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Attenuation and Persistence of and Ability To Induce Protective Immunity to a *Staphylococcus aureus aroA* Mutant in Mice

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Staphylococcus aureus **is the most important etiological agent of bovine mastitis, a disease that causes significant economic losses to the dairy industry. Several vaccines to prevent the disease have been tested, with limited success. The aim of this study was to obtain a suitable attenuated *aro* mutant of *S. aureus* by transposon mutagenesis and to demonstrate its efficacy as a live vaccine to induce protective immunity in a murine model of intramammary infection. To do this, we transformed *S. aureus* RN6390 with plasmid pTV1*ts* carrying Tn*917*. After screening of 3,493 erythromycin-resistant colonies, one mutant incapable of growing on plates lacking phenylalanine, tryptophan, and tyrosine was isolated and characterized. Molecular characterization of the mutant showed that the affected gene was *aroA* and that the insertion occurred 756 bp downstream of the *aroA* start codon. Complementation of the *aroA* mutant with a plasmid carrying *aroA* recovered the wild-type phenotype. The mutant exhibited a 50% lethal dose (1×10^6 CFU/mouse) higher than that of the parental strain (4.3×10^4 CFU/mouse). The *aroA* mutant showed decreased ability to persist in the lungs, spleens, and mammary glands of mice. Intramammary immunization with the *aroA* mutant stimulated both Th1 and Th2 responses in the mammary gland, as ascertained by reverse transcription-PCR, and induced significant protection from challenge with either the parental wild-type or a heterologous strain isolated from a cow with mastitis.**

Bovine mastitis is one of the most important diseases of dairy cows throughout the world. It is also a major cause of economic losses to the dairy industry because it leads to decreased milk production and low-quality milk (17). *Staphylococcus aureus* is the most prevalent infectious agent that affects the bovine udder. After entering the mammary gland through the teat canal and adapting to the udder environment, *S. aureus* multiplies rapidly, and an inflammatory reaction ensues, leading to tissue damage (61). Staphylococcal mastitis is extremely difficult to control by treatment alone. However, effective programs of postmilking use of germicidal teat dips, strict milking time hygiene, dry cow therapy, and culling can result in a markedly reduced incidence of *S. aureus* (14). A number of vaccines to prevent the disease and reduce the severity of intramammary (ima) infection have been described. These vaccines, however, have failed to prevent the development of staphylococcal mastitis (29, 58, 63), thus making other strategies for preventing ima infection indispensable. Although a number of molecules have been suggested as potential useful antigens for single-component vaccines, none of these approaches have been entirely successful so far (8, 36). The use of live attenuated vaccines may be considered an alternative approach. Indeed, these vaccines may have the advantage that they represent a greater pool of antigens, which may induce a broader and perhaps more intense protective immune response against bacterial aggression (5).

Bacterial attenuation can be achieved by different mechanisms. One is to introduce mutations into a key metabolic pathway whose function is essential for bacteria to survive and grow in vivo to cause disease. Several virulent strains have been attenuated by inactivation of genes in the aromatic amino acid biosynthesis pathway. Aromatic-dependent mutants of *Salmonella enterica* serovar Typhimurium (38), *Yersinia pestis* (40), *Bordetella pertussis* (50), *Corynebacterium pseudotuberculosis* (53), *Pseudomonas aeruginosa* (44), and *Listeria monocytogenes* (1) have been shown to be avirulent and to stimulate protective immunity in different hosts. Requirement of *p*-aminobenzoic acid (PABA), a precursor of folic acid that is not synthesized by mammals, has been singled out as the likely reason for reduced virulence of these bacterial strains (25). Since bacteria are unable to take up exogenous folate and the availability of PABA is limited in vertebrate tissues, the growth of *aro* mutants in vivo is severely restricted.

In the present study, an *aroA* mutant of *S. aureus* was generated by transposon mutagenesis, and experiments were conducted to test its reduced virulence, ability to colonize the mammary gland, and efficacy to induce protective immunity in a murine model of ima infection. The utilization of bacterial auxotrophs in the development of alternative immunoprophylactic approaches to prevent *S. aureus* infection is supported by this study.

MATERIALS AND METHODS

Bacterial strains, phage, and growth conditions. *S. aureus* laboratory virulent strain RN6390 (12) was kindly provided by A. L. Cheung (Darmouth Medical School, Hanover, NH). *S. aureus* RN4220 (a mutant of the 8325-4 strain that accepts foreign DNA) was used as a genetic intermediate to deliver the temperature-sensitive plasmid pTV1*ts* (64). *S. aureus* clinical strain MB319 (55) was utilized in heterologous challenge experiments. Bacteriophage ϕ11 was used to

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produce a phage lysate of strain RN4220 containing pTV1*ts* as previously described (11). The lysate was used to infect parental strain RN6390. Transductants were selected on brain heart infusion (BHI) (Difco, Detroit, MI) agar with chloramphenicol (Cm) (10 μg/ml). All strains were grown in BHI medium or in the defined minimum medium (DMM) for *S. aureus* described by Patee and Neveln (42). When necessary, Cm (10 μg/ml) or erythromycin (Em) (10 μg/ml) (Sigma, St. Louis, MO) was added. In certain experiments, colonies were replicated onto DMM agar plates minus different combinations of tryptophan (Trp) (0.05 mM), phenylalanine (Phe) (0.24 mM), tyrosine (Tyr) (0.28 mM), PABA (0.05 mg/liter), and 2,3-dihydrobenzoic acid (DHB) (10 mg/liter) (Sigma). *S. aureus* wild-type (wt) and *aroA* mutant strains were grown in BHI broth (supplemented with 10 μg/ml Em for the *aroA* mutant) to exponential phase, extensively washed with physiologic saline solution (PSS), and suspended in PSS to the desired density for inoculation to mice.

Transposon mutagenesis and screening for auxotrophic mutants. Transposition of Tn*917* carried by pTV1*ts* was performed essentially as previously described (24). *S. aureus* RN6390 carrying pTV1*ts* was grown in BHI broth containing Cm (10 μg/ml) at 30°C overnight. The culture was diluted into BHI broth containing Em (15 μg/ml), grown overnight at 42°C, and plated at 42°C on BHI agar containing Em to select for transposon mutants. These mutants were screened for the aromatic amino acid auxotrophic phenotype. To do this, Em-resistant (Em^r) colonies were replicated onto DMM agar plates without Trp, Phe, Tyr, PABA, or DHB.

DNA manipulations and Southern hybridization. Chromosomal DNA was purified from *S. aureus* strain RN6390 or the auxotrophic mutant FB306 (obtained in this study) after bacterial lysis with lysostaphin (5 mg/ml) and lysozyme (10 mg/ml) by the method of Pitcher et al. (43). Restriction endonucleases (Promega, Madison, WI) were used as recommended by the manufacturer. Chromosomal DNA was digested with EcoRI and separated by electrophoresis, transferred to and hybridized on a Zeta-Probe GT membrane (Bio-Rad, Hercules, CA) and blotted with a 1.8-kb Tn*917* BglII fragment used as a probe labeled with digoxigenin by using the DIG DNA labeling kit (Boehringer Mannheim, Germany) (31).

Complementation. A 1.4-kb fragment encompassing the *aroA* gene from *S. aureus* RN6390 was amplified by PCR using primers 5′-CTC TCT AGA ACA TTA CAA CAT GCA TGT GAA C-3′ and 5′-ACG CGT CGA CTG CGT CAT CGT TGT CAG TAG T-3′. Restriction sites for XbaI and SalI (underlined) were introduced into the fragment at the 5′ and 3′ ends, respectively. The PCR fragment was restricted and ligated into vector pALC1743 (kindly provided by A. L. Cheung) after deletion of the *gfp* gene and then transformed into *Escherichia coli* DH5α (Invitrogen, Carlsbad, CA) (28). Restriction analysis and DNA sequencing confirmed the orientation and authenticity of the cloned gene. The recombinant plasmid was electroporated into strain FR306, and Cm- and Em-resistant colonies were selected. Transformants were tested for restoration of the wild-type phenotype.

DNA amplification and sequencing. The junction fragment comprising the Tn*917* right end and the flanking chromosomal DNA (see Fig. 1A and B) was amplified from mutant FB306 using inverse PCR. Briefly, genomic DNA from the mutant was digested with HindIII and ligated at a concentration of <2 ng/ml. PCR was performed using 20 to 30 ng of ligated template DNA and 50 pmol each of primer P3 (identical to nucleotides 5485 to 5503 of Tn*917*, GenBank database accession number M11180) and primer P4 (complementary to nucleotides 4393 to 4410 of Tn*917*) for 30 cycles, with 1 cycle consisting of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The product was sequenced using the chain termination method of Sanger et al. (52) on an ABI 373A automated DNA sequencer (Applied Biosystems, Foster City, CA). Chromatograms were analyzed using the SeqEd package (Applied Biosystems).

RNA isolation and RT-PCR. Total RNA from the mammary glands of immunized and control mice was extracted using the Trizol reagent (Gibco-BRL, Life Technologies) according to the manufacturer's instructions. Chloroform-isoamyl alcohol purification was performed as described previously (13). Total RNA was quantified by spectrophotometry at 260 nm, and RNA quality was determined by the ratio of optical density at 260 nm/optical density at 280 nm. Ratios higher than 1.8 were considered acceptable. The levels of gamma interferon (IFN-γ) and interleukin 4 (IL-4) transcripts were determined by reverse transcription-PCR (RT-PCR) from 3 μg of template RNA using the Access RT-PCR system (Promega, Madison, WI). Primers for IFN-γ and IL-4 and conditions for RT-PCR have been described elsewhere (59). RT-PCR products were separated on 2% agarose gels with a 100-bp ladder as size marker (Promega, Madison, WI) and visualized by staining with ethidium bromide. PCR using primers for β-actin (59) was performed on each individual sample as a positive-control standard. Analysis was performed using the Scion Image Beta software (Scion Corp., Frederick, Md.).

Determination of virulence for mice. Swiss outbred mice were bred and maintained in the vivarium of the Department of Microbiology, School of Medicine, University of Buenos Aires, Buenos Aires, Argentina. Animal care was done in accordance with the guidelines set forth by the U.S. National Institutes of Health (37). For 50% lethal dose (LD_{50}) studies, 6-week-old, male Swiss mice were injected intraperitoneally (i.p.) with 0.5 ml of a suspension containing the bacterial strain and 2% (wt/vol) brewer's yeast in BHI broth (35). Four groups each comprising 10 mice received serial log dilutions of bacteria, and the LD_{50} was determined after 3 days by the method of Reed and Muench (47).

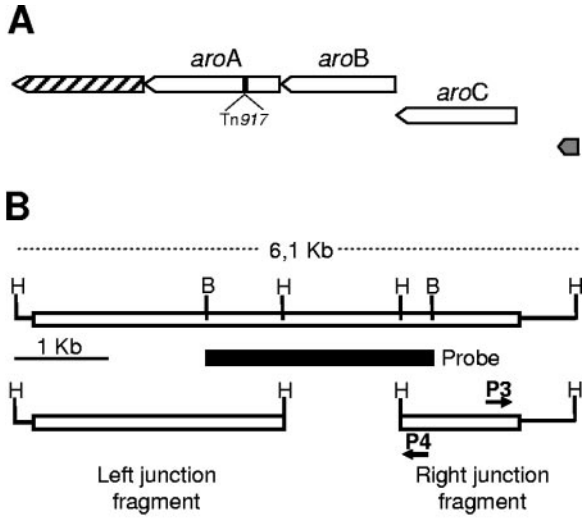
Persistence studies in vivo. Eight to 10 male, 6-week-old Swiss mice were inoculated with each strain. For intravenous (i.v.) inoculation, mice were injected with 0.2 ml of the bacterial suspension in PSS (6×10^7 CFU/mouse) into the tail vein. After different times (1 and 24 h), the lungs were excised and homogenized separately in 2 ml of sterile distilled water (16). After injection (6.5×10^6 CFU/0.5 ml of 2% brewer's yeast in BHI) by the i.p. route, the lungs were removed at different times (3 and 5 h) and homogenized. For ima inoculation, six female Swiss mice received 0.05 ml of the bacterial suspension in PSS (2×10^5 CFU/gland) into the left fourth (L4) and right fourth (R4) mammary glands as described previously (10, 21). After 24 h and 96 h, the L4 and R4 glands were removed and homogenized. Viable counts were performed on these homogenates by plating samples on BHI agar.

Immunization and wild-type challenge. Approximately 7 days before parturition, female Swiss mice were immunized with 0.05 ml of a suspension of *S. aureus* (5×10^5 CFU/gland) by ima inoculation into the L4 and R4 mammary glands. The suspension contained *S. aureus* FB306 or heat-killed (30 min at 60°C) RN6390 *S. aureus*. A booster injection containing the same dose was administered 7 days later by the same route. Fourteen days after the second injection, mice were challenged by the ima route with 0.05 ml of an RN6390 suspension (5×10^5 CFU/gland) or with a suspension containing 1×10^6 CFU/gland of *S. aureus* MB319 (23). Viable bacterial counts were performed on mammary gland homogenates.

Statistical analysis. Nonparametric data were analyzed with the Mann-Whitney test using GraphPad software (PRISM, version 2.2). Fisher's exact test was used for statistical comparison of proportions.

RESULTS

Characterization of the transposon mutant. After Tn*917* mutagenesis of *S. aureus* virulent strain RN6390, aromatic amino acid-dependent mutants were screened. To do this, 3,493 Em^r colonies were replicated onto different DMM agar plates without Trp, Phe, Tyr, and PABA or on DHB containing Em. Ten mutants exhibited auxotrophic phenotype, but only one aromatic amino acid-dependent mutant (named FB306) did not grow on DMM agar plates without the three aromatic amino acids as well as PABA and DHB. Therefore, the nutritional requirement of mutant FB306 to grow in vitro suggested that the affected enzyme responsible for the observed auxotrophic phenotype is one of those required for the synthesis of shikimic acid and chorismic acid (2). By transduction using ϕ11, the Tn*917* insertion of mutant FB306 was moved back into the wild-type RN6390 strain. Over 100 Em^r transductants were demonstrated to be dependent of the three aromatic amino acids as well as of PABA and DHB. The in vitro growth rates of mutant FB306 and parental strain RN6390 in DMM supplemented with aromatic amino acids, plus PABA and DHB, under antibiotic-free conditions did not differ significantly from each other (ca. 60 min). The reversion frequency of the mutant was lower than 2×10^{-14}. In order to perform Southern blot analysis of mutant FB306, DNA was digested with EcoRI or HindIII, subjected to electrophoresis, and probed with a 1.8-kb BglII internal fragment from Tn*917* (Fig. 1B). DNA from wild-type strain RN6390 did not hybridize with the probe (data not shown). One hybridization band was observed after EcoRI digestion, indicating that there was a single transposon insert in the chromosome. Digestion of FB306



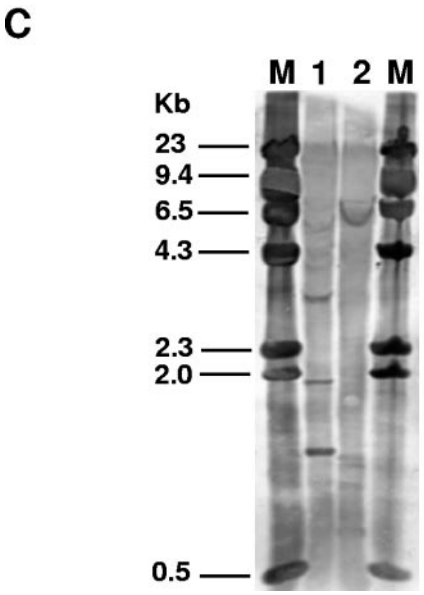


FIG. 1. (A) Schematic representation of the *aro* operon and surrounding genes according to the published sequences of *S. aureus* COL (GenBank). The gene downstream of *aroA* (similar to the *B. subtilis* gene that codes for hypothetical protein YpiA) is represented by the striped arrow, the *aroCBA* operon is represented by open arrows, and the gray arrow represents an unknown gene. The product of the *aroA* gene is 3-phosphoshikimate 1-carboxyvinyltransferase; the *aroB* product is 3-dehydroquinate synthase, and the product of the *aroC* gene is chorismate synthase. (B) Schematic representation of the Tn*917* insertion (open box) in the 6.1-kb HindIII fragment of the FB306 strain (black lines). Relevant restriction sites in Tn*917* are shown. The left and right HindIII transposon/chromosomal DNA junction fragments, the probe (black box), and the primers (arrows) are indicated. H, HindIII; B, BglII. (C) Southern blot analysis of *S. aureus aroA* mutant FB306. Chromosomal DNA was digested with HindIII (lane 1) or EcoRI (lane 2) and probed with a 1.8-kb BglII internal Tn*917* fragment. Molecular size markers are shown in lanes M.

DNA with HindIII yielded a 1.29-kb band that represented the HindIII fragment within Tn*917* plus two additional bands (~3 and 1.8 kb) which represented the junction fragment between the transposon and chromosomal DNA (Fig. 1C).

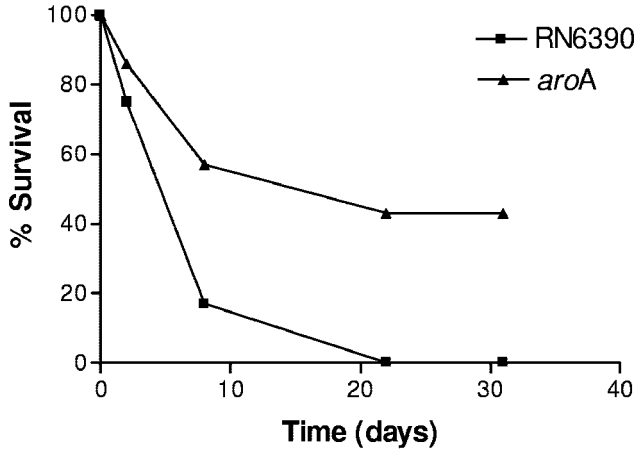


FIG. 2. Survival of mice infected with *S. aureus aroA* mutant FB306. Groups of 10 Swiss mice were challenged by the i.v. route with 2×10^7 CFU/mouse of wild-type RN6390 or *aroA* mutant suspended in PSS. Each point represents the percent survival of mice infected with the parental strain RN6390 or the *aroA* mutant. The levels of significance by Fisher's exact test were $P = 0.04$ at day 8 and $P = 0.01$ for days 22 and 31.

The DNA flanking one side of the inserted transposon in the FB306 mutant was amplified by inverse PCR using primers P3 and P4 (Fig. 1B). Approximately 700 bp, including the HindIII fragment encompassing the region marked by the Tn*917* insertion, was sequenced. The nucleotide sequence exhibited 99% identity with the 3-phosphoshikimate 1-carboxyvinyltransferase (*aroA*) gene of *S. aureus* COL, Mu50, N315, MSSA476 and MW2, as well as 98% identity with the *aroA* gene of *S. aureus* MRSA252. Subsequent similarity searches of available *S. aureus* genome sequences (www.ncbi.nlm.nih.gov) revealed the presence of a single copy of the *aroA* gene, indicating that it is widely conserved in *S. aureus*. Nucleotide sequence analysis revealed that insertion of transposon Tn*917* had occurred 756 bp downstream from the *aroA* (ca. 1,300 bp) start codon in FB306. To confirm that the lesion in *aroA* is responsible for the observed phenotype, a genetic complementation study was performed. As expected, complementation of the FB306 mutant with the plasmid carrying the *aroA* gene recovered the wild-type phenotype.

The *S. aureus aroA* mutant is attenuated in mice. Introduction of the *aroA* mutation into *S. aureus* RN6390 increased the log LD_{50} for Swiss mice from 4.8 to 6.0. In addition, 43% of mice inoculated by the i.v. route with a high dose (2×10^7 CFU/mouse) of the *aroA* mutant were in good health by day 31 postchallenge, when the experiment was terminated. In contrast, 100% of the animals injected by the i.v. route with an identical dose of wild-type *S. aureus* RN6390 died by day 10 after challenge (Fig. 2). Moreover, histopathological analysis of kidneys from mice inoculated by the i.v. route with 2×10^7 CFU/mouse of wild-type *S. aureus* or the *aroA* mutant revealed that the mutant induced histopathological changes of lesser magnitude compared with those found in mice challenged with wild-type *S. aureus* (Fig. 3).

Persistence studies. Both the *S. aureus* parental strain and the FB306 *aroA* mutant were investigated for their abilities to colonize the lungs, spleens, and mammary glands of mice.

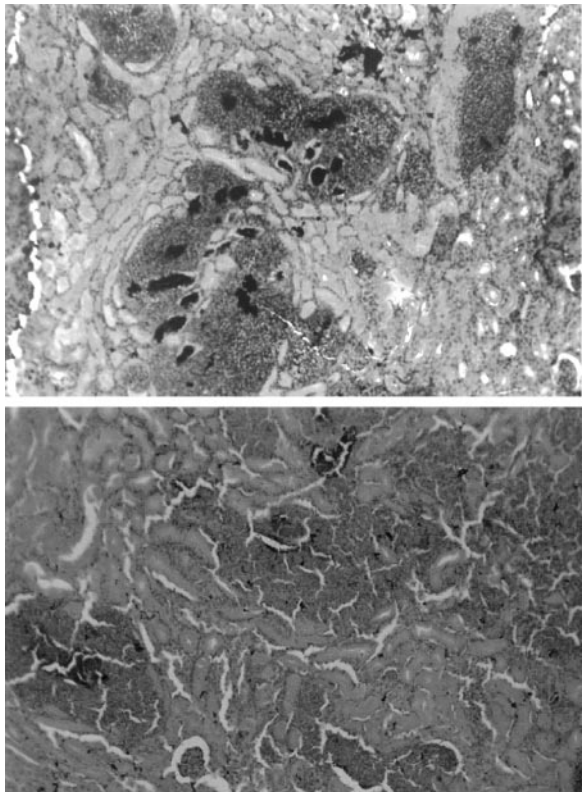


FIG. 3. Histopathological analysis of kidney tissue 3 days after i.v. bacterial challenge with lethal doses of *S. aureus* RN6390 (top) or the *aroA* mutant (bottom). Parenchyma is replaced by confluent large abscesses and inflammatory infiltrate. Tubular necrosis and reduction and/or absence of glomeruli can also be seen (stained with hematoxylin-eosin; magnification, ×100). Histological changes of lesser magnitude were induced by the *aroA* mutant, compared with those found in mice challenged with the wild-type *S. aureus*.

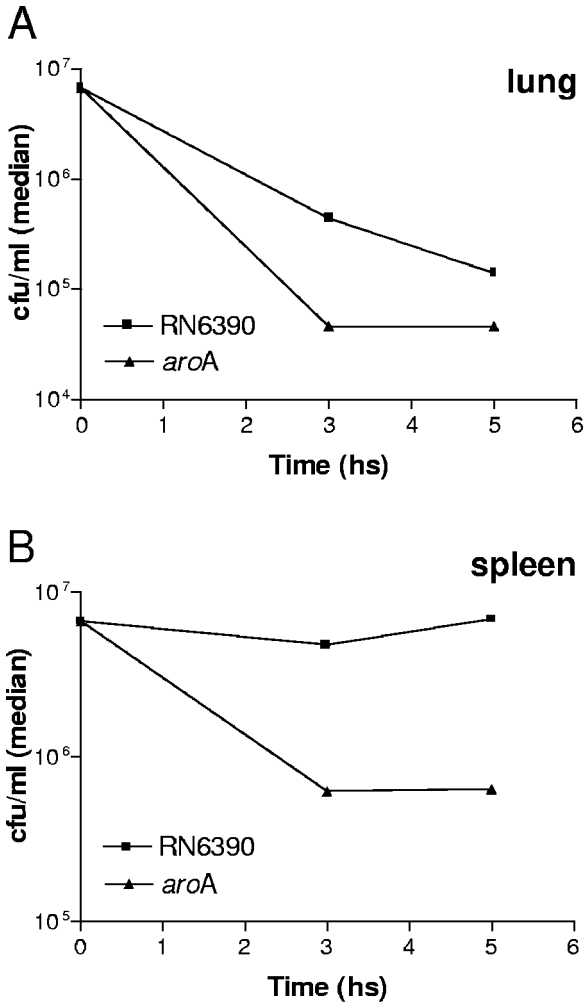


FIG. 4. Persistence of *S. aureus aroA* mutant FB306 after i.p. inoculation. In vivo growth of wild-type RN6390 and the *aroA* mutant in the lungs and spleens of Swiss mice. Each point represents the median value for 8 to 12 mice. For each time point (hours), the CFU of RN6390 versus CFU of the *aroA* mutant recovered from different tissues is depicted. The levels of significance by the Mann-Whitney test were $P = 0.002$ at 3 h (A) and $P = 0.04$ at 3 h (B).

Previous results from our laboratory were considered to choose the different times postchallenge (21, 23). Groups of mice were infected i.p. with 6.7×10^6 CFU/mouse of wild-type RN6390 or the FR306 *aroA* mutant. Bacterial counts in lungs and spleens were determined at 3 and 5 h postchallenge. Viable counts of the *aroA* mutant in the lungs decreased significantly compared with those of *S. aureus* RN6390 at 3 h postchallenge (Fig. 4A). Similar results were observed in the spleens of mice inoculated by the i.p. route (Fig. 4B).

In other experiments, groups of mice were challenged by the i.v. route with a suspension of the *aroA* mutant (6.5×10^7 CFU/mouse) or the wild-type RN6390 *S. aureus*. In blood the inoculated bacteria are rapidly and efficiently phagocytosed. Mice were sacrificed at 1 and 24 h, and the numbers of CFU in the lungs were determined. One hour after challenge, the number of CFU of the *aroA* mutant decreased significantly compared with that of the RN6390 strain (3.3×10^4 CFU/ml for the *aroA* mutant versus 1.3×10^5 CFU/ml for the wt; $P = 0.04$) (Fig. 5A). Viable counts of the *aroA* mutant were significantly reduced 24 h postinfection (3.3×10^4 CFU/ml at 1 h versus 2.4×10^2 CFU/ml at 24 h; $P = 0.017$). These results demonstrate that the *aroA* mutant has a reduced ability to multiply within lungs and spleens. All FB306 colonies recovered from mice conserved their *aroA* phenotype.

In experiments to ascertain the survival of the *aroA* mutant in the mammary gland, groups of female mice were inoculated by the ima route with 1.9×10^5 CFU/gland of wild-type RN6390 or the *aroA* mutant. At 1 and 4 days after challenge, mice were sacrificed, and the mammary glands were removed and homogenized. Aliquots from homogenates were obtained to determine the bacterial viable counts. At 1 day after inoculation, the number of CFU of the *aroA* mutant recovered from mammary glands was significantly decreased compared with that of the wild-type RN6390 (9.4×10^3 CFU/ml for the *aroA* mutant versus 4.8×10^5 CFU/ml for the wt; $P = 0.004$). Similar results were obtained by 4 days after ima challenge (9.3×10^2 CFU/ml for the *aroA* mutant versus 4.1×10^4 CFU/ml for the wt; $P = 0.03$) (Fig. 5B). These results show that the *S. aureus* FB306 *aroA* mutant has reduced ability to persist in the mouse mammary gland.

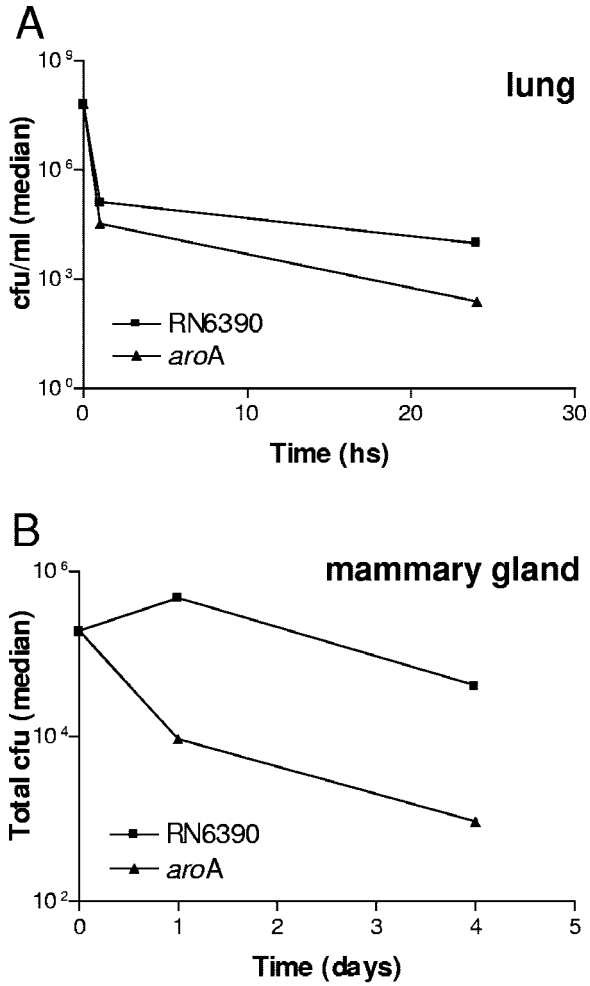


FIG. 5. Persistence of *S. aureus aroA* mutant FB306 in vivo. In vivo growth of wild-type RN6390 and the *aroA* mutant in the lungs and mammary glands of Swiss mice. (A) Each strain under study (6.5×10^7 CFU/mouse) was injected intravenously. (B) Bacteria (1.9×10^5 CFU/gland) were administered by the ima route. Each point represents the median value for 8 to 12 mice. For each time point (hours or days), RN6390 CFU versus the *aroA* mutant CFU recovered from different tissues are shown. The levels of significance by the Mann-Whitney test were as follows: $P = 0.04$ at 1 h (A) and $P = 0.004$ at day 1 and $P = 0.03$ at day 4 (B).

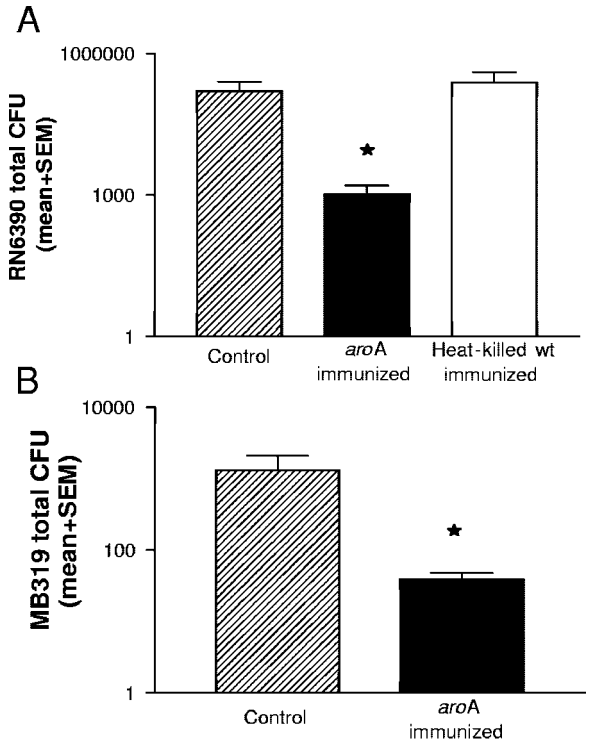


FIG. 6. Protective efficacy against intramammary challenges by local immunization with the *aroA* mutant. Groups of female mice received two injections of the *aroA* mutant (5×10^5 CFU/gland) or heat-killed RN6390 (5×10^5 CFU/gland) by the ima route. Fourteen days after the second injection, mice were challenged by the same route with 0.05 ml of a suspension of (A) parental wild-type *S. aureus* RN6390 (5×10^5 CFU/gland) or (B) *S. aureus* MB319 (heterologous strain isolated from milk of a cow with mastitis) (1×10^6 CFU/gland). Each bar represents the mean ± standard error of the mean (SEM) (error bar) of CFU recovered from 10 mammary glands. The levels of significance by the Mann-Whitney test are as follows: $P = 0.01$ for mice immunized with heat-killed-RN6390 versus mice immunized with the *aroA* mutant; $P = 0.02$ (A) and $P = 0.01$ (B) for control mice versus mice immunized with the *aroA* mutant (*).

Immunization studies. In vaccination studies using *S. aureus* live attenuated mutants, it is important to consider mucosal immunity induced by deposition of the antigen. For this reason, to determine whether vaccination with the *S. aureus aroA* mutant induced protection, mice were immunized with the *aroA* mutant by the ima route. Another group of mice was immunized with heat-killed RN6390 by the same route. Immunized mice and age-matched nonimmunized controls were challenged 14 days later with either the parental wild-type RN6390 strain (5×10^5 CFU/gland) or a heterologous virulent clinical strain (namely, MB319) (1×10^6 CFU/gland) isolated from milk of a cow with mastitis. Ninety-six hours postchallenge, viable counts were assessed in the mammary glands to determine whether immunization affected colonization or clearance of the virulent strains. The number of RN6390 CFU recovered from mammary glands of mice immunized with the *aroA* mutant (*aroA*-immunized mice) was significantly reduced compared with that of nonimmunized controls (1,052 ± 538 total CFU for *aroA*-immunized mice versus $1.6 \times 10^5 \pm 9.5 \times 10^4$ total CFU for control mice; $P = 0.02$). Similar differences were observed when the number of RN6390 CFU recovered from mammary glands of *aroA*-immunized mice was compared with that of heat-killed-RN6390-immunized mice (1,052 ± 538 total CFU for *aroA*-immunized mice versus $2.5 \times 10^5 \pm 1.5 \times 10^5$ total CFU for heat-killed-RN6390-immunized mice; $P = 0.01$) (Fig. 6A). In addition, ima immunization with the *aroA* mutant significantly decreased the number of CFU of heterologous strain MB319 compared with the viable counts of MB319 recovered from control mice (nonimmunized and MB319 challenged) (38 ± 9.4 total CFU for *aroA*-immunized mice versus 1,316 ± 801 total CFU for control mice; $P = 0.01$) (Fig. 6B). Therefore, immunization with the *aroA* mutant conferred significant protection from challenge with homologous and heterologous virulent *S. aureus*.

By 96 h after challenge with *S. aureus* RN6390 (3×10^5

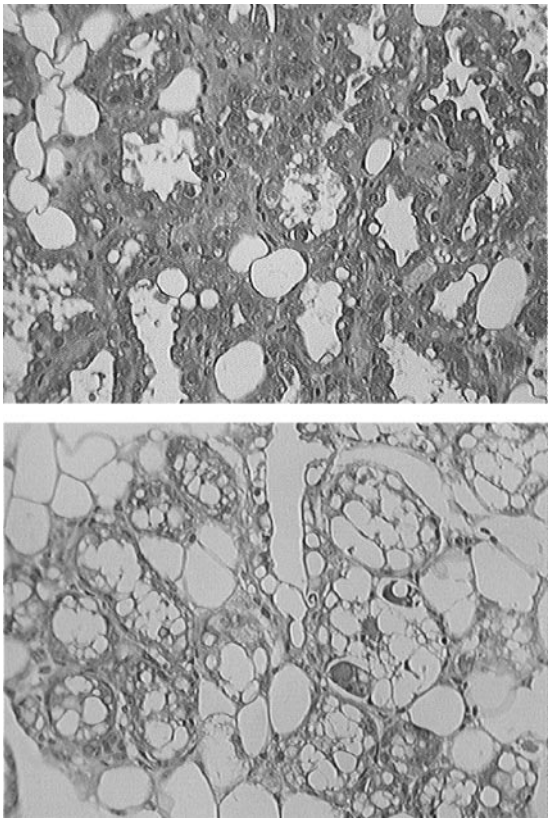


FIG. 7. Histopathology of the mammary glands of mice immunized with the *aroA* mutant and nonimmunized mice 96 hours after local challenge with wild-type *S. aureus*. (Top) Moderate polymorphonuclear leukocyte and mononuclear cell infiltration and mild vascular congestion in the mammary glands of unvaccinated and RN6390-challenged mice (stained with hematoxylin-eosin; magnification, ×400). (Bottom) The mammary glands of mice immunized with the *aroA* mutant and mice challenged with RN6390 did not exhibit infiltration or vascular congestion (hematoxylin-eosin; magnification, ×400).

CFU/gland), histological studies of mammary glands locally immunized with the *aroA* mutant and nonimmunized controls were performed. The mammary glands of unvaccinated and challenged mice showed moderate polymorphonuclear leukocyte and mononuclear cell infiltration and mild vascular congestion. Conversely, the mammary tissue of vaccinated and challenged mice did not exhibit infiltration (Fig. 7).

Immune responses induced by the *aroA* mutant. To establish whether ima immunization with the *aroA* mutant induced adaptive responses in mice, production of IFN-γ and IL-4 mRNAs was determined as an indirect measurement of activation of different subsets of T cells (Th1 and Th2, respectively). Ninety-six hours after challenge with the wild-type strain RN6390 or heterologous strain MB319, the relative mRNA levels of IFN-γ and IL-4 were determined in mammary glands by RT-PCR. Mammary glands from mice immunized with the *aroA* mutant showed an increase in the level of IFN-γ transcripts compared with control, unvaccinated mice (Fig. 8). Similar results were observed when IL-4 mRNA levels in the vaccinated group were compared with those found in the control group (Fig. 8). Moreover, the increase observed in cytokine gene expression was independent of the *S. aureus* challenge strain (RN6390 or MB319) (data from mice challenged with MB319 not shown). Therefore, the results suggest that ima immunization with the *aroA* mutant induced activation of Th1 and Th2 cell subsets in the mammary gland.

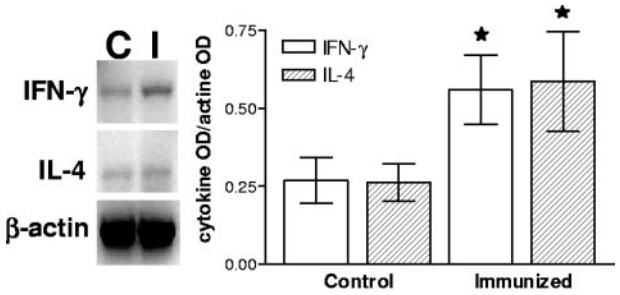


FIG. 8. IFN-γ and IL-4 mRNA responses in the mammary glands of mice immunized with the *aroA* mutant and nonimmunized mice. At 96 hours after local challenge with wild-type *S. aureus*, the mammary glands from mice immunized with the *aroA* mutant (I) and control nonimmunized mice (C) were excised, and total RNA was extracted. RT-PCR was performed using cytokine-specific primers. Amplification of the housekeeping gene β-actin was performed to ensure that similar amounts of input RNA and similar efficiencies of reverse transcription are being compared. Results are presented as the means ± standard errors of the means (SEM) (error bars) of the ratio of cytokine optical density [OD]/actin OD. The values for immunized mice were significantly different from those for control mice ($P = 0.02$ by the Mann-Whitney U test [★]).

DISCUSSION

Mutations in the basic branch of the aromatic amino acid biosynthesis pathway proved to be efficient in attenuating virulence, e.g., in *Listeria monocytogenes* (57), *Shigella dysenteriae* (62), *Pseudomonas aeruginosa* (44), *Neisseria gonorrhoeae* (9), and *Bacillus anthracis* (27), but they failed to do so in *Mycobacterium tuberculosis* (41). The main objective of this study was to ascertain whether an *aro* mutant of *S. aureus* could be attenuated and immunogenic. To do this, mutagenesis by transposition was chosen as a preliminary method. The results obtained in this investigation encourage us to begin the construction of an unmarked *aroA* deletion mutant of *S. aureus* to be utilized in field trials.

Target genes may be inactivated by integration of a transposable element. Such inactivation is usually the consequence of transcriptional interruption or of a negative polar effect on the expression of genes located downstream (15, 20). In this report, we describe the isolation and characterization of an *S. aureus* strain with a Tn*917* insertion mutation in the *aroA* gene. Whereas aromatic amino acid-dependent mutants of other gram-positive bacteria were constructed by transposon mutagenesis (1, 27, 53), this is the first report of an attenuated *aro* mutant of *S. aureus* that is evaluated for its protective efficacy as a potential vaccine. The *aro* mutants are auxotrophs for aromatic amino acids, PABA (a precursor of folic acid), and DHB (a precursor for ubiquinone). *S. aureus* FB306 is phenotypically an *aro* mutant, because it proliferates only in minimal medium supplemented with Trp, Phe, and Tyr as well as PABA and DHB. The auxotrophic phenotype of mutant FB306 was stable both in vitro and in vivo. Indeed, its reversion frequency was $<2 \times 10^{-14}$. This result is consistent with previous obser-

vations concerning the effect of Tn*917* as a chromosomal mutagen: precise excision of insertions is extremely rare, and deletions occur in about 10% of the insertion events (51). Finally, the idea that an undefined point mutation selected at 42°C (pTV1*ts* curing temperature) could have been responsible for the FB306 mutant *aro* auxotrophic phenotype might be hypothesized. In our experience, however, point mutations in *S. aureus* have a reversion frequency in the range from 10^{-6} to 10^{-8}, much higher than the value of less than 2×10^{-14} observed in this study. This finding makes unlikely that a potential point mutation could have been responsible for the *aro* auxotrophic phenotype found in *S. aureus* FB306.

The FB306 mutant erythromycin marker was mobilized into the wild-type strain RN6390 by using phage ϕ11, and the same auxotrophic phenotype was observed. It is suggested, therefore, that the dependence of aromatic amino acids, as well as PABA and DHB, of the FB306 mutant was due to Tn*917* insertion. Southern blot analysis of FB306 mutant DNA digested with EcoRI exhibited one hybridizing band, which indicated that a single copy of the transposon was inserted into the chromosome. The site of the Tn*917* insertion was confirmed by sequence analysis. The transposon interrupted the *aroA* gene (756 bp downstream from start codon), which codes for the 3-phosphoshikimate 1-carboxyvinyltransferase of the chorismic acid biosynthesis pathway (39). The identified nucleotide sequence was 98 to 99% identical to the *aroA S. aureus* published sequences. These deviations could be due to strain variation. Moreover, genetic complementation confirmed that the lesion in *aroA* was responsible for the phenotype observed in the FB306 *aroA* mutant. While *aroA* is the last gene in the *aroCBA* operon, there is a gene immediately downstream of *aroA* whose function is unknown but is similar to that coding *Bacillus subtilis* hypothetical protein YpiA (GenBank). The location of *ypiA* is conserved in many organisms, indicating that it may be important to the aromatic acid pathway. Indeed, the genes involved in the shikimate pathway and folate, ubiquinone, and aromatic amino acid synthesis are known in many bacteria, fungal pathogens, and apicomplexan parasites (49). Whether *ypiA* is involved in any enzymatic step of the shikimate pathway or major branches from chorismate should be investigated.

We hypothesized that introduction of a nonreverting mutation into the *S. aureus* chromosome causing dependence on aromatic metabolites may result in an attenuated mutant which would have reduced ability to multiply in mammalian tissues. Our results showed that the *aroA* mutant was indeed attenuated, as demonstrated by an increase in its LD_{50} and increased survival of mice compared with those of the parental wild type. The results also demonstrated that the mutation in the 3-phosphoshikimate 1-carboxyvinyltransferase restricted the in vivo growth of the *aroA* mutant compared with the wild-type RN6390 *S. aureus* counterpart. Similar findings were reported in *aroA* mutants of *Listeria monocytogenes* (57), *Bordetella bronchiseptica* (7, 25, 34), *Bordetella pertussis* (50), *Aeromonas salmonicida* (60), *Pasteurella multocida* (26), and *Yersinia enterocolitica* (3). In contrast, *Salmonella enterica* serovar Typhimurium *aroA* mutants persisted for several weeks in the livers and spleens of orally infected mice (25, 32). Although milk is an excellent culture medium for many bacteria (4), the *aroA* mutant was much less efficient at colonizing the murine mammary gland compared with the wild-type parental RN6390 strain. It is likely that attenuation was due to starvation for essential aromatic metabolites rather than indirect effects on the expression of putative virulence factors.

Hemin-auxotrophic small-colony variants have been isolated from bovine *S. aureus* ima infections (54). These auxotrophic mutants can appear after apparently successful antimicrobial therapies (45). In view of these findings, it can be speculated that auxotrophic *S. aureus* could become a potential pathogen in the mammary gland, since small-colony variants frequently revert to the wild-type phenotype in vitro (33). Such would not be the case of the auxotrophic *aro* mutant described here, because its reversion frequency is extremely low and the nutritional requirement to restore the normal phenotype is not found in mammals, thus making its growth restricted in vivo. The latter is one of the facts that makes the use of an *aro* mutant as a potential vaccine attractive. Indeed, the *S. aureus aroA* mutant obtained in this study was cleared faster than the wild-type bacteria from different tissues (lung, spleen, and mammary gland) within a time frame suitable to make an *S. aureus aroA* mutant an attractive vaccine prospect.

It is generally accepted that attenuated strains are more potent than nonliving bacteria in stimulating immune responses (5). Indeed, live attenuated bacteria produce most of the antigens normally expressed during natural infection. An important issue of the study of auxotrophic mutants is to reach the right balance between attenuation and immunogenicity, since overattenuated bacteria may not produce certain key antigens necessary for the induction of protective immunity in vivo. A point of concern is the fact that an increase in the somatic cell count could be induced as a result of an *S. aureus aroA* mutant inoculation of cows. However, low levels of polymorphonuclear leukocytes and mononuclear cells and less damage were observed in the mammary gland after administration of the *aroA* mutant compared with the wild-type strain in the mouse mammary gland. Furthermore, in previous experiments, we have seen that the number of leukocytes was not increased in milk after ima administration of an attenuated *S. aureus* strain (23). Recently, Brouillette and Malouin (4) have demonstrated that after bacterial inoculation in the mouse mammary gland, polymorphonuclear infiltration, tissue damage, and *S. aureus*-host cell interactions are similar to those found in the bovine mammary gland. Therefore, even though certain differences may exist between murine and bovine hosts, the results obtained for mouse mammary gland infection may provide valid experimental data to support final testing of a vaccine strain in cows. In any event, the potential increase in the milk somatic cell counts needs to be assessed in cows immunized by the ima route using the desired auxotrophic *S. aureus* strain under construction.

The choice of the appropriate immunization route and scheme to obtain protective immune responses should be of concern (30), because it can determine failure or success of vaccination. In this regard, we demonstrated that ima but not i.p. application of live attenuated *S. aureus* strains stimulates murine mucosal responses against the wild type (21). In practice, ima administration of the vaccine in cows is laborious and needs trained personal. However, the efficacy of local immunization of cattle against *Streptococcus uberis* experimental ima challenge was demonstrated (18, 19). In the present study we

utilized the same route of administration (ima) and immunization scheme defined in a previous study with temperature-sensitive mutants (23), and we were able to obtain significant protection after immunization with the *aroA* mutant. Interestingly, significant reduction in the number of CFU of virulent challenging *S. aureus* strains (RN6390 and MB319, both producers of hemolysins) was observed in the mammary glands of mice immunized with the *aroA* mutant. Moreover, ima immunization of mice with the *S. aureus aroA* mutant induced high levels of both IFN-γ and IL-4 transcripts in the mammary gland. In previous studies, we have demonstrated the feasibility of inducing Th1 and Th2 responses against *S. aureus* in the mouse mammary gland by local immunization with temperature-sensitive mutants of *S. aureus* during late pregnancy (22).

Raupach and Kaufmann observed that IFN-γ plays a central role in the early bacterial control of infection with *Salmonella enterica* serovar Typhimurium *aroA* strains (46). Previous evidence suggests that IFN-γ could elicit functional changes in phagocytic cells of the mammary gland that could make it effective in the control of bovine mastitis (56). Riollet et al. (48) have detected IFN-γ transcripts sporadically in cells derived from milk of cows immunized with alpha-hemolysin by ima injection. Conversely, IL-4 mRNAs were not detected in any of the samples at any time by the same authors. This observation suggests that an orientation towards a Th1-type response was induced by immunization with a single staphylococcal component, such as alpha-hemolysin. Ima immunization of mice with the *aroA* mutant under study induced high levels of both IFN-γ and IL-4 transcripts, which agrees with the fact that multiple antigens are involved in the adaptive response to a live attenuated vaccine. It can be speculated that although the *S. aureus aroA* mutant was able to grow poorly in vivo, it could still produce important virulence factors to induce an appropriate immune response. Interestingly, mice immunized with the *S. aureus aroA* mutant were protected from ima heterologous challenge with the most prevalent clone of *S. aureus* (MB319 strain) recovered from milk of cows with mastitis in Argentina (6). Since the genotypic background of the *aroA* mutant can be discriminated from those of bovine field isolates from the same region (7), our results support the performance of controlled field studies on isolated and small herds in Argentina to evaluate the protective efficacy of an *aroA* mutant.

This is the first time an *aroA* mutant of *S. aureus* was tested for its protective ability to be used as a vaccine. Although differences may exist between the bovine and murine mammary glands, the results of the present study may contribute to the rational design of a live attenuated vaccine to prevent mastitis caused by *S. aureus* in dairy cows.

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