Cloning of *dapD*, *aroD* and *asd* of *Leptospira interrogans* serovar *icterohaemorrhagiae*, and nucleotide sequence of the *asd* gene

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Metabolites such as diaminopimelate and some aromatic derivatives, not synthesized in mammalian cells, are essential for growth of bacteria. As a first step towards the design of a new human live vaccine that uses attenuated strains of *Leptospira interrogans*, the *asd*, *aroD* and *dapD* genes, encoding aspartate β -semialdehyde dehydrogenase, 3-dehydroquinase and tetrahydrodipicolinate *N*-succinyltransferase, respectively, were cloned by complementation of *Escherichia coli* mutants. The complete nucleotide sequence of the *asd* gene was determined and found to contain an open reading frame capable of encoding a protein of 349 amino acids with a calculated M_r of 38007. Comparison of this deduced *L. interrogans* aspartate β -semialdehyde dehydrogenase amino acid sequence with those of the same enzyme from *Saccharomyces cerevisiae* and *Corynebacterium glutamicum* revealed 46% and 36% identity, respectively. By contrast, the identity between the *L. interrogans* enzyme and the *Streptococcus mutans* or *E. coli* enzymes was less than 31%. Highly conserved sequences within aspartate semialdehyde dehydrogenase from the five organisms were observed at the amino and carboxyl termini, and around the cysteine of the active site.

Introduction

Leptospirosis, the disease resulting from infection with *Leptospira interrogans*, is one of the most widespread of the zoonoses. The major reservoirs for the pathogenic leptospires are rodents and domestic animals. The human vaccine, used in France, is composed of bacteria (*L. interrogans* serovar *icterohaemorrhagiae* strain Verdun) that have been killed by formalin. An alternative would be to use a live vaccine based on attenuated organisms that are auxotrophic for metabolites not available in mammalian cells, such as aromatic amino acids or diaminopimelic acid (DAP). The genus *Leptospira* is an exception among the spirochaetes since DAP is found in its peptidoglycan while ornithine is found in the genera *Spirochaeta*, *Borrelia* and *Treponema* (Johnson, 1977). These flexible, helically shaped bacteria are very heterogeneous in regard to other characteristics.

Leptospires are aerobic and have 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, 1977).

The biosynthetic pathways for amino acids known for bacteria are used in leptospires except for isoleucine, which is not exclusively derived from threonine (Charon *et al.*, 1974). Studies on the organization and regulation of the amino acid biosynthetic genes in *Leptospira* are still in their infancy. Three biosynthetic genes (*argE*, *trpG* and *trpE*) from the non-pathogenic species *L. biflexa* were cloned by complementation of the corresponding *Escherichia coli* mutants and then sequenced (Zuerner & Charon, 1988; Yelton & Charon, 1984; Yelton & Cohen, 1986). The *proA*, *proB* and *leuB* genes from the pathogenic species *L. interrogans* were cloned by Richaud *et al.*, 1990; the organization of the *leu* genes seems to differ from that in *E. coli*. The *trpE* gene from *Spirochaeta aurantia*, which also belongs to the order Spirochaetales, has also been cloned (Brahamsha & Greenberg, 1987).

Our strategy to obtain mutations in pathways leading to the biosynthesis of DAP and aromatic compounds in *L. interrogans* consists of cloning the biosynthetic genes

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Abbreviation: DAP, diaminopimelate.

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by complementation of *E. coli* mutations, inactivating the cloned genes and then introducing the mutated genes back into *L. interrogans*, replacing the wild-type.

Here we report the cloning of the *aroD*, *asd* and *dapD* genes from *L. interrogans* serovar *icterohaemorrhagiae* strain Verdun. The nucleotide sequence of the *asd* gene specifying aspartate β -semialdehyde dehydrogenase (EC 1.2.1.11), an enzyme common to the biosynthetic pathways of threonine, methionine and DAP, is reported.

Methods

Bacterial strains, plasmids and media. *L. interrogans* serovar *icterohaemorrhagiae* strain Verdun (National Reference Centre, France) was grown in EMJH medium (Johnson, 1977). *E. coli* was grown in Luria Broth in liquid medium or 0.75% agar (Sambrook *et al.*, 1989) supplemented with 50 μ g DAP ml⁻¹, 50 μ g ampicillin ml⁻¹ or 25 μ g chloramphenicol ml⁻¹. Minimal medium 63 (Sambrook *et al.*, 1989) supplemented with 0.2% glucose, 50 μ g ampicillin ml⁻¹ or 25 μ g chloramphenicol ml⁻¹ and 1 μ g thiamin ml⁻¹ was used to characterize *E. coli* transformants. Supplements for growth of *E. coli aroD* mutants were 40 μ g ml⁻¹ phenylalanine, tryptophan and tyrosine, 10 μ g ml⁻¹ *p*-aminobenzoic acid and dihydroxybenzoic acid. Other amino acids such as arginine or threonine were added at 40 μ g ml⁻¹. The cloned *E. coli dapD* gene was inactivated by insertion of a chloramphenicol cassette from pSKS114 (Shapira *et al.*, 1983) into its unique *Pst*I site (Richaud *et al.*, 1984). The *E. coli asd* gene was inactivated by insertion of a kanamycin cassette from pUC4K (Pharmacia) into the unique *Nru*I site of the gene (Haziza *et al.*, 1982). After transformation of the *recBC sbcB E. coli* strain JC7623 (Winans *et al.*, 1985) by linearized plasmids carrying the inactivated genes, cells carrying the gene replacement were selected by their ability to grow in the presence of the antibiotic and DAP. The *asd*::Km or *dapD*::Cm markers were then transferred to *E. coli* strain GT869 (Parsot, 1986) by *P1vir* transduction to give GT2000 (*asd*) and GT2003 (*dapD*), respectively. *E. coli* G6MD3 is (Δ *asd glpR glyD malA*) *his* (M. Schwartz, Institut Pasteur, Paris, France).

Cloning of *L. interrogans* genes by complementation of *E. coli* mutants. Restriction enzyme digests, ligations, agarose gel electrophoresis, transformation, electroporation and small-scale plasmid isolations were by standard procedures (Sambrook *et al.*, 1989).

Two different colony banks of, respectively, 8000 and 11000 individual Ap^r transformants in *E. coli* strain GT869 were obtained from *L. interrogans* serovar *icterohaemorrhagiae* in the *E. coli* multicopy vector pUC13 as described previously (Richaud *et al.*, 1990). In order to clone biosynthetic genes of the DAP and aromatic pathways from *L. interrogans*, complementation of the corresponding *E. coli* mutants was sought. A sample (1 μ g) of total plasmid DNA isolated from either clone bank was used to transform the *E. coli dapD*, *aroD* and *asd* mutants.

Sequencing strategy for the *asd* gene. The 0.7 kb *Eco*RI fragment from pAIL8 and the 2.7 kb *Eco*RI fragment from pAIL4 (see Fig. 1c) were cloned in both orientations into *Eco*RI-digested pTZ18R. A sequential series of overlapping deletions of the *asd* gene were obtained using an IBI Cyclone kit as described by Sambrook *et al.* (1989). The overlap of the internal *Eco*RI site was confirmed by sequencing the first 200 nucleotides of the 3 kb *Bam*HI-*Hind*III fragment originating from pAIL4. The nucleotide sequence from the whole *Pvu*I-*Sma*I insert from pAIL8 was determined by the dideoxy chain-termination method (Sanger *et al.*, 1977) using a BRL sequencing kit. The sequencing vector

pTZ18R and helper phage M13K07 were purchased from Pharmacia. Acrylamide was purchased from BDH, urea from Schwartz, and [α -³⁵S]dATP (14.8 TBq mmol⁻¹) from Amersham. All other chemicals were analytical grade or purer.

Results and Discussion

*Cloning of *L. interrogans* genes able to complement *E. coli aroD*, *dapD* and *asd* mutants*

Complementation of *E. coli* mutants was used to isolate molecular clones of *L. interrogans* genes encoding enzymes of the DAP and aromatic pathways.

With the *aroD E. coli* strain GMS343 (Novel & Novel, 1973) as recipient, 10 out of 40000 Ap^r transformants were Aro⁺ (able to grow without aromatic acid supplements). Analysis of the plasmid DNA (pADL1) of five of these clones by agarose gel electrophoresis after *Bam*HI digestion (Fig. 1a) indicated the same orientation of the 9.5 kb *Bam*HI insert. Further subcloning and gene inactivation experiments allowed us to locate the *aroD* gene more precisely (see Fig. 1a). Other *E. coli aroD* mutants (AB1360 and AB2848) (Pittard & Wallace, 1966) are complemented by pADL1, suggesting that the cloned gene is most likely the structural gene of the 3-dehydroquinase, the third step of the aromatic pathway.

With the *dapD E. coli* strain GT2003 as recipient, 17 out of 17000 Ap^r transformants were Dap⁺ (able to grow on LB medium without DAP). The plasmid DNA of nine of these clones was analysed by agarose gel electrophoresis after *Bam*HI digestion. Six contained pDDL1 (Fig. 1b) and the other three contained the same 9 kb *Bam*HI insert in the other orientation (pDDL2). Subcloning of pDDL1, first by *Pst*I digestion and religation to yield pDDL11 (Fig. 1b), then by *Bgl*II digestion of pDDL11 and religation, gave pDDL12, which was also able to complement the *dapD* mutation in GT2003. The plasmid pDDL12 also complemented another *dapD* mutant, RDD32, which carries a *Mu*cts insertion in the *E. coli dapD* gene (Richaud *et al.*, 1984). The *L. interrogans* DNA in pDDL12 is most likely the structural gene of tetrahydrodipicolinate *N*-succinyltransferase.

With the *asd E. coli* strain GT2000 as recipient, 5 out of 10000 Ap^r transformants were able to grow on LB not supplemented with DAP. Each of these five carried a plasmid (pAIL1, see Fig. 1c) with a 19 kb insert that contained an internal *Bam*HI site. Efficient complementation of an *E. coli* strain deleted for *asd* (G6MD3) by pAIL1 confirmed the presence of the *asd* structural gene on this plasmid. The *asd* complementing activity was still carried by a 1.8 kb *Sma*I-*Pvu*I insert (plasmid pAIL8, see Fig. 1c). A Cm^r cassette inserted at the internal *Bam*HI site inactivated the gene.

Fig. 1. Restriction maps of *L. interrogans* genes carried by plasmids. (a) Subclone analysis of *aroD* plasmids. pADL1 contains a 9.5 kb *Bam*HI insert in pUC13. pADL5 was obtained by deletion of the internal *Bgl*II fragment of pADL1, reducing the insert size to 4.8 kb. The insert carried by pADL5 was ligated in the vector Bluescript (Stratagene) via *Sac*I and *Sal*I bordering sites to give pADL6. Inactivation of the *aroD* gene was performed by inserting a *Cm*^r cassette from pSKS114 into the unique *Pst*I site of pADL6. (b) Subclone analysis of *dapD* plasmids. pDDL1 and pDDL2 correspond to both orientations of the 8.8 kb *Bam*HI insert in pUC13. Total digestion of pDDL1 with *Pst*I and self ligation gave rise to pDDL11; pDDL12 was obtained from pDDL11 by deletion of the internal *Bgl*II fragment. Inactivation of the *dapD* gene was performed by insertion of a *Cm*^r cassette from pSKS114 into the unique *Hinc*II site of pDDL12. (c) Subclone analysis of *asd* plasmids. The plasmid pAIL1 contains a *Bam*HI–*Bam*HI insert of 19 kb (with a supplementary *Bam*HI site) in pUC13. A 5.8 kb *Bgl*II–*Bgl*II fragment from pAIL1 was cloned into the *Bam*HI site of pUC18, giving rise to pAIL4. A 3.9 kb *Cla*I–*Hind*III fragment from pAIL4 was cloned between the *Acc*I and *Hind*III sites of pSU18, giving rise to pAIL5. pAIL8 was obtained by two internal deletions (*Sma*I and *Pvu*I) reducing the insert into the deleted pSU18 to 1.7 kb. B, *Bam*HI; P, *Pst*I; Bg, *Bgl*II; H2, *Hinc*II; H3, *Hind*III; Sal, *Sal*I, SacI, *Sac*I; E, *Eco*RI; BBg, *Bam*HI/*Bgl*II; CAC, *Cla*I/*Acc*I; S, *Sma*I. pSU18 encodes chloramphenicol resistance (Martinez *et al.*, 1988); pUC13, pUC18 and Bluescript encode ampicillin resistance.

Hybridization analysis according to Southern (1975) was used to demonstrate that the DNA inserts of the Asd⁺, Dap⁺ and Aro⁺ recombinant plasmids originated from *L. interrogans*. The sizes of the *Bam*HI fragments which hybridized with *L. interrogans* chromosomal DNA were the same as those originating from the inserts (data not shown). The results thus indicate that no rearrangement had occurred in the *dapD*, *aroD* or *asd* cloning experiments. Cloning of three *L. interrogans* genes involved in amino acid biosynthesis was thus possible by complementation of *E. coli* mutants. Characterization of one of them, the *asd* gene, was pursued further.

Nucleotide sequence of the *L. interrogans* *asd* gene and flanking regions

The sequence of the 1.8 kb *Sma*I–*Pvu*I fragment from pAIL8 which complemented an *asd* *E. coli* mutant was determined on both strands and is presented in Fig. 2. Two large open reading frames (ORFs) were identified on one DNA strand. The first, ORF1, is located between nucleotides 1 and 392. The second, ORF2, later identified as *asd*, extends from nucleotide 393 to nucleotide 1493.

Codon usage for *L. interrogans* ORF1 and ORF2 is

quite similar to that for the *trpE* and *trpG* genes from the saprophytic *Leptospira biflexa* (Yelton & Peng, 1989) and for the sphingomyelinase gene from the pathogenic *L. interrogans* (Segers *et al.*, 1990). The codon usage of *L. interrogans* is highly influenced by the low G+C content (34–39 mol%) of its genome. A or T is the preferred nucleotide in the wobble position, except for Thr and Arg, where G is preferred, and Ala, where all four nucleotides are equally present.

ORF1 is obviously incomplete since it does not contain an ATG or a GTG start codon. Comparison of the translated product of the 392-nucleotide ORF1 with the contents of the EMBL data bank did not show any significant homology with other known proteins. However, ORF1 could be the 3' end of a gene translated in the same direction as ORF2 and could form a potential operon with ORF2. The promoter of the operon would not be present on the *Sma*I–*Pvu*I insert from pAIL8.

Comparison of Asd from *L. interrogans*, *Corynebacterium glutamicum*, *Saccharomyces cerevisiae*, *Streptococcus mutans* and *E. coli*

Comparison of the translated product of ORF2 with the contents of the EMBL data bank showed significant

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1      10      20      30      40
CCCCGGTTAGGTTACTTGTGCACTCAAGGATGCGAACT
  R V R L L V A L K D A K L
      50      60      70      80
TAGAATTCGGGAAGAAAAATTTGTTCCAGTTTACACTTT
  R I R E E K F V P V L H F
      90     100     110     120
CAATATTTGCGAAAGTTTCTAGTTTTTAAAAAGAAAAAA
  Q Y L R K F S S F L K E K
      130     140     150     160
AAGTCCCTCTTTGGATCATAAACAATCCTGAAAATCCGAT
  K V P L W I I N N P E N P I
      170     180     190     200
TAGTTTAGATTGGTATGTAAGTCAAATTGGTATAAAGAT
  S L D W Y V K S N W Y K D
      210     220     230     240
CATCTTTTATTTTTTAAAAAGAACTTTCCGGGAGATTTAGTTT
  H L L F L K E L S G D L V
      250     260     270     280
TCTTTAGTGATTTAAAGATTCTTTGTCTATGCAGGATTT
  F F S D L K D S L S M Q D F
      290     300     310     320
TAGTGACTATCATCTTTTACATTTCCGGGTATGATGAAA
  S D Y H H F T F P G M M K
      330     340     350     360
ATGAGTCCAATTTACGCGAACGAATTTGTTAAAAATTTCTG
  M S P I Y A N E F V K I S
      370     380     390     400
AAAGACAGAGTAAGAATCTTTTTAAACCATGATCTATAGC
  E R Q S K N L L K P
      410     420     430     440
CGTATTTGGTGAATTACAGTTTGAGAGGCGTTTCATTTTA
  450     460     470     480
TTTATGAGTAGGGTCAAAGTTGCCGTTTTAGGCGCCACTG
  M S R V K V A V L G A T
      490     500     510     520
GTTCCGTTGGTCAGAGGTTTCATTCAATTGTTGGATCATCA
  G S V G Q R F I Q L L D H H
      530     540     550     560
TCCTTTTTTTCGAAATTACGCATCTTTGCGCTTCTGAGAAT
  P F F E I T H L C A S E N
      570     580     590     600
AGTGCCGGTAAAACATACGGTGAAGTAATGAAAACCCGGT
  S A G K T Y G E V M K T R
      610     620     630     640
GGAAAAATTTCTTCCGATATTCCTGCTTATGCTAAAAATTT
  W K I S S D I P A Y A K N L
      650     660     670     680
AGTAATCACTACTCCTGATCCTGCTAAAACGAAAGACGTT
  V I T T P D P A K T K D V
      690     700     710     720
GTATTAGCCTTTTCTGGTTTAGATTGCAATGTGCGCGGTG
  V L A F S G L D S N V A G
      730     740     750     760
AAGTGGAAAAAATTTATGCAACGCCGGTATTCATATCAT
  E V E K N Y A N A G I H I I
      770     780     790     800
TTCTAATTCTAAAAATCATAGAATGGATCCGACGGTTCGG
  S N S K N H R M D P T V P
      810     820     830     840
ATTCTTTCTGCGGAGGTGAATTCTTCTCATTGGAAGTTC
  I L S A E V N S S H L E V
      850     860     870     880
TGAATTCTCAAAAAACGAAAGGTAAGATTATTACCAATTC
  L T S Q K T K G K I I T N S

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890      900      910      920
GAACTGTACGATCATGGGTGTTACAATTTCTCTCAAACCT
  N C T I M G V T I S L K P
      930      940      950      960
CTTTTGGATCGTTTTTGGAAATTGAGTCCGTTATGCTCTTTT
  L L D R F G I E S V M L F
      970      980      990     1000
CTATGCAGGCGATCAGCGGCGCTGTTTATCCTGGAGTTCC
  S M Q A I S G A G Y P G V P
      1010     1020     1030     1040
AACGATGGATATTTTAGGAAACGTAATTCCTCATATCGGA
  T M D I L G N V I P H I G
      1050     1060     1070     1080
GGTGAAGAAGAAAAAGCGGAGATAGAACCCTCTTAAATGTT
  G E E E K A E I E P L K C
      1090     1100     1110     1120
TGGGTAAAGTCGAGAATGGAATAATTTTACACGCCGATTT
  L G K V E N G K I L H A D F
      1130     1140     1150     1160
TTCAATTTCCGCACATTGTAACCGTGTTCCTGTTTTTGAC
  S I S A H C N R V P V F D
      1170     1180     1190     1200
GGTCATACGTTTTGTGTTTTCCGTTAAGTTTAAAAAGAAAC
  G H T V C V S V K F K K K
      1210     1220     1230     1240
CTTCTAGGGAAGAAATTATTTCTCTTGGAAAGATTTTTC
  P S R E E I I S S W K D F S
      1250     1260     1270     1280
CGGAGAACCACAGACGTTAGGTCTTCTTTAGCACCAAAT
  G E P Q T L G L P L A P N
      1290     1300     1310     1320
CCGGTTATCCTTTTTAGAGAAGAAGAAGATAGGCCGCAAC
  P V I L F R E E E D R P Q
      1330     1340     1350     1360
CTAGGTTAGATTTAGATACCGGCAAAGGAATGACTACGGT
  P R L D L D T G K G M T T V
      1370     1380     1390     1400
GATAGGTCGTCTCAGGCCAGATCCTATTTTAGATTGGA
  I G R L R P D P I L D W K
      1410     1420     1430     1440
TATGTCGTTTTTAAGCCATAATACAATTCGAGGAGCGCGG
  Y V V L S H N T I R G A A
      1450     1460     1470     1480
GTGCTGCATTATTAATGCAGAACTTCTTTACAAAAA
  G A L L N A E L L Y K K K
      1490     1500     1510     1520
ATTCTTGGGATAATAATGTCATTCCATGCTGGAACCTCAA
  F L G
      1530     1540     1550     1560
TCCCCTGAACTCAAAGTGGCGGATTACAATACCGCACTAC
  1570     1580     1590     1600
AATTGACTCAGTCGTTGAGGCCAGAAATGACTTTCAATA
  1610     1620     1630     1640
TAAGAAAATTCATAAGCTCTTACTTATCATCTCTGATTGG
  1650     1660     1670     1680
ACAGATAAGTTCGTAGCAAATAAAGTGCTTCTTAATGTGG
  1690     1700     1720     1730
ATCAATTATCCAAGGAATTGGGTCTTGAGAAAGACAAGAC
  1740     1750     1760     1770
GTTGCAATTTCTGAAAGAACTTTGTACAAAATACAACCCC
  1780     1790     1800     1810
CCGATCATTAAAAAATCTGCATGGTGGATTGTAATCCTA
  1820     1830
ACGGGGATATATCTGACGGTACGATCG

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Fig. 2. Nucleotide sequence of the *L. interrogans* *asd* gene and its flanking regions. The one-letter abbreviations for the deduced amino acid sequence of ORF1 and *asd* (starting at nucleotide 444) are shown below the DNA sequence.

homology with *Strep. mutans* aspartate β -semialdehyde dehydrogenase (Cardineau & Curtiss, 1987). The *asd* gene has also been sequenced from *E. coli* (Haziza *et al.*,

1982), *Sacch. cerevisiae* (Thomas & Surdin-Kerjan, 1989) and *C. glutamicum* (Kalinowski *et al.*, 1990). Optimal alignment of the five deduced polypeptide

<i>S. c.</i>	<u>MAGKKIAGVIGATGSGVGRFILLANHPH.FELKVLGASSRSAGKKYVDVN..WKQTDLLPESATDIIVSECKSEFFKECDIVSF</u>	83
<i>L. i.</i>	<u>MSRVKVA.VLGATGSGVGRFIQLDHHPF.FEITHLCASENSAGKTYGEVMKTRWKISSIPAYAKNLVITTPDAKTQDVVLAFS</u>	84
<i>S. m.</i>	<u>MGYT..VAIYGATGAVGTRMIQOLEQSTLVRDKVRLSSRSAGKVL.....QYKDQDVTV.....LTKDSFEAVDIALE</u>	70
<i>C. g.</i>	<u>MT.T..IAVVGATGQVQVMRTLLERNFADTVRFASFPSRAGAKI.....EFRGTEIEVED.....ITQATEESLKDIDVALF</u>	72
<i>E. c.</i>	<u>M...KNVGFIGWRGMVGSVLMGRMVEERDHDIAIRPVFFSTSGLGQA.....APSFGGTTGTLQDAFDL.....EALKALDIIVT</u>	71
<i>S. c.</i>	<u>GLDADYAGAIEKEFMEAGIAI..VSNAKNYRREQDVPLIVPVNPEHLDIVAQKLDATAQOGKPRPGFICISNCSTAGLVAPLKP</u>	167
<i>L. i.</i>	<u>GLDSNVAGEVEKNYANAGIHI..ISNSKNHRMDPTVPILSAEVNSSHLEV...LTSQKTGK...IITNSNCTIMGVTISLKP</u>	159
<i>S. m.</i>	<u>SAGGSVSAKFAPYAVKAGAVV..VDNYSHFRONPDVPLVPEVNAYAMDAN.NG.....LIACPNCTSIQMVALEP</u>	139
<i>C. g.</i>	<u>SAGGTASKQYAPLFAAAGATV..VDNSSAWRKDDEVPLIVSEVNPSDKDSL VKG.....IIANPNCTTMAAMPVLKP</u>	142
<i>E. c.</i>	<u>CQGGDYTNEIYPKLRSEGWQGYWIDAASSLRMKDDAII...LDPVNQDVITDGLNNGI.....RTFV..GNCTVSLMLMSLGG</u>	146
<i>S. c.</i>	<u>LIEKEGPIDALTTTTLQATSGAGFSPGVPGIDILDNIIPIY.....IGGE..EDK...MEWETKKILA</u>	224
<i>L. i.</i>	<u>LLDRFG.IESVMLFMSQAISGAGY.PGVPTMDILGNVIPH.....IGGE..EEK...AEIEPLKCLG</u>	214
<i>S. m.</i>	<u>IRQKWG.LSRVIYSTYQAVSAGQSAINETVREIKEYVNDGVDPKAVHADI.....FPSGGD..KKHY..PIAFNALA.QI</u>	209
<i>C. g.</i>	<u>LHDAAG.LVKLHVSSYQAVSGSLAGVETLAKQVAAGVDHNVE..FVHDGQ.....AADAGD..VGPYVSPAIYVNLFPAG</u>	213
<i>E. c.</i>	<u>LFAN.DLVDWVSVATYQAASGGGARHMRRELTQMGLYGHVADELATPSSAILDIERKVTTLTRSGELPVDNFGVPLAGSLIPWID</u>	231
<i>S. c.</i>	<u>PLAEDKTHV.....KLLTPEE.IKVSQCNRAVSDGHTTECISLREKNR..PAPSVEQVKTCLKEYVCDAYKLGCHSAPK</u>	296
<i>L. i.</i>	<u>KVENGK.....ILHAD.FSISAHCNRPVFDGHTVCVSVKFKKK.PSR..EEIISWKFSGEPQTLGLPLAPN</u>	279
<i>S. m.</i>	<u>DVFTDNDYTY..EEMKMTNETKKIMEEPE.LPVSAHCVRVPIILFHSSEAVYIETKD...VAPIEEVKAIAAFPGAV.....</u>	280
<i>C. g.</i>	<u>NLVDGTFETDEEQKLNRNESRKILGLPD.LKVSCTCVRVPVFTGHTLTIHAEFDK...AITVDQAEILGAASGVK.....</u>	285
<i>E. c.</i>	<u>KQLDNGQSR..EEWKQGAETNKILNTSSVIPVDGLCVRVGALRCHSQAFTIKLKKDVS IPTVEEL.....LAAHNPWA</u>	302
<i>S. c.</i>	<u>QTIHVLEQPDPRPRLDRNRDSGYGVSVGRIREDPDLD...FKMVVLSHNTIIGAAGSGVLIAETLLARNLI</u>	365
<i>L. i.</i>	<u>PVILFREEDPRPRLDLDTGKGMTTIGRLRPDPILD...WKYVVLSHNTIRGAAGAALLNAELLYKK.....KF.LG</u>	349
<i>S. m.</i>	<u>LEDDIKHQIYPOAANAVGSRFTFVGRIKQDLDIEN..GIHMWVSDNLLKGAANSIITANRLHERGLVRSTSELKFELK</u>	357
<i>C. g.</i>	<u>LVDVPT...PLAAAGIDE.SLVGRIQDSTVDDNRLVLVSGDNLKGAALNTIQIAELL.....VK</u>	344
<i>E. c.</i>	<u>KVVPNDREITMRELTPAAVTGT.LTTPVGRLRK.....LNMGPFLSAFTVGAQPLRRMLRQLALLWGAAE</u>	367

Fig. 3. Alignment of the deduced amino acid sequences of the *L. interrogans* (*L. i.*) *asd* gene product and other published aspartate semialdehyde sequences from *Strep. mutans* (*S. m.*), *Sacch. cerevisiae* (*S. c.*), *C. glutamicum* (*C. g.*) and *E. coli* (*E. c.*). Gaps have been introduced to maximize alignments with respect to identical and functionally similar amino acids. The positions of identical residues among all sequences are designated with stars. Identity among amino acid residues of the aspartate semialdehyde of *L. interrogans* and *Sacch. cerevisiae* on the one hand and of *Strep. mutans* and *C. glutamicum* on the other is represented by underlining in the upper sequence of the respective pair. The cysteine of the active site of the *E. coli* aspartate semialdehyde dehydrogenase (Biellmann *et al.*, 1980; Haziza *et al.*, 1982) is indicated by an arrow.

sequences was obtained by the CLUSTAL computer program (Higgins & Sharp, 1988) and revealed homology between the five proteins (Fig. 3). Pairwise comparisons revealed that *L. interrogans* Asd is more closely related to *Sacch. cerevisiae* Asd than to the other bacterial Asd proteins. While the *L. interrogans* enzyme shows 46% identity with the enzyme from yeast, the value falls to 36% with the *C. glutamicum* enzyme, 31% with that from *Strep. mutans* and 25% with that from *E. coli*. The *Strep. mutans* and *C. glutamicum* enzymes share 40% of their amino acid residues but those of *Sacch. cerevisiae* and *Strep. mutans* share only 32%. The polypeptide from *E. coli* is less similar to any of the other four (21 to 26% identity). Extensive homology between the whole Asd sequences from the five organisms provided unequivocal evidence that the genes derive from a common ancestor.

There are five regions of close similarity between the five proteins: residues 10–16, 147–148, 175–181, 232–235 and 308–311 (numbering refers to the *L. interrogans*

protein, see Fig. 3). The choice of the methionine initiator codon for *L. interrogans asd* at position 444 (Fig. 2) seems reasonable given the similarity at the N-terminus of the five sequences and is in agreement with the determined N-terminus of the *E. coli* aspartate semialdehyde dehydrogenase (Haziza *et al.*, 1982). No likely candidate ribosome-binding site (Shine & Dalgarno, 1974) complementary to the 3' end of the *L. interrogans* 16S rRNA (Fukunaga *et al.*, 1990) could be found by analysis of the DNA sequence immediately upstream from the predicted ATG translational start codon of *asd*. The *L. interrogans asd* gene encodes a polypeptide of 349 amino acid residues with an M_r of 38007, in good agreement with the estimated sizes of the other four Asd polypeptides.

The high degree of conservation of the amino acid sequences observed for several segments of these proteins probably reflects structural and functional requirements. In particular, the active site for the *E. coli*

aspartate β -semialdehyde dehydrogenase has been identified as the amino acid sequence Phe-Val-Gly-Gly-Asn-Cys-Thr-Val-Ser (Biellmann *et al.*, 1980; Haziza *et al.*, 1982) between amino acid residues 130 and 138. The predicted amino acid sequence for the *L. interrogans* aspartate β -semialdehyde dehydrogenase contains a cysteine residue in the same position, with the Asn-Cys sequence that is present in the other four enzymes (see arrow in Fig. 3).

Expression of the *asd* gene

The *asd* gene of *L. interrogans* is expressed at a low level in whole *E. coli* cells since growth is possible in LB medium without DAP but enhanced when DAP is added. Confirmation of this low activity was given by the assay of aspartate semialdehyde dehydrogenase from crude extracts of *E. coli* cells (*asd*::Km) carrying the recombinant plasmid pAIL1. The resulting activity was not enhanced compared to the activity from prototrophic *E. coli* cells expressing a single chromosomal copy of *asd* (data not shown). Similarly low expression is observed for the *L. interrogans proA*, *proB* and *leuB* genes previously cloned, which are not expressed detectably in minicells (Richaud *et al.*, 1990). The low expression of *L. interrogans* genes in *E. coli* could be due to translation problems (absence of canonical ribosome-binding site) and different codon usage.

The synthesis of Asd in *E. coli* is independently repressed by methionine, threonine and lysine, although derepression is greatest upon lysine limitation (Haziza *et al.*, 1982). Attenuator-like regions have been found upstream of the *asd* gene of *Strep. mutans* (Cardineau & Curtiss, 1987). The corresponding gene (*HOM2*) from *Sacch. cerevisiae* is regulated by the general control of amino acid synthesis. Upon initial inspection, the sequence upstream of the *L. interrogans asd* structural gene does not resemble the reported arrangement of attenuation signals in amino acid biosynthetic operons in Gram-negative bacteria (Kolter & Yanofsky, 1982). This is consistent with the fact that no such signals have been found for the biosynthetic genes from *L. biflexa* that have been sequenced (Yelton & Peng, 1989).

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