Characterization of an Aromatic Amino Acid-Dependent
Listeria monocytogenes Mutant: Attenuation, Persistence,
and Ability To Induce Protective Immunity in Mice

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A transposon insertion mutant of Listeria monocytogenes was shown to be deficient in prephenate dehydratase, an enzyme acting late in the pathway for biosynthesis of phenylalanine. This mutant had reduced virulence in mice. The mutant and parent strains persisted to the same extent in the tissues of infected mice and elicited similar degrees of splenomegaly. Mice vaccinated with the mutant were protected significantly from subsequent challenge with virulent L. monocytogenes.

In farm animals, especially sheep, losses due to deaths from listeriosis can be considerable (21). To protect against infection, killed and live attenuated strains have been used as vaccines in animals, but with little or no success (1, 3, 8, 13). The failure of previous live attenuated strains as vaccines is probably due to the method of attenuation used (8, 13).

An alternative to the procedures previously used for making attenuated strains is the generation of strains with a defined mechanism of attenuation. This approach is the basis of the suggestion that hemolysin-deficient strains of Listeria monocytogenes could be suitable as attenuated strains for vaccines (15). However, use of these strains would exclude protection from the effects of hemolysin, an important virulence factor (18). Therefore, we decided to assess the potential of aromatic amino acid-dependent, hemolytic strains of L. monocytogenes for use as live vaccines. Aromatic amino acid-dependent (aro) strains of Salmonella typhimurium and Salmonella typhi are very successful examples of the use of defined mutants as vaccines (12, 17, 19) against salmonellosis. We assessed an aromatic amino acid-dependent transposon insertion mutant of L. monocytogenes as a candidate vaccine in a mouse model of listeriosis.

L. monocytogenes Lm.918.6 is an aromatic amino acid-dependent mutant of the wild-type strain Lm.1070138 (2). Both strains were obtained from D. Portnoy, University of Pennsylvania, and maintained as described before (20). When necessary, minimal medium (6), supplemented with the appropriate amino acid at 20 μg/ml, was used. Cells for inoculation into mice were grown at 30°C to late log phase in tryptose soya broth (Difco), supplemented when appropriate with erythromycin (25 μg/ml). The cells were then resuspended in tryptose soya broth containing 10% (vol/vol) glycerol and stored at −20°C for several months without significant loss of viability. When required, the bacteria were thawed rapidly, harvested by centrifugation, and resuspended in sterile distilled water. The specific activity of prephenate dehydratase was assayed by the method of Nester and Jensen (16). Female MF1 outbred mice, ca. 30 g in weight (Harlan Olac Ltd., Shaw’s farm, Bicester, United Kingdom), were used throughout. Virulence was estimated by determining the 50% effective dose (ED50). Doses of bacteria between 105 and 109 CFU in 100 μl of distilled water were administered intravenously to groups of five mice. The number of mice per group that reached the end point of the assay (i.e., became moribund) between days 0 and 6 was recorded and used to calculate the ED50 by the log-probit method (the percentage of mice surviving to end point versus the log of the CFU inoculated) (5).

In experiments to ascertain the numbers of bacteria in the livers and spleens, infected mice were killed by cervical dislocation at the desired times after infection. The spleens and livers were removed, weighed, and homogenized separately in 10 ml of sterile distilled water in a Stomacher-Lab blender (Seward Medical), and viable counts on tryptose agar were obtained (20). Results were expressed as mean counts of viable listeriae per gram of tissue. To determine whether vaccination with Lm.918.6 conferred resistance to subsequent infection, mice were first vaccinated (intravenously) with 104 CFU of Lm.918.6 in water. The booster vaccination was an identical dose of Lm.918.6 given 14 days after the initial vaccination. At 28 days after the initial vaccination with Lm.918.6, the ED50 of Lm.1070138 for the mice was determined as described above. Data were analyzed by the Mann-Whitney U test (10) or the Kolmogorov-Smirnov test (10).

Lm.918.6 had been described previously as a Tn917 insertion mutant that required aromatic amino acids for growth (2). To ensure that Lm.918.6 contained a single Tn917 insertion, a Southern blot was performed to EcoRI-digested chromosomal DNA with a radiolabelled Tn917-specific probe as described in reference 2. Uncut plasmid pTV32 was included as a positive control, as it carries a single copy of Tn917 (22). The probe hybridized to a single EcoRI fragment (Fig. 1). Since there are no recognition sites for EcoRI within Tn917 (2), Lm.918.6 appears to contain a single Tn917 insertion. To characterize more precisely the biochemical defect, aromatic amino acids and intermediates in the aromatic amino acid pathway were assayed for their ability to support the growth of Lm.918.6 in minimal medium. While phenylalanine and its immediate precursor, phenylpyruvic acid, supported growth, neither tyrosine nor prephenate (a precursor of both tyrosine and phenylpyruvic acid) did. To explain this, the activity of prephenate dehydratase, the enzyme that catalyzes the conversion of pre-
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with either phenic acid to phenylpyruvic acid, in both Lm.1070138 and Lm.918.6 was determined. In Lm.918.6, the activity was 0.02 U/mg of protein, whereas the wild-type level was 7.00 U/mg of protein. This suggested that the single transposon insertion in Lm.918.6 was affecting expression of the pheA gene, which encodes prephenate dehydratase. As measured by ED$_{50}$, strain Lm.918.6 was significantly ($P < 0.05$) attenuated compared with the wild-type strain, Lm.1070138, in the mouse model of listeriosis (Table 1). Lm.918.6 multiplied to significantly ($P < 0.05$) lower numbers in both the liver and the spleen than Lm.1070138 (Fig. 2A and B). After a dose of $10^4$ viable cells of Lm.1070138, peak counts of $6 \times 10^6$ CFU/g of spleen tissue and $7 \times 10^7$ CFU/g of liver tissue were obtained after 3 days. At the same dose, Lm.918.6 grew more slowly, reaching peak counts of $2 \times 10^6$ and $9 \times 10^5$ CFU/g of spleen and liver tissue, respectively, at day 5 (Fig. 2A and B). No significant difference ($P > 0.05$) in the clearance of the two strains between days 1 and 9 was noted, and by day 11, both strains had been cleared (Fig. 2A and B). Maximum splenomegaly, as measured by the mean spleen weight of the five animals at each time point, occurred 4 days after maximum numbers of listerialae were detected, i.e., on day 7 postinfection with the wild-type strain Lm.1070138 and on day 9 postinfection with Lm.918.6 (Fig. 2C). The extent of splenomegaly induced by Lm.918.6 was not significantly

![Image](http://iai.asm.org/)  
**FIG. 1.** Southern blot of EcoRI-digested Lm.918.6 chromosomal DNA (a) and uncut plasmid pTV32 DNA (b), probed with a 1.5-kb transposon Tn917-specific DNA probe (2). Lambda DNA digested with either XhoI or HindIII was used as molecular weight markers.

<table>
<thead>
<tr>
<th>Strain</th>
<th>ED$_{50}$ (CFU)</th>
<th>Mean$^*$</th>
<th>SEM</th>
<th>95% CL$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lm.1070138</td>
<td>$5.51 \times 10^4$</td>
<td>$4.97 \times 10^2$</td>
<td>$1.28 \times 10^4$</td>
<td>$2.37 \times 10^5$</td>
</tr>
<tr>
<td>Lm.918.6</td>
<td>$2.61 \times 10^7$</td>
<td>$3.67 \times 10^3$</td>
<td>$7.00 \times 10^5$</td>
<td>$9.71 \times 10^7$</td>
</tr>
</tbody>
</table>

$^a$ Mean dose required to render 50% of the mice moribund. $^*$, significantly different ($P < 0.05$) from value for Lm.1070138.

$^b$ CL, confidence limits.

**TABLE 1.** Comparison of ED$_{50}$ of Lm.1070138 and Lm.918.6

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**FIG. 2.** (A and B) Concentrations of viable Lm.1070138 (---) and Lm.918.6 (---) in spleens (A) and livers (B) of MF1 mice over 15 days after intravenous infection with $10^6$ CFU. Each point (A and B) represents the geometric mean for five mice. (C) Splenomegaly (expressed as mean spleen weight, in grams) induced over 15 days by infection with $10^6$ CFU of Lm.1070138 (---) and Lm.918.6 (---). Each point represents the arithmetic mean for five mice.
TABLE 2. Effect of vaccination with Lm.918.6 on the ED₅₀ of wild-type Lm.1070138 in mice

<table>
<thead>
<tr>
<th>Lm.918.6 vaccination</th>
<th>Lm.1070138 ED₅₀ (CFU)</th>
<th>Mean*</th>
<th>SEM</th>
<th>95% CL*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5.51 × 10⁶</td>
<td>4.79 × 10⁵</td>
<td>1.28 × 10⁵-2.37 × 10⁵</td>
<td></td>
</tr>
<tr>
<td>1 dose</td>
<td>1.73 × 10⁴***</td>
<td>5.35 × 10³</td>
<td>1.73 × 10³-8.82 × 10³</td>
<td></td>
</tr>
<tr>
<td>2 doses</td>
<td>6.00 × 10⁴***</td>
<td>6.09 × 10³</td>
<td>1.37 × 10³-2.62 × 10³</td>
<td></td>
</tr>
</tbody>
</table>

* Each vaccinating dose contained 10⁶ CFU of Lm.918.6, given intravenously.
** Mean dose required to render 50% of the mice moribund. * Significantly different (P < 0.05) from value for unvaccinated mice; ** significantly different (P < 0.05) from value for mice receiving one dose.
* CL, confidence limits.

different (P > 0.05) from that induced by Lm.1070138 (Fig. 2C), but the duration was significantly reduced, with spleens returning to normal weight 11 days postinfection (Fig. 2C). In contrast, with Lm.1070138, splenomegaly was maintained for at least 15 days postinfection (Fig. 2C). In all these experiments, the phenotype and genotype of Lm.918.6 recovered from the infected animals were confirmed by growth on minimal medium and by Southern blotting with Tn917-specific DNA probes. In all cases, Lm.918.6 remained PheA⁺ with a single transposon insertion (data not shown).

To determine whether vaccination with Lm.918.6 conferred protection against subsequent infection, mice were first vaccinated with Lm.918.6 and then challenged 28 days later with Lm.1070138. The ED₅₀ of Lm.1070138 was significantly (P < 0.05) greater in mice vaccinated with one dose of Lm.918.6 than in nonvaccinated mice (Table 2). In addition, a significant (P < 0.05) booster effect was seen when mice were given a second dose of Lm.918.6 14 days after the initial vaccination (Table 2). Therefore, strain Lm.918.6 with a transposon insertion which affects pheA expression is attenuated in the mouse model for listeriosis. In the same system, it confers significant protection against infection by virulent L. monocytogenes, with a pronounced booster effect upon second vaccination. This is the first evidence that an auxotrophic mutant of L. monocytogenes can be attenuated in a manner similar to that described for ara mutants of S. typhimurium (17). Why a marked reduction in pheA gene expression should result in attenuation is not yet clear. It has been hypothesized that in ara4 mutants of S. typhimurium attenuation may be due to an inability to synthesize p-amino-benzoate and dihydroxybenzoate, attenuation in this case may reflect the poor availability of phenylalanine in mammalian cells. In addition, feedback inhibition of earlier aromatic pathway enzymes by the accumulation of prephenate, the substrate of prephenate dehydratase (9), may also be occurring. Reduced virulence of pheA mutants of gram-negative or gram-positive bacteria has not, to our knowledge, been reported previously. Studies of ara mutants of Salmonella sp. (4, 11) suggest that a strain of L. monocytogenes with multiple lesions in the pathway of aromatic amino acid synthesis might prove to be more attenuated than Lm.918.6. Future studies will attempt to introduce such additional mutations into Lm.918.6. This should serve to increase the attenuation and also reduce the risk of possible reversions in vivo. It is entirely feasible that such strains could be useful as vaccines in farm animals.

In addition to their potential as vaccine strains, nonreverting aromatic amino acid-dependent mutants of L. monocytogenes may be exploited as carriers of cloned gram-positive virulence factors and protective antigens of other pathogens, as has been reported for similar strains of Salmonella (4, 14).

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REFERENCES


