

Growth, Virulence, and Immunogenicity of *Listeria monocytogenes* *aro* Mutants

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Mutants of *Listeria monocytogenes* with deletions in genes of the common branch of the biosynthesis pathway leading to aromatic compounds were constructed as possible virulence-attenuated carrier strains for protein antigens or vaccine DNA. *aroA*, *aroB*, and in particular *aroE* mutants showed strongly reduced growth rates in epithelial cells and even in rich culture media. The metabolism of the *aro* mutants under these conditions was predominantly anaerobic. Aerobic metabolism and a wild-type growth rate were, however, regained upon the addition of vitamin K₂, suggesting that the *aro* mutants are deficient in oxidative respiration due to the lack of menaquinone. Replication of the *aro* mutants in the host cell's cytosol and cell-to-cell spread were drastically slowed down, and all *aro* mutants showed high virulence attenuation in mice, i.e., the 50% lethal dose in BALB/c mice was increased at least 10⁴-fold for the *aroA*, *aroB*, and *aroA/B* mutants and >10⁵-fold for the *aroE* mutant compared to the parent strain. Nevertheless, mice preimmunized with *aro* mutant bacteria elicited good T-cell response and full protection against a subsequent challenge with the virulent wild-type strain. A total of 5 × 10⁶ *aroA*, *aroB*, and *aroA/B* mutant bacteria were sufficient to obtain a protective T-cell response, while 5 × 10⁸ *aroE* or *aroA/E* mutants were necessary to achieve comparable numbers of antigen-specific T cells. These numbers were well tolerated without causing any signs of disease, indicating that *Listeria* strains with deletions in genes of the basic branch of the aromatic amino acid pathway could be useful vaccine carriers for inducing T-cell immunity.

Listeria monocytogenes is a gram-positive facultative intracellular pathogen which specifically infects professional antigen-presenting cells, like monocytes, macrophages, and dendritic cells (18, 25, 36, 48). After uptake into these cells, the bacteria escape into the cytosol by disrupting the phagosomal membrane by the action of the cytolysin listeriolysin (LLO) (40). In the cytoplasm, efficient bacterial replication occurs, and listeriae spread into neighboring cells without being released into the extracellular environment. Hence, cytotoxic T cells play a major role in the adaptive immune response against *L. monocytogenes* infections (27). For this reason, *L. monocytogenes* has received increasing attention as a carrier strain for the generation of live vaccines against infectious and neoplastic diseases (reviewed in references 17 and 37).

Since these bacteria have the potential to cause severe systemic infections with symptoms such as sepsis and encephalomeningitis, particularly in immunocompromised and pregnant patients (48), *L. monocytogenes* vaccine strains must be avirulent but still strongly immunogenic. In the past several years, attenuated strains of *L. monocytogenes* which lacked essential virulence genes were introduced for this purpose (4, 5, 9–11, 16, 19, 24, 28, 33, 38). These strains are basically avirulent and were even used in a safety study with human volunteers (2). Auxotrophic mutations successfully applied for virulence at-

tenuation in other bacterial carrier systems were also used for obtaining attenuated *L. monocytogenes* strains (1, 29, 45, 47). However, these mutations either led to insufficient virulence attenuation (1, 29, 45) or required coinjection of the impaired metabolite for the induction of an efficient immune response against *L. monocytogenes* (47).

In a number of bacterial pathogens, mutations in the basic branch of the aromatic amino acid biosynthesis pathway proved to be particularly efficient in attenuating virulence, e.g., in *Salmonella enterica* serovar Typhimurium (21), *Shigella flexneri* (26), *Shigella dysenteriae* (49), *Pseudomonas aeruginosa* (42), *Bordetella pertussis* (43), *Neisseria gonorrhoeae* (7), and *Bacillus anthracis* (22), but failed in *Mycobacterium tuberculosis* (35).

Here, we describe mutants with deletions in *aroA* (encoding the first enzyme in aromatic amino acid biosynthesis, 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase), *aroB* (encoding the second enzyme, 3-dehydroquinate synthase), and *aroE* (encoding 5-enolpyruvylshikimate-3-phosphate synthase). All three single mutants, as well as *aroA/B* and *aroA/E* double mutants, still contained the entire set of virulence genes but were strongly attenuated and remained highly immunogenic.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1. *Escherichia coli* DH10b was used as a host for all DNA manipulations.

Bacteria were cultivated either in brain heart infusion (BHI) or in modified Welshimer's broth minimal medium (41) containing 2% glucose. Additional supplements (from Sigma, Steinheim, Germany) consisted of 0.1 g (each) of

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TABLE 1. Listerial strains used

Strain	Genetic property	Reference or source
<i>L. monocytogenes</i> EGDe	Wild-type strain	15
Mutants		
<i>aroA</i>	Deletion of <i>aroA</i>	J. Stritzker and W. Goebel, submitted for publication
<i>aroB</i>	Deletion of <i>aroB</i>	This work
<i>aroE</i>	Deletion of <i>aroE</i>	This work
<i>aroA/B</i>	Deletion of <i>aroA</i> and <i>aroB</i>	This work
<i>aroA/E</i>	Deletion of <i>aroA</i> and <i>aroE</i>	This work
<i>pheA trpEG tyrA</i>	Deletion of <i>pheA</i> , <i>trpEG</i> , and <i>tyrA</i>	This work
<i>aroE</i> revertant	<i>aroE</i> integration into the <i>aroE</i> mutant	This work

L-tryptophan, L-phenylalanine, and L-tyrosine/liter; 0.3 g of shikimate/liter; 0.3 g of *p*-aminobenzoic acid/liter; 0.1 g of folic acid/liter; and 50 mg of vitamin K₂/liter.

For anoxic BHI cultures, the medium was degassed at 60°C for 45 to 60 min under vacuum and overlaid with mineral oil. For infections, *Listeria* strains were grown to a late-logarithmic growth phase (180 Klett units for *L. monocytogenes* EGDe, 150 Klett units for *aroA*, *aroB*, and *aroA/B* mutants; and 90 Klett units for *aroE* and *aroA/E* mutants) at 37°C in BHI, washed with phosphate-buffered saline (PBS) (pH 7.4), resuspended in 20% (vol/vol) glycerol-PBS, and stored at -80°C.

Construction of *Listeria* mutant strains. All deletion mutageneses were constructed using *L. monocytogenes* EGDe as a parental strain and were performed by a homologous-recombination technique (50) using constructs derived from the mutagenesis vector pLSV101 (derived from pLSV1, kindly provided by T. Fuchs).

For in-frame deletion of *aroB*, a 424-bp fragment (primers *aroB*5'-1 [5'-AA AGGTGCTGAATTCGGTGTGC-3'] and *aroB*5'-2 [5'-TGTAATCCCGGGCAT GTTTAAAACCTCTTC-3']) that was localized upstream of the deletion locus and a 269-bp fragment (primers *aroB*3'-3 [5'-GGGAATCCCGGGTGTAGAGC GATTCGAG-3'] and *aroB*3'-4 [5'-ACATAGGAAGCGAATTCGGCA-3']) downstream of the deletion locus were amplified. Both fragments were digested with PspAI, ligated, and further amplified with primers *aroB*5'-1 and *aroB*3'-4, mentioned above. The recombination fragment was then cloned into pLSV101 using EcoRI and BamHI restriction, resulting in pLSV101 Δ *aroB*, which was transformed into *L. monocytogenes* EGDe (for *aroB* mutant construction) or *aroA* (for *aroA/B* mutant construction). After cultivation at 42°C with 5 μ g of erythromycin/ml, clones with a chromosomally integrated knockout plasmid could be selected. The resulting clones were further cultivated at 30°C without erythromycin to obtain the *aroB* chromosomal deletion mutants.

Deletion of *aroE* in *L. monocytogenes* EGDe and *aroA* was performed by the same method described above. The following primers were used (nomenclature as indicated for *aroB* primers): *aroE*5'-1 (5'-GCTTGGGATCCTTGAAAGTG AAGATGC-3'), *aroE*5'-2 (5'-GTAATCCCGGGCATCGTCAATTCGCT-3'), *aroE*3'-3 (5'-CACTACCCGGGTAACAACCTTACCAG-3'), and *aroE*3'-4 (5'-TGATAGAATTCAGCTAACACGAGCAAA-3').

From the *aroE* mutant, an *aroE* reversion mutant was constructed by using the same method. Therefore, *aroE* was amplified using *aroE*5'-1 and *aroE*3'-4 with wild-type chromosomal DNA as a template, and a pLSV101 *aroE* reversion was obtained after BamHI and EcoRI restriction and ligation.

Construction of the *pheA trpEG tyrA* mutant was performed by first making an in-frame deletion of *trpEG* (primers *trpEG*5'-1 [5'-TAAATGAATTCATCAA ACAAGAATAGTACTTT-3'], *trpEG*5'-2 [5'-TTTCATCCCGGGCATCTAAA TCATCCCTTCTT-3'], *trpEG*3'-3 [5'-CACATCCCGGGTGTTTTGAAGT GTTAGCG-3'], and *trpEG*3'-4 [5'-ACTATTGGATCCATCTCCGCTGTCC CGCA-3']), followed by in-frame deletion of *tyrA* in *trpEG* (primers *tyrA*5'-1 [5'-CAATTAGAATTCAGCTTTTATGTAGGCATGC-3'], *tyrA*5'-2 [5'-GTCCTACCCGGGCATTCAAAAACGCCTCCAAAC-3'], *tyrA*3'-3 [5'-TT GAATGCCCGGGTAGGAGCGAATGACGATGA-3'], and *tyrA*3'-4 [5'-AT GGATGGATCCCCTAAAATACTGTATC-3']), and finally by in-frame deletion of *pheA* in *trpEG tyrA* (primers *pheA*5'-1 [5'-GTACACAGAATCTTG TAGCTAATAAAATGGAT-3'], *pheA*5'-2 [5'-ATAAGCCCGGGCATTTTC AACTCTCCTTTTT-3'], *pheA*3'-3 [5'-TTACACCCGGGTAAATAAAGAG TTATCGCAATT-3'], and *pheA*3'-4 [5'-CTAATCGGATCCAAGGGTTTAA ACTCGGATC-3']).

All in-frame deletion mutants were confirmed by PCR analysis and sequencing of the corresponding sections of the chromosome.

Metabolic analysis. Listerial strains were grown in BHI containing 2% glucose. Ten milliliters of sterile filtered incubation medium was adjusted to pH 2 by adding 5 ml of 6 N HCl. The acidified medium was successively extracted twice with 40 ml of ethyl ether and 40 ml of dichloromethane (all solvents were distilled before use). The combined organic layers were dried over sodium sulfate and filtered. The solvent was removed at 42°C using a 25-cm-long Vigreux distillation column. Finally, the residue was filled up to a standardized volume of 1 ml, and 1 μ l was injected for high-resolution gas chromatography-mass spectrometry (HRGC-MS) analysis.

HRGC-MS analysis was performed using an Agilent 5973 mass selective detector coupled to an Agilent 6890 gas chromatograph with split injector (1:20) and an Agilent 7683 automatic liquid sampler. The system was equipped with Chem Station software. A J&W DB Wax fused-silica capillary column (30-m by 0.25-mm inside diameter; 0.25- μ m film thickness) was employed. The temperature program was 3 min isothermal at 50°C and then increased at 4°C/min to 220°C, using a flow rate of 1.0 ml of helium/min. The MS operating values were as follows: ionization voltage, 70 eV (electron impact ionization); ion source and interface temperatures, 230°C.

Identifications were carried out by comparison of mass spectral and chromatographic retention data of the target compounds with those of authentic reference substances (lactic acid, acetic acid, and acetoin were obtained from Fluka, Steinheim, Germany).

Cell culture, infection experiments, and plaque assay. Caco-2 (human colon adenocarcinoma) and 3T3 (mouse fibroblast) cells were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine (Gibco) and 10% fetal calf serum (Biochrom, Berlin, Germany).

For infection, cells were seeded in 24-well plates 1 day prior to infection. After a wash step with RPMI 1640 containing 2 mM L-glutamine, the cells were infected with 10 bacteria per cell for 1 h. The cells were washed with RPMI 1640 containing 2 mM L-glutamine and cultivated with gentamicin-containing medium (100 μ g/ml), which was replaced with medium containing 10 μ g of gentamicin/ml after 1 h. Counts of viable intracellular bacteria were determined by plating serial dilutions of mechanically lysed cell suspensions on BHI agar.

Plaque formation by *L. monocytogenes* in 3T3 cells was performed as described earlier (46). In brief, a confluent layer of 3T3 fibroblasts was infected with 1 bacterium per 10 cells. Instead of replacing medium containing 100 μ g of gentamicin/ml with medium containing 10 μ g of gentamicin/ml, the cells were overlaid with medium containing 0.5% agarose, as well as 10 μ g of gentamicin/ml. After 2 days, plaques were visualized by the addition of an overlay of medium containing 0.5% agarose, 10 μ g of gentamicin/ml, and 0.1% neutral red (1% stock solution was filter sterilized). Plaques were visualized after 4 to 8 h, and their diameters were measured (at least 50 plaques/strain).

Animal experiments. All animal experiments were approved by the government of the district of Unterfranken and conducted according to the German animal protection guidelines. Female BALB/c mice (6 to 8 weeks of age) were purchased from Charles River Laboratories, Sulzfeld, Germany.

Mice were infected via intravenous (i.v.) injection of 100 μ l of an endotoxin-free PBS (pH 7.4) bacterial suspension. The mean number of viable bacteria \pm standard deviation in liver and spleen homogenates for five mice were determined by plating serial dilutions on BHI agar plates, which were incubated at 37°C for 2 days before CFU were counted.

For protection assays, 7-week-old BALB/c mice were infected with either PBS alone (negative control), 10³ CFU of *L. monocytogenes* EGDe (positive control), or 5 \times 10⁶ CFU of the corresponding *aro* mutants. After 4 weeks, the mice were challenged with 10³ CFU of *L. monocytogenes* EGDe bacteria, and the bacterial loads in the spleen and liver were determined 4 days after challenge.

Ex vivo enumeration of peptide-specific CD8 T cells. The frequency of peptide-specific CD8 T lymphocytes was determined in a gamma interferon (IFN- γ)-specific enzyme-linked immunosorbent assay (ELISPOT) as described previously (14). Mice were immunized either with 10³ CFU of *L. monocytogenes* EGDe or with graded doses between 5 \times 10² and 5 \times 10⁸ CFU of the various *L. monocytogenes* deletion mutants. Unseparated splenocytes (6 \times 10⁵/well) were stimulated for 6 h in round-bottom 96-well microtiter plates in the presence of 10⁻⁶ M peptide. Subsequently, activated cells (4 \times 10⁵ or 4 \times 10⁴/well) were transferred to anti-IFN- γ -coated (clone RMMG-1; Biosource, Camarillo, Calif.) membrane-backed 96-well ELISPOT plates (Nunc, Wiesbaden, Germany), which after 18 h of incubation were developed with biotin-labeled rat anti-mouse IFN- γ monoclonal antibody (clone XMG1.2; BD Biosciences), horseradish peroxidase-streptavidin conjugate (Dianova, Hamburg, Germany), and aminoethyl-carbazole dye solution (Sigma, Deisenhofen, Germany). The frequency of antigen-specific cells was calculated as the number of spots per splenocyte seeded. In

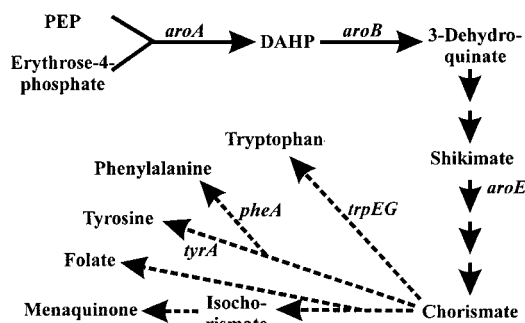


FIG. 1. Common amino acid biosynthesis pathway in *L. monocytogenes*. PEP, phosphoenolpyruvate; DAHP, 3-deoxy-arabino-heptulonate 7-phosphate. Each solid arrow represents one enzyme. Dashed arrows represent several enzymes, one of which is indicated in some cases.

addition, the total number of antigen-specific T cells per spleen was calculated from the T-cell frequency and the total number of cells per spleen. The specificity and sensitivity of the ELISPOT assay were controlled with IFN- γ -secreting CD8 T-cell lines specific for amino acids 217 to 225 and 449 to 457 of p60 and amino acids 91 to 99 of LLO. Recovery of seeded T cells was generally >90%.

RESULTS

Growth of *aro* mutants in culture media. Mutants with deletions in different genes of the common biosynthesis pathway (*aroA*, *aroB*, *aroA/B*, *aroE*, and *aroA/E*) (Fig. 1) leading to the aromatic amino acids were constructed. As expected, these mutants were unable to grow in minimal medium without aromatic amino acids. Growth of the *aroA*, *aroB*, and *aroA/B* mutants, but not the *aroE* mutant, was restored after the addition of shikimate. Only partial growth of the *aroE* and *aroA/E* mutants was obtained when all aromatic amino acids, including *p*-amino benzoate, were provided (Table 2). Unexpectedly, the growth rates of all *L. monocytogenes aro* mutants were markedly lower than that of the wild-type *L. monocytogenes* EGDe or the *aroE* revertant strain when the *aro* mutants were cultured in rich BHI medium (Table 2).

To investigate whether the reduced growth rate is due to impaired uptake of the three aromatic amino acids or to the lack of folate and/or ubiquinone-menaquinone biosynthesis, a

triple-deletion *pheA trpEG tyrA* mutant was constructed which is unable to produce phenylalanine, tyrosine, and tryptophan but is not hampered in folate and ubiquinone-menaquinone biosynthesis (Fig. 1). The growth rate of this mutant strain in BHI was similar to that of the wild-type strain (Table 2).

The lack of folate was excluded as a cause for the reduced growth of the *aro* mutants in BHI, as the addition of trimethoprim or sulfanilamide (both drugs inhibit folate biosynthesis) to BHI led to growth inhibition of the wild-type strain, but also of the *aro* mutants (data not shown), and supplementation of the BHI medium with additional folate did not increase the growth rate of the *aro* mutants (Table 2).

Ubiquinone (and/or the prokaryote-specific menaquinone) is an essential component of the respiratory chain, with precursors derived from the common branch of the aromatic amino acid pathway, and a defect in ubiquinone (menaquinone) biosynthesis is expected to strongly impair oxidative phosphorylation even in the presence of oxygen. Indeed, growth of the *L. monocytogenes* wild-type strain in BHI under anoxic conditions resulted in a replication rate which was similar to that observed for the *L. monocytogenes aro* mutants under oxic conditions. In contrast, no change in the growth rates of the *aro* mutants was observed when these mutants were grown in BHI under oxic or anoxic conditions (Table 2). Furthermore, the observed accumulation of lactate and the reduced acetate concentration, as well as the absence of acetoin, a typical overflow substance secreted by *L. monocytogenes* growing aerobically, in the culture supernatant of the *aroE* mutant (Table 3) suggest that *L. monocytogenes aro* mutants carry out a predominantly anaerobic metabolism (44). Finally, addition of vitamin K₂ (menaquinone 4) restored the growth rate of the *aroA* and *aroE* mutants (Table 2) and led to the disappearance of lactate and reappearance of acetoin in the supernatant of the *aroE* mutant, which was also observed in the *aroE* revertant grown in BHI alone (Table 3).

Reduced cytosolic replication and cell-to-cell spread of listerial *aro* mutants in vitro. Internalization of all *aro* mutants by nonphagocytic epithelial (Caco-2) and endothelial (HBMEC) cells is significantly enhanced due to increased internalin A and internalin B production in these mutants compared to the wild-type strain (J. Stritzker, C. Schoen, and W. Goebel, sub-

TABLE 2. Generation times of *L. monocytogenes* strains under different broth culture conditions

Medium	Generation time (min) ^a							
	EGDe	<i>aroA</i>	<i>aroB</i>	<i>aroA/B</i>	<i>aroE</i>	<i>aroA/E</i>	<i>pheA trpEG tyrA</i>	<i>aroE</i> revertant
BHI	43 ± 4	74 ± 3	79 ± 5	73 ± 4	151 ± 21	164 ± 23	44 ± 4	43 ± 3
BHI folate ^b	45 ± 5	79 ± 6	ND	ND	135 ± 16	ND	ND	ND
BHI anoxic ^c	77 ± 5	70 ± 7	ND	ND	93 ± 2	ND	ND	ND
BHI vitamin K ₂ ^b	47 ± 6	50 ± 7	ND	ND	45 ± 5	ND	ND	ND
MWB ^d	105 ± 5	NG	NG	NG	NG	NG	NG	ND
MWB aaa ^b	102 ± 7	367 ± 124	ND	ND	>1,000	ND	ND	ND
MWB aaa PABA ^b	101 ± 6	354 ± 5	ND	ND	>1,000	ND	ND	ND
MWB Shikimate ^b	103 ± 10	94 ± 3	ND	ND	NG	ND	ND	ND
MWB aaa Vitamin K ₂	94 ± 7	98 ± 24	ND	ND	118 ± 32	ND	ND	ND

^a Generation time mean values ± standard deviations (three samples). ND, not determined; NG, not grown.

^b Supplements were added in the following concentrations: folate, 0.1 g/liter; vitamin K₂, 50 mg/liter; aaa (aromatic amino acids), 0.1 g/liter (each); PABA (*para*-aminobenzoic acid), 0.1 g/liter; shikimate, 0.3 g/liter.

^c Anoxic growth conditions were achieved by degassing of BHI containing 2% glucose and an overlay of mineral oil.

^d Modified Welshimer's broth (MWB) is a defined minimal medium for *L. monocytogenes* (41).

TABLE 3. Relative amounts of acetoin, acetate, and lactate produced compared to *L. monocytogenes* EGDe grown in BHI

Strain	Medium	Amt produced		
		Acetoin ^a	Acetate ^a	Lactate ^b
<i>aroE</i>	BHI ^c	0.00 ± 0.00	0.03 ± 0.03	0.74 ± 0.57
	Vitamin K ₂ ^d	1.44 ± 0.18	1.41 ± 0.95	0.07 ± 0.06
	Anoxic ^e	0.00 ± 0.00	0.03 ± 0.01	0.71 ± 0.16
EGDe	BHI	1.00	1.00	0.02 ± 0.02
	Vitamin K ₂	1.03 ± 0.29	1.14 ± 0.50	0.05 ± 0.00
	Anoxic	0.05 ± 0.04	0.08 ± 0.05	1.00
<i>aroE</i> revertant	BHI	1.24 ± 0.29	1.53 ± 0.14	0.02 ± 0.01

^a Acetoin and acetate values are given as relative amount ± standard deviation (for three independent measurements) compared to the amount produced by the wild-type strain under aerobic growth conditions.

^b Lactate amounts ± standard deviations (for three independent measurements) compared to the amount produced by the wild-type strain under anoxic growth conditions.

^c BHI containing 2% glucose.

^d BHI containing 2% glucose and vitamin K₂ at a final concentration of 50 µg/ml.

^e Degassed BHI containing 2% glucose overlaid with mineral oil.

mitted for publication). While release of the *aro* mutants from the primary phagosome into the host cell cytosol seemed to be comparable to that of the wild-type strain, growth of the *aro* mutants in the cytosol was significantly reduced. The *aroA*, *aroB*, and *aroA/B* mutants grew in this cellular compartment with a generation time of ~90 min, which is about twice as long as that of the wild-type strain; the *aroE* and *aroA/E* mutant strains replicated even more slowly and stopped intracellular growth ~3 h postinfection (Fig. 2A). Similar behavior of the *aro* mutants was observed in plaque assays with 3T3 fibroblasts (Fig. 2B). The plaque size of the *aroA*, *aroB*, and *aroA/B* mutants in these assays was about half that of the wild-type strain, indicating a reduced spreading capacity of these *aro* mutants in comparison to the wild-type strain. No plaques were observed in this assay after infection with the *aroE* or *aroA/E* mutant, although actin tails were formed (data not shown).

***L. monocytogenes* with mutations in the aromatic amino acid pathway is strongly attenuated in vivo.** The growth characteristics of the *L. monocytogenes* *aro* mutants in BHI and in mammalian cell cultures described above imply that the virulence of these mutant strains will be attenuated in vivo. To further investigate the extent of virulence attenuation, groups of 7-week-old female BALB/c mice (three individuals per group) were infected i.v. with 10⁸ viable bacteria of the corresponding strains (which is 4 orders of magnitude higher than the 50% lethal dose of *L. monocytogenes* EGDe). No signs of disease were observed after infection. To test whether the attenuated bacteria were still able to replicate in vivo, counts of viable bacteria in the liver and spleen were determined at various time points postinfection (i.v. injection with 5 × 10⁷ *aro* mutant bacteria per mouse). Since the 50% lethal dose of *L. monocytogenes* EGDe is 3.5 orders of magnitude lower, no wild-type bacteria were included in this experiment. As indicated by the increasing bacterial load between 4 and 24 h postinfection (Fig. 3), *aroA*, *aroB*, and *aroA/B* mutants replicated in the liver and spleen and were present at rather high levels without causing any obvious signs of disease besides enlargement of the liver and spleen (data not shown). Infection

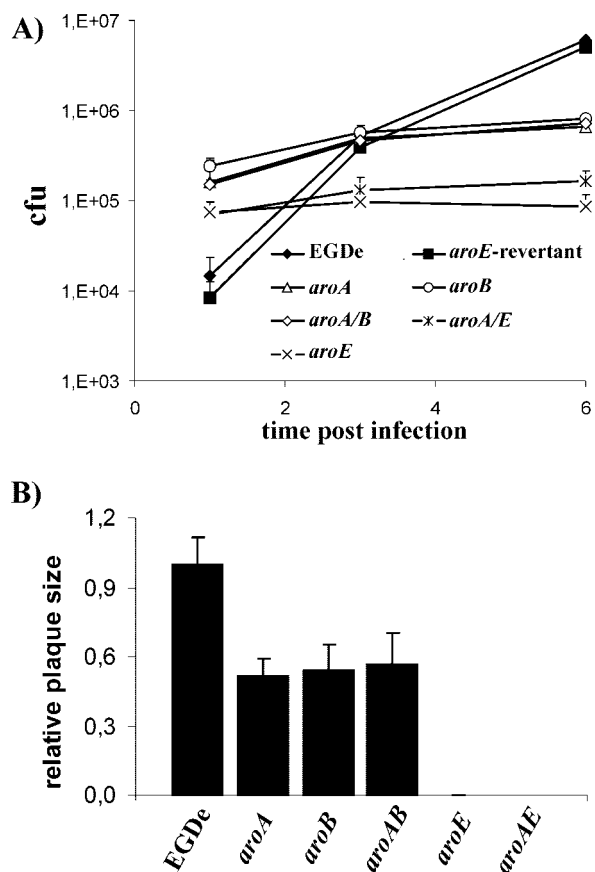


FIG. 2. (A) Mean bacterial counts plus standard deviations for three wells after infection of 2 × 10⁵ Caco-2 cells using 2 × 10⁶ *L. monocytogenes* EGDe or *aroA*, *aroB*, *aroA/B*, *aroE*, or *aroA/E* mutant organisms. (B) For plaque assays, a confluent layer of 3T3 fibroblasts was infected with 1 bacterium per 10 cells, and the diameters of at least 50 plaques were measured; their mean diameters plus standard deviations are compared to those after infection with *L. monocytogenes* EGDe.

with 10³ *L. monocytogenes* organisms, however, resulted in mild symptoms of disease. In contrast to the other *aro* mutants, the *aroE* and *aroA/E* mutants did not replicate in the liver or spleen, as indicated by the rapid decrease in the numbers of viable bacteria in the infected organs (Fig. 3). However, these strongly attenuated mutants survived at a low level for up to 7 days in the liver and spleen.

Attenuated *aro* mutant *L. monocytogenes* strains induce a protective CD8 T-cell response. In order to evaluate the immunogenic potential of *L. monocytogenes* strains with mutations in the aromatic amino acid biosynthetic pathway, we measured the T-cell response against the well-defined H-2^d-restricted CD8 T-cell epitopes p60 217 to 225, p60 449 to 457, and LLO 91 to 99 of *L. monocytogenes* (14). Priming of an *L. monocytogenes*-specific CD8 T-cell response by the *aroA/B* mutant, which appears to be best suited to be a possible vaccine carrier strain due to its putative low reversion rate to the wild type (since *aroA* and *aroB* are located in different parts of the genome and may not be complemented in one step by recombination) and high attenuation, required at least 5 × 10⁶ CFU (Fig. 4A). After immunization, a significantly stronger CD8⁺-T-cell response was obtained after i.v. immunization with 5 ×

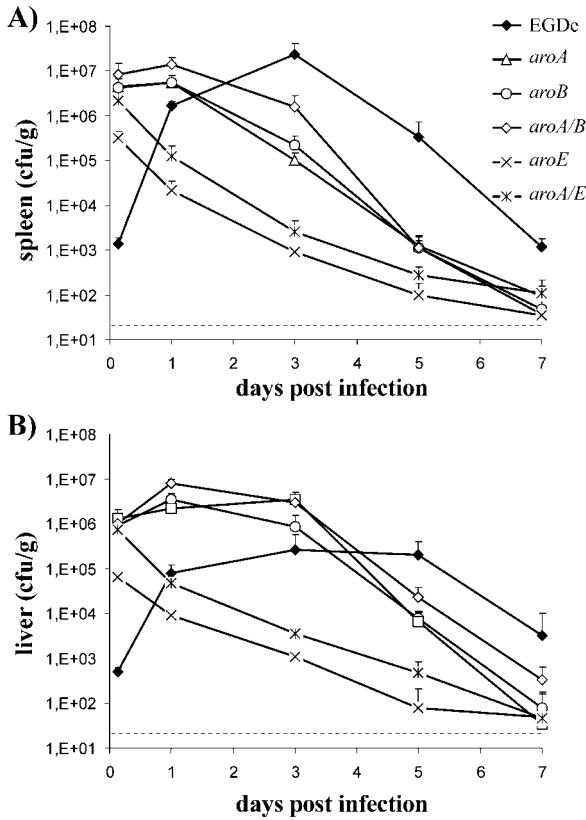


FIG. 3. Colonization of spleens (A) and livers (B) of BALB/c mice following i.v. infection with 5×10^7 CFU of *aroA*, *aroB*, *aroA/B*, *aroE*, or *aroA/E* mutant or 10^3 *L. monocytogenes* EGDe bacteria. Each point represents the mean number of CFU/g of organ plus standard deviation for five mice. Dashed lines represent the limit of detection.

10^6 CFU of the *aroA*, *aroB*, and *aroA/B* mutants compared to immunization of mice with the *aroE* or *aroA/E* mutant (Fig. 4B). While *aroA*, *aroB*, and *aroA/B* mutants primed a significant CD8 T-cell response against all three epitopes tested, the *aroE* or *aroA/E* mutant strain selectively primed a T-cell response only against the immunodominant epitope LLO 91 to 99. Thus, the *aroA/B* mutant combines excellent immunogenicity with high biosafety due to the double deletion of two independent, widely separated genes. In contrast to the *aroA*, *aroB*, and *aroA/B* mutants, the *aroE* and *aroA/E* mutants were significantly less immunogenic. The CD8 T-cell response induced by immunization with the *aroE* and *aroA/E* mutants was enhanced when more bacteria were inoculated (Fig. 4C); however, CD8 T cells never reached the frequency which was obtained after immunization with the *aroA/B* mutant (Fig. 4A and B). The *Listeria*-specific CD8 T-cell response observed after immunization with the *aroA/B* mutant, expressed both as frequency of antigen-specific CD8 T cells per splenocyte (Fig. 4, top graphs) and as total T-cell response per spleen (Fig. 4, bottom graphs), was superior to those of the other mutants tested.

The protection obtained after immunization of mice with the different *aro* mutant *Listeria* strains correlated well with the frequency of peptide-specific CD8 T cells in vivo. Immunization with 5×10^6 CFU of the *aroA*, *aroB*, and *aroA/B* mutants protected mice at least as well as immunization with 10^3 wild-type bacteria (Fig. 5), and in only some mice, very few bacteria could be detected (detection limits, 25 CFU/g of liver and 100 CFU/g of spleen). In correlation with the lower frequencies of *L. monocytogenes*-specific CD8 T cells observed after immunization with either the *aroE* or *aroA/E* mutant, significantly less protection against challenge with wild-type *L. monocytogenes* was obtained with these mutants compared to mice infected

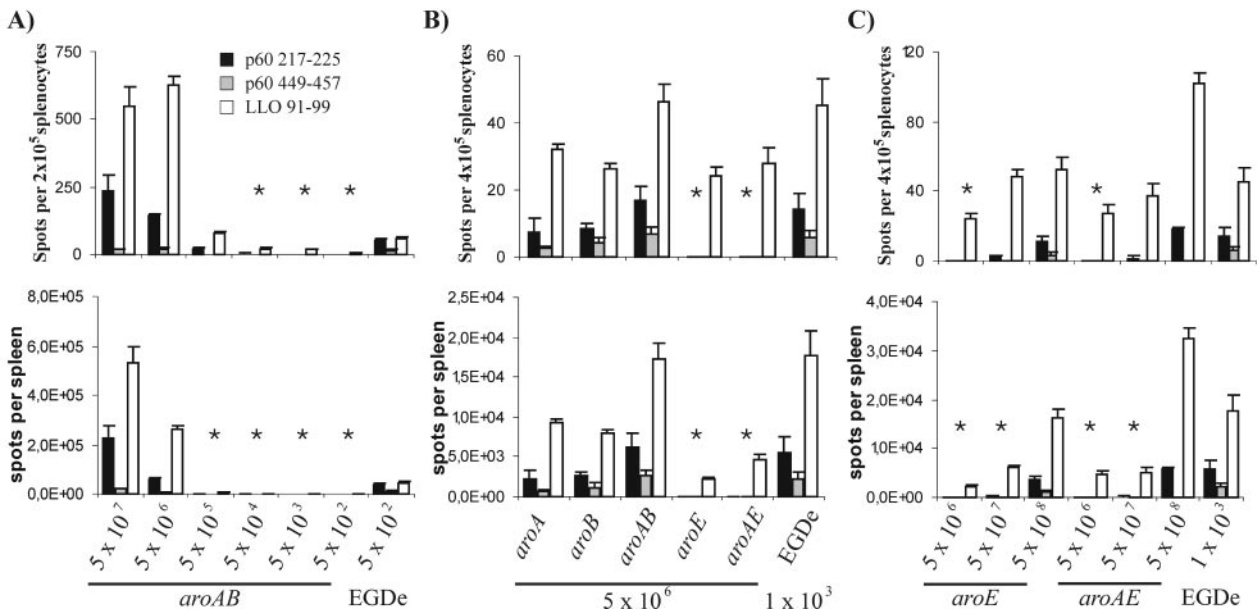


FIG. 4. ELISPOT assay of spleen cells after i.v. infection of 7-week-old BALB/c mice with different numbers of CFU of *aro* mutants and *L. monocytogenes* EGDe. The results are shown as spots per splenocyte plus standard deviation (top graphs) and mean spots per spleen plus standard deviation (bottom graphs) for three mice. The asterisks indicate *P* values of <0.05 between wild-type and mutant strains for all three epitopes, determined using a one-sided Student *t* test. (A) Spots per spleen are shown, depending on the applied infective dose of the *aroA/B* mutant. (B) CD8 T-cell response when 5×10^6 CFU of the *aro* mutants and 10^3 CFU of the wild-type strain were applied. (C) For efficient T-cell responses, at least 5×10^7 CFU of the *aroE* or *aroA/E* mutant is necessary.

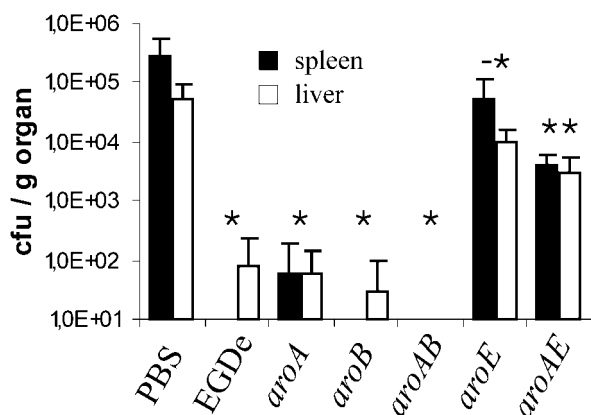


FIG. 5. Colonization of spleen and liver in immunized mice 4 days after i.v. infection with 10^3 CFU of *L. monocytogenes* EGDe bacteria. Immunization was performed with 5×10^6 CFU of *aro* mutants, 10^3 CFU of *L. monocytogenes* EGDe (positive control), or PBS (negative control). Each bar represents the mean number of CFU/g of organ plus standard deviation for five mice. *, $P < 0.05$ between mice immunized with bacteria and PBS controls; *, $P < 0.05$ between *aroE* mutant-immunized animals and *L. monocytogenes* EGDe-immunized mice, and $P < 0.07$ between *aroE* mutant-immunized animals and PBS-immunized controls; **, $P < 0.05$ between *aroA/E*-immunized animals and PBS- and *L. monocytogenes* EGDe-immunized animals, respectively, determined using a one-sided Student *t* test.

with the *aroA*, *aroB*, or *aroA/B* mutant. Thus, the *L. monocytogenes aroA/B* mutant combines high attenuation and biosafety with high immunogenicity, which points to this strain as a possible vaccine carrier strain.

DISCUSSION

In this study, we investigated the impacts of *aroA*, *aroB*, and *aroE* mutations on replication under extra- and intracellular conditions and on the virulence and immunogenicity of *L. monocytogenes*.

Although all *aro* mutants should be unable to produce the aromatic amino acids, including *p*-amino benzoate, as well as menaquinone, the results obtained showed unexpectedly major differences in the growth rates in culture media and inside mammalian cells and in the degree of virulence attenuation between the *aroA*, *aroB*, and *aroA/B* mutants (with deletions in genes determining the first two enzymes of the common pathway) and the *aroA/E* and *aroE* mutants [with a deletion in the gene determining the enzyme for the conversion of shikimate-3-phosphate to 5-*O*-(1-carboxyvinyl)-3-phosphoshikimate]. The growth phenotypes of all *L. monocytogenes aro* mutants were also different from those of the intensively studied *aroA* mutants of *S. enterica* serovar Typhimurium and other *Enterobacteriaceae* (note that the *aroA* mutation in the latter gram-negative bacteria corresponds to the *aroE* mutation in *L. monocytogenes* and other gram-positive bacteria). The *Salmonella aroA* mutant grows like wild-type *Salmonella* in rich BHI medium, indicating that all essential aromatic compounds which this mutant lacks, can be provided by the culture medium (31). In contrast, the *aro* mutants of *L. monocytogenes*, and in particular the *aroE* mutant, showed strongly reduced growth in these media compared to the wild-type strain. This growth reduction is not due to impaired uptake of the aromatic

amino acids by the *aro* mutants, since a triple mutant with specific mutations in the biosynthesis pathways leading to Phe, Tyr, and Trp grows at the same rate as the wild-type strain in BHI and minimal media supplemented with the aromatic amino acids. Our results rather suggest that the lack of menaquinone biosynthesis is the major cause of the impaired growth of the *aro* mutants, since *L. monocytogenes* can apparently synthesize only menaquinone but not ubiquinone (<http://genolist.pasteur.fr/ListiList/>), whereas in *S. enterica* serovar Typhimurium, all genes for ubiquinone and menaquinone biosynthesis have been identified in the genome sequence. Ubiquinone and menaquinone are major electron carriers in the aerobic respiration chain, receiving electrons from reduced NAD generated via oxidation of reduced carbon sources by the corresponding dehydrogenases.

Indeed, all *L. monocytogenes aro* mutants carried out predominantly anaerobic metabolism, as indicated by the low concentration of acetoin and acetate and the increased accumulation of lactate in the culture media (44), as well as the nearly identical growth rates under oxic and anoxic conditions and the restored aerobic growth of the *aro* mutants if vitamin K₂ was added to the culture medium. Preliminary data from comparative transcriptome and proteome patterns of the *L. monocytogenes* wild-type strain and the *aroA* mutant also indicate a switch to anaerobic metabolism in the *aroA* mutant, even under aerobic growth conditions (Stritzker et al., unpublished results).

One would expect that the *S. enterica* serovar Typhimurium *aroA* mutant should also be unable to produce these two central electron carriers essential for NADH-driven oxidative respiration unless *S. enterica* serovar Typhimurium can circumvent this defect by a respiration mechanism that does not require ubiquinone-menaquinone to be absent in *L. monocytogenes*. Alternatively, ubiquinone (which *S. enterica* serovar Typhimurium is able to synthesize) or ubiquinone precursors (e.g., 2,3-dihydroxybenzoate or *p*-hydroxybenzoate) are present in the rich culture media used, like BHI, and complement the ubiquinone defect of the *aroA* mutant.

The reason for the significant growth difference between the *aroA*, *aroB*, and *aroA/B* and the *aroE* mutants is less clear. A fortuitous additional mutation in the *aroE* deletion mutant can be ruled out, since the same growth phenotype was obtained with independently constructed *aroE* deletion mutants and the *aroA/E* double mutant, and an *aroE* revertant showed wild-type replication in extracellular and intracellular growth and also produced wild-type amounts of acetoin, acetate and lactate under aerobic growth conditions. Toxic effects of the possible accumulation of intermediates from the common pathway, e.g., dehydroquinone or shikimate, or the additional ATP consumption in the reaction leading to shikimate-3-phosphate (possible in the *aroE* mutant but not in the *aroA*, *aroB*, and *aroA/B* mutants) also seem to be unlikely causes of the observed growth difference between the two mutants, since an *aroA/E* mutant shows the same phenotype as the *aroE* mutant. Possibly a yet-unknown side reaction leads to low-level generation of a component in the common pathway including menaquinone (downstream of the first intermediate, 2-dehydro-3-deoxy-D-arabino-heptulosonate), which can be converted at low efficiency to the end products of the aromatic amino acid pathway by the *aroA*, *aroB*, and *aroA/B* mutants but not by the *aroE* and *aroA/E* mutants. In addition, excess oxygen under oxic growth conditions may harm the *aroE* and *aroA/E* mutants

but not the *aroA*, *aroB*, and *aroA/B* mutants, since the assumed low-level synthesis of menaquinone in the latter group of mutants may suffice to detoxify the oxygen. This assumption is supported by the enhanced growth rate of the *aroE* mutant under nonoxic growth conditions, which resembles those of the *aroA* mutant and the wild-type strain (Table 2).

Similar to the *aroA* mutants of *S. enterica* serovar Typhimurium and other *Enterobacteriaceae* (6, 21, 30, 42, 49), all *aro* mutants of *L. monocytogenes* described here are strongly virulence attenuated. However, the physiological basis for the virulence attenuation of the two intracellular bacteria may be different. A shortage of aromatic amino acids in the vacuolar compartment in which *S. enterica* serovar Typhimurium replicates seems to be a major reason for its virulence attenuation (31). This is most likely not the case for the *L. monocytogenes aro* mutants. *L. monocytogenes* replicates in the cytosol of infected host cells, and all *aro* mutants reach this cellular compartment at least as efficiently as the wild-type strain, since the internalin A (B)-mediated internalization of these mutants by mammalian cells is significantly enhanced and listeriolysin production necessary for escape into the cytosol is as high as that of the wild-type strain (data not shown). For listeriae residing in mammalian-cell cytosol, the supply of aromatic amino acids does not appear to be a limiting factor for the following reasons: (i) *L. monocytogenes* mutants with single, double, and even triple mutations in phenylalanine, tyrosine, and/or tryptophan biosynthesis are not significantly hampered in intracellular growth (data not shown); (ii) no mutants with defects in any of these pathways were ever isolated in previous studies screening for virulence-defective *L. monocytogenes* mutants (13, 23; K. Knuth, C. Stuehler, T. Fuchs, and W. Goebel, unpublished data); and (iii) previous studies using *L. monocytogenes* mutants auxotrophic for aromatic amino acids showed only marginal virulence attenuation and no intracellular growth reduction (1, 29).

We assume that the predominant anaerobic metabolism of the *aro* mutants is the major cause of the reduced intracellular growth and, hence, the virulence attenuation of these mutants. The stronger attenuation of the *aroE* and *aroA/E* mutants than of the *aroA*, *aroB*, and *aroA/B* mutants is in line with this assumption. Efficient replication of *L. monocytogenes* in the cytosol requires aerobic metabolism, as indicated by the strongly reduced growth rate of a mutant with inactive pyruvate dehydrogenase (32) and of mutants with defects in either the oxidative branch of the citrate cycle or the oxidative respiration chain (Knuth et al., unpublished).

As reported recently, disruption of the basic branch of the aromatic amino acid pathway failed in *M. tuberculosis* (35). Since *M. tuberculosis*, which has a strict aerobic metabolism, is also able to synthesize only menaquinone (but, like *L. monocytogenes*, is unable to produce ubiquinone) (<http://www.genome.ad.jp/kegg/>), this compound cannot be produced when genes in the basic branch of the aromatic amino acid pathway are deleted. However, since *M. tuberculosis* is unable to switch to anaerobic metabolism, *aro* mutants will not survive.

The CD8 T-cell response in BALB/c mice after immunization with different *L. monocytogenes aro* mutants correlated well with their replication potential in the host cell's cytosol. Compared to the *aroE* and *aroA/E* mutants, immunization with the *aroA*, *aroB*, or *aroA/B* mutant primed a significantly stronger

p60- and LLO-specific CD8 T-cell response. The CD8 T-cell response and the protection obtained after immunization with 5×10^6 CFU of the *aroA/B* mutant were at least as strong as those after immunization with a sublethal dose of the *L. monocytogenes* wild-type strain (10^3 bacteria). However, in the case of the *aroA/B* mutant, immunization was accompanied by only weak pathological side effects, such as slight enlargement of the liver and spleen. Thus, the *L. monocytogenes aroA/B* mutant combines high attenuation and biosafety due to an expected extremely low reversion rate with high immunogenicity, which points to this strain as a good vaccine carrier strain.

Some of the known virulence factors of *L. monocytogenes*, e.g., LLO, are required for efficient major histocompatibility complex class I-restricted antigen presentation in infected antigen-presenting cells, *L. monocytogenes* strains attenuated by the knockout of virulence factors, like *hly*, *actA*, or a combination of *actA* and *plcB*, therefore, have impaired immunogenicity. The LLO-deficient mutant, although highly attenuated in virulence, is strongly hampered in antigen presentation via the major histocompatibility complex class I pathway (4) and in delivery of DNA vaccines (20) and is also missing an important antigen (3, 8, 12, 34). In a previous study, it was shown that an *actA* mutant shows highly reduced release of vaccine DNA, even in the presence of a phage lysin used as a tool to disrupt the listerial carrier (39), and compared to the wild-type strain, was inferior to a vaccine carrier expressing the complete set of *L. monocytogenes*-specific virulence factors. In the same study, elevated plasmid DNA transfer and antigen presentation using an *aroA* mutant compared to wild-type *L. monocytogenes* were shown.

The *aro* mutations described were also introduced into a previously developed listerial lethal balanced system stabilizing introduced antigen-encoding plasmid DNA (39). The resulting plasmid-stabilized *aro* strains may be valuable tools for eliciting cellular immune responses using either DNA or protein delivery strategies.

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