Selective requirement of the shikimate pathway of *Legionella pneumophila* for intra-vacuolar growth within human macrophages but not *Acanthamoeba*

1Snake C. Jones, 1Christopher T. D. Price, 2Marina Santic, and 1Yousef Abu Kwaik*

1Department of Microbiology and Immunology and Center for Predictive Medicine, College of Medicine, University of Louisville, Louisville, KY, 40202; 2Department of Microbiology University of Rijeka, Rijeka, Croatia

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* For correspondence: abukwaik@louisville.edu
Abstract

Legionella pneumophila utilizes the Dot/Icm type IV translocation system to proliferate within a vacuole in a wide variety of natural amoebal hosts and in alveolar macrophages of the human accidental host. Although L. pneumophila utilizes host amino acids as the main sources of carbon and energy, it is not known whether de novo synthesis of amino acids by intra-vacuolar L. pneumophila contributes to its nutrition. The aroB and aroE genes encode enzymes for the shikimate pathway that generates the aromatic amino acids Phe, Trp, and Tyr. Here we show the aroB and aroE mutants of L. pneumophila to be defective in growth in human monocyte-derived macrophages (hMDMs) but not in Acanthamoeba. The aroB and aroE mutants are severely attenuated in intrapulmonary proliferation in the A/J mouse model of Legionnaires’ disease, and the defect is fully complemented by the respective wild type allele. The two mutants grow normally in rich media but do not grow in defined media lacking aromatic amino acids, and the growth defect is rescued by inclusion of the aromatic amino acids, which are essential for production of the pyomelanin pigment. Interestingly, supplementation of infected hMDMs with the three aromatic amino acids or with Trp alone rescues the intra-macrophage defect of the aroE but not the aroB mutant. Therefore, the shikimate pathway of L. pneumophila is differentially required for optimal growth within human macrophages, which are auxotrophic for Trp and Phe, but is dispensable for growth within Acanthamoeba that synthesize the aromatic amino acids.
Introduction

*Legionella pneumophila* is an environmental organism of aquatic reservoirs, where the bacteria replicate within selected species of amoebae and other unicellular protists (1-4). Upon transmission to the human accidental host, *L. pneumophila* causes Legionnaires’ disease, an often fatal pneumonia. The route of infection in humans is through inhalation of aerosolized water droplets that reach the alveolar spaces. Upon entry into human alveolar macrophages, *L. pneumophila* is localized within a vacuole designated as the *Legionella*-containing vacuole (LCV), which evades the endocytic pathway and is rapidly remodeled by the rough endoplasmic reticulum (5, 6). High throughput analyses of the proteome of the LCV have shown selective acquisition of numerous host proteins (7-9). Remodeling of the LCV is mediated by ~300 bacterial effector proteins that are injected into the host cell via the Dot/Icm type IVb secretion system (5, 10-12). These effectors modulate various cellular processes, including evasion of the endocytic pathway, innate immunity, pro- and anti-apoptotic and signaling pathways, and modulation of protein transcription and translation (5, 10, 13). During late stages of the infection, the bacteria exit the LCV and finish the last few rounds of replication within the cytosol followed by lysis of the host cell (14-16). Remarkably, the intracellular life cycle of *L. pneumophila* within human macrophages and amoebae is similar at the cellular and molecular levels (2, 3, 17).

Amino acids are the main sources of carbon and energy for intra-vacuolar proliferation of *L. pneumophila* (18-24). However, the basal cellular levels of amino acids are below the threshold needed for proliferation of many intracellular pathogens, including *Anaplasma*, *Francisella*, and *Legionella* (25-27). Therefore, intracellular bacterial pathogens have evolved efficient strategies for nutrient acquisition within host cells, such as acquiring biosynthetic genes,
or by manipulating host cell processes to elevate the cytosolic cellular levels of major sources of carbon and energy (25, 27). One primary example is *L. pneumophila* that utilizes the Dot/Icm-translocated AnkB effector to promote decoration of the LCV with polyubiquitinated proteins (28-30). Host-mediated proteasomal degradation of the LCV-decorated polyubiquitinated proteins generates a surplus of amino acids above the threshold needed for intra-vacuolar proliferation of *L. pneumophila* and its virulence *in vivo* (31). The growth defect exhibited by the *ankB* mutant within human monocyte-derived macrophages (hMDM) or amoebae, or *in vivo*, is totally rescued by supplementation with amino acids (31). Amino acids are the preferred sources of carbon and energy for *L. pneumophila*, but 7 amino acids (Cys, Met, Arg, Thr, Val, Ileu, and Leu) are essential for the organism (18-24). It has been shown by 13C-labeling that *L. pneumophila* residing within *Acanthamoeba* or macrophages imports amino acids from the host cell cytosol into the LCV to be utilized by intra-vacuolar *L. pneumophila* (23, 32), but the import mechanisms involved remain unknown.

The requirement for specific amino acids for intra-vacuolar bacterial pathogens varies according to the host cell environment, as the nutritional resources that can be accessed by the invading pathogen can vary between different hosts (25, 27, 33). *L. pneumophila* invades and proliferates within a variety of amoebal species, and therefore, the bacteria have likely adapted to the idiosyncratic nutritional resources within various protozoan hosts in various aquatic environments (2, 34). Therefore, many bacterial metabolic and biosynthetic pathways may not be needed within a certain host, depending on the nutritional resources within that host (26). It has been shown that when *L. pneumophila* is grown for hundreds of generations in mouse macrophages, unique clones arise with auxotrophy for lysine, which is sufficiently available in macrophages (35). However, the evolved lysine auxotrophic clones grow less efficiently than
the parental strain within *Acanthamoeba* and *Hartmannella*, suggesting less availability or less efficient import of lysine within amoebae compared to macrophages (35).

Although *L. pneumophila* triggers elevation of the cellular levels of amino acids (31) that are imported into the LCV (23, 32), very little is known about the role of bacterial amino acid biosynthetic pathways in intra-vacuolar proliferation. The tryptophan auxotroph of *L. pneumophila* Philadelphia-1 strain grows at a rate similar to wild type *L. pneumophila* within human monocytes (36), indicating that the host proteasomal degradation generates sufficient levels of Trp to support intra-vacuolar bacterial growth. Aromatic amino acids, whether supplied by the host cell or produced endogenously by the bacteria, are among the nutrients required for growth of *Legionella* (18, 19, 23).

Here we characterize two mutants of *L. pneumophila* strain AA100/130b that have been shown to have a defect in intra-vacuolar proliferation in human macrophages, but their proliferation in amoebae is comparable to the parental strain (37). We show one mutant is defective in the *aroB* gene, which codes for shikimate dehydrogenase, while the other mutant is defective in the *aroE* gene, which codes for 3-dehydroquinate synthase (Fig. 1). These enzymes are part of the shikimate pathway for synthesis of aromatic amino acids (L-phenylalanine, L-tryptophan, and L-tyrosine) and other compounds (Fig. 1) (38). We show that *L. pneumophila* requires an intact shikimate pathway for selective intra-vacuolar growth within human macrophages and in mouse lungs. This is the first example for the role of *de novo* amino acid biosynthesis and the shikimate pathway in intra-vacuolar proliferation of *L. pneumophila*, despite the bacteria-triggered elevation of host cellular amino acids.
Materials and Methods

Bacterial strains and vectors. The virulent *L. pneumophila* strain AA100 has been described previously (39). Selection and initial characterization of the strains GE88 and GK79, isogenic mil mutants of AA100, have been previously described (37). Sequence analysis showed that the mutated genes were homologs of *aroB* (GE88) and *aroE* (GK79). Complementing plasmids pAB2 (*aroB*) and pAE7 (*aroE*) were constructed by amplifying segments of the AA100 genome containing AroE (lpg2808 in the *L. pneumophila* Philadelphia genome) and AroB (lpg0933), with enough flanking sequence of ~1kb to include their promoters and termination signals, using primers XbaI_aroE_R, CGCTCTAGAGCAATGCCCGGATGA; BamHI_aroE_F, CGCGGATCCATCCTCTTAA; XbaI_aroB_F, GCGTATTCTAGACGGGTGGCGGAGTCG; and BamHI_aroB_R, CGCATTGGATCCATTCGCAACATCA. Amplified segments were cloned in pBC SK+ (Stratagene Inc., La Jolla, CA) using the enzymes indicated in the primer names. Complementing plasmids were introduced into the mutant strains by electroporation with an ECM 630 (Harvard Apparatus).

Medium and growth conditions. CDM was prepared as previously described (39). CDMs were also prepared with L-phenylalanine (Phe, F), L-tryptophan (Trp, W), or L-tyrosine (Tyr, Y), or all three aromatic amino acids (AAAs), omitted. *L. pneumophila* strains were grown at 37°C for 3 days on BCYE agar, with antibiotics when appropriate, and suspended in CDM or CDM without Tyr, Trp and Phe for inoculating liquid cultures, or in RPMI for infection of macrophages.
Growth and pigment production in CDM. CDM cultures were incubated at 37°C with shaking at 180 rpm, and protected from light. At various time points, samples were removed and OD$_{550}$ was read for each culture, and then for each culture supernatant after centrifugation to remove bacteria. Supernatant OD (pigment) was subtracted from the OD of the culture to estimate growth.

Intracellular growth kinetics within U937 macrophages and hMDMs

U937 macrophage-like cells were grown in complete RPMI (RPMI 1640 with 200 mM L-glutamine, 25 mM HEPES, and 10% fetal bovine serum) at 37°C and 5% CO$_2$ as we described previously (28). Two days prior to infection, U937 cells were adjusted to 5x10$^5$/ml in fresh medium. Phorbol 12-myristate 13-acetate was added at 50 ng/ml, and 10$^5$ cells per well were placed in 96-well plates. Human monocyte-derived macrophages (hMDMs) were prepared as we described previously (28). Briefly, blood was drawn from healthy human donors, heparinized, and separated on a Ficoll-Hypaque gradient. The monocyte fraction was collected, washed 3 times with Hanks’ balanced salt solution, then resuspended at about 10$^7$ cells/ml in RPMI with 20% FBS. The cells were incubated 3 days in 6-well, ultra low attachment plates (Corning) at 37°C and 5% CO$_2$. Cells were scraped, macrophages were counted, and cells were suspended at 5x10$^5$ macrophages/ml in RPMI plus 10% FBS; 10$^5$ cells per well were placed in 96-well plates and incubated 2 days. The medium was replaced with RPMI plus 5% FBS, and plates were incubated 1 day. The medium was replaced with RPMI plus 1% FBS, and plates were incubated 1 day. Two hours prior to infection, U937 cells were washed 3 times with RPMI to remove PMA. Both U937 and hMDMs were placed in 200 µl complete RPMI, or in complete RPMI supplemented with aromatic amino acids (obtained from Sigma) at the concentrations...
used in CDM (Phe 0.45 g/l, 2.7 mM; Trp 0.4 g/l, 2 mM; and Tyr 0.075 g/l, 0.4 mM). Infections with *L. pneumophila* were done in triplicate at an MOI of 1. After addition of the bacteria, plates were centrifuged 5 min at 210 RCF, and incubated at 37°C with 5% CO2 for 1 h. Cells were washed 3 times with fresh medium to remove extracellular bacteria, and incubated at 37°C with 5% CO2. Cells were lysed in sterile dd-H2O at 1, 24, 48, and 72 hours post-infection, and dilutions were plated on BCYE for counting.

Additional experiments were done as above, but with some modifications. Medium was replaced at 1 hour prior to infection; after 1 hour of infection, cells were treated with gentamicin, 50 µg/ml, for 1 hour, washed 3 times, and medium replaced; time points were 2, 24, and 48 hours post-infection. Amino acid supplements were standardized to 1 mM each, and for demonstrating a dose response, concentrations were 1, 0.75, 0.5, and 0.25 mM.

**Infection of A/J mice with *L. pneumophila***

Female pathogen-free, 6-8 week old A/J mice from our own colony that we maintain were used for infection by intra-tracheal inoculation with 50 µl containing the bacterial dose, as we described previously (28, 40). Mice were humanely euthanized at various times, the lungs were removed, homogenized and dilutions were cultured on BCYE agar for 72 h, as described previously (28, 40).
Results

Characterization of the GK79 and GE88 mutants and their virulence in A/J mice

Most of the intra-macrophage defective mutants of *L. pneumophila* exhibit similar phenotypes within amoebae, and in particular the Dot/Icm translocation-defective mutants (41, 42). However, a collection of *L. pneumophila* mutants have been isolated and shown to be selectively defective for replication within macrophages but not amoebae, and the defective loci in these mutants have been designated as macrophage infectivity loci (*mil*) (37). One of the *mil* mutants has been characterized to be defective in *htrA*, indicating higher level of exposure to stress conditions within human macrophages compared to amoebae (43). In this study, we characterized the two *mil* mutants designated GK79 and GE88 (37). Sequence analyses of the defective loci within both mutants showed that the GE88 mutant was defective in the *aroB* gene, which codes for shikimate dehydrogenase, while the GK79 mutant was defective in the *aroE* gene, which codes for 3-dehydroquinate synthase (Fig. 1). Both genes appear to be monocistronic and are located in different regions of the genome.

Since the *aroB* and *aroE* mutants are defective in intracellular proliferation within human macrophages (37), we determined whether the two mutants were defective in intrapulmonary proliferation of *L. pneumophila* in vivo. We inoculated A/J mice intra-tracheally with $10^6$ CFUs of the wild type (WT) *L. pneumophila* strain, the *aroB* or *aroE* mutants, or the *aroB* and *aroE* mutants complemented with the respective WT allele. Proliferation of *L. pneumophila* in the lungs of infected mice was assessed by enumeration of the CFUs in lung homogenates up to 3 days post-infection. As expected, there was robust intrapulmonary proliferation by the WT strain observed by 2 days post-infection (Fig. 2). In contrast, there was no detectable intrapulmonary proliferation for either of the two mutants, compared to the WT strain (Student $t$-
test, \( p < 0.003 \). Importantly, intrapulmonary proliferation of the \( aroB \) and \( aroE \) mutants complemented with the respective WT allele was indistinguishable from the WT strain (Student \( t \)-test, \( p > 0.6-0.9 \)) (Fig. 2). We conclude that \( aroB \) and \( aroE \) are indispensable for intra-
macrophage and intrapulmonary proliferation of \( L. pneumophila \) in vivo in the mouse model, but are dispensable for growth within \( Acanthamoeba \) (37).

**Growth of \( L. pneumophila \) mutants in chemically defined medium**

We determined whether the two mutants were auxotrophic for the aromatic amino acids (Phe, Trp, and Tyr) that are the products of the shikimate pathway (Fig. 1). The WT strain and both mutants grew similarly in rich BYE broth within the 24h period examined (Fig. 3A). Therefore, we determined growth in chemically defined medium (CDM) (39). The WT strain grew to a maximum OD\(_{550}\) of 1 after 48h in CDM (Fig. 3B). However, both the \( aroB \) and \( aroE \) mutants grew slower than the WT strain in CDM, but the slower growth was efficiently rescued by genetic complementation of the two mutants by a plasmid harboring the respective WT gene (Fig. 3B).

**Auxotrophy of \( L. pneumophila aroB \) and \( aroE \) mutants for aromatic amino acids**

Since the enzymes encoded by \( aroB \) and \( aroE \) function in the shikimate synthesis pathway, which leads through chorismate to synthesis of the three aromatic amino acids (Fig. 1), we determined whether the two mutants were auxotrophic for the three aromatic amino acids. The data showed that the WT strain did not grow in CDM lacking all three aromatic amino acids, but grew in CDM lacking Tyr or Trp, similar to the complete CDM (Fig. 4A). However, the WT
strain grew slower to a maximum $OD_{550}$ of ~0.5 in CDM lacking Phe (Fig. 4A). This indicates that *de novo* synthesis of Phe is not sufficient for optimal growth of *L. pneumophila* *in vitro*. Both the *aroB* and *aroE* mutants grew somewhat slower than the wild type strain in CDM (Fig. 3) and in CDM lacking Tyr (Fig. 4B and D). However, both mutants failed to grow in CDM lacking either Phe or Trp (Fig. 4B and D). Complementation of each mutant with a plasmid carrying the respective wild type gene enabled the mutants to grow in CDM at a rate similar to wild type (Fig. 3). Complementation of the *aroB* mutant restored growth in CDM lacking Trp (Fig. 4C), but not for the *aroE* mutant (Fig. 4E). However, complementation did not restore growth to the mutants in CDM lacking Phe (Fig. 4C and E). This may not be surprising since Phe is required for optimal growth of the WT strain. We conclude that the *aroB* and *aroE* mutants are auxotrophic for Phe and Trp but not Tyr as they have the capacity to convert phenylalanine to tyrosine.

**Pigment production by *L. pneumophila* *aro* mutants**

Upon growth transition of *L. pneumophila* into the stationary phase (44, 45), bacterial catabolism of Tyr produces homogentisic acid (HGA), which oxidizes and polymerizes to become a pyomelanin, a brown pigment (Fig. 1) (46-50). It has been shown that abundance of either tyrosine or phenylalanine in the medium increases pigment production (51), and that conversion of Phe into Tyr by the phenylalanine hydroxylase (PAH, Fig. 1) provides the excess Tyr needed to produce the pigment (51). The pigment plays no role in intracellular survival, but has been shown to confer resistance of *L. pneumophila* to ordinary light, and thought to enable ecological adaptation of *Legionella* species in the environment (47-49). HGA and HGA-pyomelanin are involved in iron acquisition, reducing ferric iron to ferrous iron, which can be...
taken up by FeoB of Legionella (46). Since both mutants are defective in the shikimate pathway that synthesizes Tyr, the catabolism of which produces the pyomelanin pigment, we determined whether the two aro mutants produced the brown pigment. The data showed that the two mutants produced pigment in CDM, albeit slightly less than the WT strain, which correlated with the slower growth of the two mutants in CDM compared to the WT strain (Fig. 5). At 72 h, the aroB mutant produced about 65% of the WT strain level, and aroE mutant produced about 48% of the WT level of the pigment (Fig. 5B, C). The WT strain did not produce pigment when all three amino acids (FYW) were omitted from the CDM (Fig. 5). The pigment level produced by the WT strain was not affected when Trp was omitted from the CDM medium; while omission of Tyr caused ~45% reduction, and omission of Phe caused >70% reduction in pigment production (Fig. 5A).

Neither of the two aro mutants produced pigment when all three amino acids (FYW) were omitted from the CDM, similar to the WT strain (Fig. 5). The aroB and aroE mutants produced no pigment if Phe or Trp was omitted from the CDM medium, but production was not affected when Tyr was omitted for the mutants (Fig. 5B, C). This supports the notion that pigment production is largely driven by the first step of converting phenylalanine to tyrosine, and not merely by the presence of tyrosine.

**Effect of aromatic amino acid supplementation on rescue of the intra-macrophage defect of the aroB and aroE mutants**

The aroB and aroE mutants are auxotrophic for the aromatic amino acids and are defective in intra-macrophage growth. Therefore, we determined whether supplementation with excess aromatic amino acids would rescue the aroB and aroE mutants. The data showed that the
WT strain increased by ~1000-fold cfu within 48 hours of infection of hMDMs (Fig. 6).

Supplementation of the tissue culture media with the three aromatic amino acids had no effect on intracellular growth of the WT strain (Fig 6A and B). Both mutants exhibited a partial intra-macrophage defect at 24-48h and never approached the level of cfus for the wild type strain.

Supplementation with aromatic amino acids (1 mM) in *L. pneumophila*-infected hMDMs did not rescue the intra-macrophage defect of the *aroB* mutant (Fig. 6A). However, upon supplementation of the three aromatic amino acids, intracellular growth of the *aroE* mutant was completely restored, similar to the wild type strain levels (Fig. 6B).

Since the three aromatic amino acids rescued the *aroE* but not the *aroB* mutant for the intra-macrophage growth defect, we determined whether a single aromatic amino acid supplementation could rescue the *aroE* mutant within hMDMs. We observed that supplementation of cell culture media with Tyr or Phe alone did not rescue the intracellular replication defect of the *aroE* mutant in hMDMs (data not shown). In contrast, supplementation with Trp alone rescued growth of the *aroE* mutant in hMDMs to the same extent as the combination of all three aromatic amino acids, and similar to the WT strain (Fig. 7A).

Importantly, rescue of the intracellular growth defect of the *aroE* mutant within hMDMs by Trp supplementation was dose-dependent at concentrations of 0.25-1 mM (Fig. 7B). When Trp was supplemented at 0.25 mM, *aroE* mutant replication was not restored, but supplementation at ≥0.5 mM was sufficient for optimal growth similar to the WT strain.

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L. pneumophila is auxotrophic for seven amino acids (Leu, Ile, Met, Val, Thr, Cys, and Arg) (18, 22, 23). Therefore, L. pneumophila is capable of importing sufficient levels of the essential amino acids from the host cytosolic pool (23, 32) whose threshold is elevated through proteasomal degradation within both human macrophages and Acanthamoeba (31). Our data indicate that in addition to the LCV-mediated import of the aromatic amino acids from the host cell cytosol (23, 32), de novo synthesis of aromatic amino acids through the shikimate pathway is required for optimal intra-vacuolar growth of L. pneumophila within mammalian macrophages. Using chemical mutagenesis, a tryptophan auxotroph of L. pneumophila Philadelphia-1 strain was reported in 1988 and the mutant strain grows within human monocytes at a rate similar to wild type L. pneumophila (36). The genetic defect of the mutant is not known but it is likely to be at a metabolic step that has no effect on the synthesis of the other two aromatic amino acids. Since the aroB and aroE mutants are defective within human macrophages but not within Acanthamoeba, the two auxotrophic mutants obtain sufficient amounts of the three aromatic amino acids within Acanthamoeba but not in human macrophages, and the aroB/E-encoded enzymes are potential targets for therapy (52).

Upon growth transition of L. pneumophila into the stationary phase (44), the HGA-pyomelanin brown pigment, which is derived from the catabolism of Tyr, is produced (46). Interestingly the phhA-encoded PAH of L. pneumophila converts Phe into Tyr in a single step, which provides the excess Tyr needed to produce the pigment (51). Both HGA and pyomelanin are involved in iron acquisition, reducing ferric iron to ferrous iron (53). That the two aro mutants produce the pyomelanin pigment in the absence of Tyr is likely due to the ability of L. pneumophila to convert Phe into Tyr by the phhA-encoded PAH (51).
Human cells are auxotrophic for nine amino acids (Leu, Ile, Met, Val, Thr, Phe, Trp, His, Lys), which include the two aromatic amino acids Trp and Phe, but not Tyr. However, all three aromatic amino acids are not essential for, and are synthesized by *Acanthamoeba*, which has an intact shikimate pathway (32, 54). Therefore, the ability of *Acanthamoeba* to synthesize the three aromatic amino acids correlates with the normal replication of the two mutants within *Acanthamoeba*. The defective phenotype of the *aroB* and *aroE* mutants within mammalian macrophages compared to *Acanthamoeba* is likely impacted by the auxotrophy of macrophages for two of the three aromatic amino acids. It is possible that differential levels of each of the aromatic acids in the cytoplasm of macrophages vs. *Acanthamoeba* are a contributing factor for the defect of the two *aro* mutants within mammalian macrophages but not *Acanthamoeba*. It is also possible that the LCV-mediated import of aromatic amino acids may be more efficient in *Acanthamoeba* compared to mammalian macrophages.

Although import of aromatic amino acids by the LCV membrane and its subsequent utilization by intra-vacuolar *L. pneumophila* has been already documented (23, 32), and expression of many host SLC amino acid transporters is triggered upon infection of human macrophages by *L. pneumophila* (55), the mechanisms of import of amino acids by the LCV remain unknown. Since many host SLC amino acid transporters have been detected in the proteome of the LCV within macrophages (7-9), it is likely that import of amino acids is mediated by some of the SLCs that are incorporated into the LCV membrane. The SLC1A5 neutral amino acid transporter has been shown to be required for intra-vacuolar growth of *L. pneumophila* (56), but its potential LCV localization remains to be validated. Different phenotypes of the two *aro* mutants in macrophages vs. *Acanthamoeba* may be also explained by
possible different levels or efficiency of LCV-mediated import of the amino acids in the two
evolutionarily distant hosts.

The *aroE* mutant of *L. pneumophila* is rescued for its defect in intracellular proliferation
within hMDMs upon supplementation with Trp. In contrast, the *aroB* mutant was not rescued by
supplementation with any of the three aromatic amino acids, despite the fact that the two
enzymes encoded by *aroB* and *aroE* are essential for the synthesis of these amino acids (Fig. 1).
However, there is a metabolic pathway that branches off the main shikimate pathway between
the two steps catalyzed the *aroB* - and *aroE*-encoded enzymes, and this branch pathway leads to
the formation of protocatechuate (Fig. 1). The PRODORIC database shows that *L. pneumophila*
is capable of synthesis of protocatechuate from 3-dehydroshikimate. Protocatechuate is
important in plants and fungi as a precursor or metabolite of a number of phenolic compounds,
and is itself an antioxidant (57). Protocatechuate is catabolized by the β-ketoadipate pathway,
which is found in a wide variety of soil microbes (57, 58), but the *L. pneumophila* AA100/130b
genome does not show the presence of any putative enzyme for this pathway. Therefore, the
pathway branch off the shikimate pathway prior to the formation of shikimate is likely required
for intra-macrophage replication of *L. pneumophila*, independent of the aromatic amino acids.
However, future studies must verify directly the potential role of protocatechuate synthesis in
intra-vacuolar growth of *L. pneumophila* in human macrophages.

Our data show that the *aroE* mutant is rescued for its intracellular growth defect within
hMDMs by supplementation of Trp but not Phe or Tyr. This is despite the prediction that the
*aroE* mutant is defective in synthesis of the three aromatic amino acids (Fig. 1). Thus, the
shikimate pathway and *de novo* synthesis of Phe and Tyr is required for optimal intra-vacuolar
growth of *L. pneumophila*. However, *de novo* synthesis of Trp is not required for intra-vacuolar
growth of *L. pneumophila* within human macrophages, if a sufficient exogenous level of Trp is provided, in addition to the major source of host-mediated proteasomal degradation (31). *L. pneumophila* has the capacity to convert Trp into chorismic acid, which is the precursor for the synthesis of Tyr and Phe (Fig. 1) and therefore Trp metabolism may alleviate the loss of these two amino acids. The inability to rescue the intra-macrophage defect of the *aroE* mutant by supplementation of Tyr or Phe may possibly be due to a negative feed-back regulation of the first enzymatic step in the shikimate pathway (Fig. 1) (59). We conclude that the import of Tyr and Phe by the LCV (23, 32) is not sufficient to compensate for the lack of *de novo* synthesis of the two amino acids by intra-vacuolar *L. pneumophila* within mammalian macrophages.

In conclusion, our data show that the shikimate pathway is dispensable for intra-amoeba growth of *L. pneumophila* but is selectively required for optimal intra-macrophage growth and for intrapulmonary proliferation in mice. The *aroB* and *aroE* mutants are auxotrophic for the aromatic amino acids, two of which are also essential for macrophages but not *Acanthamoeba*. This is the first example of requirement of an amino acid biosynthetic pathway in intracellular growth of *L. pneumophila*, despite the AnkB-mediated generation of high levels of cellular amino acids in the infected cells.
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Figure legends

**Fig. 1.** The shikimate pathway of *L. pneumophila*.  
**Fig. 2.** Intra-pulmonary growth of *L. pneumophila* in A/J mice. Three A/J mice for each time point were infected with $10^6$ cfus of *L. pneumophila* WT strain, or one of the *aro* mutant mutants, or their complemented counterparts (/c). At each time point, three mice were sacrificed and lungs were obtained, homogenized and dilutions were plated on agar for CFU enumeration. The results are the mean of 3 mice/time point. These results are representative of two independent experiments.  
**Fig. 3.** Growth of *L. pneumophila* and the *aroB* and *aroE* mutants in BYE or their complemented counterparts (/c) (A) or in chemically defined medium (CDM) (B). The data are representative of 4 independent experiments.  
**Fig. 4.** Growth of WT (A) or *aro* mutants (B and D) of *L. pneumophila* in CDM or CDM deficient in aromatic amino acids. The *aroB/c* (C) and *aroE/c* (E) are the mutants complemented with the respective WT allele. ‘-’ indicates CDM deficient in the indicated aromatic amino acid; -F is lacking Phe, -Y is lacking Tyr, and -W is lacking Trp. The data are representative of 5 independent experiments.  
**Fig. 5.** Pigment production by *Legionella pneumophila* WT strain (A), the *aroB* mutant (B), and the *aroE* mutant (C) in CDM with or in CDM lacking aromatic amino acids. The Y-axis is the %
normalization of pigment production to the WT strain (100%) in CDM. +FYW contains all 3 aromatic amino acids, -FYW is lacking all 3 aromatic amino acids, -F is lacking Phe, -Y is lacking Tyr, and -W is lacking Trp. Error bars are the standard deviation of triplicate samples and the data shown are representative of three independent experiments.

Fig. 6. Effect of supplementation of aromatic amino acids on rescue of the intracellular growth defect of the aroB and aroE mutants in hMDMs. The hMDMs were infected for 1h with either the WT strain or the aroB (A) or aroE (B) mutants at an MOI of 10, followed by 1h gentamicin treatment to kill remaining extracellular bacteria. Intracellular growth was assessed by enumeration of CFUs on agar plates and the data are shown as the log_{10} fold increase in CFUs at 24, 48 and 72h relative to the 2h post-infection. ‘FWY’ indicates supplementation with 1mM of the three aromatic amino acids. Error bars represent standard deviation of triplicate samples and the data shown are representative of three independent experiments. NS, indicates no statistically significant (student t-test) difference between the aroB mutant in presence or absence of aromatic amino acids supplementation at 48h post-infection. The * represents a statistically significant increase in CFUs recovered from the aroE mutant with FWY supplementation versus without supplementation at 48h post-infection (student t-test, p <0.05).

Fig. 7. Tryptophan supplementation rescues the aroE mutant intracellular growth defect in hMDMs. A) hMDMs supplemented with all three aromatic acids (FWY), or Trp (W) alone were infected with the WT strain or the aroE mutant as described in figure 6. B) Dose-dependent rescue of the aroE mutant by increasing concentrations of Trp. Intracellular growth was assessed by enumeration of CFUs on agar plates and the data are shown as the log_{10} fold increase in CFUs.
at 24, 48 and 72h relative to 2h post-infection. Asterisks represent statistically significant increases in CFUs of the aroE mutant with Trp supplementation at 48h post-infections versus without supplementation (students t-test, \( p < 0.05 \)). The data points are averages of 3 replicates for each treatment at each time point and the results were reproducible in three independent experiments.
References


47. **Steinert M, Engelhard H, Flugel M, Wintermeyer E, Hacker J.** 1995. The Lly protein protects *Legionella pneumophila* from light but does not directly influence its


Fig. 1.
Fig. 2.

![Graph showing log_{10} CFU/ml over time for different strains.]

- wt
- aroE
- aroB
- aroB/c
- aroE/c

Time:
- 0h
- 2h
- 24h
- 48h
- 72h
Fig. 3.
Fig. 4.
Fig. 5.
Fig. 6.  

**Log**\textsubscript{10} CFU/ml, T/T\textsubscript{0}  

- **WT**  
- **WT+FWY**  
- **aroB**  
- **aroB+FWY**  

**Log**\textsubscript{10} CFU/ml, T/T\textsubscript{0}  

- **WT**  
- **WT+FWY**  
- **aroE**  
- **aroE+FWY**  

*ns*
Fig. 7.