Identification of the Aspartate-β-Semialdehyde Dehydrogenase Gene of *Legionella pneumophila* and Characterization of a Null Mutant

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The ability of *Legionella pneumophila* to cause Legionnaires' disease is dependent on its capacity to survive in the intracellular environment of its host cells. Furthermore, outbreaks of this disease have been associated with contaminated water sources where *L. pneumophila* survives as a parasite of protozoa. In this study, we determined the effect of nutritional auxotrophy on the ability of *L. pneumophila* to survive in the intracellular environment of its host cells. We generated a diaminopimelic acid (DAP) auxotroph (AA400) of *L. pneumophila* by disruption of the aspartate-β-semialdehyde (*asd*) gene. The ability of AA400 to survive within macrophages and protozoa was found to be defective. This defect was due solely to the *asd* disruption since complementation of the mutant with the wild-type *asd* gene restored its capacity for intracellular survival. Furthermore, the defect was not completely complemented by DAP supplementation to the culture media. Thus, our results suggest that disruption of the *asd* gene may prove to be useful in the design of attenuated vaccines against Legionnaires' disease.

Legionella pneumophila, a gram-negative facultative intracellular bacterium, is a major cause of community-acquired and nosocomial pneumonia and is associated with a high level of mortality, particularly among immunocompromised individuals (14, 17, 32). The ability of *L. pneumophila* to cause disease is dependent on its ability to survive and replicate within a specialized membrane-bound phagosome in human macrophages and epithelial cells (29, 34). At the ultrastructural and molecular levels, the life cycle of *L. pneumophila* within human phagocytic cells is very similar to its life cycle in its environmental host, the protozoa (1, 13, 21). We have recently described the expression of type IV pili by *L. pneumophila* and their role in adherence to both mammalian and protozoan cells (41). Although some of the attachment and uptake mechanisms of the two hosts are similar, there are also distinct differences (4, 13, 21–23, 25, 31, 45).

Protection against bacterial pathogens has become increasingly urgent due in part to the alarming rise in drug resistance among these pathogens (43). Interestingly, it has been shown that *L. pneumophila* becomes 1,000-fold more resistant to antibiotics after intracellular growth (11). This rise in antibiotic resistance may be partly due to the expression of unique bacterial genes in the intracellular environment (2, 3, 5, 6, 42). Furthermore, treatment of patients infected with *L. pneumophila* with the commonly used antibiotics often fails to eradicate the disease (35). Thus, alternative methods for the treatment of Legionnaires' disease may be necessary.

The most effective means to generate a protective immune response against intracellular bacterial pathogens is by live attenuated bacteria that deliver particulate antigens to antigen-processing cells (27, 30). One promising method of bacterial attenuation is the generation of auxotrophic strains that are unable to survive intracellularly due to the absence of the essential nutrient that they require within the host cell. Examples of these are the aromatic amino acid auxotrophs of *Salmonella typhimurium* and the leucine and diaminopimelic acid (DAP) auxotrophs of mycobacteria (10, 20, 38).

As in most gram-negative bacteria, a major constituent of the peptidoglycan layer of *L. pneumophila* is DAP (8, 24). DAP is involved in the peptide cross-linking of the peptidoglycan. Since mammalian cells do not utilize or synthesize DAP, intracellular bacterial pathogens that are auxotrophic for DAP would not be able to survive in the mammalian host. Therefore, it may be possible to develop chemotherapeutic agents that target enzymes of the DAP biosynthetic pathway. Such an auxotroph may also be a potential live attenuated vaccine candidate or a vehicle for the delivery of immunogenic antigens of other intracellular pathogens. To identify a possible target of chemotherapeutic agents and to determine the feasibility of using *L. pneumophila* as a particulate antigen delivery system, we cloned and characterized the aspartate-β-semialdehyde dehydrogenase (*asd*) gene of *L. pneumophila* and examined the phenotype of a null mutant.

MATERIALS AND METHODS

Bacterial strains and vectors. The virulent AA100 strain of *L. pneumophila* has been described previously (3). *Escherichia coli* DH5α (Bethesda Research Laboratories [BRL], Gaithersburg, Md.) was used for the majority of cloning experiments. *E. coli* K-12 derivative strain χ2981 carries an *asd* insertion mutation that renders it auxotrophic for DAP (5, 7). Plasmid pUC-4K was purchased from Pharmacia (Piscataway, N.J.) and was the source of the kanamycin (*kan*) resistance gene used as a probe for Southern hybridization. Plasmids pBC-SK⁺ and pBlueScript were purchased from Stratagene (La Jolla, Calif.). Plasmid pBOC20 is a chloramphenicol-resistant plasmid that contains *oriT* and the *sacB* gene from *Bacillus subtilis* (6). The *sacB* gene encodes levansucrase and is lethal to *L. pneumophila* grown in the presence of sucrose (16). The conjugative plasmid pRK212.1 was used as a helper plasmid to mobilize plasmid pBOC20 by conjugation as described previously (6).

DNA manipulations. Chromosomal DNA preparations, transfection, restriction enzyme digestion, and DNA ligation were performed as described elsewhere (39) unless specified otherwise. Restriction enzymes and T4 DNA ligase were obtained from BRL.

Plasmid DNA preparations were performed by using a Qiagen (Chatsworth, Calif.) plasmid kit according to the manufacturer's recommendations. Transformations were carried out by electroporation using a Gene Pulser as recom-

mended by the manufacturer (Bio-Rad, Hercules, Calif.). Purification of DNA fragments from agarose gels for subcloning or labeling for Southern hybridization was done by using a Qiaex kit as recommended by the manufacturer (Qiagen). Transfer of DNA from agarose gels into membranes, fluorescein labeling of DNA probes, hybridizations, and detection were performed as previously described (5).

Tissue culture and protozoan culture. Macrophage-like U937 cells were maintained at 37°C and 5% CO_2 in RPMI 1640 tissue culture medium supplemented with 10% heat-inactivated fetal calf serum (Sigma Chemical Co., St. Louis, Mo.). Prior to infection, cells were differentiated with phorbol 12-myristate 13-acetate (PMA) for 48 h as described previously (5). Differentiated cells are nonreplicative adherent macrophage-like cells. Monolayers were washed three times with the tissue culture medium prior to infection. For infection of monolayers, *L. pneumophila* grown for 48 h at 37°C on buffered charcoal-yeast extract (BCYE) agar plates was resuspended in RPMI 1640. The infection was carried out as described for each experiment.

Hartmannella vermiformis CDC-19 (ATCC 50237) has been cloned and grown in axenic culture as a model for the study of the pathogenesis of *L. pneumophila* (19). The amoebae were maintained in ATCC culture medium 1034, and infection with *L. pneumophila* was done in assay medium as previously described (4).

Gene cloning and amplification of the *L. pneumophila asd* gene. To generate an *L. pneumophila* chromosomal DNA library, AA100 DNA was partially digested with *Sau*3A and sucrose gradient size-selected fragments of 35 to 50 kb were ligated into a *Bam*HI site in cosmid pLTP6, a derivative of pLTP5 (9). The ligation mixture was packaged into λ phage with the use of a λ packaging mix (Stratagene). The packaged DNA was transfected into *E. coli* HB101. To isolate clones that carry the *asd* gene of *L. pneumophila*, the cosmid DNA library was conjugated from HB101 into the *E. coli* DAP auxotroph χ2981. Clones that complemented the χ2981 defect were selected. A common 4.3-kb *Hin*dIII fragment from these clones was found to complement the *asd* defect in χ2981. The 4.3-kb fragment was subcloned into pBlueScript (pOH1) and into pBOC20 (pOHBOC1).

To amplify the *L. pneumophila asd* gene, two primers complementary to sequence 200 bp upstream (BAM-1) and downstream (X-1) (5′-GCGGGATC CCTGCGTGGG-3′ and 5′-GCGCTCGAGAAGTAAGACA-3′, respectively) of the *asd* gene were used for PCR. BAM-1 was designed to generate a *Bam*HI restriction site and X-1 was designed to generate an *Xho*I restriction site to facilitate subcloning. A 1.4-kb fragment was isolated and subcloned into pBOC20 (pOHBOC2).

DNA sequencing. To locate and partially sequence the *asd* gene of *L. pneumophila*, unidirectional deletion with exonuclease III was used as described elsewhere (26, 37). The dideoxy-chain termination method of Sanger et al. (40) was employed, using a Sequenase kit (United States Biochemical, Cleveland, Ohio). The T3 promoter primer of the vector was used for sequencing of the exonuclease III-digested DNA. Once homology to other known *asd* sequences was found, oligonucleotides were synthesized (BRL) and sequencing of both strands of the gene was performed. The DNA sequences were compared to other sequences at the National Center for Biotechnology Information databases by using blastX and blastP programs.

Transposon mutagenesis and allelic exchange into the *L. pneumophila* chromosome. A 4.3-kb fragment containing the *asd* gene of *L. pneumophila* was subcloned into pBOC20, designated pOHBOC1, and transformed into the *E. coli* χ2981. Mini-Tn*10*::*kan* mutagenesis was performed exactly as described previously (6). An insertion within the *asd* gene in pOHBOC1 was conjugated into *L. pneumophila*, and allelic exchange was performed exactly as described previously (6). Confirmation of the chromosomal insertion was obtained by Southern hybridization analysis.

Cytopathogenicity of *L. pneumophila* mutants to U937 cells. Infection of PMA-differentiated U937 monolayers was performed in triplicate wells of 96-well plates containing 5×10^4 CFU/well at a multiplicity of infection of 1. The infected monolayers were incubated at 37°C for 24, 48, or 72 h. For measurements of the number of remaining viable cells in the monolayer, the monolayers were treated with alamar blue dye as described previously (5). The relative degree of cytopathogenicity of *L. pneumophila* to the monolayers was expressed as the optical density of the reduced alamar blue as an indicator of the remaining viable cells that reduced the dye.

Growth kinetics of *L. pneumophila* mutants in U937 cells and in *H. vermiformis*. Differentiated 5×10^4 U937 cells in 96-well plates were infected with *L. pneumophila* in triplicate at a multiplicity of infection of 10 for 30 min at 37°C. Wells were then washed three times with tissue culture medium and incubated in the presence of 50 μg of gentamicin per ml at 37°C for 1 h to kill extracellular bacteria. Wells were washed three times with tissue culture medium to remove gentamicin. Time zero was harvested at this point by hypotonic lysis of the monolayers and plating dilutions onto BCYE plates for colony enumeration. The rest of the monolayers were incubated at 37°C for several time intervals. At the end of each time interval, the supernatant of the monolayer was transferred to a sterile well and monolayers were lysed hypotonically. The lysate was combined with the supernatant, and aliquots were plated onto BCYE agar plates for enumeration of bacteria.

To determine the infectivity of the *L. pneumophila* strain to *H. vermiformis*, 10^5 amoebae/ml were infected with 10^3 CFU of *L. pneumophila*/ml in 5 ml of culture
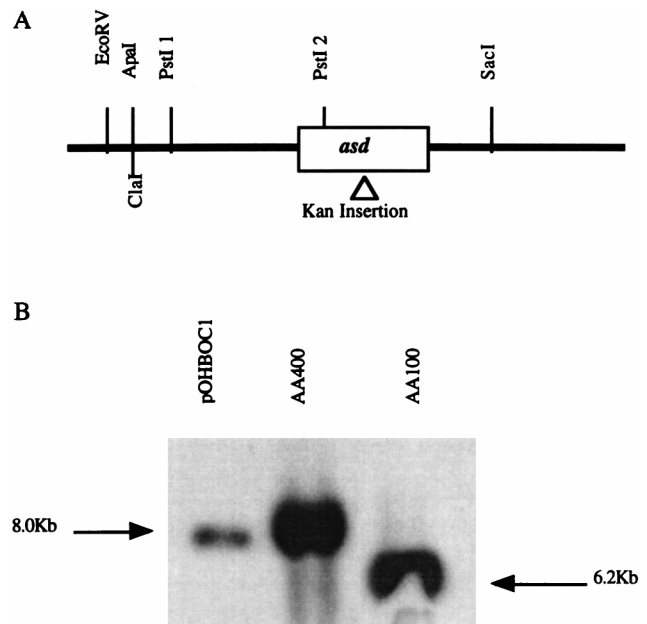

FIG. 1. (A) Restriction map of the *L. pneumophila* 4.3-kb fragment containing the *asd* gene. The relative location of a mini-Tn*10*::*kan* insertion in the *asd* gene is indicated. (B) Southern hybridization of DNA from wild-type *L. pneumophila* (AA100), Δ*asd L. pneumophila* (AA400), and plasmid pOHBOC1K harboring the 4.3-kb *L. pneumophila* fragment with the mini-Tn*10*::*kan* insertion. All DNA samples were digested with *Pst*I and probed with pOH1 harboring the 4.3-kb *L. pneumophila* fragment. The 1.8-kb increase in the size of the band in pOHBOC1K and AA400 corresponds to the size of the *kan* cassette insertion.

in triplicate as described previously (4). At several time points, aliquots were plated on BCYE agar plates for enumeration of bacteria.

Nucleotide sequence accession number. The sequence shown in Fig. 2 has been assigned GenBank accession no. AF034213.

RESULTS

Cloning of the *L. pneumophila asd* gene. The Asd enzyme plays a central role in the biosynthesis of several amino acids in addition to DAP (44). Since the *asd* gene is conserved across several species, our strategy to clone the *asd* gene of *L. pneumophila* was to complement an *asd* defect in *E. coli* χ2981 with an *L. pneumophila* cosmid library for growth without DAP supplementation. Twelve clones that grew without a supplement of DAP were isolated. The cosmid DNA from these clones was purified, and a common 4.3-kb *Hin*dIII fragment was purified, subcloned into pBlueScript, and designated pOH1. pOH1 complemented the *asd* mutation of χ2981 for growth without DAP. Thus, pOH1 contained the *asd* gene of *L. pneumophila*.

Sequencing and analysis of the *L. pneumophila asd* gene. Restriction mapping of pOH1 was used to further localize the *L. pneumophila asd* gene. Several digestion products were subcloned into pBlueScript and tested for the ability to complement the *asd* defect in χ2981. The *L. pneumophila asd* gene was localized to a 2.6-kb region of the 4.3-kb fragment between the *Pst*I and *Sac*I sites (Fig. 1A). Sequencing of the fragment revealed the presence of a potential 1,041-nucleotide open reading frame (Fig. 2).

Alignment of the predicted protein sequence of the open reading frame to other protein sequences in the databases revealed that it was most similar to the Asd of *Vibrio cholerae*, with 61% identity and 80% similarity. Several conserved amino acids among all known *asd* sequences were also present in the

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            10         20         30         40         50         60         70         80         90
       *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
ACGTTTCAAA ATATAGCCAT ATCGTAGTTC GTTTTCTGCT TCGCAAGCAG GAATTGAACT TGACTAGCAC ATTGTTAATT GATGGTAAGA
            100        110        120        130        140        150        160
       *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
TGAGGTCATA ATG AGC AGA CAT TTG AAT GTA GCT ATC GTA GGT GCC ACC GGC GCT GTT GGA GAA ACC TTT TTA ACC
            M   S   R   H   L   N   V   A   I   V   G   A   T   G   A   V   G   E   T   F   L   T>
       170        180        190        200        210        220        230        240
       *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
GTT TTG GAA GAA AGA AAT TTT CCA ATT AAG TCC CTT TAC CCA TTA GCG AGT TCT CGC TCA GTA GGT AAG ACA GTG
 V   L   E   E   R   N   F   P   I   K   S   L   Y   P   L   A   S   S   R   S   V   G   K   T   V>
       250        260        270        280        290        300        310
       *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
ACT TTT AGA GAT CAG GAA TTG GAT GTT TTG GAT TTA GCA GAA TTT GAT TTT AGC AAA GTC GAT CTT GCT TTA TTC
 T   F   R   D   Q   E   L   D   V   L   D   L   A   E   F   D   F   S   K   V   D   L   A   L   F>
       320        330        340        350        360        370        380        390
       *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
TCT GCA GGC GGC GCT GTT TCA AAA GAG TAT GCA CCA AAA GCT GTG GCT GCT GGG TGC GTG GTG GTT GAT AAT ACC
 S   A   G   G   A   V   S   K   E   Y   A   P   K   A   V   A   A   G   C   V   V   V   D   N   T>
       400        410        420        430        440        450        460
       *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
TCC TGT TTT CGT TAT GAG GAT GAT ATT CCT CTT GTT GTC CCT GGA AGT GAA TCC TCA TCC AAT CGC GAT TAT ACT
 S   C   F   R   Y   E   D   D   I   P   L   V   V   P   G   S   E   S   S   N   R   D   Y   T>
       470        480        490        500        510        520        530        540
       *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
AAA AGA GGC ATT ATT GCT AAC CCT AAT TGC TCT ACC ATT CAG ATG GTC GTT GCT CTA AAG CCA ATC TAT GAC GCT
 K   R   G   I   I   A   N   P   N   C   S   T   I   Q   M   V   V   A   L   K   P   I   Y   D   A>
             550        560        570        580        590        600        610
       *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
GTT GGT ATT AGT CGT ATT AAT GTG GCG ACT TAC CAG TCT GTT TCC GGG ACT GGC AAA AAA GCC ATC AGC GAG CTG
 V   G   I   S   R   I   N   V   A   T   Y   Q   S   V   S   G   T   G   K   K   A   I   S   E   L>
             620        630        640        650        660        670        680        690
       *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
GTT GCT CAG GTA GGC GAT CTT TTA AAT GGG AGG CCA GCT AAT GTT CAA GTT TAT CCT CAG CAA ATT GCT TTT AAT
 V   A   Q   V   G   D   L   L   N   G   R   P   A   N   V   Q   V   Y   P   Q   Q   I   A   F   N>
             700        710        720        730        740        750        760
       *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
GCG CTT CCT CAT ATT GAT CAG TTC GAA GAC AAT GGT TAT ACC CGA GAA GAG ATG AAG ATG GTC TGG GAA ACC CGC
 A   L   P   H   I   D   Q   F   E   D   N   G   Y   T   R   E   E   M   K   M   V   W   E   T   R>
             770        780        790        800        810        820        830        840
       *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
AAG ATT ATG GAA GAT GAC AGC ATT ATG GTT AAC CCT ACA GCC GTC AGA GTT CCT GTT ATT TAT GGG CAT TCT GAA
 K   I   M   E   D   D   S   I   M   V   N   P   T   A   V   R   V   P   V   I   Y   G   H   S   E>
             850        860        870        880        890        900        910
       *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
GCG GTT CAT CTG GAG TTA AAA AAG CCT TTG ACG GCT GAC GAT GCT CGC GCG CTT TTG GCA AAG GCA CCC GGC GTT
 A   V   H   L   E   L   K   K   P   L   T   A   D   D   A   R   A   L   L   A   K   A   P   G   V>
       920        930        940        950        960        970        980        990
       *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
ACT GTA GTG GAT AAT CTT TCT AAA GCA AGT TAT CCC ACA GCA ATT AAG AAT GCA GTC GGG CAT GAT GAT GTT TTT
 T   V   V   D   N   L   S   K   A   S   Y   P   T   A   I   K   N   A   V   G   H   D   D   V   F>
             1000       1010       1020       1030       1040       1050       1060
       *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
GTA GGG CGC ATA AGA CAG GAT ATT TCT CAT CCT TGT GGA CTA AAT TTG TGG ATA GTC GCA GAT AAC ATT CGC AAA
 V   G   R   I   R   Q   D   I   S   H   P   C   G   L   N   L   W   I   V   A   D   N   I   R   K>
       1070       1080       1090       1100       1110       1120       1130       1140
       *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
GGT GCG GCA ACT AAC GCG GTA CAG ATC GCT GAA ATA CTG CAA AGA GAG TTT CTG TTG AAG CTC TCT TTG CCA CAA
 G   A   A   T   N   A   V   Q   I   A   E   I   L   Q   R   E   F   L   L   K   L   S   L   P   Q>
             1150       1160       1170       1180       1190       1200       1210       1220       1230
       *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
TAA CAGAGA TTCAGTATCT TGATAGCAAA CTTGTATGTC CTCCATCGGC CATGCAGGTT ATGTTAGAAA AAATGCCATA CTTTTTATAT
...
             1240       1250       1260       1270       1280       1290       1300       1310       1320
       *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
CCTAAGTTAT ACTTCTATTA TGACTGTTAA ATTTTGAAAA AAGATGGCGA AAGAATATTA TTTATCCGAT GAGATCGAAG AAATAGTGCT
             1330       1340       1350       1360       1370       1380       1390       1400       1410
       *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
CGCCGAGTACT GTATTGTCTT ACTCTCGACC CAATTTATCA TCAAAACGCT TCAATGATTT AAAAAATGGA AAAACTCCCT GGGAAGCGGA
             1420       1430       1440
       *     *     *     *     *     *
ACTGGATTTA AACGGTTTAA ACGTTGAATC TGC
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FIG. 2. DNA sequence of the *L. pneumophila asd* gene. The start codon is indicated in bold.

predicted *L. pneumophila asd* gene product. Most notably were glycines 11, 14, and 17 involved in coenzyme binding, cysteine 132 and glutamine 159 in the active subunit of the enzyme, and arginine 238 important for substrate binding (36).

Generation of an *asd* insertion mutant of *L. pneumophila.* To facilitate allelic exchange into the *L. pneumophila* chromosome, the 4.3-kb fragment harboring the *L. pneumophila asd* gene was subcloned into pBOC20, designated pOHBOC1, and
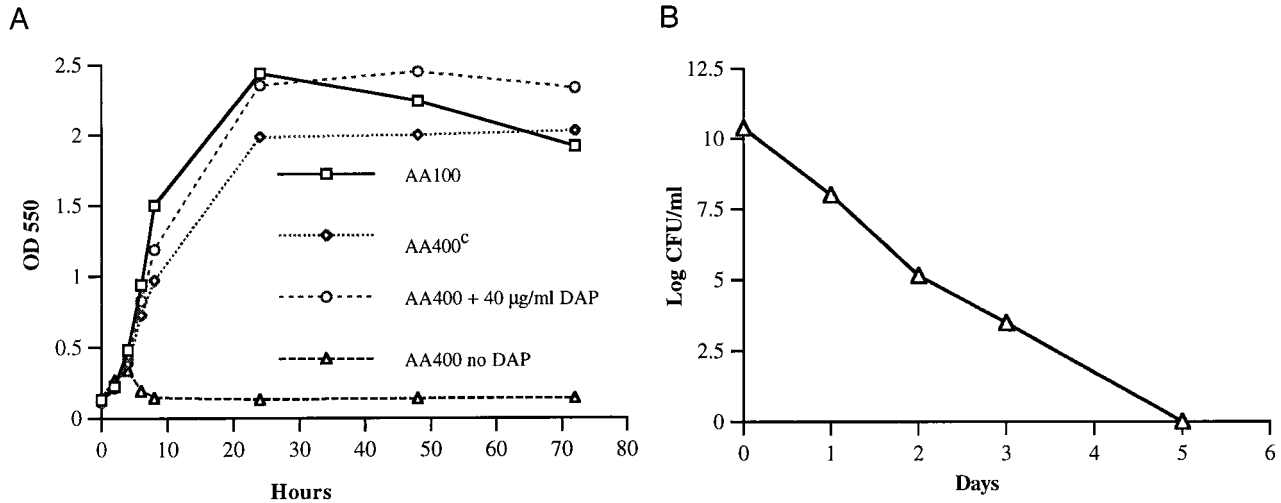
A
B

FIG. 3. (A) In vitro growth kinetics of AA400 in BYE medium. Cell density was determined at an optical density of 550 nm (OD 550). (B) AA400 grown in BYE medium in the absence of DAP. Error bars cannot be seen due to their small values.

subjected to mini-Tn*10*::*kan* mutagenesis. Selection for clones that did not complement the χ2981 *asd* defect was used to screen for potential *asd* mini-Tn*10*::*kan* insertion mutants. One of the mini-Tn*10*::*kan asd* insertion mutants, designated pOHBOC1K, was mobilized into *L. pneumophila* AA100 by conjugation, and an *asd* mutant designated AA400 was isolated (Materials and Methods) (Fig. 1A). Allelic exchange into the *L. pneumophila* chromosome was confirmed by Southern hybridization, which showed a 1.8-kb increase in the size of the fragment that contained the *asd* gene (Fig. 1B). AA400 was auxotrophic for DAP and exhibited DAP dose-dependent growth in culture medium (data not shown). A DAP concentration of 40 μg/ml was the lowest concentration sufficient for optimal *in vitro* growth of AA400 (Fig. 3A). Growth of AA400 in BYE medium without a supplement of DAP was restored by introduction of pOHBOC1 into AA400 (AA400^c [AA400 complemented with wild-type *asd*]; Fig. 3A). AA400 did not grow in the absence of DAP supplement, and viable bacteria were absent from the culture 6 days after incubation without DAP (Fig. 3B).

Cytopathogenicity of AA400 to U937 macrophage-like cells. AA100 was completely cytopathogenic to U937 cells 48 and 72 h postinfection (Fig. 4). In contrast, AA400 was completely defective in cytopathogenicity to U937 cells (Fig. 4). A 40-μg/ml supplement of DAP to the infection with AA400 only partially restored the cytopathogenicity defect of AA400 (Fig. 4). Increasing the concentration of DAP to 200 μg/ml did not enhance the cytopathogenicity of AA400 (data not shown). AA400^c exhibited wild-type cytopathogenicity to U937 cells (Fig. 4). A supplement of DAP had no effect on the cytopathogenicity of AA100 (data not shown). Thus, DAP auxotrophy in *L. pneumophila* rendered it defective for cytopathogenicity to U937 macrophages, and a supplement of DAP was unable to completely rescue the mutant.

***In vivo* growth of AA400 in U937 macrophage-like cells.** The ability of AA400 to survive and replicate in U937 macrophage-like cells was determined. In the absence of DAP, AA400 did not replicate and reached undetectable levels at 11 h postinfection in U937 cells (Fig. 5A). A supplement of DAP to U937 cells infected with AA400 resulted in a constant number of bacterial counts over the time course of the experiment, although a slight increase in bacterial numbers was observed 48 h

postinfection (Fig. 5A). Furthermore, preincubation of the U937 cells with DAP prior to infection for up to 24 h or increasing the concentration of DAP to 200 μg/ml did not enhance the viability of AA400 (data not shown). AA400^c exhibited a wild-type growth pattern in U937 cells (Fig. 5A).

In vivo growth of AA400 in protozoa. Since protozoa are the environmental hosts of *L. pneumophila*, we determined the ability of AA400 to survive within the protozoan *H. vermiformis*. In the absence of DAP, AA400 did not replicate within *H. vermiformis* and was undetectable 4 days postinfection (Fig. 5B). The viability of AA400 was slightly enhanced in the presence of DAP, although bacteria were undetectable 7 days postinfection (Fig. 5B). AA400^c exhibited a growth pattern similar to that of AA100 (Fig. 5B). Preincubation of *H. vermiformis* or another protozoan host (*Acanthamoeba polyphaga*)
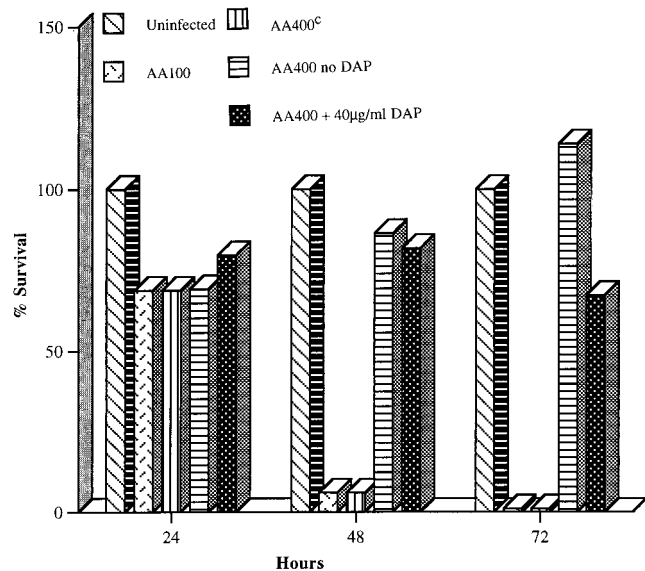

FIG. 4. Cytopathogenicity of *L. pneumophila* AA400 (Δ*asd*), AA400^c, and AA100 (wild type) to U937 macrophage-like cells. Error bars cannot be seen due to their small values.
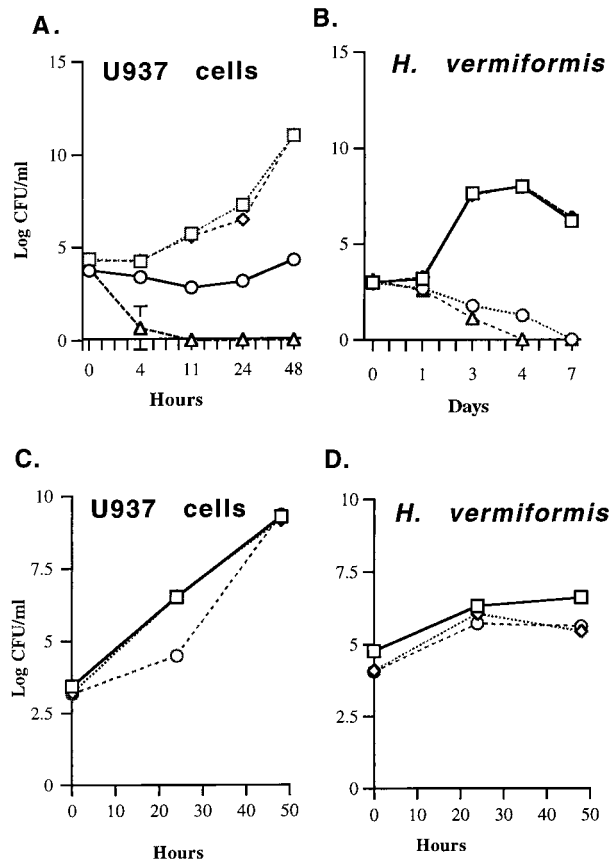
A.

B.

C.

D.

FIG. 5. (A and B) Intracellular growth kinetics of *L. pneumophila* AA400 (Δ*asd*), AA400^c, and AA100 (wild type). ○, AA400 plus 40 μg of DAP per ml; □, AA100; ◇, AA400^c; △, AA400, no DAP. (C and D) Comparison of intracellular survival of AA400 complemented with the PCR-generated *asd* (AA400^{cc}) to that of AA100 and AA400^c. CFU levels were determined by plating dilutions from each time point on BCYE agar. Some error bars cannot be seen due to their small values. □, AA100; ◇, AA400^{cc}; ○, AA400^c.

with DAP for 24 h prior to infection had no effect on the viability of AA400 (data not shown).

Does the insertion in *asd* have a polar effect? Since DAP did not rescue the mutant in U937 macrophages or *H. vermiformis*, it is possible that the mini-Tn*10*::*kan* insertion in AA400 has polar effects on genes downstream of *asd* which may be important for intracellular survival of *L. pneumophila*. Thus, to determine if the *asd* defect of AA400 is the sole contributor to its inability to survive intracellularly, we complemented this strain with a PCR-generated *asd* gene. A 1.4-kb region from pOH1 harboring the *L. pneumophila asd* gene alone was amplified by PCR. This fragment was subcloned into pBOC20 and designated pOHBOC2. pOHBOC2 was found to complement the *asd* defect of χ2981. pOHBOC2 was mobilized into AA400, and colonies were selected for kanamycin resistance and the ability to grow without DAP supplement.

The ability of one of the *asd*-complemented clones of AA400, designated AA400^{cc}, to survive and replicate within *H. vermiformis*, *A. polyphaga*, and U937 cells was compared to that of AA100 and AA400^c. pOHBOC2 complemented AA400 for intracellular survival and growth in all cell lines tested (Fig. 5C and D and data not shown). Thus, the inability of AA400 to grow intracellularly was due solely to the lack of a functional *asd* gene.

DISCUSSION

Nutritional auxotrophs of several facultative intracellular pathogens have been generated and shown to be defective in intracellular survival and avirulent in animal models (12, 20, 28). Furthermore, targeting bacterium-specific metabolic pathways with antibiotics may prove to be an effective means of interrupting a pathogen's intracellular life cycle. To determine the effect of nutritional auxotrophy on the intracellular life cycle of *L. pneumophila* and the feasibility of using the Asd enzyme as a potential target for treatment, we generated an aspartate-β-semialdehyde dehydrogenase (*asd*) mutant of *L. pneumophila*.

The *asd* mutant (AA400) was found to be completely defective in cytopathogenicity to U937 cells. In addition, AA400 was defective in intracellular growth within U937 macrophages, *H. vermiformis*, and *A. polyphaga*. In contrast to the thymidine and tryptophan auxotrophs of *L. pneumophila* that can be rescued by the respective nutrient, AA400 was not rescued by DAP (33). The inability of a DAP supplement to complement the intracellular growth of AA400 may be explained by several possibilities. First, in U937 cells the bacterial counts remained constant in the presence of DAP, which may indicate that some DAP reached the *L. pneumophila* phagosome, thereby prolonging the intracellular survival of AA400, or that the rate of replication of AA400 in the presence of DAP is equal to its rate of death in the intracellular environment. Second, although eukaryotic cells do not utilize DAP, it is possible that DAP is degraded by the cell lines examined in this study before it reaches the *L. pneumophila* phagosome. Third, it is possible that DAP is unable to cross the cell membrane of the cell lines tested or, if it does, may not reach the *L. pneumophila* phagosome in sufficient quantities, especially since 40 μg of DAP per ml was required for optimal *in vitro* growth. However, recently an *asd* mutant of *S. typhimurium* was shown to be capable of taking up DAP in the intracellular environment of Int-407 cells (15). This may indicate a difference between the phagosomal compartments of *L. pneumophila* and *S. typhimurium*, the lack of a DAP transport mechanism in the tested cell lines, or both.

It is possible that the insertion mutation in the *L. pneumophila asd* gene resulted in a polar effect, thereby altering the expression of downstream genes that may be necessary for intracellular survival. However, complementation of AA400 with the wild-type *asd* gene restored its ability to grow within all the tested cell lines. This finding indicates that the *asd* defect alone in AA400 is sufficient to disrupt its intracellular growth.

The ability of *L. pneumophila* to cause disease in humans has been closely associated with its ability to survive within protozoa in the contaminating water sources (18). The inability of AA400 to survive within *H. vermiformis* and *A. polyphaga* even in the presence of DAP in the culture media may indicate the possibility of targeting bacterium-specific metabolic pathways with antimicrobial agents in water systems. However, it should be cautioned that the inability of AA400 to survive intracellularly in tissue culture conditions may not reflect its ability for intracellular survival in environmental water reservoirs.

In summary, a DAP auxotroph of *L. pneumophila* was generated and found to be unable to survive in the intracellular environment of mammalian and protozoan cells. Our data indicate that the DAP biosynthetic pathway is a potential target for antibiotic therapy, and its disruption may be useful in the design of attenuated *L. pneumophila* vaccines. Furthermore, the inability of this mutant to survive in its environmental host may extend the idea of antimicrobial treatment of

infected individuals to the treatment of water supplies that are associated with Legionnaires' disease outbreaks.

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