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Selective Requirement of the Shikimate Pathway of *Legionella pneumophila* for Intravacuolar Growth within Human Macrophages but Not within *Acanthamoeba*

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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 intravacuolar *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* spp. 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 alleles. 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 intramacrophage 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 the *Acanthamoeba* spp. that synthesize the aromatic amino acids.**

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 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 antiapoptotic 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 cycles of *L. pneumophila* within human macrophages and amoebae are similar at the cellular and molecular levels (2, 3, 17).

Amino acids are the main sources of carbon and energy for intravacuolar 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* spp. (25–27). Therefore, intracellular bacterial pathogens have evolved efficient strategies for nutrient acquisition within host cells, such as by acquiring biosynthetic genes or 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*, which 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 intravacuolar 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 ^{13}C labeling that *L. pneumophila* residing within *Acanthamoeba* spp. or macrophages imports amino acids from the host cell cytosol into the LCV to be utilized by intravacuolar *L. pneumophila* (23, 32), but the import mechanisms involved remain unknown.

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The requirement for specific amino acids for intravacuolar bacterial pathogens varies according to the host cell environment, as the nutritional resources that can be accessed by the invading pathogen can differ 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* spp., 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 intravacuolar proliferation. The tryptophan auxotroph of the *L. pneumophila* Philadelphia-1 strain grows at a rate similar to that seen with wild-type (WT) *L. pneumophila* within human monocytes (36), indicating that the host proteasomal degradation generates sufficient levels of Trp to support intravacuolar bacterial growth. Aromatic amino acids (AAAs), 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 intravacuolar proliferation in human macrophages but whose proliferation in amoebae is comparable to that of the parental strain (37). We show that 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 [Phe {F}], L-tryptophan [Trp {W}], or L-tyrosine [Tyr {Y}]) and other compounds (Fig. 1) (38). We show that *L. pneumophila* requires an intact shikimate pathway for selective intravacuolar growth within human macrophages and in mouse lungs. This is the first example demonstrating the role of *de novo* amino acid biosynthesis and the shikimate pathway in intravacuolar proliferation of *L. pneumophila*, despite the bacterium-triggered elevation of host cellular amino acid levels.

MATERIALS AND METHODS

Bacterial strains and vectors. The virulent *L. pneumophila* AA100 strain has been described previously (39). Selection and initial characterization of 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 ~1 kb to include their promoters and termination signals, using primers XbaI_aroE_R (CGCTCTAGAGCAATGCCCGGATGA), BamHI_aroE_F (CGCGGATCCATCCTCTTCCCTTAA), 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 generator (Harvard Apparatus).

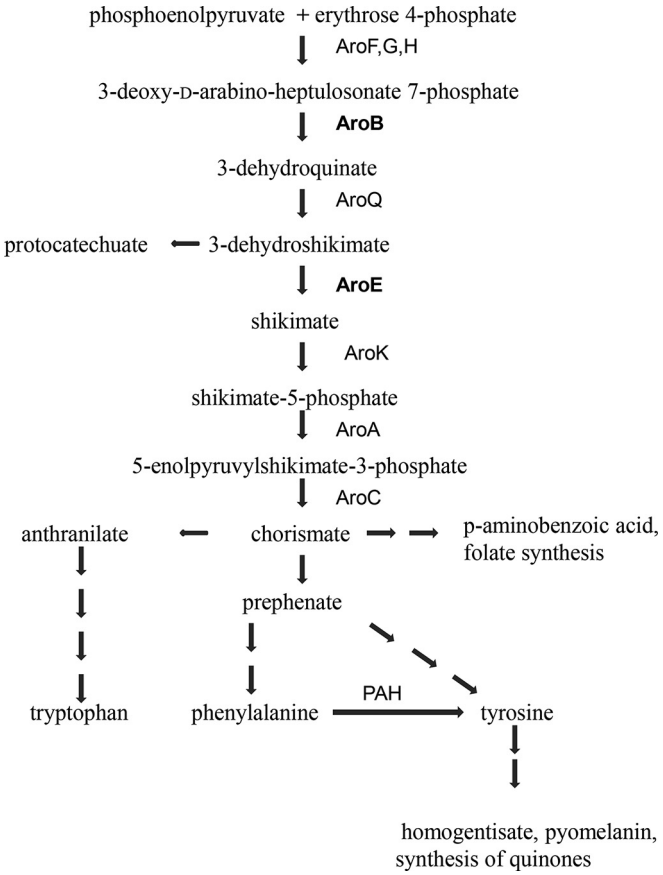

FIG 1 The shikimate pathway of *L. pneumophila*.

Medium and growth conditions. Chemically defined medium (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 with all three aromatic amino acids (AAAs) omitted. *L. pneumophila* strains were grown at 37°C for 3 days on buffered charcoal yeast extract (BCYE) agar, with antibiotics when appropriate, and suspended in CDM or CDM without Tyr, Trp, and Phe for inoculating liquid cultures or in RPMI medium for infection of macrophages.

Growth and pigment production in CDM. CDM cultures were incubated at 37°C with shaking at 180 rpm and were protected from light. At various time points, samples were removed and the optical density at 550 nm (OD_{550}) was read for each culture and then for each culture supernatant after centrifugation to remove bacteria. The supernatant OD (pigment) value was subtracted from the OD value of the culture to estimate growth.

Intracellular growth kinetics within U937 macrophages and hMDMs. U937 macrophage-like cells were grown in complete RPMI medium (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 5×10^5/ml in fresh medium. Phorbol 12-myristate 13-acetate (PMA) 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, hep-

arinized, and separated on a Ficoll-Hypaque gradient. The monocyte fraction was collected, washed 3 times with Hanks' balanced salt solution, and then resuspended at about 10^7 cells/ml in RPMI medium with 20% fetal bovine serum (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 5×10^5 macrophages/ml in RPMI medium plus 10% FBS; 10^5 cells per well were placed in 96-well plates and incubated 2 days. The medium was replaced with RPMI medium plus 5% FBS, and plates were incubated 1 day. The medium was replaced with RPMI medium plus 1% FBS, and plates were incubated 1 day. Two hours prior to infection, U937 cells were washed 3 times with RPMI medium to remove PMA. Both U937 and hMDMs were placed in 200 μl complete RPMI medium or in complete RPMI medium supplemented with aromatic amino acids (obtained from Sigma) at the concentrations used in CDM (0.45 g/liter Phe, 2.7 mM; 0.4 g/liter Trp, 2 mM; and 0.075 g/liter Tyr, 0.4 mM). Infections with *L. pneumophila* were done in triplicate at a multiplicity of infection (MOI) of 1. After addition of the bacteria, plates were centrifuged for 5 min at a relative centrifugal force (RCF) of 210 and incubated at 37°C with 5% CO_2 for 1 h. Cells were washed 3 times with fresh medium to remove extracellular bacteria and incubated at 37°C with 5% CO_2. Cells were lysed in sterile double-distilled water (ddH_2O) at 1, 24, 48, and 72 h postinfection, and dilutions were plated on BCYE for counting.

Additional experiments were done as described above but with some modifications. Medium was replaced at 1 h prior to infection; after 1 h of infection, cells were treated with gentamicin (50 μg/ml) for 1 h and washed 3 times and the medium was replaced; the time points were 2, 24, and 48 h postinfection. 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-to-8-week-old A/J mice from our own colony that we maintain were used for infection by intratracheal 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 and 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 intramacrophage-defective mutants of *L. pneumophila*, in particular, the Dot/Icm translocation-defective mutants, exhibit similar phenotypes within amoebae (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 macrophage infectivity loci (*mil*) (37). One of the *mil* mutants has been characterized to be defective in *htrA*, indicating a 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 the two mutants showed that the GE88 mutant was defective in the *aroB* gene, which codes for shikimate dehydrogenase, whereas the GK79 mutant was defective in the *aroE* gene, which codes for 3-dehydroquinate synthase (Fig. 1).

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 intratracheally with 10^6 CFU of the wild-type (WT) *L. pneumophila* strain, the *aroB* or *aroE* mutants, or the *aroB* and *aroE* mutants complemented with the respective WT alleles. Proliferation of *L. pneumophila* in the lungs of infected mice was assessed by enumeration of the CFU in lung homogenates at up to 3 days postinfection. As expected, there was robust intrapulmonary proliferation by the WT strain observed by 2 days postinfection (Fig. 2). In contrast, there was no intrapulmonary proliferation detectable 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 alleles was indistinguishable from that of the WT strain (Student *t* test, $P > 0.6$ to 0.9) (Fig. 2). We conclude that *aroB* and *aroE* are indispensable for intramacrophage and intrapulmonary proliferation of *L. pneumophila in vivo* in the mouse model but are dispensable for growth within *Acanthamoeba* spp. (37).

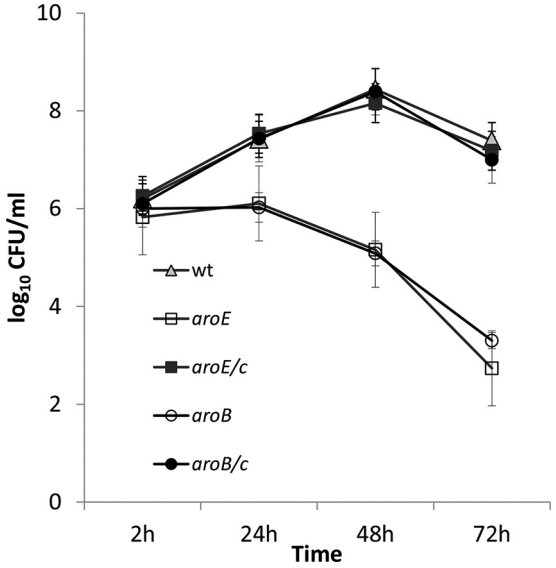

FIG 2 Intrapulmonary growth of *L. pneumophila* in A/J mice. Three A/J mice for each time point were infected with 10^6 CFU of *L. pneumophila* WT strain or one of the *aro* mutant mutants or their complemented counterparts (/c). At each time point, three mice were sacrificed, lungs were obtained and homogenized, and dilutions were plated on agar for CFU enumeration. The results are the means of the data determined from 3 mice/time point. These results are representative of two independent experiments.

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 the two mutants grew similarly in rich buffered yeast extract (BYE) broth within the 24-h 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 48 h 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 plasmids harboring the respective WT genes (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

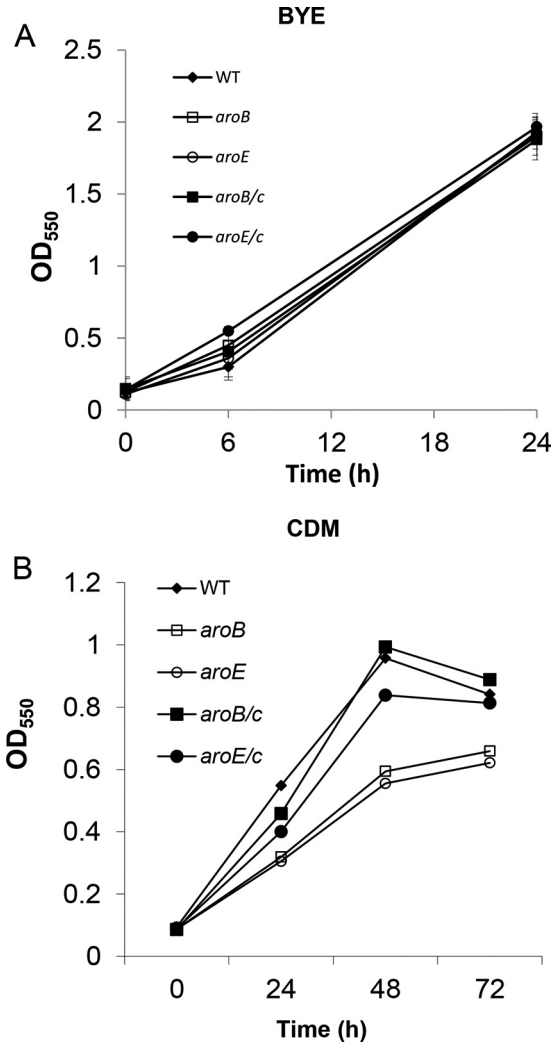

FIG 3 Growth of *L. pneumophila* and the *aroB* and *aroE* mutants or their complemented counterparts (/c) in BYE medium (A) or in chemically defined medium (CDM) (B). The data are representative of the results of 4 independent experiments.

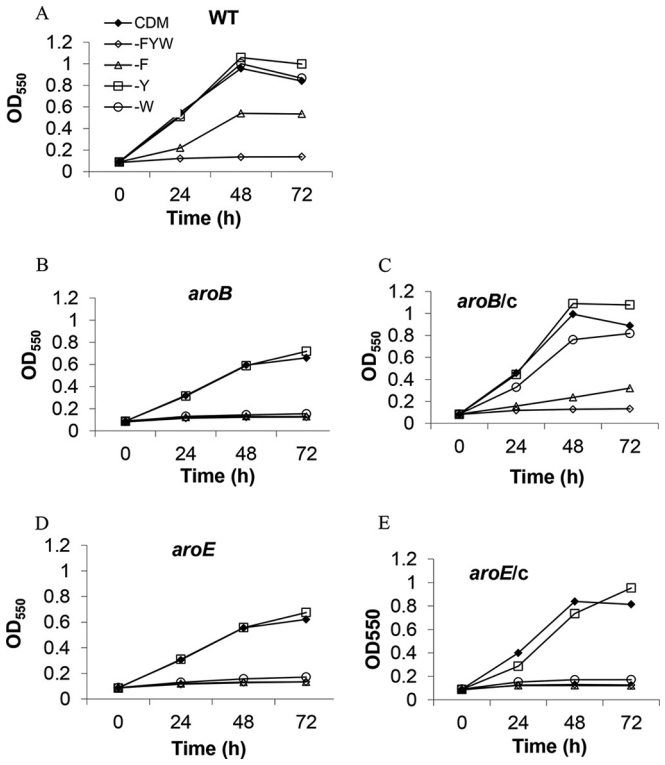

FIG 4 Growth of WT (A) or *aro* mutants (B and D) of *L. pneumophila* in CDM or CDM deficient in aromatic amino acids. *aroB*/*c* (C) and *aroE*/*c* (E) are the mutants complemented with the respective WT alleles. CDM deficient in the indicated aromatic amino acid: -F lacked Phe, -Y lacked Tyr, and -W lacked Trp. The data are representative of the results of 5 independent experiments.

WT strain did not grow in CDM lacking all three aromatic amino acids but grew in CDM lacking Tyr or Trp similarly to the growth seen with 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 that seen with the 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) (46–49). It has been shown that an abundance of either tyrosine or phenylalanine in the medium increases pigment production (50) and that conversion of Phe into Tyr by the phenylalanine hydroxylase (PAH; Fig. 1) provides the excess Tyr needed to produce the pigment (50). The pigment plays no role in intracellular survival but has been shown to confer resistance of *L. pneumophila* to ordinary light and is thought to enable ecological adaptation of *Legionella* species in the environment (47, 48). HGA and HGA-pyomelanin are involved in iron acquisition, reducing ferric iron to ferrous iron, which can be taken up by FeoB of *Legionella* spp. (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 than the WT strain in CDM (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 and C). The WT strain did not produce pigment when all three amino acids (FYW) were omitted from the CDM (Fig. 5).

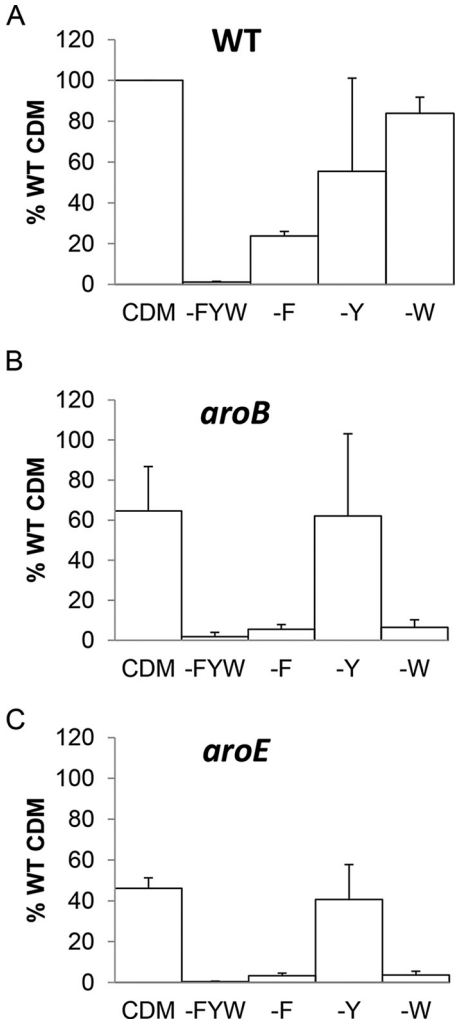

FIG 5 Pigment production by the *Legionella pneumophila* WT strain (A), the *aroB* mutant (B), and the *aroE* mutant (C) in CDM with or in CDM lacking aromatic amino acids. The data corresponding to the *y* axis represent percent normalization of pigment production to the WT strain (100%) in CDM. +FYW contained all 3 aromatic amino acids, -FYW lacked all 3 aromatic amino acids, -F lacked Phe, -Y lacked Tyr, and -W lacked Trp. Error bars represent the standard deviations of the results determined with triplicate samples, and the data shown are representative of the results of three independent experiments.

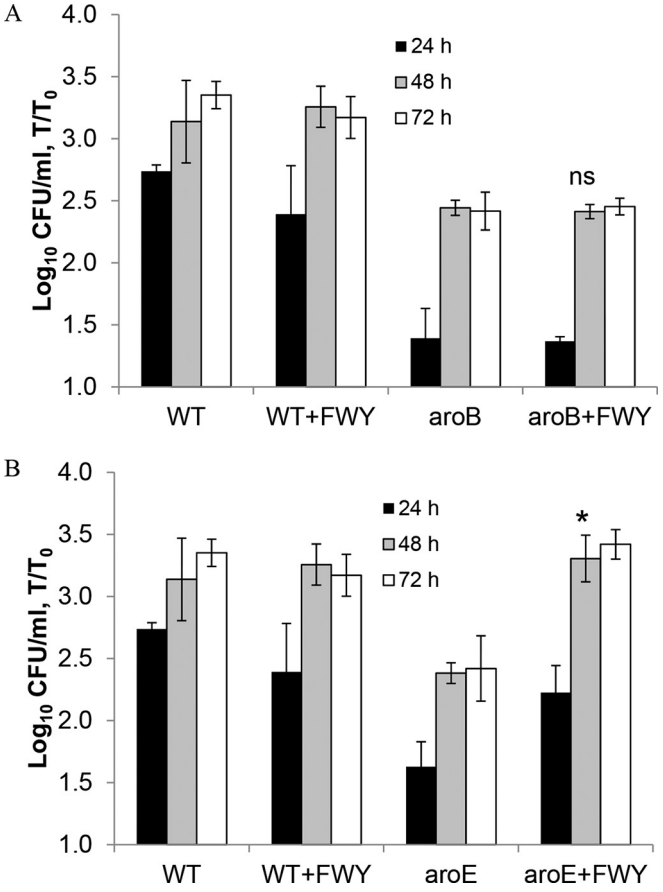

FIG 6 Effect of supplementation of aromatic amino acids on rescue of the intracellular growth defect of the *aroB* and *aroE* mutants in hMDMs. The hMDMs were infected for 1 h with either the WT strain or the *aroB* (A) or *aroE* (B) mutants at an MOI of 10, followed by 1 h of gentamicin treatment to kill the remaining extracellular bacteria. Intracellular growth was assessed by enumeration of CFU on agar plates, and the data are shown as the $\log_{10}$ fold increase in CFU at 24, 48, and 72 h relative to 2 h postinfection. +FWY, supplementation with a 1 mM concentration of each of the three aromatic amino acids. Error bars represent standard deviations of the results determined with triplicate samples, and the data shown are representative of the results of three independent experiments. ns, no statistically significant (Student *t* test) difference between the results determined with the *aroB* mutant in the presence or absence of aromatic amino acid supplementation at 48 h postinfection. *, statistically significant increase in CFU recovered from the *aroE* mutant with FWY supplementation versus without supplementation at 48 h postinfection (Student *t* test, $P < 0.05$).

The pigment level produced by the WT strain was not affected when Trp was omitted from the CDM medium, while omission of Tyr caused an ~45% reduction and omission of Phe caused a >70% reduction in pigment production (Fig. 5A).

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

Effect of aromatic amino acid supplementation on rescue of the intramacrophage defect of the *aroB* and *aroE* mutants. The *aroB* and *aroE* mutants are auxotrophic for the aromatic amino acids and are defective in intramacrophage 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 CFU level increased by ~1,000-fold within 48 h 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. 6). Both mutants exhibited a partial intramacrophage defect at 24 to 48 h and never approached the level of CFU seen with the wild-type strain. Supplementation with aromatic amino acids (1 mM) in *L. pneumophila*-infected hMDMs did not rescue the intramacrophage defect of the *aroB* mutant (Fig. 6A). However, upon supplementation of the three aromatic amino acids, intracellular

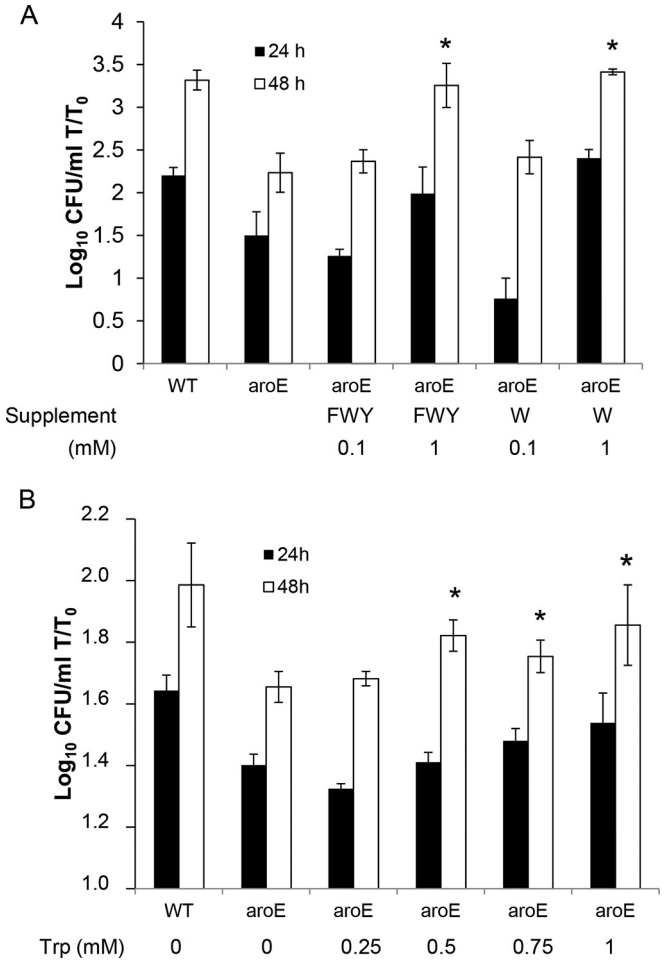

FIG 7 Tryptophan supplementation rescues the *aroE* mutant intracellular growth defect in hMDMs. (A) hMDMs supplemented with all three aromatic acids (FWY) or Trp (W) alone were infected with the WT strain or the *aroE* mutant as described in the Fig. 6 legend. (B) Dose-dependent rescue of the *aroE*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

growth of the *aroE* mutant was completely restored to levels 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 intramacrophage 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 similarly to the level seen with 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 to 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 that of the WT strain.

DISCUSSION

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* spp. (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 intravacuolar growth of *L. pneumophila* within mammalian macrophages. A tryptophan auxotroph of the *L. pneumophila* Philadelphia-1 strain determined by chemical mutagenesis was reported in 1988, and the mutant strain grows within human monocytes at a rate similar to that seen with 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* spp., the two auxotrophic mutants obtain sufficient amounts of the three aromatic amino acids within *Acanthamoeba* spp. but not in human macrophages, and the *aroB*/*E*-encoded enzymes are potential targets for therapy (51).

Upon growth transition of *L. pneumophila* to 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 that provides the excess Tyr needed to produce the pigment (50). Both HGA and pyomelanin are involved in iron acquisition, reducing ferric iron to ferrous iron (52). 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 activity of *phhA*-encoded PAH (50).

Human cells are auxotrophic for nine amino acids (Leu, Ile, Met, Val, Thr, Phe, Trp, His, and Lys), including 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* spp., which have an intact shikimate pathway (32, 53). Therefore, the ability of *Acanthamoeba* spp. to synthesize the three aromatic amino acids correlates with the normal replication of the two mutants within *Acanthamoeba* spp. The defective phenotype of the *aroB* and *aroE* mutants within mammalian macrophages compared to *Acanthamoeba* spp. 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 versus *Acanthamoeba* spp. are a contributing factor for the defect of the two *aro* mutants within mammalian macrophages but not *Acanthamoeba* spp. It is also possible that the LCV-mediated import of aromatic amino acids may be more efficient in *Acanthamoeba* spp. than in mammalian macrophages.

Although import of aromatic amino acids by the LCV membrane and the subsequent utilization by intravacuolar *L. pneumophila* have been already documented (23, 32) and expression of many host solute carrier (SLC) amino acid transporters is triggered upon infection of human macrophages by *L. pneumophila*

(54), 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 intravacuolar growth of *L. pneumophila* (55), but its potential LCV localization remains to be validated. Different phenotypes of the two *aro* mutants in macrophages versus *Acanthamoeba* spp. may also be explained by possible different levels or efficiencies of LCV-mediated import of the amino acids in the two evolutionarily distant hosts.

The *aroE* mutant of *L. pneumophila* was 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 by 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 (56). Protocatechuate is catabolized by the ß-ketoadipate pathway, which is found in a wide variety of soil microbes (56, 57), 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 intramacrophage replication of *L. pneumophila*, independently of the aromatic amino acids. However, future studies must verify directly the potential role of protocatechuate synthesis in intravacuolar 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 are required for optimal intravacuolar growth of *L. pneumophila*. However, *de novo* synthesis of Trp is not required for intravacuolar 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 intramacrophage defect of the *aroE* mutant by supplementation of Tyr or Phe may possibly be due to negative-feedback regulation of the first enzymatic step in the shikimate pathway (Fig. 1) (58). 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 intravacuolar *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 intramacrophage 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* spp. This is the first example of a 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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