Cloning of genes required for amino acid biosynthesis from *Leptospira interrogans* serovar *icterohaemorrhagiae*

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Leptospira interrogans belongs to a large family of important pathogens, which is part of the order Spirochaetales, a distinct group of eubacteria. In order to obtain a better understanding of the genetic organization of this species, we have constructed a DNA library of the serovar *icterohaemorrhagiae*, using the *Escherichia coli* vector pUC13. We have isolated *Leptospira* DNA fragments containing the genetic information required to complement strains of *E. coli* with defects in proline and leucine biosynthesis. While a 3.9 kb fragment which complemented *proA* also complemented *proB*, a 15 kb fragment complementing *leuB* could not complement other *leu* mutations. The *L. interrogans* origin of the cloned DNA fragments was confirmed by DNA–DNA hybridization. The hybridization was specific to the pathogenic species and was not seen with the saprophytic species *L. biflexa*.

Introduction

Leptospira, a member of the order Spirochaetales, is a genus composed of thin, motile, aerobic, spiral-shaped bacteria (Johnson, 1977). The two species, *L. interrogans*, which is pathogenic for man and animals, and *L. biflexa*, which is saprophytic, contain many different serovars. 16S rRNA phylogenetic studies suggest that the spirochaetes represent a group of eubacteria of ancient origin (Paster *et al.*, 1984). *Leptospira* has simple nutritional requirements. The only necessary organic compounds are vitamins B12 and B1, and long-chain fatty acids, which serve as the obligate carbon and energy sources (Johnson & Gary, 1962; Johnson *et al.*, 1969).

Radiotracer studies (Charon *et al.*, 1974) similar to those done on *Escherichia coli* (Roberts *et al.*, 1955) indicate that the known biosynthetic pathways for amino acids are used in *Leptospira*. One exception is that isoleucine is not exclusively derived from threonine (Charon *et al.*, 1974; Westfall *et al.*, 1983). Studies on the organization and regulation of the amino acid biosynthetic genes in *Leptospira* are still in their infancy. Feedback inhibition of anthranilate synthase and threonine deaminase by tryptophan and isoleucine respectively has been demonstrated (Yelton & Peng, 1989; Westfall, 1980). No support for any mechanism of regulation at the level of gene expression (by repression or attenuation, for example) has been given. Three biosynthetic genes (*argE*, *trpG* and *trpE*) from the non-pathogenic species *L. biflexa* were cloned by complementation of the corre-

sponding *E. coli* mutants and then sequenced (Zuerner & Charon, 1988; Yelton & Charon, 1984; Yelton & Cohen, 1986). The *trpE* gene from *Spirochaeta aurantia*, which also belongs to the order Spirochaetales, has also been cloned (Brahamsha & Greenberg, 1987).

Since *Leptospira* has a unique morphology, physiology and evolutionary peculiarity, we were interested in its genetics. Here we report the cloning of *L. interrogans* genes which complemented mutations in the *proA*, *proB* and *leuB* genes of *E. coli*. Similarities and differences in their organization relative to the corresponding *E. coli* genes are discussed.

Methods

Bacterial strains, plasmids and media. *Escherichia coli* strains and plasmids are listed in Table 1. *E. coli* strains were grown in Luria broth in liquid or 0.75% agar (Miller, 1972). The following *Leptospira* strains (national reference centre, France) were used: *L. interrogans* serovar *icterohaemorrhagiae* strain Verdun and *L. biflexa* serovar *patoc* strain Patoc 1. Minimal 63 medium (Miller, 1972) supplemented with 0.2% glucose, 50 µg ampicillin ml⁻¹ and 1 µg thiamin ml⁻¹ was used to characterize *E. coli* transformants; the medium allowing selection for Pro⁺ and Leu⁺ transformants additionally contains leucine and proline at concentrations of 2 mM. *Leptospira* strains were grown in EMJH medium (Ellinghausen & McCullough, 1965; Johnson & Harris, 1967). *Borrelia burgdorferi* strain B31 was grown in BSKII medium (Barbour, 1984).

Recombinant DNA techniques. *Leptospira* chromosomal DNA was isolated by the method of Silhavy *et al.* (1984). Restriction enzyme digests, ligations, agarose gel electrophoresis, and small-scale plasmid isolations were by standard procedures (Maniatis *et al.*, 1982). The

Table 1. *E. coli* strains and plasmids

Relevant genotype		Source or reference
<i>E. coli</i> strains		
GT869	<i>thrB1004 pro thi rpsL hsdS lacZAM15 (F' lacZAM15 lac^h traD36 proA⁺B⁺)</i>	Parsot (1986)
HB101	<i>F⁻ hsdS recA13 ara-14 proA2 leuB6 lacY1 galK2 rpsL20 xyl-15 mtl-1 supE44 λ</i>	Boyer & Roulland-Dussoix (1969)
AR1062	<i>thr leu lac gal xyl mal mtl min hsdS</i>	Rambach & Hogness (1977)
JM83	<i>ara Δ(lac-pro) rpsL thi φ80 dlacZAM15</i>	Vieira & Messing (1982)
X340	<i>proB28 metB1 relA1 spoT1 λ⁻</i>	B. Bachmann (Yale University)
DJ105	<i>F⁻ leu proC trp his ilv argH purD met ara lac mal gal str</i>	J. H. Miller (Los Angeles, USA)
CV512	<i>F⁺ leuA321</i>	I. M. Calvo (Ithaca, NY, USA)
CV514	<i>F⁺ leuB401</i>	
CV516	<i>F⁺ leuB061</i>	
CV520	<i>F⁺ leuC171</i>	
CV522	<i>F⁺ leuC222</i>	
CV524	<i>F⁺ leuD211</i>	
CV526	<i>F⁺ leuD101</i>	
MC1061	<i>araD139 Δ(ara, leu)7697 ΔlacX74 galU galK hsdR rpsL</i>	Casadaban & Cohen (1980)
Plasmids		
pUC9	Ap ^R	Vieira & Messing (1982)
pUC13	Ap ^R	Messing (1983)
pSKS114	Ap ^R Cm ^R	Shapira <i>et al.</i> (1983)

method of Hanahan (1983) was used for *E. coli* transformation. Ap^r transformants were selected at 37 °C on plates containing 50 µg ampicillin ml⁻¹, 20 µg 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside ml⁻¹ and 5 mM-isopropyl thiogalactopyranoside. DNA from *L. interrogans* was partially digested with restriction endonuclease *Bam*HI and mixed with an approximately equimolar amount of pUC13. The pUC13 DNA was purchased from Pharmacia, already digested to completion with *Bam*HI and dephosphorylated. The DNA mixture was incubated with T4 DNA ligase for 18 h at 4 °C. The entire ligation mixture (0.5 µg of total DNA) was used to transform *E. coli* strain GT869. By use of this procedure, a total of 8000 transformants (i.e. 30 transformants per ng of vector DNA) were obtained, 75% of which contained inserts with an average size of 8–9 kb. Total plasmid DNA was extracted from the pool of Ap^r transformants by the alkaline method of Birnboim & Doly (1979) (adjusted for a large amount of cells) with a recovery of 5 mg of plasmid DNA.

DNA–DNA hybridizations. For blot analysis according to Southern (1975), DNA was transferred to a nylon membrane. Probes were radiolabelled with [³⁵S]dCTP (37 TBq mmol⁻¹) by the multiprimer method (Feinberg & Vogelstein, 1983). Labelled DNA (50 ng) was added to the filter and allowed to hybridize at 65 °C for 18 h. The filter was washed at 65 °C in 6 × SSC then 2 × SSC, air dried and exposed to X-ray film (Kodak X-Omat AR2) for 48 h at –80 °C (1 × SSC is 0.15 M-NaCl, 0.015 M-trisodium citrate, pH 7.0).

Results

A colony bank of 8000 individual Ap^r transformants in *E. coli* strain GT869 was obtained from *L. interrogans* serovar *icterohaemorrhagiae* using the *E. coli* multicopy vector pUC13. The serovar corresponds to one used in France to prepare human vaccine.

Cloning of Leptospira interrogans genes encoding *proA*- and *leuB*-complementing activities

A sample (1 µg) of total plasmid DNA isolated from the clone bank was used to transform *E. coli* HB101, which

carries mutations in the *proA* and *leuB* genes. About 10000 Ap^r transformants were obtained on LB rich medium. These clones were then screened by direct replica plating on synthetic medium allowing selection for Pro⁺ or Leu⁺. Twenty Pro⁺ clones (0.2%) and five Leu⁺ (0.05%) were thus obtained. Ten Pro⁺ and the five Leu⁺ clones were studied further.

The plasmid DNA of the 10 Pro⁺ and five Leu⁺ clones was analysed by agarose gel electrophoresis after *Bam*HI digestion (Fig. 1). The Pro⁺ plasmids (pPL1 and pPL4) and the Leu⁺ plasmids (pLL1) contained inserts of 6.4 and 15 kb respectively. *Eco*RI restriction enzyme analysis of all these plasmids gave two patterns corresponding to both orientations for the inserts present in the Pro⁺ plasmids, indicating possible expression from *Leptospira* promoters or from *Leptospira* sequences functioning as such in *E. coli*; however, in the case of the five Leu⁺ plasmids, only one *Eco*RI pattern was obtained, indicative of a unique orientation of the 15 kb *Bam*HI insert (see below).

To determine whether the acquired characters were plasmid-borne, the Pro⁺ and Leu⁺ plasmids were used to transform *E. coli* HB101 selecting for ampicillin resistance. The clones obtained were then screened for proline or leucine independence: all the Ap^r transformants obtained with pPL1 and pPL4 were simultaneously Pro⁺ and all those obtained with pLL1 were simultaneously Leu⁺. These results confirm that complementing activities are associated with the recombinant plasmids.

In order to localize the *proA* and the *leuB* genes more precisely on the corresponding plasmids and to reduce the *Leptospira* DNA fragment, different subclonings were performed in each case as described in Fig. 1. These reductions resulted in the following plasmids: pPL11

Fig. 1. Subclone analysis of *Pro*⁺ and *Leu*⁺ plasmids. (a) Restriction map of *leuB*-complementing plasmids. pLL1 was isolated from the *L. interrogans* genomic library. Elimination of two *Pst*I fragments gave rise to pLL11. Subsequent elimination of a 2.5 kb *Sac*I fragment gave rise to pLL12. Subcloning of a *Hind*III fragment from pLL12 into the corresponding site of pUC9 generated pLL13. pLL131 was obtained by *Hind*III digestion and religation of pLL13 in order to reverse the orientation of the insert. pLL14 and pLL15 were obtained by inserting into *Cla*I-digested pLL13 a *Cm*^r 'cassette' from *Acc*I-digested pSKS114, creating at the same time a 0.5 kb deletion in the *Leptospira* insert. (b) Restriction maps of *proA/B*-complementing plasmids. pPL1 and pPL4 were isolated from the *L. interrogans* genomic library. Total digestion of pPL1 with *Eco*RI and self-ligation of the large *Eco*RI-*Eco*RI fragment obtained gave rise to pPL11. pPL12 was obtained by insertion of the *Cm*^r 'cassette' of *Bam*HI-digested pSKS114 into the unique *Bgl*II site of pPL11. Thin and heavy lines represent pUC vectors and *Leptospira* DNA inserts respectively; dashed lines represent the *Cm*^r 'cassette' from pSKS114. B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; P, *Pst*I; H, *Hind*III; S, *Sac*I.

carrying a 3.9 kb *Eco*RI-*Bam*HI fragment complementing the *proA2* allele and plasmid pLL13 carrying a 3.2 kb *Hind*III fragment complementing the *leuB6* allele. The orientation of the *Hind*III insert of pLL13 (Fig. 1a) has been reversed and the resulting plasmid (pLL131) still complements the *leuB* mutant, suggesting an expression of the gene from *Leptospira* DNA as was the case for the proline gene.

Fig. 2. Southern blot analysis of *Leu*⁺ and *Pro*⁺ recombinant plasmids (part A and part B, respectively) and chromosomal DNAs from different bacterial sources. Unlabelled chromosomal and plasmid DNAs were digested with restriction enzymes and electrophoresed through a 0.7% (w/v) agarose gel, denatured and transferred to a nylon membrane. Hybridizations were performed at 65 °C with 50 ng of labelled pLL13 (A) or pPL11 (B) as probes. Part A: 5 µg of *L. interrogans* DNA digested with *Bam*HI (lane 1) or *Hind*III (lane 2); 5 µg of *B. burgdorferi* (lane 3) and *E. coli* (lane 4) DNA digested with *Hind*III; 10 ng of pLL13 digested with *Hind*III (lane 5). Part B: 5 µg of *L. interrogans* DNA digested with *Bam*HI (lane 1) or *Bam*HI + *Eco*RI (lane 2); 5 µg of *B. burgdorferi* (lane 3) and *E. coli* (lane 4) DNA digested with *Bam*HI + *Eco*RI; 10 ng of pPL11 digested with *Bam*HI + *Eco*RI (lane 5).

Leptospira origin of cloned fragments

Hybridization analysis according to Southern (1975) was used to demonstrate that the DNA inserts of the *Pro*⁺ and *Leu*⁺ recombinant plasmids originated from *L. interrogans*. As can be seen from Fig. 2, pPL11 used as a probe hybridizes with a 6.4 kb *Bam*HI fragment (lane B1) and a 3.9 kb *Eco*RI-*Bam*HI fragment (lane B2); pLL13 when used as a probe hybridizes with a 15 kb *Bam*HI fragment (lane A1) and a 3.2 kb *Hind*III fragment (lane A2) of *L. interrogans* chromosomal DNA. These results indicate that no rearrangement occurred during the cloning experiment. Hybridization is seen only with the *L. interrogans* serovar *icterohaemorrhagiae* DNA and neither with *E. coli* nor with *Borrelia* DNA.

Furthermore, no hybridization is seen with the saprophytic species *L. biflexa* (data not shown). This indicates that the cloned complementing DNAs were of *L. interrogans* origin.

Gene organization of the cloned fragments

To determine whether the *Leptospira* inserts present in the recombinant plasmids could encode genes complementing activities other than *proA* and *leuB*, we used the corresponding plasmids to transform other mutant strains of *E. coli*.

Proline genes. In *E. coli*, conversion of glutamate to proline is mediated by the sequential action of three enzymes: γ -glutamyl kinase, γ -glutamyl phosphate reductase and Δ -pyrroline-5-carboxylate reductase (for a review, see Leisinger, 1987). These enzymes are the products of the *proB*, *proA* and *proC* genes, respectively, which have been sequenced (Deutch *et al.*, 1982, 1984). The first enzyme (γ -glutamyl kinase) is subject to allosteric feedback inhibition by proline. No regulation by repression of the proline genes has been reported. The *proB* and *proA* genes form an operon at 6 min on the *E. coli* chromosome while the *proC* gene is unlinked at 9 min.

Besides strain HB101, several proline auxotrophs were transformed with plasmids pPL1, pPL4 and pPL11: strain JM83, which is deleted for both *proB* and *proA* genes, strain X340 which carries a *proB* mutation and strain DJ105 which carries a *proC* mutation. The transformants were then assayed for growth in the absence of proline. Strains JM83 and X340 were complemented by all recombinant plasmids while DJ105 was complemented by none of them; i.e. the 3.9 kb of *Leptospira* DNA encodes both *proB* and *proA* activities but *proC*, if present, is not expressed. In *E. coli*, a 2.5 kb fragment is sufficient to encode the entire *proBA* locus (Deutch *et al.*, 1984). This would suggest a genetic linkage in *Leptospira* similar to that in *E. coli*.

To examine whether *Leptospira proB* and *proA* genes were organized in an operon as in *E. coli*, we inserted a chloramphenicol-resistance 'cassette' in the *Leptospira* insert of pPL11 (see details of the constructs in the legend of Fig. 1). The resulting plasmid, pPL12, no longer complemented the different *proA,B E. coli* strains: this result suggests that insertion of the 'cassette' is polar and that *Leptospira proB* and *proA* genes constitute an operon. However, further data are necessary to demonstrate that the *proA* and *proB* genes form a single transcriptional unit with two distinct translational products.

The *leuB* gene. The oxidative decarboxylation of β -isopropylmalate is the penultimate reaction in the

leucine biosynthetic pathway in *E. coli* as well as in other micro-organisms (reviewed by Umbarger, 1987). The enzyme β -isopropylmalate dehydrogenase (LeuB) which catalyses this reaction, together with the two other enzymes, isopropylmalate synthetase (LeuA) and isopropylmalate isomerase (LeuCD), which are specifically involved in L-leucine biosynthesis are the products of an operon, *leuABCD*, which is subject to regulation by attenuation. Only the nucleotide sequence of the *leuD* gene has been reported (Friedberg *et al.*, 1985).

In order to examine whether the *Leptospira leu* genes were also contiguous, we transformed several leucine auxotrophs mutated in *leuA* (CV512), *leuC* (CV520 and CV522) or *leuD* (CV524 and CV526) as well as a strain (MC1061) deleted for the whole leucine operon with plasmids pLL1 (15 kb insert), pLL13 and pLL131 (3.2 kb insert). None of the recombinant plasmids complemented any of the defined mutations tested.

Discussion

Few DNA sequences from the pathogenic species *L. interrogans* have been cloned and maintained in *E. coli*: DNA probes usable in taxonomic identification (LeFebvre, 1987; Van Eys *et al.*, 1988; Zuerner & Bolin, 1988) isolated from serovar *hardjo* colony libraries; antigen genes from a serovar *canicola* colony library (Yamaguchi *et al.*, 1988); two antigen genes from serovar *pomona* (Doherty *et al.*, 1989); and a 23S ribosomal RNA gene from serovar *canicola* (Fukunaga *et al.*, 1989).

This paper reports the first cloning of genes involved in amino acid biosynthetic pathways from a pathogenic *Leptospira* strain. As already mentioned, such genes (involved in tryptophan and arginine biosynthesis) have been isolated from a saprophytic species of *Leptospira* (Yelton & Charon, 1984; Zuerner & Charon, 1988). Our results of DNA hybridization are in agreement with those of these authors in indicating a genetic divergence between the pathogenic and saprophytic species of *Leptospira*, a divergence previously noted by Brendle *et al.* (1974).

The *L. interrogans* DNA clone bank constructed here seems to be representative. The *E. coli* strain (HB101) used for cloning the biosynthetic genes is auxotrophic for leucine and proline, and *Leptospira* genes encoding both complementing activities were cloned. Indeed, assuming that the size of the *Leptospira* genome is around 5000 kb, (C. Baril & I. Saint Girons, unpublished results) and that the average size of the inserts is 8 kb, our DNA clone bank represents 10 times the genome.

The *Leptospira* gene which complements the *E. coli leuB* allele is most likely the structural gene for

isopropylmalate dehydrogenase since the cloned gene complements three different alleles of the *leuB* gene (strains HB101, CV514 and CV516). The positive complementation of more than one allele is crucial. For example, instead of the actual *leuB* structural gene, an allele-specific suppressor of the *leuB6* mutation present in HB101 has been cloned from *Lactobacillus bulgaricus* (Hottinger *et al.*, 1987). No other *Leptospira* leucine gene was cloned, although the *leuB* gene is located in the middle of the insert and the size of the insert is large enough (15 kb) to encode several genes. Different explanations for these results are possible. The other *Leptospira* leucine genes could be unlinked to the *leuB* gene, which would indicate an organization different to that found in *E. coli* and in *Bacillus subtilis* (Piggot & Hoch, 1985), where the leucine biosynthetic genes form an operon. These leucine genes could also be present on the plasmid but not expressed in *E. coli*.

The *Leptospira* genes encoding proline-complementing activity consist of two genes, *proB* and *proA*. A fragment of 3.9 kb is sufficient to encode these two activities. In *E. coli*, the *proB* and *proA* genes are organized as an operon *proBA* on a 2.5 kb DNA fragment (Deutch *et al.*, 1984). Our data suggest that in *Leptospira*, the *proB* and *proA* genes could also be organized as an operon, although other possibilities such as for example a single gene encoding a bifunctional protein are not ruled out. The linkage of the *proA* and *proB* genes in organisms such as *E. coli*, *Salmonella typhimurium* (Sanderson & Roth, 1988) *Campylobacter* (Lee *et al.*, 1985) and *Leptospira* is perhaps related to the fact that γ -glutamyl kinase (*proB* product) is active only in association with γ -glutamyl phosphate reductase (*proA* product), the second enzyme of the proline biosynthetic pathway (Smith *et al.*, 1984). However, the situation is different for *Pseudomonas* and *Saccharomyces* (Adams & Frank, 1980), where the three proline genes, *A*, *B* and *C*, are scattered.

In conclusion, our experiments show that *L. interrogans* amino acid biosynthetic genes can be expressed in *E. coli*. Attempts to identify the proteins derived from the cloned *Leptospira* genes (intact or inactivated by a chloramphenicol cassette) by minicell analysis failed. The products of the *leuB*, *proA* and *proB* genes, although expressed at a level sufficient for complementation of the corresponding *E. coli* mutants, are not visible as distinct bands on a gel. Expression has already been demonstrated for genes from the saprophytic species *L. biflexa*, although in none of these cases has any evidence been given that this expression occurs from *Leptospira* signals. These cloned genes are useful material for genomic mapping and the chloramphenicol insertions in these genes are powerful tools for obtaining mutations on the *Leptospira* chromosome.

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