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Intracytoplasmic Growth and Virulence of *Listeria monocytogenes* Auxotrophic Mutants

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The intracellular growth of several auxotrophic mutants of *Listeria monocytogenes* was examined in cell culture, and virulence was evaluated in mice by intravenous injection of log-phase bacteria. *L. monocytogenes* transposon insertion mutants requiring either uracil, phenylalanine, glycine, proline, or nicotinic acid for growth were fully virulent and grew similarly to the parental strain as shown by their growth rates in cell culture. Those requiring all three aromatic amino acids (phenylalanine, tryptophan, and tyrosine) or adenine were 1.5 $\log_{10}$ less virulent than the wild type. A threonine auxotroph, which showed enhanced growth in the presence of threonine-containing peptides as compared with that in the presence of free threonine, was approximately 1 $\log_{10}$ less virulent than the wild type. When host cells were deprived of specific amino acids required by both the host cell and *L. monocytogenes*, the bacteria continued to grow intracellularly. These studies suggest that the cytoplasm of eucaryotic cells behaves like rich medium, facilitating the growth of an intracellular bacterial pathogen with complex growth requirements. In addition, results related to amino acid deprivation during intracellular growth and specific extracellular growth requirements of a threonine auxotroph suggest that *L. monocytogenes* may utilize intracellular peptides as a source of amino acids.

Microbial acquisition of nutrients is a central feature of host-parasite relationships, and bacterial pathogenicity is in part dependent on the availability and acquisition of nutrients. Therefore, one may ask which nutrients are available extracellularly and in distinct intracellular compartments? Bacon et al. correlated the avirulence of purine and *p*-aminobenzoic acid auxotrophs of *Salmonella typhi* in mice with their inability to grow in minimal medium supplemented with peritoneal fluid (4), suggesting that these nutrients were limiting extracellularly. Fields et al. identified *Salmonella typhimurium* mutants with defective survival capacity in macrophages (11), while Leung and Finlay selected replication-defective *S. typhimurium* mutants in nonphagocytic cell lines (17). Among these mutants were auxotrophs requiring purines, pyrimidines, aromatic amino acids, and histidine, suggesting intracellular nutritional restrictions. These auxotrophs were all attenuated in mice. Thymine auxotrophs of *Shigella flexneri*, a pathogen which can grow within the cytoplasm, do not form plaques in tissue culture and are avirulent in monkeys (1, 23), suggesting a low intracytoplasmic thymine content.

The phagosomal milieu is generally inherently nonpermissive for bacterial growth. Therefore, bacteria which grow in vacuoles must develop mechanisms to acquire nutrients. Indeed, *Legionella pneumophila*, in spite of having complex growth requirements, replicates within the phagosome (14). Horwitz observed that bacterial-containing phagosomes were of elevated pH, failed to fuse with lysosomes, and were surrounded by mitochondria and ribosomes (13), suggesting an active role of the bacterium in modifying the vacuolar environment. However, all nutrients are not available since thymidine auxotrophs fail to multiply in human monocytes (20). As a second example, the chlamydiae, which are obligate intracellular pathogens with complex growth requirements, multiply within a vacuole (21), suggesting that they have developed mechanisms to acquire nutrients. More recently, it has been reported that the parasitophorous vacuolar membrane of both *Toxoplasma gondii* and *Plasmodium falciparum* contains a pore which allows diffusion of small cytosolic constituents (10a, 29).

There is evidence that the cytoplasm is a more permissive growth environment than the phagosome. Wild-type *Listeria monocytogenes*, which is naturally auxotrophic for several amino acids and vitamins (27), replicates within the cytoplasm, but hemolysin-minus mutants, which are unable to escape from the phagosome, do not grow (11a, 26). *Escherichia coli* K-12 harboring the *Shigella* virulence plasmid (28) and *Bacillus subtilis* expressing the *L. monocytogenes* hemolysin (5) enter and replicate within the host cell cytoplasm.

Availability of nutrients may not be the only factor regulating intracellular bacterial growth as the intracellular concentration of specific amino acids and the bacterial capacity to compete with host cells may be limiting factors. The growth of *Rickettsia prowazekii* depends on the intracellular concentration of serine, glycine, and proline, which varies among cell lines (2, 3). A latent chlamydial infection becomes activated when host cell protein synthesis is inhibited, increasing the availability of amino acids arising from host protein turnover (12).

Additional elements may regulate intracellular growth since growth rates vary among bacterial species. Intracytoplasmic growing *Rickettsia* spp. have a doubling time of 8 to 10 h (32), whereas *L. monocytogenes* grows with a doubling time of approximately 1 h (31) and *S. flexnerii* has a doubling time of about 40 min (28). The potential use of complex forms of nutrients may account for these growth rate differences.

L. monocytogenes, a facultative intracellular bacterial

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TABLE 1. Bacterial strains and relevant characteristics

Strain	Growth requirement	Growth rate in:		LD_{50} in mice
		J774[a]	Henle 407[b]	
10403S	WT[c]	64.6 ± 4.2	80.0 ± 12.5	$10^{4.2}$
DP-L1764	Adenine	79.7 ± 11.6 (<0.001)[d]	102.0 ± 10.5 (0.003)	$10^{5.7}$
DP-L1775	Phe, Trp, and Tyr	59.6 ± 3.4	83.2 ± 10.5	$10^{5.9}$
DP-L1777	Phe	66.5 ± 2.2	ND[e]	$<10^{4.7}$
DP-L1786	Gly	70.0 ± 7.5	ND	$<10^{4.8}$
DP-L1809	Thr	72.0 ± 3.2 (0.005)	89.6 ± 15.9	$10^{5.0}$
DP-L1822	Pro	66.5 ± 3.5	ND	$<10^{4.8}$
DP-L1839	Niacin	63.2 ± 2.4	ND	$<10^{4.8}$
DP-L1851	Uracil	67.8 ± 6.8	ND	$<10^{4.8}$

[a] Expressed as the doubling time in minutes ± standard deviation between 2 and 8 h of intracellular growth. Data represent the means of at least three independent experiments.
[b] Expressed as the doubling time in minutes ± standard deviation between 2.5 and 8.5 h of intracellular growth. Data represent the means of at least four independent experiments.
[c] Wild-type *L. monocytogenes* is an auxotroph for seven amino acids (Arg, Cys, Gln, Ile, Leu, Met, and Val) and four vitamins (biotin, riboflavin, thiamine, and thioctic acid).
[d] *P* values (shown in parentheses) were determined by the Student's *t* test.
[e] ND, not determined.

pathogen, is an ideal system for studying intracellular parasitism because there are excellent models of infection in tissue culture and in mice. We were interested in determining the nutritional limitations of intracytoplasmic growth for *L. monocytogenes*. Therefore, we analyzed the intracellular growth of several auxotrophic mutants of *L. monocytogenes*. We also addressed whether there was a correlation between intracellular bacterial growth in vitro and virulence levels in mice. Results from in vitro experiments indicated that the cytoplasm behaves like a rich bacterial growth medium. In vivo virulence assays revealed that auxotrophs requiring either all three aromatic amino acids, adenine, or threonine were attenuated in mice, but those requiring either uracil, phenylalanine, glycine, proline, or nicotinic acid were as virulent as the wild type. In addition, results related to amino acid deprivation during intracellular growth and specific extracellular growth requirements of a threonine auxotroph suggested that *L. monocytogenes* may utilize intracellular peptides as a source of amino acids.

MATERIALS AND METHODS

Bacterial strains and in vitro growth. *L. monocytogenes* 10403S (6) is the wild-type parental strain used in these studies. Auxotrophic *L. monocytogenes* strains were obtained after mutagenesis with Tn*917*-LTV3 (9) and identified by lack of growth on modified Welshimer's medium, a defined minimal medium for *L. monocytogenes* (27). Auxotrophic requirements were initially determined as described by Davis et al. (10) and are listed in Table 1. Minimal growth requirements of auxotrophic mutants were determined by supplementing modified Welshimer's medium with various concentrations of required nutrients and by monitoring the optical density at 600 nm.

Intracellular growth. The mouse macrophage-like cell line J774 and the human epithelial cell line Henle 407 were propagated in Dulbecco's modified Eagle's medium (DME) with 10% fetal calf serum (FCS) as described by Portnoy et al. (26).

Intracellular growth assays were performed as described previously (26) with certain modifications. J774 cells were seeded onto coverslips 24 h before infection, while Henle cells were split 1/10 from a confluent monolayer 2 days before infection. At 15 h before infection, cells were washed three times with phosphate-buffered saline (PBS) and then incubated in minimal essential medium with Earle's balanced salts (EMEM) and 10% dialyzed FCS (10,000-molecular-weight cutoff) purchased from Hyclone Laboratories, Inc. In some assays, the EMEM was deficient for either aromatic amino acids or threonine, as specified in the text. The cells were kept in this medium for the entire infection period.

Bacteria were grown overnight in brain heart infusion broth at 30°C and washed once in PBS. J774 cells were infected for 30 min with 10^5 bacteria ml^{-1}, which resulted in the infection of approximately one bacterium per 20 cells. Henle cells were infected for 1 h with 2×10^6 bacteria ml^{-1}, which also resulted in the infection of approximately one bacterium per 20 cells. After the initial infection, cells were washed three times with PBS at 37°C, and prewarmed culture medium was added. Gentamicin sulfate was added to a final concentration of 5 $\mu g\ ml^{-1}$ for infection in J774 cells or 50 $\mu g\ ml^{-1}$ for infection in Henle 407 cells. At specific time points, the number of intracellular bacteria was determined in triplicate as described previously (26).

Plaque formation in L2 cells. The plaque formation assay was performed as described previously (31) with certain modifications. Confluent monolayers of L2 cells in DME–10% FCS were infected for 1 h, washed three times with PBS, and overlaid with DME–5% FCS containing 0.7% agarose and 10 μg of gentamicin ml^{-1}. Plaques were stained with neutral red 3 days later, and the mean plaque diameter formed by each strain was compared with the mean plaque diameter of strain 10403S.

LD_{50} determination. Fifty percent lethal doses (LD_{50}) were determined in BALB/c mice by intravenous injection of log-phase bacteria as described previously (26).

RESULTS AND DISCUSSION

In the present study, we investigated the nutritional requirements for the intracellular growth of *L. monocytogenes* in the host cell cytoplasm by using two cell lines, the J774 mouse macrophage-like cell line and the Henle 407 human epithelial cell line. The results indicated that the cytoplasm is highly permissive since all of the auxotrophs tested were able to grow intracellularly in J774 and Henle 407 cells (Table 1). In J774 cells, the doubling times of most auxo-

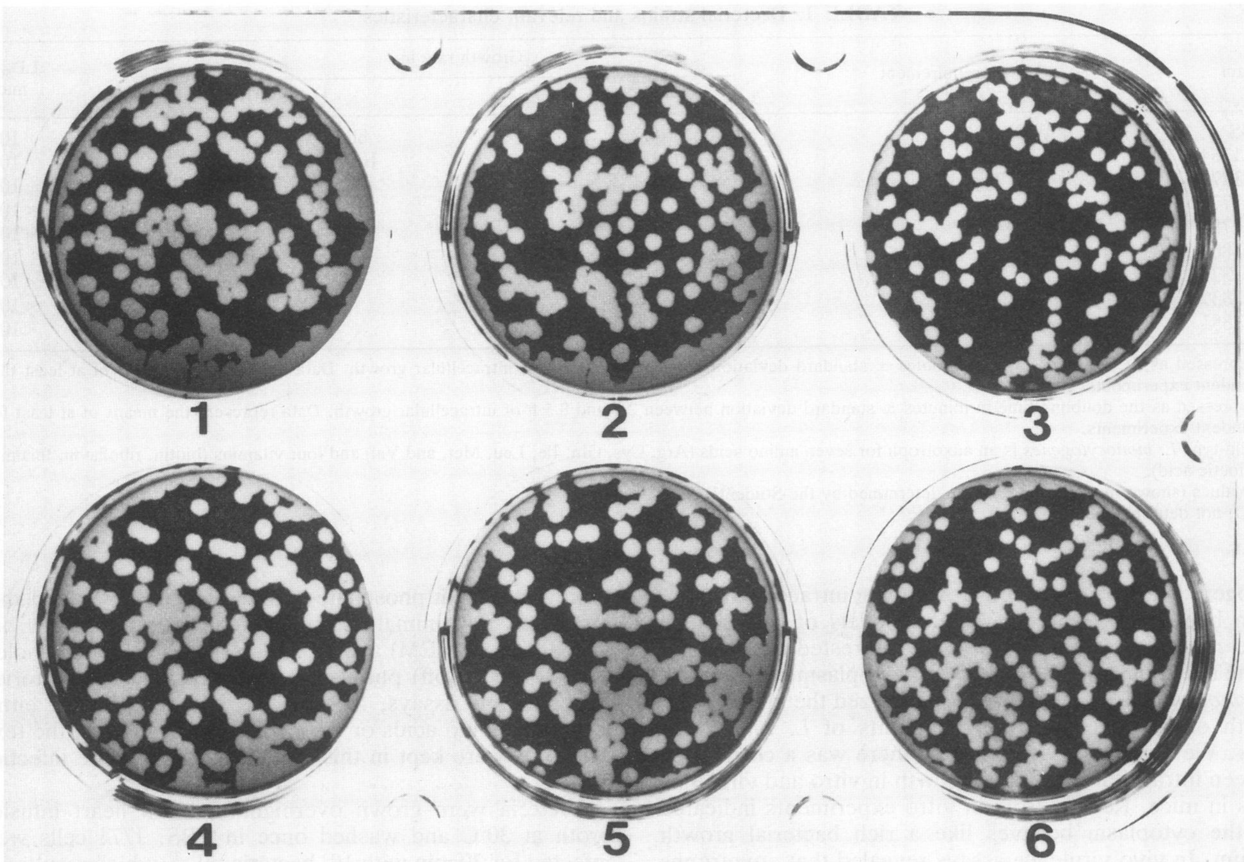

FIG. 1. Plaque formation in infected L2 cells. Wells: 1, 10403S, wild-type *L. monocytogenes*; 2, DP-L1764, an adenine auxotroph; 3, DP-L1775, a phenylalanine, tryptophan, and tyrosine auxotroph; 4, DP-L1777, a phenylalanine auxotroph; 5, DP-L1786, a glycine auxotroph; 6, DP-L1809, a threonine auxotroph.

trophs were not statistically different than that of the parental strain, except for the adenine and threonine auxotrophs. Henle 407 cells were more restrictive than J774 cells as shown by the longer doubling times, and, as in J774, the adenine auxotroph had the slowest intracellular growth rate. These results suggested that all of the nutrients required by these *L. monocytogenes* auxotrophs were present in the cell cytoplasm, but the concentration and/or the availability of these nutrients might vary between cell types. Moreover, the ability of the bacteria to invade, grow, and spread cell to cell was not impaired in these mutants as reflected by the formation of plaques in L2 cells, except for the auxotroph requiring all three aromatic amino acids and the auxotroph requiring threonine, which made plaques equivalent to 81 to 85% of the size of the wild type (Fig. 1).

The intracellular growth rate of *L. monocytogenes* and its auxotrophs was difficult to reconcile with the concentration of free intracellular amino acids as reported for HeLa cells (25) and the content of cell culture medium (Table 2). Neither meet the minimal bacterial growth requirements for cysteine, methionine, or threonine. One could speculate that *L. monocytogenes* secretes proteases that degrade intracellular proteins, increasing the pools of free amino acid, or that *L. monocytogenes* feeds on more complex forms of nutrients.

When tissue culture cells are starved of an essential amino acid, the intracellular concentration of the free amino acid is undetectable after 24 h (25). We tested wild-type *L. monocytogenes* and an aromatic amino acid auxotroph for growth in aromatic-amino-acid-starved cells. Starvation did not alter growth rates of 10403S in either cell line and did not prevent the growth of the aromatic amino acid auxotroph, although the rate of growth was slower (Fig. 2). However, starvation retarded initiation of growth in Henle cells. These results suggest that *L. monocytogenes* was capable of using nutrients other than free amino acids but does not exclude that free amino acids may contribute to growth in complete medium and in the cytoplasm.

The threonine auxotroph had an unusually high threonine requirement (10 mM) for in vitro growth (Table 2). In contrast, when the only source of threonine was contained in a peptide, the minimum growth requirement was decreased to 2.5 mM threonine (Table 2, footnote *d*). Other threonine auxotrophs from independent transposon libraries were found to have identical nutritional characteristics (data not shown). These results revealed that *L. monocytogenes* used threonine-containing peptides more efficiently than free threonine. Consistent with this premise, starvation did not alter bacterial growth rates of 10403S and DP-L1809 in threonine-starved J774 cells (Fig. 3). In Henle cells starved for threonine, the rate of growth of 10403S and DP-L1809 was not significantly different, even though it was slightly decreased when compared with growth in complete EMEM. Considering that *L. monocytogenes* threonine auxotrophs require a high concentration of free threonine to grow, these results suggested that, in threonine-starved cells, *L. monocytogenes*

TABLE 2. Comparison of specific amino acid concentration (mM) in distinct media and *L. monocytogenes* minimal growth requirements

Amino acid	Amino acid concn (mM)			Bacterial phenotype
	Intracellular milieu[a]	EMEM	Bacterial requirement[b]	
Arg	0.03	0.6	0.6	WT
Cys	<0.05	0.1	0.8	WT
Gln	8.1	2.0	4.1	WT
Ile	1.00	0.4	0.8	WT
Leu	0.73	0.4	0.8	WT
Met	0.19	0.1	0.7	WT
Val	0.79	0.4	0.8	WT
Gly	0.79	0.0	≤0.1	Gly auxotroph[c]
Phe	0.52	0.2	0.1	Phe auxotroph[c]
Pro	0.80	0.0	≤1.0	Pro auxotroph[c]
Thr	0.96	0.4	10.0[d]	Thr auxotroph[c]
Trp	<0.1	0.05	0.05	Trp auxotroph[c]
Tyr	0.81	0.2	0.2	Tyr auxotroph[c]

[a] Free amino acid pools determined for HeLa cells (25).
[b] Determined for wild-type *L. monocytogenes* (27) and auxotrophic mutants (this study) as the minimal concentration required to obtain maximum optical density in modified Welshimer's medium.
[c] Tn*917*-LTV3 insertion mutant (9).
[d] The threonine auxotrophic mutant could be complemented either with 2.5 mM of a tripeptide (Thr-Val-Leu), 2.5 mM of a tetrapeptide (Val-Thr-Lys-Gly), or 5 mM of a pentapeptide (Val-His-Leu-Thr-Pro).

utilized nutrients other than free amino acids, most likely peptides. It has long been recognized that peptides are a valuable form of nutrients, especially for fastidious microorganisms (24). The competition that exists for uptake of free amino acids sharing common transport systems has not been observed when amino acids are linked to peptides. In some instances, the growth rate of an amino acid auxotroph can be enhanced with peptides (24). On the basis of these criteria, we hypothesize that peptides are an important source of amino acids for *L. monocytogenes* intracytoplasmic growth.

The virulence of *L. monocytogenes* auxotrophs was examined after intravenous infection in BALB/c mice. The LD_{50}s of phenylalanine, glycine, proline, niacin, and uracil auxotrophic mutants of *L. monocytogenes* were similar to

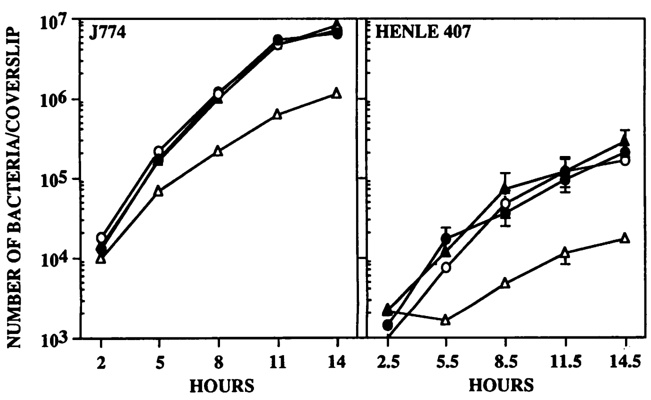

FIG. 2. Growth of *L. monocytogenes* 10403S (circles) and DP-L1775 (triangles), an aromatic amino acid auxotroph, in J774 and Henle 407 cells. Cells were kept in complete (closed symbols) or aromatic-amino-acid-deprived (open symbols) EMEM supplemented with 10% dialyzed FCS. Datum points and error bars represent the mean and standard deviations of the number of viable bacteria recovered from three coverslips.

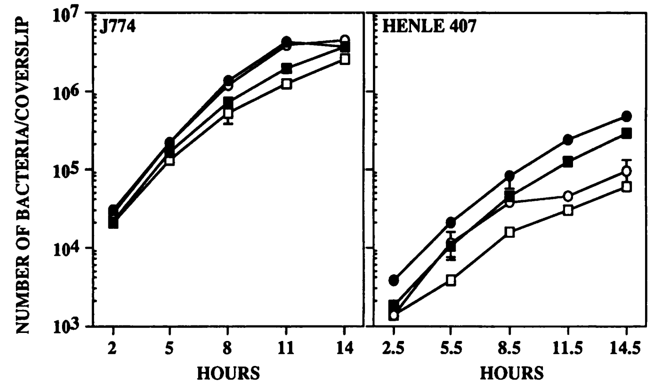

FIG. 3. Growth of *L. monocytogenes* 10403S (circles) and DP-L1809 (squares), a threonine auxotroph, in J774 and Henle 407 cells. Cells were kept in complete (closed symbols) or threonine-deprived (open symbols) EMEM supplemented with 10% dialyzed FCS. Datum points and error bars represent the mean and standard deviations of the number of viable bacteria recovered from three coverslips.

that of the wild type ($<10^{4.8}$; Table 1). Auxotrophs requiring either adenine or all three aromatic amino acids had LD_{50}s of $10^{5.7}$ and $10^{5.9}$, respectively, which is approximately 1.5 $\log_{10}$ less virulent than the LD_{50} of the wild type, and the threonine auxotroph had an LD_{50} of $10^{5.0}$, which is 1 $\log_{10}$ less virulent lower than the LD_{50} of the wild type. These results are in contrast to the results with *Salmonella* and *Yersinia* adenine and aromatic amino acid auxotrophs, which are 4 to 6 $\log_{10}$ less virulent than the wild type (7, 8, 11, 17, 19, 22). This may be consistent with the growth of *L. monocytogenes* within the host cytoplasm, while *Salmonella* and *Yersinia* spp. reside in vacuoles. Alternatively, the requirements of *Salmonella* and *Yersinia* spp. may reflect extracellular growth in vivo (15, 30).

Our results do not eliminate the possibility that auxotrophic mutants of *L. monocytogenes* would be less virulent during the intestinal phase of a natural infection. For example, an aromatic-dependent mutant of *S. flexneri* is avirulent in human and monkeys, even though intracellular growth in cell cultures is normal (16, 18). Until investigated by oral infection, it would be premature to conclude that auxotrophs of *L. monocytogenes* are not attenuated during natural infection.

The results of this study indicate that the cytoplasm is a rich source of nutrients. Therefore, why are more pathogens not taking advantage of this environment? In fact, some vacuolar pathogens do take advantage of cytoplasmic nutrients by modifying the vacuole, thus gaining access to cytoplasmic nutrients, as recently shown for *T. gondii* and P. falciparum (10a, 29). Other examples include the chlamydiae and *L. pneumophila*, which in spite of having complex growth requirements, replicate inside the phagosome, suggesting that they have developed mechanisms to acquire nutrients (14, 21). Therefore, it is reasonable to speculate that all intracellular pathogens which grow intracellularly (i.e., in a vacuole and in the cytoplasm) have access to cytoplasmic nutrients, whereas those which do not grow intracellularly may use the cell as a transient refuge or as an efficient way to be transported to alternate body locations.

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