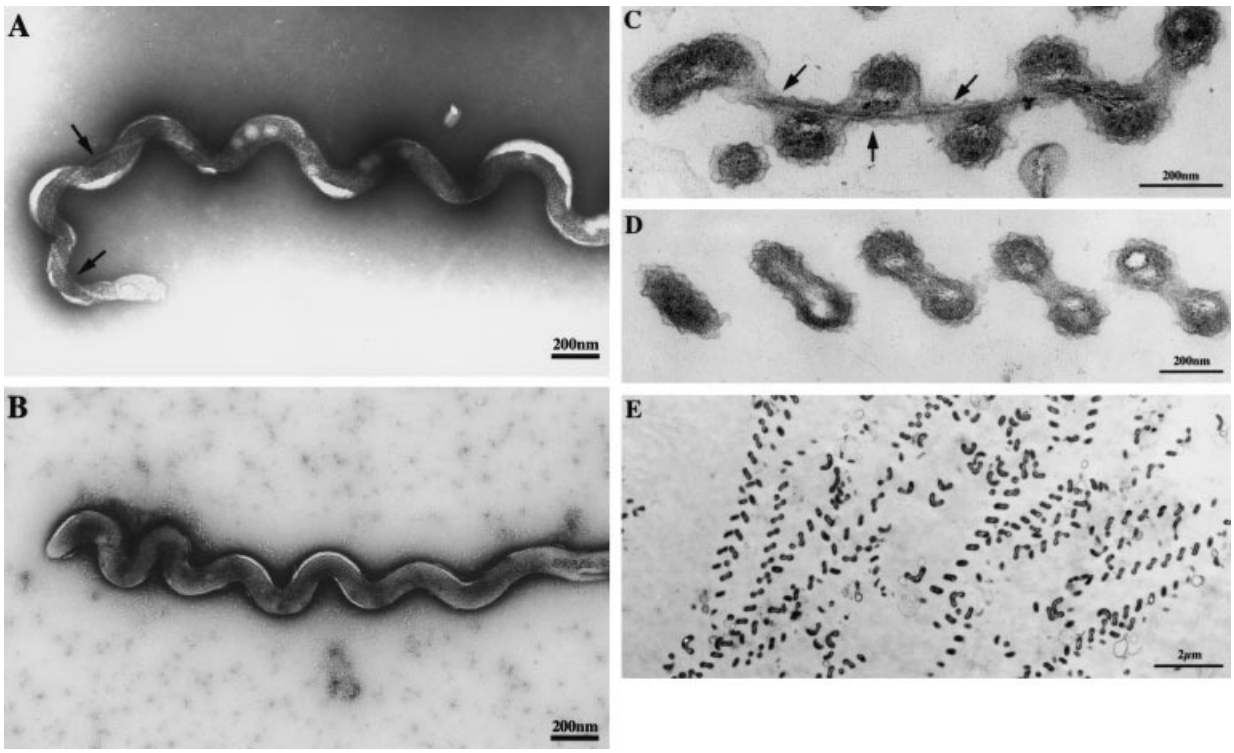


Fig. 6. Microscopic analysis of *L. biflexa* wild-type and *flaB* double recombinant mutant. Electron microscopy of *L. biflexa* wild-type strain (A) and *flaB* double cross-over mutant strain (B). Thin-section electron micrographs of wild type (C) and Str^r *flaB* mutant (D and E) of *L. biflexa* strain. Arrows point to the endoflagella.

plus Str. This should allow the double cross-over events to accumulate. Indeed, this two-step selection led to an enrichment for Km^r Str^r colonies by a factor of 10–50. Again, genetic analysis of clones is consistent with gene replacement of the *flaB* chromosomal copy by the Km gene-disrupted allele carried on the plasmid. However, when a high number of transformants was obtained ($>$ 100 Km^r Str^r colonies on a plate), a significant proportion resulted from spontaneous Str^r mutation in the plasmid-borne *rpsL*$^+$ allele (data not shown). Although spontaneous mutations of the *rpsL*$^+$ allele occurred at low frequency, this does not interfere significantly with the ability to select for allelic exchange directly if $< 10^5$ Km^r bacteria are spread on counterselective medium.

Evidence for non-motility and deficiency in endoflagella of the flaB *mutants*

We selected one *flaB* double recombinant from both wild-type and Str^r *L. biflexa* for further analysis. We noted that cultures of *L. biflexa flaB* mutants in EMJH liquid medium rapidly sank to the bottom of the tube, whereas wild-type strains produced homogeneous suspensions. Motility assays on soft agar plates show a dramatic difference between mutants and wild-type strain, as both double recombinant mutants failed to swim on soft agar plates, whereas single cross-over mutants and the wild-type strain spread quite readily (Fig. 5; data not shown). Examination of *flaB* double recombinants by dark-field microscopy revealed low motility compared with the high velocity of the wild-type strain. Furthermore, the mutants could not rapidly reverse the direction of swimming like the wild-type strain in exponential phase cultures (data not shown). In *Leptospira* spp., the endoflagella are more rigid than the cell cylinder, causing the end of the cell to conform to the shape of their flagella; thus, depending on the direction of rotation of flagella, ends of the cell are hook or spiral shaped (Li *et al*., 2000a). As viewed by both dark-field and phase-contrast microscopy, the cell cylinder of mutants was not as bent and flexible as those of the wild-type strain, and mutants were longer. Phase-contrast microscopy of fixed cells shows that 60/62 bacteria of the wild-type strain have hook or spiral shaped ends. In contrast, 50/60 bacteria of the *flaB* mutant clearly lacked the bent-end morphology (data not shown). Because of the small diameter of *Leptospira* spp. (cell diameter 0.1–0.15 μm), their ultrastructure could not be distinguished by dark-field microscopy. Examination by electron microscopy of wild-type strains negatively stained with 2% uranyl acetate shows that endoflagella were visible through the outer membrane. A single endoflagellum was observed starting from the cell ends. The length of the endoflagella appears to be similar to the length of the bent ends (Fig. 6A); endoflagella have never been observed in the centre of the cell, consistent with the evidence that they do not overlap in the centre of the cell (Goldstein and Charon, 1988). Mutants have retained their body helices but have lost their hook-shaped ends (Fig. 6B). In addition, we were not able to distinguish endoflagella in the *flaB* mutants (Fig. 6B). Thin sections of embedded cells confirm that, although the wild-type strain contained one short endoflagellum along the axis of the cell at each end (Fig. 6C), mutants retained their helical body but lacked endoflagella (Fig. 6D and E).

Discussion

Previous attempts to inactivate genes in *Leptospira* spp. have been unsuccessful. We have applied a method used recently in mycobacteria and *Streptomyces* spp. (Oh and Chater, 1997; Hinds *et al*., 1999) to carry out gene targeting by homologous recombination in *L. biflexa*. This methodology uses the organism's endogenous machinery of DNA repair and/or recombination to integrate DNA at a target locus as directed by DNA sequence homology. The rationale is that UV-irradiated or alkali-denatured DNA molecules may provide a more recombinogenic substrate for DNA repair and/or recombination systems of the cell host.

We first determined the nucleotide sequence of the *L. biflexa flaB* region and then constructed a plasmid suicide vector with *flaB*::Km to deliver the inactivated target gene into the *L. biflexa* chromosome. Successful gene replacement was only achieved by using UV- or alkali-treated suicide plasmid vector. Using a similar methodology, successful *flaB* gene replacement has also been achieved using shorter flanking DNA in the *flaB* construct (0.35 kb and 0.45 kb compared with 0.9 kb and 0.8 kb; data not shown). We can easily differentiate single cross-overs (integrated plasmids) from double cross-overs (gene replacement) by Southern and PCR analysis of colonies. The stimulatory effect of homologous recombination was maximum after UV irradiation of DNA at 25 mJ cm^{-2} and alkali denaturation (0.2 M sodium hydroxide) at 37°C for 30 min. The number of transformants was reduced when UV or alkali treatment was increased, probably because of severe DNA damage imposed on the plasmid vector. In comparison with other bacteria, *L. biflexa* is relatively resistant to UV (Stamm and Charon, 1988). It should be noted that repair-deficient mutants, such as *uvrA* and *recA* mutants, of *E. coli* and *B. subtilis* are dramatically more sensitive to UV than the wild-type strains (Howard-Flanders, 1968; Friedman and Yasbin, 1983). In addition, the *recA* gene was identified previously in *L. biflexa* and was found to complement *recA* mutations of *E. coli* functionally (Stamm *et al*., 1991). Taken together, these results indicated that *L. biflexa* may possess an efficient DNA repair system. Introduction of altered DNA into the

bacterial cell may recruit the recombination machinery for initiating homologous pairing and strand exchange. Because no spontaneous Km^r colonies were found, the Km marker, which is expressed in *L. biflexa* recombinants, is a very convenient marker. To date, we have not seen aberrant integration events (illegitimate recombination), i.e. UV-irradiated or alkali-denatured DNA specifically enhanced homologous recombination. The methodology of using UV-irradiated and alkali-denatured DNA was also applied to inactivate the *Leptospira meyeri metY* that encodes for an *O*-acetylhomoserine sulphydrylase involved in methionine biosynthesis (Belfaiza *et al*., 1998). Again, we obtained several recombinants with a plating efficiency even higher than that of *L. biflexa flaB* (data not shown). Among the Km^r colonies, 14% (5/36) had undergone a double cross-over event. This result suggests that other genes as well as other *Leptospira* spp. may behave similarly to *L. biflexa flaB* for gene replacement experiments.

Our data indicate that the *rpsL* gene is a suitable counterselectable marker in *L. biflexa*, but the *sacB* system cannot be applied to *L. biflexa*. The *rpsL* system is based on the well-known phenomenon that Str^r mediated by mutations in the *rpsL* gene is recessive to the wild-type *rpsL* gene. Allelic exchange using the *rpsL* system was first demonstrated in *E. coli* (Rusell and Dahlquist, 1989) and, more recently, in *Mycobacterium smegmatis* (Sander *et al*., 1995) and *Streptomyces roseosporus* (Hosted and Baltz, 1997). Double recombinant mutants can be selected directly on Km plus Str plates after electroporation. Although this experiment worked well using a one-step selection with plasmid DNA pretreated as described above, a two-step procedure can be applied in cases where the double recombination frequency is too low. In the first step, single recombinants are selected using the antibiotic gene marker used for gene disruption (e.g. the Km gene). These recombinants are grown to stationary phase, thus allowing secondary recombination events to accumulate, then plated on medium containing Km and Str. All the tested *L. biflexa* transformants selected on counterselective plates (Str^r Km^r colonies) were indeed allelic exchange mutants. However, when the two-step procedure is applied, mutations of the *rpsL* allele carried on the plasmid also lead to the appearance of spontaneous Str^r colonies. It should be possible to adapt the *rpsL* system to other species of *Leptospira* and other spirochaetes. The only prerequisites needed are the existence of a spontaneous Str^r host (owing to a mutation within *rpsL*) and cloning of the $rpsL^+$ gene of that organism. Either a replicative or a suicide vector can be used for the delivery vector. In the latter case, a one-step procedure is applied. Such an *rpsL* vector can also be used to develop an efficient insertional mutagenesis system by delivering a transposon element into the chromosome. Insertional mutants are then selected after inducing the loss of the delivery system.

Motility of leptospires is dependent on the presence of two endoflagella (or periplasmic flagella), one arising at each end of the spirochaete, located between the protoplasmic cylinder and the outer membrane sheath. The motility of the spirochaete allows these organisms to swim through gel-like media, such as connective tissues, which inhibit the motility of most other bacteria (Li *et al*., 2000a), suggesting that it could play an important role as a virulence factor. *Leptospira* spp. differ from other spirochaetes in having hook-shaped and/or spiral-shaped ends. The *flaB* gene encodes one of the two major proteins of the flagella (Faine *et al*., 1999). The FlaB proteins of *Leptospira* spp. show significant similarities to flagellin proteins of other spirochaetes as well as both Gram-positive and Gram-negative bacteria. In the spirochaete *B. hyodysenteriae*, targeted mutagenesis of *flaB1*, closely related to *L. biflexa flaB* (closest homologue in a BLAST search), *flaB2* or *flaB3*, did not affect the synthesis of flagella, and mutants were morphologically normal. This suggested that specific inactivation of one FlaB species could be substituted for another FlaB species (the three FlaB proteins share 37–51% sequence identity) and therefore yielded mutants capable of assembling intact flagella (Rosey *et al*., 1995; Li *et al*., 2000b). Along the same lines, *Treponema* spp. also contained three class B proteins for their endoflagella (FlaB1, FlaB2 and FlaB3) (Ruby *et al*., 1997; Fraser *et al*., 1998). In contrast, in *B. burgdorferi*, inactivation of the unique *flaB* gene prevented the filament from assembling. Mutants were non-motile, deficient in endoflagella and rod shaped (Motaleb *et al*., 2000). Likewise, *L. biflexa flaB* mutants were not able to form functional endoflagella, but their cell body remained intact and helical. Therefore, endoflagella in *Leptospira* spp. did not dictate the helical shape of the cell body. FlaB does not have skeletal functions as observed in the *B. burgdorferi flaB* mutant (Motaleb *et al*., 2000). It should be noted that, although *Borrelia* endoflagella extend and wrap along the cell body (Li *et al*., 2000a), this is not the case for *Leptospira* spp. However, the *L. biflexa flaB* mutants differ from the *L. biflexa* wild-type strain in that they are relatively straight, not flexible and without hook-shaped ends. Such linear mutants were also observed in *Leptonema illini*, belonging to the same Leptospiraceae family as *Leptospira* spp., after nitrosoguanidine treatment (Bromley and Charon, 1979). The characteristic hook- and spiral-shaped ends of *Leptospira* spp. were thought to be caused by the rotation of the endoflagella (rotary motors are located at their bases; Goldstein and Charon, 1988). Therefore, when the endoflagella were not rotating (or not present), these bent ends disappeared. The non-motility of our *flaB* mutants should not be the result of polar effects on downstream or upstream genes. Indeed, only an ORF encoding the N-terminus of a putative threonine dehydratase (with more than 35% identity with *Bacillus halodurans*

and *Mycobacterium leprae* putative threonine dehydratases) was found 350 bp downstream the *L. biflexa flaB*. Therefore, gene inactivation of *L. biflexa flaB* demonstrates that FlaB is involved in flagella assembly and motility.

In conclusion, construction of isogenic mutants of *L. biflexa* by genetic techniques is now feasible. We anticipate that virtually any non-essential genes of the *L. biflexa* genome could be targeted by the methodologies described in this paper. *Leptospira* spp. are among the easiest spirochaetes to grow, and their motility is structurally the least complex (Li *et al*., 2000a). Therefore, the availability of an efficient mutagenesis system in *L. biflexa* will facilitate the inactivation of genes involved in motility and should yield a better understanding of the complex geometry and motility mechanism of spirochaetes. The use of a suicide delivery vector does not require complex vector constructions and provides the advantage that it can be used in pathogenic species, for which no shuttle vectors are available. We could therefore envisage carrying out gene replacement experiments of putative virulence factors to define their role precisely. For example, we are interested in assessing the virulence of flagellin or haemolysin (Lee *et al*., 2000) mutants of pathogenic species in *in vivo* models.

Experimental procedures

Bacterial strains and culture conditions

L. biflexa serovar patoc strain Patoc 1 (National Reference Centre, Institut Pasteur, France) was grown at 32°C in EMJH (Ellinghausen and McCullough, 1965; Johnson and Harris, 1967) liquid medium or on EMJH 1% agar plates. Colonies were also stabbed in 0.3% agar of EMJH medium with or without kanamycin for motility assays. A streptomycin-resistant mutant of *L. biflexa* serovar patoc strain Patoc 1 was isolated spontaneously as described previously (Fukunaga and Mifuchi, 1988). *E. coli* XL10-Gold (Stratagene) was grown at 37°C on solid or liquid Luria–Bertani (LB) medium. When necessary, antibiotics were used at the following concentrations: kanamycin, 50 μg ml^{-1}; streptomycin, 50 μg ml^{-1}. For the *sacB* system, solid medium was supplemented with 1%, 2%, 5% or 10% sucrose.

PCR

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), and a final cycle of extension for 10 min at 72°C. Primer pairs used for PCR are indicated in Table 1.

Southern blotting

Genomic DNA of *L. biflexa* was isolated as described previously (Meier *et al*., 1985). Genomic DNA was digested with *Sac*II or *Cla*I and separated overnight by electrophoresis through 1% agarose gels. DNA was then transferred to a nylon membrane (N$^+$-Hybond; Amersham) (Sambrook *et al*., 1989). Probes for *flaB* and Km were amplified from *L. biflexa* (primers FlB and FlC) and recombinant plasmid pAT21 (Trieu-Cuot and Courvalin, 1983) (primers Kan3 and Kan5), respectively, then labelled with [α-^{32}P]-dCTP using the Megaprime DNA labelling kit (Amersham). Membranes were hybridized overnight at 65°C in Rapid hybridization buffer (Amersham), followed by stringent washing.

Cloning of L. biflexa flaB

After nucleotide sequence alignment of the *flaB* loci of *L. interrogans* serovar autumnalis (accession number AF064055), *Leptospira borgpetersenii* (accession number AF064057) and *Leptospira* sp. strain Akiyami (accession number AB027157), degenerate primers FlaN and FlaT were designed to amplify the corresponding region in *L. biflexa*. A faint amplified product of the expected size (744 bp) was obtained by PCR and cloned into pCR2.1 (Invitrogen) for sequencing. We deduced nucleotide sequences of the flanking DNA of *L. biflexa flaB* by LM-PCR (Prod'hom *et al*., 1998). Briefly, double-stranded linkers (composed of primers LK1and LK2) were ligated to cohesive ends of *Eco*RI-digested genomic DNA of *L. biflexa*, and PCR was performed using one primer specific for the *L. biflexa flaB* (primers flaAM and FlA are directed upstream and downstream from *flaB* respectively; see Fig. 1 and Table 1) and a second primer specific for the *Eco*RI-adapted linker (primer LK2). Both sides of *flaB* were thus amplified, and PCR products (351 bp and 944 bp) were cloned into pCR2.1 (Invitrogen) for sequencing.

Suicide and replicative plasmid constructs for allelic exchange

The 1683 bp fragment of the *L. biflexa flaB* region amplified by PCR with primers FlB and FlC was first cloned into pCR2.1 (Invitrogen). The *flaB* fragment was then released by *Spe*I and *Xho*I digestion, blunt ended and cloned into *Pvu*II-cut pUC19; the resulting vector was called pP16. The *E. faecalis* kanamycin cassette was amplified from plasmid pAT21 (Trieu-Cuot and Courvalin, 1983) with primers Kan3 and Kan5. The resulting 1062 bp amplified product and the *Hin*dIII-cut pP16 (*Hin*dIII cuts once in *flaB* of pP16) were blunt ended and ligated. After deletion of the ampicillin cassette of pUC19 by *Sca*I and *Aat*II digestion, blunt ending and religation, the resulting pPA2 was used as a suicide vector to deliver the inactivated *flaB* in *L. biflexa*. To obtain allelic exchange in *L. biflexa*, we also constructed a replicative vector containing the *rpsL* gene as a counter-selectable marker (*E. coli–L. biflexa* shuttle vector pGKLrL described below) by transferring a 3 kb *Hae*II fragment containing *flaB*::Km from pPA2 into *Fsp*I-cut pGKLrL (this step also removes the kanamycin cassette of pGKLep4), thus generating pPAR1 (Table 1).

Assessment of counterselectable markers in L. biflexa

The *B. subtilis sacB* gene was amplified from vector pPR29 (Pelicic *et al*., 1997) with primers sacB1 and sacB2 (Table 1).

Amplified products were cloned into pCR2.1 (Invitrogen), and the resulting inserts were released by *Eco*RV and *Bam*HI digestion and then inserted into the *Sma*I and *Bam*HI site of pGKLep4 (Saint Girons *et al*., 2000). Plasmid constructs were introduced into *E. coli* XL10-Gold (Stratagene) by electroporation (Gene Pulser unit; Bio-Rad), and transformants were selected on LB agar supplemented with kanamycin. Plasmids were recovered using a Qiaprep Spin miniprep kit (Qiagen). Plasmid constructs were tested for *sacB* expression by plating *E. coli* transformants onto LB medium containing kanamycin and 10% sucrose, then introduced in *L. biflexa*. Electrocompetent cells of *L. biflexa* were prepared as described previously (Saint Girons *et al*., 2000). Briefly, *L. biflexa* cultures were grown to exponential phase, and pellets were washed twice in water and resuspended in water at room temperature. The competent cells were electroporated in the presence of 2–5 μg of vector DNA and then transferred to 1 ml of EMJH liquid medium, in which they were incubated overnight at 32°C before plating.

The *rpsL*$^+$ gene was amplified from *L. biflexa* (Zuerner and Charon, 1990) with primers LpR1 and LpR2, using *Pfu* polymerase (Stratagene), and cloned into the *Sma*I site of pGKLep4 (Saint Girons *et al*., 2000) to produce pGKLrL. Str^r *L. biflexa* cells were electroporated with pGLKrL as described above; individual kanamycin-resistant colonies were then inoculated into kanamycin-containing EMJH liquid medium and incubated at 32°C to stationary phase. Cultures were then spread onto EMJH–kanamycin plates with or without streptomycin. For *flaB* gene replacement using the *rpsL* system, a one- or two-step procedure was performed. *L. biflexa* competent cells (Str^r strain) were electrotransformed with pPAR1 (pGKLrL with *flaB*::Km), incubated overnight in EMJH liquid medium, and then transformants were selected on EMJH–kanamycin plates with (one-step procedure) or without streptomycin. For the two-step procedure, individual kanamycin-resistant colonies, appearing in 10–14 days, were picked, inoculated into kanamycin-containing EMJH liquid medium and incubated at 32°C to stationary phase. Cultures were then spread onto EMJH–kanamycin plates supplemented with streptomycin and incubated at 32°C to select the double recombination events.

Gene replacement protocols

Double-stranded DNA was irradiated by UV or denatured by alkali treatment (Hinds *et al*., 1999) as follows. DNA was denatured in a total volume of 20 μl containing 0.2 mM EDTA−0.2 M NaOH for 15–30 min at 37°C or 42°C. Two microlitres of 3 M sodium acetate, pH 4.8, was added, and the mixture was purified using Qiaquick mini-columns (Qiaquick PCR purification kit; Qiagen). DNA was eluted with 18 μl of H_2O, and DNA was kept on ice before electroporation. For UV treatment, 18 μl of DNA solution in a droplet on a plate was subjected to 25, 50, 150 or 250 mJ cm^{-2} UV irradiation in a UV chamber (GS Gene linker; Bio-Rad), and DNA was kept on ice before electroporation.

Microscopy

Cell motility of exponential phase cultures was analysed using dark-field microscopy. We also used phase-contrast microscopy of fixed cells for analysis of cell morphology. For electron microscopy, cells were centrifuged at 2000 *g* for 4 min, and the pellet was washed in 1× PBS. To visualize bacteria by negative staining, cells were resuspended in 10 mM Tris-HCl, pH 8, then placed on a carbon-coated grid for 1 min and stained with 2% uranyl acetate for 30 s. For electron microscopy of thin sections, bacteria were resuspended with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 1 h at room temperature. Cells were collected by centrifugation and embedded in agarose type VII (Sigma) using microvette CB300 (Sarstedt). Small pieces of agar containing bacteria were post-fixed with 1% osmium tetroxide in cacodylate buffer for 1 h, then rinsed. Samples were embedded in Poly/Bed 812 (Polysciences) after ethanol dehydration. Blocks were then sectioned conventionally, stained and observed.

Accession number

The GenBank accession number for the *L. biflexa flaB* nucleotide and amino acid sequence is AF320637.

Acknowledgements

The Institut Pasteur supported this work. M.P. thanks the Fondation de France (Prix Jacques Monod) for financial support. It represents a portion of a thesis submitted by A.B. (recipient of Pasteur–Weizmann fellowship) to the University of Paris VII for the PhD degree. We are grateful to Evelyne Couture-Tosi and Pierre Gounon for electron microscopy analysis. We thank Tony Pugsley and Guy Baranton for helpful comments on the manuscript.

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