Cloning and analysis of the *leuB* gene of *Leptospira interrogans* serovar *pomona*

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The *leuB* gene of *Leptospira interrogans* serovar *pomona* strain kenniwicki has been cloned on a 9.5 kb plasmid, pWVL1, by complementation of *Escherichia coli leuB* mutants. Subcloning and Tn5 mutagenesis showed that the region required for complementation was approximately 1.2 kb in length. Enzyme assays showed that the product of the cloned gene was a β -isopropylmalate dehydrogenase. Defects in the *leuA*, *leuC* and *leuD* genes of *E. coli* were not complemented by pWVL1. The nucleotide sequence of the *leuB*-complementing region and surrounding DNA has been determined. Three open reading frames were found which encode proteins of 40.9, 38.8 and 15 kDa. Analysis of subclones containing nucleotide deletions of varying sizes showed that only the 38.8 kDa protein was necessary to obtain complementation of *E. coli leuB* mutations. The PIR data base was searched and the enzyme 3-isopropylmalate dehydrogenase from six different micro-organisms was found to share significant amino acid sequence similarity (43–57%) with the 38.8 kDa *L. interrogans leuB* gene product. The organization of the leucine biosynthetic genes in *L. interrogans* differs from that found in *E. coli*, *Salmonella typhimurium* and *Bacillus subtilis*.

Introduction

Leptospira is a genus composed of long, slender, helical, motile bacteria (Faine & Stallman, 1982; Holt, 1978; Johnson, 1977, 1981). Phylogenetic analysis based on oligonucleotide cataloguing of bacterial 16S rRNAs suggests that the spirochaetes, including *Leptospira*, represent an early evolutionary divergence within the eubacteria (Fox *et al.*, 1980; Paster *et al.*, 1984). A distinguishing feature of leptospires is the use of long-chain fatty acids or alcohols as their chief carbon and energy source (Holt, 1978; Johnson, 1977). Leptospires synthesize all of their amino acids except isoleucine by well-established biosynthetic pathways (Charon *et al.*, 1974; Westfall *et al.*, 1983).

Since *Leptospira* is a member of a group of bacteria which show an early evolutionary divergence from other eubacteria, the organization and control of genetic information in these bacteria may well differ from that in the better-studied eubacteria. Previous investigations

have demonstrated that, at least in some instances, the genetic organization seen in *Leptospira* does differ from that in other well-studied bacteria (Fukanaga & Mifuchi, 1989; Richaud *et al.*, 1990; Yelton & Peng, 1989; Zuerner & Charon, 1988). The goal of this study was to expand our understanding of leptospiral gene structure and organization.

Leucine biosynthetic genes were chosen as our investigative model because the leucine operon has been well studied in *Escherichia coli*, *Salmonella typhimurium* and *Bacillus subtilis*. The major biosynthetic pathway leading to leucine is the isopropylmalate pathway. In *E. coli*, *S. typhimurium* and *B. subtilis* there are three enzymes unique to leucine biosynthesis: 2-isopropylmalate synthase, the product of the *leuA* gene; isopropylmalate dehydratase, which consists of two subunits encoded by the *leuC* and *leuD* genes; and the NAD-dependent β -isopropylmalate dehydrogenase, the *leuB* gene product (Bartholomew & Calvo, 1971; Parsons & Burns, 1969; Umbarger, 1978; Ward & Zahler, 1973). The four contiguous structural genes are transcribed as a unit and thus comprise an operon (Burns *et al.*, 1966). Attenuation of transcription is the major, and probably only, control mechanism by which leucine regulates expression of the leucine operon (Gemmell *et al.*, 1979, 1983; Searles *et al.*, 1983; Ward & Zahler, 1973).

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The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M59431.

Little is known about the organization and control of the leucine biosynthetic genes in *Leptospira* (Richaud *et al.*, 1990; Zuerner, 1986). The *leuB* genes have been cloned from *L. interrogans* serovar *icterohaemorrhagiae* (Richard *et al.*, 1990) and *L. biflexa* serovar *patoc* (Zuerner, 1986), but these cloned genes have not been characterized or sequenced. The *leuB* gene cloned from *L. interrogans* serovar *icterohaemorrhagiae* was present on a 17.7 kb plasmid; subcloning localized the region necessary for *leuB* complementation to a 3.2 kb *HindIII* fragment obtained from the centre of the original plasmid (Richaud *et al.*, 1990). Despite its size, the original plasmid was not able to complement mutations in other genes required for leucine biosynthesis (Richaud *et al.*, 1990), suggesting that the genes necessary for such complementation were not located adjacent to the *leuB* gene. The *leuB* gene cloned from *L. biflexa* was localized to a 1.6 kb fragment of DNA by subcloning and transposon mutagenesis (Zuerner, 1986). This fragment of DNA was shown to encode a 43 kDa protein by maxicell analysis (Zuerner, 1986). In this paper we report the cloning, characterization and nucleotide sequence of a gene from *L. interrogans* serovar *pomona* which complements a *leuB* defect in *E. coli*.

Methods

Materials. Restriction endonucleases and a nick-translation reagent kit were purchased from Bethesda Research Laboratories. T₄ DNA ligase was purchased from New England Biolabs. Calf alkaline phosphatase was purchased from Boehringer-Mannheim. ³⁵S-dATP (> 1000 Ci mmol⁻¹, 10 mCi ml⁻¹) and L-[³⁵S]methionine (> 37 TBq mmol⁻¹, > 1000 Ci mmol⁻¹, 10 mCi ml⁻¹) were purchased from Amersham. A ³²P-sequencing kit was obtained from Pharmacia. En³Hance was obtained from DuPont. All enzymes were used as recommended by the manufacturer.

Bacterial strains and phage. *E. coli* strain JA221 [Δ *trpE5 leuB6 hsdR* (*r*⁻*m*⁺)] was used as a recipient for the initial cloning experiments (Chinault & Carbon, 1979; Yelton & Charon, 1984) and was obtained from Dr John Carbon (University of California at Santa Barbara). Other *E. coli* strains used in this study were CV512 (*leuA371*); CV514 (*leuB410*); CV516 (*leuB61*), CV422 (*leuC222*); CV526 (*leuD101*); HB101 [*hsdS* (*r*⁻*m*⁻), *recA*, *proA*, *leuB6*]; SE5000 (*recA*); and TB1 (*hsdR*, *proA*). The leucine auxotrophs were obtained from Dr Barbara Bachmann at the *E. coli* Genetic Stock Center; SE5000 was obtained from Dr Lola Stamm (University of North Carolina), and TB1 from Dr Thomas Baldwin (Texas A & M University). Strains of *L. interrogans* serovar *pomona* type kennewicki and *L. biflexa* serovar *patoc* were used as sources of DNA and were obtained from Dr Richard Zuerner (National Animal Disease Center, Ames, Iowa) and Dr Nyles Charon (West Virginia University), respectively. The λ ::Tn5 hybrid, λ 467 (*b221 rex*::Tn5, *c1857*, *Oam29*, *Pam80*), was utilized for transposon mutagenesis studies and was obtained from Dr Nancy Kleckner (Harvard University).

Media. *E. coli* strains were routinely propagated in LB broth at 37 °C, except when acting as hosts for screening the *leuB* gene, or for Tn5 mutagenesis studies, in which case they were incubated at 30 °C. M9 medium was prepared according to Maniatis *et al.* (1982) and used

to characterize transformants. YM broth was used to grow *E. coli* recipients for λ 467 infections (de Bruijn & Lupski, 1984). *Leptospira* strains were propagated on the modified Tween-80 albumin salt medium (EMJH) of Johnson (Johnson & Harris, 1967).

DNA extraction and cloning. *Leptospira* DNA was extracted and purified as previously described (Yelton & Charon, 1984). Plasmid DNA was purified from *E. coli* cells grown overnight in 250 ml cultures (Davis *et al.*, 1980). DNA from *L. interrogans* serovar *pomona* was partially digested with *EcoRI* and ligated to *EcoRI*-digested, calf-alkaline-phosphatase-treated pUC13 (Vieira & Messing, 1982). Ligated DNA was used to transform *E. coli* JA221 using the procedure of Kushner (1978). Ampicillin-resistant (Amp^r) transformants were selected at 30 °C.

Gel electrophoresis and DNA-DNA hybridization. Electrophoresis was performed as described by Maniatis *et al.* (1982). Specific DNA fragments were purified by electroelution from agarose followed by passage through Elutip-d columns (Schleicher and Schuell). Purified DNA fragments were radiolabelled by nick-translation using ³⁵S-dATP. DNA-DNA hybridizations were done as described by Maniatis *et al.* (1982). The conditions used for DNA-DNA hybridization allowed approximately 6% (high stringency) or 40% (low stringency) base mismatch.

Transposon mutagenesis. Mutagenesis was performed with Tn5 contained in λ 467 using the procedure described by de Bruijn & Lupski (1984). *E. coli* HB101 harbouring pWVL5 was transduced to kanamycin resistance (Kan^r) with λ 467. The plasmid DNA was extracted from Kan^r transformants and then transformed into *E. coli* JA221. The phenotypes of the Tn5-modified plasmids in *E. coli* JA221 were determined by replica plating to selective media. The Tn5 insertion sites were located by restriction enzyme mapping of the mutated plasmid DNAs (de Bruijn & Lupski, 1984; Lawn *et al.*, 1978).

Maxicell analysis. *E. coli* SE5000 containing various plasmids was used for maxicell analysis as described by Sancar *et al.* (1979). Cells were labelled with [³⁵S]methionine for 1 h at 30 °C, then harvested and lysed by boiling in electrophoresis sample buffer. For analysis, equal amounts of the resulting lysates were electrophoresed at 150 V. After electrophoresis, the gels were analysed by fluorography using En³Hance.

Measurement of enzyme activity. 2-Hydroxy-4-methyl-3-carboxyvalerate:NAD⁺ oxidoreductase (EC 1.1.1.85; β -isopropylmalate dehydrogenase) activity was measured at 37 °C in crude extracts by the procedure of Parsons & Burns (1969). Protein determinations were performed using a kit from BioRad employing the Bradford assay (Bradford, 1976). The substrate, β -isopropylmalate, was obtained from Dr John Schloss (E. I. Dupont Co., Wilmington, DE).

DNA sequencing and analysis. DNA sequencing was performed by the dideoxy chain-termination method of Sanger *et al.* (1977). Sequential, overlapping deletions of the cloned DNA molecule were constructed by exonuclease III and S1 nuclease treatment (Erase-a-base kit; Promega) as described by Henikoff (1984). Double-stranded DNA templates were prepared by the alkaline lysis procedure of Ish-Horowitz & Burke (1981). ³⁵S-dATP and T7 DNA polymerase (Pharmacia) were used for the sequencing reactions. Analysis of the sequence data was done using the DNASTAR program.

Results

Cloning the *leuB* gene of *L. interrogans*

A genomic library containing approximately 4000 Amp^r transformants on LB agar plates was constructed for *L.*

Fig. 1. Subclone and transposon mutagenic analysis of pWVL1. (a) Subclones were produced from pWVL1 by digesting the plasmid to completion with a restriction endonuclease and then self-ligating the fragment containing the vector. The phenotypes of the various subclones were determined. The single lines represent *Leptospira* DNA; the boxes represent pUC13 DNA. The deleted regions are indicated by parentheses. Abbreviations for restriction enzymes are as follows: B, *Bgl*II; E, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sal*I; X, *Xba*I. (b) *Tn5* insertions into pWVL5 were obtained as described in the text. The phenotype of the cells harbouring the plasmid containing *Tn5* was determined by plating on the appropriate media; the location of the *Tn5* insertion was determined by restriction enzyme digestion. The dots indicate insertions having a *LeuB*⁺ phenotype; the vertical lines indicate insertions having a *LeuB*⁻ phenotype.

interrogans in *E. coli* JA221 (*leuB6*) using pUC13 as the vector. Analysis of β -galactosidase activity indicated that approximately 96% (481 of 500) of the Amp^r transformants contained insertions into the *Eco*RI site of pUC13. Colonies representing the entire library were mixed together, grown in LB broth for 2 h at 37 °C, pelleted by centrifugation, washed twice with M9 medium and plated onto M9 salts agar containing tryptophan and ampicillin. These plates were incubated at 30 °C for 6 d. Approximately 300 leucine-independent (*Leu*⁺) colonies were obtained. Plasmid DNA was isolated from three of these colonies. Each of the three colonies contained a 9.5 kb plasmid; these plasmids were shown to be identical by digestion with *Eco*RI. One of these plasmids was designated pWVL1 and used for further analysis. A restriction map of pWVL1 (Fig. 1a) was generated as described (de Bruijn & Lupski, 1984;

Lawn *et al.*, 1978). To ensure that the *Leu*⁺ transformants were not revertants, pWVL1 was used to retransform *E. coli* strain JA221 to Amp^r. These colonies were then screened for leucine independence. As a control, strain JA221 transformed with pUC13 was used. All of the cells transformed with pWVL1 DNA simultaneously became Amp^r and *Leu*⁺. None of the cells transformed to Amp^r with pUC13 became *Leu*⁺.

Allele-specific complementation test

Hottinger *et al.* (1987) reported that a cloned serine tRNA gene from *Lactobacillus bulgaricus* was able to specifically complement the *E. coli leuB6* mutation, but was not able to complement the *E. coli leuB401* and *leuB61* mutations. They suggested that this allele-specific complementation of *E. coli leuB6* occurs by missense suppression. pWVL1 was introduced into different strains of *E. coli* [JA221 (*leuB6*), CV514 (*leuB401*) and CV516 (*leuB61*)] which carried independent mutations in their *leuB* genes. The leptospiral gene present on pWVL1 was able to complement all three *leuB* mutations, suggesting that we had cloned the *leuB* gene and not the gene for some suppressor tRNA molecule.

Screening for complementation of leucine auxotrophs by pWVL1

The genes for the leucine biosynthetic enzymes are organized in operons in *E. coli*, *S. typhimurium* and *B. subtilis* (Somers *et al.*, 1973; Ward & Zahler, 1973). Assuming that the corresponding genes are organized analogously in *L. interrogans*, the size of the cloned DNA fragment in pWVL1 suggested the possibility that other leucine biosynthetic genes might be found on the fragment. To test this, *E. coli* strains CV512 (*leuA*), CV422 (*leuC*) and CV526 (*leuD*) were transformed with pWVL1. The Amp^r colonies obtained following transformation were replica plated onto minimal medium which lacked leucine. No complementation of the *leuA*, *leuC* or *leuD* mutations was obtained with pWVL1.

Localization of the *leuB* gene

In *E. coli* the *leuB* gene occupies approximately 1 kb of DNA (Friedberg *et al.*, 1985). Because of the large size of pWVL1, subcloning onto smaller fragments was used to help localize the *L. interrogans leuB* gene. pWVL1 was digested to completion with *Sal*I, *Bgl*II, *Xba*I or *Pst*I. Those DNA fragments which still contained pUC13 vector were self-ligated to create subclones pWVL2, pWVL3, pWVL4 and pWVL5 (Fig. 1a). After transformation into *E. coli* JA221, Amp^r clones were selected

Fig. 2. Deletion and maxicell analysis of pWVL6. (a) Deletions were introduced into pWVL6 using exonuclease III. The resulting plasmids were introduced into *E. coli* JA221 and their ability to complement the *leuB* mutation in these cells was determined. The boxes represent pUC13 DNA and the single lines represent *Leptospira* DNA. The extent of the deletion is indicated by the dotted vertical line. Abbreviations for restriction enzymes are as follows: A, *Ava*I; B, *Bgl*II; E, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sal*I; X, *Xba*I. The letters to the left of the plasmids refer to the lanes in part (b) of the figure. (b) The number of proteins produced by the various deletion-containing plasmids was determined using maxicells. The upper arrow indicates the 42 kDa protein (lanes A and C) required for complementation of the *leuB* mutation in *E. coli*. The lower arrow indicates the 40 kDa protein (lanes A and B) encoded by ORF1. Lanes: A, pWVL8; B, pWVL9; C, pWVL11; D, pWVL12; E, pGEM-4; F, no plasmid.

Table 1. Enzyme assays

Bacterial strain/plasmid	Specific activity* of β -isopropylmalate dehydrogenase
<i>E. coli</i> TB1 (leucine prototroph)	0.20 $\pm$ 0.04
<i>E. coli</i> CV516 (<i>leuB61</i>)/pWVL11	0.19 $\pm$ 0.07
<i>E. coli</i> CV516 (<i>leuB61</i>)	0.00
<i>L. interrogans</i> (leucine prototroph)	0.055 $\pm$ 0.01

* The specific activity of the enzyme was determined from the linear portion of the reaction curve and is expressed as $\mu\text{mol } \alpha\text{-ketoisocaproic acid produced min}^{-1} (\mu\text{g protein})^{-1}$. The data represent the means $\pm$ the standard deviations of the means obtained from a minimum of three experiments performed in duplicate.

and tested for their abilities to complement the *leuB6* defect in *E. coli* JA221. Cells containing any one of the four plasmids obtained by subcloning were *Leu*⁺. This result indicated that the *leuB*-complementing activity was located within the 3.5 kb leptospiral DNA fragment found in pWVL5.

As the fragment of *L. interrogans* serovar *pomona* DNA contained in pWVL5 was still considerably larger than the corresponding coding region for *leuB* in *E. coli* (Friedberg *et al.*, 1985), transposon mutagenesis with Tn5 was performed on pWVL5 to further localize the functional region of the *leuB* gene. A total of 48

independent Kan^r and Amp^r colonies were examined for their leucine phenotypes; 38 of the colonies were *Leu*⁺ and 10 were *Leu*⁻. The locations of the Tn5 insertions were determined by digestion with restriction enzymes and subsequent sizing of the fragments (de Bruijn & Lupski, 1984). The results are shown in Fig. 1(b). The region of the leptospiral DNA required for *leuB* function was approximately 1.2 kb in length.

Southern blot analysis

To ensure that the DNA which had been cloned originated from *L. interrogans* serovar *pomona*, Southern blot analysis was performed. pWVL1 DNA was purified by two successive bandings in CsCl/ethidium bromide gradients and was then digested with *Pst*I and *Eco*RI. The resulting 2 kb fragment which contained the *leuB* complementing activity was isolated and labelled with ³⁵S-dATP by nick-translation. The labelled DNA was used to probe Southern blots which contained *Eco*RI-digested pUC13 DNA and *Eco*RI-digested DNA isolated from *E. coli*, *L. biflexa* serovar *patoc* and *L. interrogans* serovar *pomona*. Under conditions of high stringency, a single hybridizing band of 3.1 kb was detected using the probe and serovar *pomona* DNA; no hybridization with pUC13, *E. coli*, or *L. biflexa* DNAs was detected (data not shown).

TATAAACTCAGATCGCCAAAGGAAACGCTTCCAAATCCGCGTCTTCTGTTGTCTCTCA
 10 20 30 40 50 60
 TCGGACGTGATTCAAGTCATTTTAGAAGGAAATGCAAACTCACAATCCGACCTCCGGA
 70 80 90 100 110 120
 ACGAACCTAAAATTTAAATTTATCTCTCTTCAAAGCCTAAAAGCTCCTAAGTCCAAAG
 130 140 150 160 170 180
 AAGAAATTTAAATACTTACCAAGATCTTCTTCTGAAATCAAACCTCCGAGAAATAT
 190 200 210 220 230 240
 MetAsnAspAlaAspIleHisLysGluLeuPheGlnHis
 TTTTACAACCTGCGGGGCTCTCATGAATGACGACAGATCCACAAAGAACTTTTCAACA
 250 260 270 280 290 300
 ThrLysGluLeuAlaAspHisTyrLeuLeuAsnThrTyrAlaArgTyrAspValAlaPhe
 CACAAGGAATTAGCGGATCTTATCTCAATACGTATGCAAGATACGACGATGAGCTT
 310 320 330 340 350 360
 ArgTyrGlyValAsnGluLeuLeuPheAspPheAsnLysGlnTyrIleAspPheHis
 TCGTTATGGAGTTAACGAATCTTATTCGATTTTGATAATAACAATACATTGATTTCCT
 370 380 390 400 410 420
 CysGlyValAlaValThrAsnLeuGlyHisAlaAspProAspIleIleGluValValArg
 TTGCGGAGTTGCGGTTACAACTTAGGACGACGATCTGACATCATAGAGTGGTCCG
 430 440 450 460 470 480
 SerGlnAlaAspLysLeuPheHisThrSerAsnLeuPheTyrSerGluGluAlaSerLys
 TTCTCAAGCGGATAAATTTTACACTTCCAATCTATTTTATTCTGAAAGCTTCAAA
 490 500 510 520 530 540
 LeuAlaGluLeuIleIleLeuAsnSerPheProGlyLysValPheLeuThrAsnSerGly
 ACTTGCAGAACTTATCATTAATTCCTTCTCGGAAAGTGTCTTTTAAACCAATTCAGG
 550 560 570 580 590 600
 ThrGluAlaIleGluGlyAlaPheLysLeuAlaArgLysTyrAlaTyrSerLysArgIle
 AACCGAAGCATCGAAGGAGCATTTAACTCGCAAGAAATACGCATATTCAAAAGAAAT
 610 620 630 640 650 660
 ValAspProIleIleLeuSerLeuGluLysSerPheHisGlyArgSerValSerGlyMet
 CGTAGATCCTATCATCTCTTCTGGAAGAAAGTTTTCACGGAAGATCCGTTTCGGGTAT
 670 680 690 700 710 720
 SerLeuThrGlyGlnAspLysIleArgLysGlyTyrGlyGluLeuLeuLysGlyIleGlu
 GAGTTTAAACCGGACAAGATAAATCAGAAAGGTATGGAAGAACTTTCTAAAGGAATCGA
 730 740 750 760 770 780
 PheIleGluProAsnAsnAspGluAlaLeuValAlaAlaPheGluArgTyrGlnGlyArg
 ATTTATAGAACCGAACCAAGCAGCAAGCCCTTTAGCGGCATTCGAAAGATACCAAGGAG
 790 800 810 820 830 840
 IleValAlaLeuIleGluGluProIleLeuGlyGluSerGlyIleIleProLeuSerArg
 AATCGTAGCCCTTAATCGAAGAACCCATCTTAGGCGAAAGTGAATTTATACCTTTATCTAG
 850 860 870 880 890 900
 AsnPheLeuThrLeuSerArgGluLeuThrGluGluAsnGluAlaLeuLeuIlePheAsp
 AAATTTCTTACACTTTCCAGAGAACTTAACAGAAAGAAACGAGCGCTTCTAATTTTCGA
 910 920 930 940 950 960
 GluIleGlnThrGlyMetGlyArgThrGlyThrLeuPheAlaPheGluThrMetGlyPhe
 CGAAATCCAAACCGGAATGGGTAGAATCGGAACGTTATTCGCAATTTGAAACGATGGGTTT
 970 980 990 1000 1010 1020
 SerProAspAlaMetThrLeuAlaGlyLeuGlySerGlyPheProIleGlyAlaLeu
 TAGTCAGACGCAATGACACTTCCCAAGGGCTCGGATCTGGATTTCGATCGCGCTTT
 1030 1040 1050 1060 1070 1080
 IleValGlyGluLysTyrGlnAspPheThrGlnGlySerHisGlySerThrPheGly
 GATTGTAGGAGAAAAATATCAGGACCTTTTACCAAGGATCTCAGCGCTCCACATTTGG
 1090 1100 1110 1120 1130 1140
 GlyAsnHisLeuAlaAlaValAlaTyrGluThrIleArgIleIleGlnThrArgGlu
 AGGTAATCATTTGCTGCTGCTGCTGCTTATGAACGATTCGTATCATTCACAAACGAGA
 1150 1160 1170 1180 1190 1200
 IleLeuAsnAsnValAsnIleCysSerAspIleAlaPheThrArgLeuArgGluMetGln
 GATTTTAAACCAACGTAATATTTGCTCTGACATTTGCTTTTACAAGACTGAGAGAAATGCA
 1210 1220 1230 1240 1250 1260
 GluLysTyrProValIleSerGluValArgGlyLysGlyLeuHisIleGlyLeuGluLeu
 GGAAAAATATCCAGTGATTTTCAAGTAAGAGGAAAGGACTTCACATCGGACTTGAATT
 1270 1280 1290 1300 1310 1320
 LysValProSerArgProIleAlaGluAlaCysLeuSerGlnGlySer***
 GAAAGTCTCCAGCAATTTGCGGAGCTGTTTCTCGAGGGCTCGTATGATCAATGCA
 1330 1340 1350 1360 1370 1380
 ACGGCGGATAACGTAGTTGGGATTATGCCCCACTTACAATTTCCGACAGATTTTTAAAT
 1390 1400 1410 1420 1430 1440
 Met
 CAAGGATTAGACATCTTAGAATCCGTACTCAACAAAACTAAAGGATCTAAAAAGAAAT
 1450 1460 1470 1480 1490 1500
 LysAsnValAlaValLeuSerGlyAspGlyIleGlyProGluValMetGluIleAlaIle
 GAAGAATCTAGCAGTACTTTCAAGAGACGGAATCGGACCGGAAGTCTGGAGATAGCCAT
 1510 1520 1530 1540 1550 1560
 SerValLeuLysLysAlaLeuGlyAlaLysValSerGluPheGlnPheLysGluGlyPhe
 CTCGGTTTAAAAAGGCTCTCGGTGCAAAAGTTTCCGAGTTTCAATTTTAAAGAGGATT
 1570 1580 1590 1600 1610 1620
 ValGlyGlyIleAlaIleAspLysThrGlyHisProLeuProProGluThrLeuLysLeu
 TGTAGTGGGAATCGCAATCGATAAACTGGACACCCACTTCCACCGGAACTCTTAACT
 1630 1640 1650 1660 1670 1680
 CysGluGluSerSerAlaIleLeuPheGlySerValGlyGlyProLysTrpGluThrLeu
 ATGTGAAGAAATCTTCCGAATCTTCTTCCGAAGTGTGGAGGTCTTAATGGGAAACACT
 1690 1700 1710 1720 1730 1740
 ProProGluLysGlnProGluArgGlyAlaLeuLeuProLeuArgLysHisPheAspLeu
 CCCTCCGGAAGAAACACCGGAACGAGAGGGCCTCTTACCTTTGAGAAACATTTTGATCT
 1750 1760 1770 1780 1790 1800
 PheAlaAsnLeuArgProAlaIleIleTyrProGluLeuLysAsnAlaSerProValArg
 ATTTGCAAACTTAAGACCTGCGATCATTTATCCAGAGTTGAAAAATGCTTCTCAGTTCG
 1810 1820 1830 1840 1850 1860
 SerAspIleIleGlyAsnGlyLeuAspIleLeuIleLeuArgGluLeuThrGlyGlyIle
 TTCTGATATTATGGAAACGATAGATATTCTCATATTAAAGAGAGTTAACCGGAGAAAT
 1870 1880 1890 1900 1910 1920
 TyrPheGlyGlnProLysGlyArgGluGlySerGlyGlnGluPheAlaTyrAspThr
 TTATTTTGGACAACCAAGGAAGAGAGGATCAGGTGAGGAAGAAATTGCTATCAGCAC
 1930 1940 1950 1960 1970 1980
 MetLysTyrSerArgArgGluIleGluArgIleAlaLysValAlaPheGlnAlaAlaArg
 GATGAAGTATCCAGAGAGAAATCGAAAGGATTGCTAAAGTCGATTCAGGCGGCGCAG
 1990 2000 2010 2020 2030 2040
 LysArgAsnAsnLysValThrSerIleAspLysAlaAsnValLeuThrThrSerValPhe
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 2050 2060 2070 2080 2090 2100
 TrpLysGluValValIleGluLeuHisLysLysGluPheSerAspValGlnLeuAsnHis
 TTGGAAGGAAGTAGTAATCGAATTCGATAAGAAAGAAATTTTCAGACGTCCTAATTGAATCA
 2110 2120 2130 2140 2150 2160
 LeuTyrValAspAsnAlaAlaMetGlnLeuIleValAsnProLysGlnPheAspValVa
 TCTTTATGTGGCAATGCGGCGATGCGAGTTAATCGTAAATCCGAACAAATTCGACGTGGT
 2170 2180 2190 2200 2210 2220
 LeuCysGluAsnMetPheGlyAspIleLeuSerAspGluAlaSerIleIleThrGlySe
 TCTTTGTGAGAATATGTTTGGTGATATCTTTCGGACGAGGCTTCCATCATTACGGGTTC
 2230 2240 2250 2260 2270 2280
 IleGlyMetLeuProSerAlaSerLeuSerGluSerGlyPheGlyLeuTyrGluProSe
 AATCGGAATGCTTCTCTCTCTCTCTTCCGAATCTGGATTGGATTGATGAACCTTC
 2290 2300 2310 2320 2330 2340
 GlyGlySerAlaProAspIleAlaGlyLysGlyValAlaAsnProIleAlaGlnValLeu
 TGGTGGTTCTGCGCGGACATAGCCGGAAGAGAGTGGCAAAATCCGATTTGCTCAAGTATT
 2350 2360 2370 2380 2390 2400
 SerAlaAlaLeuMetLeuArgTyrSerPheSerMetGluGluGluAlaAsnLysIleGlu
 GAGTGGCGGCTGTATGTTACGTTATCTTTTCTTGAAGAAGAGCAACAGATAGA
 2410 2420 2430 2440 2450 2460
 ThrAlaValArgLysThrIleAlaSerGlyLysArgThrArgAspIleAlaGluValGly
 AACCCGCTGCGTAAACGATTTGCTCCGGAAGAGAGAGAGTATGCGGAAAGGATAGG
 2470 2480 2490 2500 2510 2520
 SerThrIleValGlyThrLysGluIleGlyGlnLeuIleGluSerPheLeu***
 ATCTACGATCTGAGGAATCAAGAAATCGGTCAATTTGATGCAATCTTCTCTTCTTAAAGAA
 2530 2540 2550 2560 2570 2580
 MetLysAlaGlyValAlaProAsnGlyArgProTyrGlnValLeuIleAla
 GGTAAGAAATATGAAGCAGGTGTAGCTCCCAACGGAAGACCGTATCAAGTATTGATCG
 2590 2600 2610 2620 2630 2640
 GluAsnSerArgPheGlnAlaLysGlnLeuAlaGlnIleLeuGluSerGluGlyTyrGlu
 CAGAAATATCCAGATTCCAGCAAAACAGTTGCGCCGATTTTGGAAATCGGAAGGTTATC
 2650 2660 2670 2680 2690 2700
 ValIleGlyPheAlaGluAsnGlyLysGluLeuValLysLeuTyrAspGluHisArgLeu
 AAGTATGTTGGTTTTCAGAGAACCGGAAGGAACTCGTCAAACTCATATGTAACACAGAC
 2710 2720 2730 2740 2750 2760
 ValAspLeuIleThrLeuAspLeuAsnLeuProValMetAspGlyTyrAlaThrPhePhe
 TTGATATTAAATTAATTTACATTGGATTAAACCTTCCAGTAAAGGACGTTATGCGACCTTT
 2770 2780 2790 2800 2810 2820
 GluIleLysGlyLysGlyValLeuProArgIleValIleValSerGluGluAsnThrPro
 TTGAAATTAAGGAAAGGAGTTCTTCCGAAATGTAATCGTTTCCGAGAGAAATACCC
 2830 2840 2850 2860 2870 2880
 AlaValLeuLysAsnLeuIleAspGluGlyAlaMetAspTyrIleProLysProIleLys
 CCGCGCTTTAAAAAATCTTATAGACGAAGGTGCAATGGATTATTTCCAAAACCGATTAC
 2890 2900 2910 2920 2930 2940
 ArgGluLysIleLeuGluLysValAsnAlaAlaIleLysLysValProLysVal***
 AACGGGAAAAAATCTTGGAAAAAGTTAACCGCAGCAATCAAAAGTATCCCAAGGTCTAAC
 2950 2960 2970 2980 2990 3000
 CTCTTTCCAAATCAAAATTCAGATTCTTCTCTACTTTCTTTTACGATGACTGAAGAAAT
 3010 3020 3030 3040 3050 3060
 CTGAATTTCTTCTTAAAGTACAAATTTTATCACATTGAAGTAAACTCGAATTC
 3070 3080 3090 3100 3110

Fig. 3. Nucleotide sequence. The nucleotide sequence of the cloned 3.1 kb *Eco*RI fragment obtained from pWVL1 is shown, with the deduced amino acid sequences of the three ORFs. Potential Shine-Dalgarno sequences are underlined.

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L. int.  M-----KNVAVLSGDGIGPEVMEIAISVLKKAALGAKVSEFQ-FK-EGF-VGGIAIDKTGHPLPPEET
C. mal.  MSVKTKTITILPGDHVGTIVNEAIKVLEAIEAATPYQKIHFDKFHHLIGGAATDATGVPLPDDA
S. cer.  MSAP-KKIVVLPDGHVGGQETAEAIKVLKASDVRSNVKFD--ENHLIGGAATDATGVPLPDEA
B. coa.  MK--MK-LAVLPDGDIGPEVMDAAIRVLKTVLDNDGHEAV-F--ENALIGGAATDEAGTLPPEET
T. aqu.  M-----K-VAVLPDGDIGPEVTEAALKVLRALDEAEGGPTY-----EVFPFGGAIDASA-PFPEET
B. sub.  MK---KRIALLPGDGIGPEVLESATDVLKSVARFNHEFE-F--EYGLIGGAATDEHNNPLPEET
B. cal.  MGN--YRIAVLPDGDIGKEVTSGAVEVLKAVGIRFGHEFT-F--EYGLIGGAATDEAGTLPPEET
          *   *   . . . . . * . . . . . *   *   . . . . . * . . . . .

L. int.  LKLCEESSAILFGSVGGPKWETLPPEKQPERGALLPLRKHFDLFLANLRPAI-IYPELKNASPVRS
C. mal.  LESAKNSDAVLLGAVGGPKWGT--GALRPEQG-LLKIRKELNLYANIRPCNFASDSLLELSPLRP
S. cer.  LEASKKVDVALLGAVGGPKWGT--GSRPEQG-LLKIRKELQLYANLRPCNFASDSLLELSPIKP
B. coa.  LDICRRSDAILLGAVGGPKWDHNPASLRPEKG-LLGLRKEMGLFANLRPVK-AYATLLNASPLKR
T. aqu.  RKGVEEAELVLLGAVGGPRLGRPSQDPPGDG-ASFLKEKPDLFANLRPAK-VFPGLERLSPLKE
B. sub.  VAACKNADAILLGAVGGPKWDQNLSELRPEKG-LLSIRKQLDLFANLRPVK-VFESLSDRSPLKE
B. cal.  VRLCRESDAVLLGAVGGPKWDDNPPHLRPEKG-LLAIRKQLDLFANLRPVV-CYDSLVSRSPLKP
          . . . . . * . . . . . * . . . . . * . . . . . * . . . . .

L. int.  DIIGNGLDILILRELTGGIYFGQPKGREGS--GQEEFAYDTMKYSRREIERIAKV-AF-QAARKR
C. mal.  EVV-KGTNLIIVRELVGGIYFGDREEQESADKQT--AWDTEKYTVDEVTRITRMAAFMALQHTP
S. cer.  QFA-KGTDFVVVRELVGGIYFGKRKEDD----GDGV-AWDSEQYTVPEVQTRITRMAAFMALQHEP
B. coa.  ERV-ENVDLVIVRELTGGLYFGRPSERRGP--GENEV-VDTLAYTREEIERIEK-AF-QLAQIR
T. aqu.  EIA-RGVDVLIVRELTGGLYFGEPRGM-----SEAE-AWNTERYSKPEVERVARV-AF-EAARKR
B. sub.  EYI-DNVDFVIVRELTGGLYFGQPSKRYVNTGEQE-AVDTLFYKRTEIERVIRE-GF-KMAATR
B. cal.  DLV-QGVDFVIVRELTGGLYFGQPSA--VVENGE-EKAVDTLLYKKEEIERIVRM-AF-ELARGR
          . .   . * . . . . . * . . . . . * . . . . . * . . . . .

L. int.  NNKVTSIDKANVLTTSVFWKEVVIELHKKEFSDVQLNHLYVDNAAMQLIVNPKQFD-VVLCENMF
C. mal.  PLPIWSLDKANVLASSRLWRRTVDKVISSEFPTLSVQHQLIDSAAMILIQNPTKLNGIITSNMF
S. cer.  PLPIWSLDKANVLASSRLWRKTVEETIKNEFPTLVQHQLIDSAAMILVKNPHTLNGIITSNMF
B. coa.  RKKLASVDKANVLESSRMWREIAEETAKK-YPDVLESHMLVDSTSMQLIANPGQFD-VIVTENMF
T. aqu.  RKHVSVVDKANVLEVEFRRTVVE-VGRGYPDVVALEHQYVDAMAMHLVRSARFD-VVVYGNIF
B. sub.  KGKVTVDKANVLESSRLWREVAED-VAQEFDPVKLEHMLVDNAAMQLIYAPNQFD-VVVTENMF
B. cal.  RKKVTVDKANVLESSRLWREVAEE-VANFPDVTLEHMLVD-MRMQLIRAPKQFD-VIVTENMF
          * . . . . . * . . . . . * . . . . . * . . . . . * . . . . .

L. int.  GDILSDEASIIITGSIGMLPSASL-S----ESGFLGYEPSGGSAPDIAGKGVANPIAQVLSAALML
C. mal.  GDIISDEASVIPGSLGLLPSASLASLPDNTAFGLYEPCHGSAPDLPANKV-NPIATILSAASML
S. cer.  GDIISDEASVIPGSLGLLPSASLASLPDKNTAFGLYEPCHGSAPDLPKNKV-DPIATILSAAMML
B. coa.  GDILSDEASVITGSLGMLPSASLRS-----DRFGMYEPVHGSAPDIAGQKANPLGTVLSAALML
T. aqu.  GDILGNLRADLPGLSLGLLPSASL-----GRGTPVFEPVHGSAPDYAGKGR-NPTAAILSAAMML
B. sub.  GDILSDEASMLTGSIGMLPSASL-S----SSGLHLFEPVHGSAPDIAGKGMANPFAAILSAMML
B. cal.  GDILSDEASMLSGSLGMLPSASL-S----ASGPSLYEPVHGSAPDIAGMKNKANPIAAILSAMML
          *** . . . . . * . . . . . * . . . . . * . . . . . * . . . . .

L. int.  RYSFSMEEANKIETAVRKTIASGKRTRDIAEVG----STIVGTKEIGQLIESFL-----
C. mal.  RLSLDCVKEAEALEEAVKQVLDKGIRTDALRGTS----STTEVGDAIVEAVTKILKEKA-----
S. cer.  KLSLNLPEEGKAIEDAVKKVLDAGIRTDGLGGSN----STTEVGDAVAEEVKKILA-----
B. coa.  RYSFGLKEAAAIEKAVDDVLQDGYCTGDLQVANGKVVSTIELTDR---LIEKLNSAARPRIFQ
T. aqu.  E-QLRPGGLARKVEDAAKALLET--PPDGLGGSE-----ARAFATV--LRH--LA-----
B. sub.  RTSFGLKEEAKAVEDAVNKLVLASGKRTRDLARSEEF--STQAITEEVKAAIMSENTISNV-----
B. cal.  RLSFGLTAEAGGRAR-VWQALALG-SGSRLLGQRRPHL-STNEMVEEIKAAVLDYTAIAQIMTVYA-
          . * . . . * . . . . . * . . . . . * . . . . . * . . . . .

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Fig. 4. Alignment of the amino acid sequences of various β -isopropylmalate dehydrogenases. The sequences have been aligned using the MACAW program (Schuler *et al.*, 1991). Where necessary, to enhance the alignments, gaps have been introduced and are indicated by dashes. An amino acid residue appearing in at least six of the seven sequences is indicated by an asterisk; a residue appearing in at least five of the seven sequences or a residue where a related amino acid has been substituted is indicated by a dot. Abbreviations: *L. int.*, *Leptospira interrogans*; *C. mal.*, *Candida maltosa*; *S. cer.*, *Saccharomyces cerevisiae*; *B. coa.*, *Bacillus coagulans*; *T. aqu.*, *Thermus aquaticus*; *B. sub.*, *Bacillus subtilis*; *B. cal.*, *Bacillus caldopenax*.

Maxicell analysis

The protein product of cloned *leuB* gene was identified by using maxicells (Sancar *et al.*, 1979). For these studies, plasmid pWVL6 was produced by subcloning the internal 3.1 kb *EcoRI* fragment of pWVL1 (Fig. 1a) into pGEM-4 (Promega). Subclones of pWVL6, containing nucleotide deletions of varying sizes, were constructed and

tested for their *leuB*-complementing activity. *E. coli* SE5000 was transformed with various plasmid subclones (Fig. 2a) and the resulting transformants were analysed to determine the number and the sizes of the proteins necessary for *leuB* function (Fig. 2b). A 42 kDa protein and a 40 kDa protein were produced by the cloned DNA. A third open reading frame (ORF), potentially encoding a 15 kDa protein, has been identified within the

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H. sap. MFSKLAHLQRFAVLSRGVHSSVASATSVATKKTVQGPPTSDDIFEREYKYGAHNYHPLPVALERG
S. cer. MSEATLSKQTI EWENKYSAHNYHPLPVVFHKA
L. int. MNDADIHKELFQHTKELADHYLLNTYARYDVAFRYG
      . * * . * * . .

H. sap. KGIYLWDVEGRKYFDFLSSYSAVNQGHCHPKIVNALKSQVDKLTLSRAFYNNVLGEYEEYITKL
S. cer. KGAHLWDPEGKLYLDFLSAYSAVNQGHCHPHI IKALTEQAQTLTLSSRAFHNDVYAQFAKFVTEF
L. int. VNELLFDFDNKQYIDFHCGVAVTNLGHADPDII EVVRSQADKLFHTSNLFYSEEASKLAELII-L
      * * . . * . * . . * * * . . * * . * . . .

H. sap. FNYH-KVLPMTGVEAGETACKLARKWGYTVKGIQKYKAKIVFAAGNFWGRTL SAISSTD-PTSY
S. cer. SGFE-TVLPMTGAEAVETALKLARRWGYMKKNIPQDKAII LGAEGNFHGRTFGAISLSTDYEDSK
L. int. NSFPGKVFLTNSGTEAIEGAFKLARKYAYSKRIVDP---IILSLEKSFHGRSVSGMSLTGQDKIRG
      . * . * . * * * * * . . . * . * * * . . .

H. sap. DGFGPFMP-----GFDIIPYNDLPALERALQDP---NVAAFMVEPIQGEAGVVVPDPGYLMGVR-
S. cer. LHFPGFPVNVASGHSVHKIRYGHAEFVPILESPEGKNVAII LEPIQREAGIVVPPADYFPKVS-
L. int. -GYGELL-----KGIEFIEPNDEALVAAFERYQGR-IVALIEEPILGESGII-PLSRNFLTLRS
      . * . . . . * . . . . . * . . * * * * * .

H. sap. ELCTRHQVLFIADEIQTGLARTGRWLAVD-Y-ENVRPDIVLLGKALSGGLYPVSAVLCDDDIMLT
S. cer. ALCRKHNVLLIVDEIQTGIGRTGELL CYDHYKAEAKPDIVLLGKALSGGVLPVSCVLSSH DIMSC
L. int. ELTEENEALLIFDEIQTGMGRTGTLFAFE-T-MGFSPDAMTLAKGLGSG-FPIGALIVGEKYQDL
      * . * . * * * * * . * . . * * . * . * * * . * . . .

H. sap. IKPGEHGSTYGGNPLGCRVAIAALEVLEEENLAENADKLGII LRNELMKL---PSDVVTVAVRGKG
S. cer. FTQGSHGSTFGGNPLASRVAIAALEVIRDEKLCQRAAQLGSSFIAQLKALQAKSNGI ISEVRGMG
L. int. FTQGSHGSTFGGNHLAAAVAYETIRIIQTREILNNVNICSDIAFTRLREMQEKP-VISEVRGKG
      . * * * * * * * * . * * . . . . * . . . . * * * *

H. sap. LLNAIVIKETK--DWDWKVCLRLRDNGLLAKPTHGDIIRFAPPLVIKEDELRESIEIINKTILSF
S. cer. LLTAIVIDPSKANGKTAWDLCLLMKDQGLLAKPTHDHIIIRLAPPLVISEEDLQTVETIAKCIDL
L. int. LHIGLELKVPS--RPIA-EACLSQGS
      * . . . * *

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Fig. 5. Alignment of the amino acid sequences of the ORF1 protein and two ornithine aminotransferases. The sequences have been aligned using the MACAW program (Schuler *et al.*, 1991). Where necessary, to enhance the alignments, gaps have been introduced and are indicated by dashes. An amino acid residue appearing in all of the sequences is indicated by an asterisk; when a residue appears in two of the three sequences and a related amino acid has been substituted in the third sequence, a dot is used. Abbreviations: *H. sap.*, *Homo sapiens*; *S. cer.*, *Saccharomyces cerevisiae*; *L. int.*, *Leptospira interrogans*.

cloned DNA through nucleotide sequencing (see below). The 42 kDa protein was found in the *Leu*⁺ strains and was missing from the *Leu*⁻ strains. Deletions within the putative *leuB* ORF inactivated the complementing activity, thus confirming that this ORF was necessary for the *leuB*-complementing activity. Deletions extending into either ORF1 or ORF2 had no effect on the *leuB*-complementing activity of the clone. These results indicated that only the 42 kDa protein was required for complementation of the *leuB* defect.

Assay of β -isopropylmalate dehydrogenase from *L. interrogans*

The product of the cloned *L. interrogans leuB* gene was tested to determine whether it could convert β -isopropylmalate to α -ketoisocaproate. Crude cellular extracts were

made from *L. interrogans* serovar *pomona*, *E. coli*, a *leuB* mutant of *E. coli*, and the *E. coli leuB* mutant transformed with pWVL11 to leucine prototrophy. These extracts were incubated at 37 °C in the presence of β -isopropylmalate. The quantity of α -ketoisocaproate produced in these experiments was determined colorimetrically by reaction with 2,4-dinitrophenylhydrazine and KOH (Parsons & Burns, 1969). The reactions were linear for at least 20 min; the specific activity of the enzyme was determined after 10 min of reaction. The results are shown in Table 1. There was no detectable enzyme activity in the *leuB* mutant strain of *E. coli*. When this mutant strain was transformed with a plasmid containing the cloned gene, the specific activity of the enzyme was similar to that found in wild-type *E. coli*, indicating that the product of the cloned gene was a β -isopropylmalate dehydrogenase. The specific activity of

the enzyme in the serovar *pomona* extract was fourfold lower than that seen in the extract from wild-type *E. coli*; this may be because the conditions used for the enzyme assay, while optimum for the enzyme from *E. coli*, may not be optimum for the enzyme from *Leptospira*. Alternatively, the leptospiral enzyme may have a lower activity, as the generation time of these cells is much longer than that of *E. coli* and there has been no evolutionary pressure to optimize the efficiency of the enzyme. The cloned enzyme in the *E. coli* extracts would show a higher specific activity due to gene dosage effects which lead to overproduction of the protein.

Nucleotide sequencing and alignment of amino acid sequences

To confirm the identity of the cloned gene and to determine the possible function of the DNA adjacent to the *leuB* gene, both strands of the leptospiral *EcoRI* DNA fragment found in pWVL6 were sequenced in their entirety. The resulting nucleotide sequence of serovar *pomona* DNA and the amino acid sequences deduced from the nucleotide data are shown in Fig. 3. Three ORFs, which encoded proteins of 40926 (ORF1), 38826 (*leuB*) and 15058 (ORF2) Da, were found. The location of the 38826 Da ORF corresponds to the *leuB* functional region as defined by transposon mutagenesis (Fig. 1*b*). Each ORF was preceded by a potential Shine–Dalgarno sequence.

The inferred amino acid sequences of *leuB*, ORF1 and ORF2 were used to search the PIR data bank for similar proteins. The amino acid sequence of the β -isopropylmalate dehydrogenase cloned from serovar *pomona* showed 43–57% identity and 19–26% related substitutions when compared with the same enzyme obtained from six different micro-organisms, *Candida maltosa* (Kawamura *et al.*, 1983; Hamasawa *et al.*, 1987), *Saccharomyces cerevisiae* (Andreadis *et al.*, 1984), *Bacillus coagulans* (Sekiguchi *et al.*, 1986), *Thermus aquaticus* (Sekiguchi *et al.*, 1986), *Bacillus subtilis* (Sekiguchi *et al.*, 1987) and *Bacillus caldopenax* (Sekiguchi *et al.*, 1987). An alignment of the amino acid sequences of the 3-isopropylmalate dehydrogenases from *L. interrogans* and the other microorganisms is shown in Fig. 4. Conservation of amino acid sequence is seen throughout the protein.

The amino acid sequence of ORF1 shows about 58% similarity (32% identity and 26% related substitutions) with ornithine–oxoacid aminotransferase obtained from either *Homo sapiens* (Inana *et al.*, 1986) or *Saccharomyces cerevisiae* (Degols, 1987). When the amino acid sequences of these proteins were aligned, several regions of strong homology were found (Fig. 5).

No proteins similar to ORF2 were found in the

database. None of the cloned proteins had amino acid sequences similar to *LeuA*, *LeuC* or *LeuD*, the other leucine biosynthetic enzymes.

Discussion

A genomic library of *L. interrogans* serovar *pomona* DNA was constructed in the plasmid vector pUC13. A clone (pWVL1) was isolated from this genomic library which complemented three independent *leuB* mutations in *E. coli*. The *leuB*-complementing activity in pWVL1 was localized to approximately 1.2 kb of DNA by subcloning and by transposon mutagenesis using Tn5. Southern blot analysis of *EcoRI*-digested pWVL1 and serovar *pomona* DNAs revealed identically sized restriction fragments homologous to the cloned *leuB* gene. These data suggest that only a single form of the cloned *leuB* gene is present on the *L. interrogans* genome. No hybridization was detected with *E. coli* or *L. biflexa* serovar *patoc* DNAs. It is understandable that no cross-hybridization occurred between *L. interrogans* and *L. biflexa* because previous studies have established that little, if any, DNA homology exists between these species (Brendle *et al.*, 1974; Yasuda *et al.*, 1987).

Tn5 insertions flanking the *leuB* gene did not inactivate gene expression, suggesting that expression of the gene depends on its own promoter rather than on a pUC13 promoter. It is unlikely that Tn5 promoters were used since Tn5 promoters are weak and generally do not allow expression of genes located outside of the element itself (Berg *et al.*, 1980). While these results suggest that promoters may be located within the cloned *Leptospira* DNA, it is not clear whether these are native *Leptospira* promoters or merely sequences which fortuitously act as promoters in *E. coli*. Analysis of the sequence data revealed an *E. coli* σ^{70} -like promoter sequence (CTTACA...TTAAAT) located about 70 nucleotides upstream of the initiation codon of the *leuB* gene (Fig. 3). Mapping of the 5' end of the *leuB* mRNA will be needed to determine whether this sequence is acting as a promoter in *E. coli* and whether it is the native promoter used by *L. interrogans*.

The close linkage of ORF1, *leuB* and ORF2 suggests that these genes might be transcribed as a unit. Consistent with such a possibility is the existence of an *E. coli* σ^{70} -like promoter sequence located upstream of ORF1. This promoter would have a –35 region of CTTCCG followed by a –10 region of TAAAT (Hawley & McClure, 1983). While the –35 region is not ideal and the spacing is only 15 nucleotides, the RNA polymerase isolated from spirochaetes has been shown to differ from that isolated from *E. coli* (Allan *et al.*, 1986). The structural alteration found in the spirochaetal RNA polymerase may allow for some differences in promoter

sequence and spacing when compared to the *E. coli* paradigm. Mapping of the promoters recognized by the leptospiral RNA polymerase will be needed to resolve this issue. Only one potential stem-loop-forming sequence is located within the sequenced DNA. It is found immediately downstream of ORF2 at nucleotides 3005 to 3084 and is followed by the sequence TTTTAT. This sequence might allow the stem-loop to function as a simple terminator (Platt, 1986). The existence of a potential promoter upstream of the sequenced DNA and of a potential terminator downstream of this DNA is consistent with the hypothesis that these genes may be transcribed as a unit.

Both strands of the 3.1 kb *Eco*RI fragment of pWVL6 (Fig. 3) were sequenced. Three open reading frames (ORFs) were present within this fragment. The predicted size of the protein encoded by the ORF spanning the *leuB* functional region as defined by Tn5 mutagenesis was 38826 Da. Further analysis, using subclones, confirmed that this ORF was necessary for complementation of a *leuB* mutation (Fig. 2).

The maxicell experiments (Fig. 2*b*) revealed that three proteins were encoded by the cloned DNA. The 42 kDa protein was essential for expression of the LeuB⁺ phenotype. However, the 358 amino acid residues found in the *leuB* coding region constituted a molecular mass of 38826 Da. This value is about 7.5% less than that determined by SDS-polyacrylamide gel electrophoresis. The cause of the discrepancy could be anomalous SDS binding to the protein as was found with the *leuB* enzymes from *S. cerevisiae* and *B. caldovenax* (Andreadis *et al.*, 1984; Sekiguchi *et al.*, 1987). The 40 kDa protein is the product of ORF1, since deletion of this ORF resulted in loss of the protein (Fig. 2*b*, lane C). The molecular mass of the protein encoded by ORF1, as calculated from its amino acid composition, is 40926 Da, which closely corresponds to the results obtained by maxicell analysis. Nucleotide sequence analysis identified another open reading frame, ORF2. The broad 14.5 kDa band seen in lanes C and D of Fig. 2(*b*) may represent the product of ORF2, as it is approximately the correct size and is only seen in cells containing an intact ORF2.

The LeuB protein from *L. interrogans* is comparable in size to the 43 kDa protein encoded by the *leuB* gene cloned from *L. biflexa* (Zuerner, 1986). Zuerner (1986) also found that his clone encoded proteins of approximately 46 and 15 kDa. The genes encoding these proteins could correspond to ORF1 and ORF2 found in the *L. interrogans* clone. If so, this would suggest a conservation of genetic organization in this region of the chromosome among the leptospires.

Analysis of the amino acid sequence of the β -isopropylmalate dehydrogenase from *L. interrogans* showed the enzyme to have substantial amino acid

sequence similarity with the β -isopropylmalate dehydrogenases from other micro-organisms (Fig. 4). The closest similarity is seen with the enzymes from the *Bacillus* species, and the least with the enzymes from the yeasts. This relationship is consistent with the evolutionary divergence of these organisms as proposed by Woese (1987).

The ORF for *leuB* begins with a methionine residue; in position 17 there is another methionine residue. The codon for the first methionine residue is likely to be the correct start codon because (a) it is preceded by a Shine-Dalgarno sequence while the second methionine codon is not, and (b) alignment of the amino acid sequences of the various LeuB enzymes demonstrates that the first 17 residues are highly conserved (Fig. 4).

In *E. coli* and *S. typhimurium* the terminal step in leucine biosynthesis is catalysed by a non-specific aminotransferase (Calvo, 1983). These organisms can use either the aromatic transaminase (TyrB) or the branched-chain amino acid transaminase (IlvE) to convert α -ketoisocaproic acid to leucine. In our *L. interrogans* clone the close genetic linkage of ORF1 and *leuB* suggested that ORF1 might encode a protein functional in leucine biosynthesis. A search of the PIR data base detected similarity between the amino acid sequence of ORF1 and the ornithine aminotransferases obtained from *H. sapiens* and *S. cerevisiae* (Fig. 5). Since ornithine aminotransferases catalyse the reaction ornithine + α -oxoacid $\rightarrow$ L-amino acid + glutamic semialdehyde, we hypothesize that the ORF1 product may function to catalyse the terminal step in leucine biosynthesis in *Leptospira* by transferring an amino group from ornithine to α -ketoisocaproic acid to produce leucine.

Our sequence data indicate that the gene organization of *L. interrogans* differs from that found in other bacteria. In *E. coli*, *S. typhimurium* and *B. subtilis*, four contiguous structural genes, encoding the three enzymes required for leucine biosynthesis, are transcribed as a unit and comprise an operon (Burns *et al.*, 1966; Somers *et al.*, 1973; Ward & Zahler, 1973). The *leuA* gene is located immediately upstream of the *leuB* gene and the *leuC* gene is located immediately downstream. In *L. interrogans* an ORF was found upstream of the *leuB* gene, but its protein product has no significant amino acid similarity to the *leuA*-encoded protein of *S. typhimurium* (Ricca & Calvo, 1990). Immediately downstream of the leptospiral *leuB* gene was a small ORF encoding a basic protein. This protein shares no amino acid sequence similarity with the *leuC* gene from *S. typhimurium* (Rosenthal & Calvo, 1990). Furthermore, the LeuC protein in other bacteria is larger and is not as basic (Umbarger, 1978). Thus the organization of the genes required for leucine biosynthesis in *L. interrogans* differs from that found in

other bacteria. This is consistent with the findings of Richaud *et al.* (1990) and Zuerner (1986), who also found that their *leuB* leptospiral clones, although containing substantial amounts of contiguous DNA both upstream and downstream of the *leuB* gene, were unable to complement defects in other *E. coli* genes required for leucine biosynthesis.

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