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Construction and Characterization In Vivo of *Bordetella pertussis aroA* Mutants

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A DNA fragment encoding a kanamycin resistance determinant was used to insertionally inactivate the cloned *aroA* gene of *Bordetella pertussis* in *Escherichia coli* K-12, and a conjugative shuttle vector system based on the suicide vector pRTP1 was used to deliver the mutations from *E. coli* back into *B. pertussis* CN2992FS and BP1. The *aroA* mutation was introduced by allelic exchange into the chromosome of *B. pertussis*, resulting in otherwise isogenic parental and *aroA* mutant pairs. The *B. pertussis aroA* mutants grew well on laboratory medium supplemented with aromatic compounds but failed to grow on unsupplemented medium. The *B. pertussis aroA* mutants expressed the normal *B. pertussis* extracellular, virulence-associated proteins; inactivated, whole-cell vaccines prepared from the mutants protected mice as efficiently as vaccines made from the parent strains against intracerebral challenge with the virulent *B. pertussis* 18323. Live *B. pertussis aroA* bacteria inefficiently colonized the lungs of NIH/S mice after they were challenged with aerosol, unlike the wild-type *B. pertussis* organism. Mice exposed to three separate aerosols of live *B. pertussis aroA* bacteria were protected against lung colonization after being exposed to an aerosol containing the virulent parental *B. pertussis* strain. High-level antibodies against *B. pertussis* rapidly appeared in the sera of mice immunized by aerosol with the *B. pertussis aroA* strains and challenged with the virulent parent.

Live vaccines based on attenuated microorganisms are effective in stimulating protective immune responses against a variety of pathogens. In the past, attenuated vaccine strains were derived empirically and the genetic changes responsible for attenuation were consequently unknown. This led to problems with the stability and quality control of certain live vaccines. Modern molecular genetic techniques can now be applied to construct stable, genetically defined, attenuated bacterial strains whose suitability for use as live vaccines in particular animal hosts can be assessed.

Attenuation can be achieved in a number of ways. One approach is to identify virulence determinants produced by the pathogen and construct strains, using genetic manipulation, which fail to produce those determinants. An example is the construction of strains of *Vibrio cholerae*, which do not express cholera enterotoxin (15, 22). When attenuated bacterial strains are constructed, it is essential to ensure that only defined, nonreverting mutations are introduced into the genome of the pathogen. In the case of *V. cholerae*, mutations in the enterotoxin were initially constructed in a cloned enterotoxin gene in *Escherichia coli* K-12 and the mutated gene was reintroduced into *V. cholerae* by using genetic crosses (15, 22).

An alternative approach to achieving attenuation is to introduce mutations into a key metabolic pathway whose function is essential for bacteria to survive and grow sufficiently in vivo to cause disease. Virulent *Salmonella* strains can be attenuated by introducing stable auxotrophic mutations affecting important metabolic pathways, including the prechorismate (*aro* mutants) and the purine biosynthetic pathways (13, 28). *Salmonella* strains harboring mutations in *aroA* (13, 19), *aroC* (7), *aroD* (23), or combinations of these mutations (7, 23) are attenuated and highly effective live oral vaccines against salmonellosis in several animal hosts (13, 19, 33). *Salmonella* spp. are invasive pathogens that can enter and grow inside eucaryotic cells (9, 10). At present, it is unknown whether *aro* mutants of noninvasive pathogens are attenuated.

Bordetella pertussis is the causative agent of whooping cough in humans. *B. pertussis* colonizes the human host by attaching to the ciliated epithelial cells lining the upper respiratory tract. There, the bacteria grow and express a variety of virulence-associated extracellular and surface-located proteins which induce disease symptoms. There have been reports that *B. pertussis* can enter eucaryotic cells, but in the human infection, *B. pertussis* is thought to predominently colonize the mucosal surface of the respiratory tract (2, 8, 17). We recently reported the cloning in *E. coli* and sequencing of the *B. pertussis aroA* gene (20). As a preliminary step towards constructing rationally attenuated strains of *B. pertussis* and other *Bordetella* species, we have constructed *B. pertussis aroA* mutants and tested their abilities to colonize the lungs of experimentally infected mice. The results of the study are described in this report.

MATERIALS AND METHODS

Bacteria, plasmids, and growth conditions. *B. pertussis* CN2992F is a derivative of CN2992, the Wellcome whole-cell vaccine strain, which is modulated not by increased concentrations of nicotinic acid but by temperature only (21). A spontaneous streptomycin-resistant mutant of CN 2992F (2992FS) was used in this study. *B. pertussis* BP1, a streptomycin-resistant derivative of strain Tohama 1, was obtained from G. Miller, Stanford University, Palo Alto, Calif. *E. coli* AB2829 *aroA* was obtained from B. Bachmann

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at the *E. coli* Genetic Stock Center, Yale University, School of Medicine, New Haven, Conn. *E. coli* SM10 λ *pir thi thr lon tonA lacY supE recA*::RP4-2-Tc::Mu (32) was obtained from G. Miller. pRTP1 has been described previously (36). pUC4K was obtained from Pharmacia, Milton Keynes, England. Plasmid pBPT*aroA*52 consists of a pUC18 vector containing a 2.2-kilobase-pair *Pst*I fragment encoding the *B. pertussis aroA* gene (20).

B. pertussis was routinely grown in Stainer-Scholte (SS) liquid medium at 37°C (35) or on Cohen-Wheeler (CW) solid medium (4) containing 10% sterile defibrinated horse blood. *B. pertussis aroA* mutants were grown on CW blood agar with or without aromatic substituents (aromix) or in SS medium with or without aromix. Aromix stock contained 4 mg of tryptophan, 4 mg of tyrosine, 4 mg of phenylalanine, 1 mg of dihydroxybenzoic acid, and 1 mg of *para*-aminobenzoic acid per ml and was used as a 1-in-100 dilution in supplemented media. *E. coli* strains were routinely cultured on L agar or in L broth (18). Antibiotic concentrations for *E. coli* strains were as follows: ampicillin, 100 μg/ml; and kanamycin, 40 μg/ml. For *B. pertussis*, concentrations were as follows: kanamycin, 50 μg/ml; and streptomycin, 200 μg/ml.

DNA manipulations. Restriction endonucleases and T4 DNA ligase were obtained from Boehringer (Lewes, England) or GIBCO BRL (Paisley, Scotland) and were used according to the instructions of the manufacturers. DNA polymerase 1 large fragment (Klenow enzyme) was fast protein liquid chromatography pure from Pharmacia.

DNA preparations and hybridization. Plasmid DNA was purified by using standard techniques (18). *B. pertussis* chromosomal DNA was prepared by using a modification (19) of the method of Hull et al. (14). DNA was transferred to nylon (Pall Biodyne) filters by the method of Southern (34). Radioactively labeled DNA probes were obtained, using nick-translation as described by Maniatis et al. (18).

Bacterial conjugation. Bacteria were grown on appropriate solid culture medium; *E. coli* was grown overnight, and *B. pertussis* was grown for 3 days. Bacterial growth was swept from the plates with Dacron swabs and suspended in phosphate-buffered saline (PBS), pH 7.2. The optical density of the suspensions at 650 nm was determined and adjusted to 0.4, and mating mixtures were set up by mixing *B. pertussis* and *E. coli* cells in the ratios 10:1 and 100:1 of *B. pertussis* to *E. coli*. These were immediately plated onto CW blood agar plates containing 10 mM $MgCl_2$. The plates were incubated at 37°C in sealed jars for 4 h. After this time, the bacteria were swabbed from the plate into PBS and plated, for selection of transconjugants, onto CW blood agar plates containing appropriate antibiotics and aromix. Plates were incubated at 37°C for up to 5 days.

Aerosol infection. Groups of female NIH/S mice (11 to 14 g and approximately 3 weeks old) were placed in cages on a rotating carousel in a plastic exposure chamber as described previously (27). A bacterial suspension in PBS was prepared from 2- to 3-day-old cultures of *B. pertussis* grown on CW blood agar plates containing aromix as appropriate. The mice were exposed to an aerosol (generated from the bacterial suspension) of 2×10^9 CFU in PBS by a Turret mouthpiece tubing operated by a System 22 CR60 high-flow compressor (Medic-Aid, Pagham, West Sussex, United Kingdom) giving a very fine mist at a dynamic flow of 8.5 liters/min. The generated mist was drawn through a chamber by a vacuum pump at a passage of ca. 12 liters of air per mist mixture per min, which maintained 70% relative humidity in the chamber. The exposure to aerosol lasted 30 min; a period of 10 min then allowed the chamber to clear.

The course of the infection was assessed by performing counts of viable bacteria in lungs. Groups of four mice were removed at intervals and killed by cervical dislocation, and their lungs were aseptically removed and homogenized in a Potter-Elvehjem homogenizer with 2 ml of PBS. Dilutions of the homogenate were spotted onto CW blood agar plates containing streptomycin (and aromix, where appropriate), and the number of CFU was determined for each set of lungs.

Preparation of whole-cell vaccines. Strains were grown on CW agar supplemented with 10% defibrinated horse blood (and aromix, where appropriate) for 72 h. The growth was washed off with PBS, and the suspensions were incubated for 24 h at 37°C in the presence of 0.25% Formalin. Formolization was terminated by centrifugal washing of the cells with PBS and suspending the pellets in PBS containing 1/1,000 merthiolate. Bacterial concentration (expressed as milligrams per milliliter [dry weight]) was determined spectrophotometrically at this stage. The final vaccines were stored at 4°C before potency determination. Potency determination was performed according to the World Health Organization requirements, using a frozen *B. pertussis* 18323 challenge strain as reported previously (26). NIH/S mice between 11 and 14 g were immunized intraperitoneally with corresponding dilutions of vaccines and challenged 14 days later intracerebrally. The results were computed by parallel line probit analysis, and the relative potency of vaccines was related to the British pertussis reference vaccine 66/84.

Adenylate cyclase and pertussis toxin determination. Adenylate cyclase activity was determined in whole cells by the method of Salomon et al. (31). The CHO cell assay was performed as described by Hewlett et al. (12), using known concentrations of pertussis toxin as reference.

Detection of filamentous hemagglutinin and 69-kilodalton outer membrane protein. Both filamentous hemagglutinin (FHA) and the 69-kilodalton outer membrane protein were detected in *B. pertussis* cells, using Western blotting (immunoblotting). Whole-cell proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (16) and transferred to nitrocellulose by the method of Towbin et al. (38). Proteins were visualized by incubating the nitrocellulose with either a rabbit polyclonal anti-FHA serum (1:500 dilution) or a mouse monoclonal anti-69-kilodalton outer membrane protein antibody (1:10 dilutions of hybridoma culture supernatant), and then the appropriate anti-species immunoglobulins horseradish peroxidase conjugate and 4-chloro-1-napthol were used as the substrate (Sigma, Poole, Dorset, United Kingdom).

Anti-*B. pertussis* antibodies. The murine serum antibody response to *B. pertussis* was measured by an enzyme-linked immunosorbent assay (ELISA), using formolized *B. pertussis* as antigen. Formolized *B. pertussis* (50 μl; 5×10^8 CFU per ml in 0.1 M carbonate buffer [pH 9.6]) were absorbed onto 96-well flat-bottomed microtiter plates (Costar Corp., Cambridge, Mass.) by incubation at 4°C overnight. The wells were aspirated and washed three times with PBS containing 0.05% Tween 20 (PBS-Tween) and incubated with 100 μl of PBS-Tween–0.1% bovine serum albumin. The wells were washed and incubated with 50 μl of test serum in PBS-Tween for 2 h at 37°C. After the wells were washed, 50 μl of goat anti-mouse immunoglobulin horseradish peroxidase conjugate of appropriate class specificity (Sigma) diluted 1 in 1,000 in PBS-Tween was added. After incubation at 37°C for 2 h, the plates were washed and incubated for 20 to 30 min at

room temperature with 50 μl of substrate (0.04% *o*-phenylenediamine dissolved in phosphate-citrate buffer [pH 5.0]–24 mM citrate–64 mM disodium hydrogen phosphate containing 40 μl of hydrogen peroxide).

The reaction was terminated by the addition of 50 μl of 1 M sulfuric acid. Plates were read in a Titertek Multiscan MCC ELISA reader at 492 nm.

Sera were obtained from groups of four mice by cardiac puncture and pooled. Sera were serially diluted and assayed for anti-*B. pertussis* immunoglobulins of the immunoglobulin G (IgG), IgM, and IgA classes, using heavy-chain-specific antibody conjugates.

RESULTS

Construction of *B. pertussis aroA* mutants. In order to construct *B. pertussis aroA* mutants, the cloned *B. pertussis aroA* gene was first inactivated in vitro. pBPT*aroA*52 consists of a pUC18 vector containing a 2.2-kilobase-pair *Pst*I fragment encoding the *B. pertussis aroA* gene (Fig. 1). This plasmid can complement the *aroA* lesion in *E. coli* K-12 AB2829, allowing the strain to grow on minimal medium in the absence of aromix (20). In order to create a single *Bam*HI site in the *aroA* coding sequence, the *Bam*HI site in the polylinker of pBPT*aroA*52 was removed by digestion with *Xba*I and *Eco*RI; the resultant single-stranded ends were then filled in with Klenow enzyme and blunt-end ligation, thus reconstructing the *Eco*RI site in the resulting plasmid, pBPT*aroA*53. Next, the kanamycin resistance cassette from pUC4K was cloned on a *Bam*HI fragment into the remaining *Bam*HI site in the *aroA* coding region of pBPT*aroA*53 to create plasmid pBPT*aroA*54. Inactivation of the *aroA* gene was checked by transforming *E. coli* AB2829 with pBPT*aroA*54; transformants could not grow on minimal medium unless it was supplemented with aromix. In preparation for returning the inactivated *aroA* gene to *B. pertussis*, the mutated *aroA* locus was removed from pBPT*aroA*54 onto the suicide shuttle vector pRTP1 as follows. pBPT*aroA*54 was cleaved to completion with *Eco*RI and then partially with *Hin*dIII. A 3.6-kilobase-pair *Eco*RI-*Hin*dIII fragment containing the cloned DNA from pBPT*aroA*54, including the Km^r cassette, was gel purified and ligated into pRTP1 previously cleaved with *Eco*RI and *Hin*dIII. *E. coli* transformants harboring the resultant plasmid were selected for resistance to Ap^r and Km^r. The resulting plasmid was called pBPT*aroA*55.

pRTP1 is mobilizable by sequences derived from RP4 on the chromosome of *E. coli* SM10 and does not replicate in *B. pertussis* because of the absence of the *pir* gene product, carried in SM10 on a lysogenic bacteriophage. Furthermore, pRTP1 encodes the S12 allele which confers streptomycin sensitivity on otherwise streptomycin-resistant bacterial strains. *E. coli* SM10(pBPT*aroA*55) was plate mated with *B. pertussis* BP1 or CN2992FS, and transconjugants were selected with CW blood agar plates containing streptomycin and kanamycin as selective antibiotics.

Any *B. pertussis* colonies appearing on the selective plates should be *aroA* mutants, since Km^r should only be expressed if the wild-type *aroA* allele is replaced on the chromosome by the mutated *aroA* gene. This recombination may take place by two general mechanisms, one involving crossover between homologous structures of *B. pertussis* DNA at a single site within or flanking the *aroA* gene (which would result in insertion of the entire recombinant plasmid) and the other involving two regions separated by the site of the kanamycin resistance cassette insert (which would result in replacement of only the *aroA* allele) with no vector integration. Because of the S12 allele on pRTP1, streptomycin in the medium selects against the single crossovers and thus selects against vector integration.

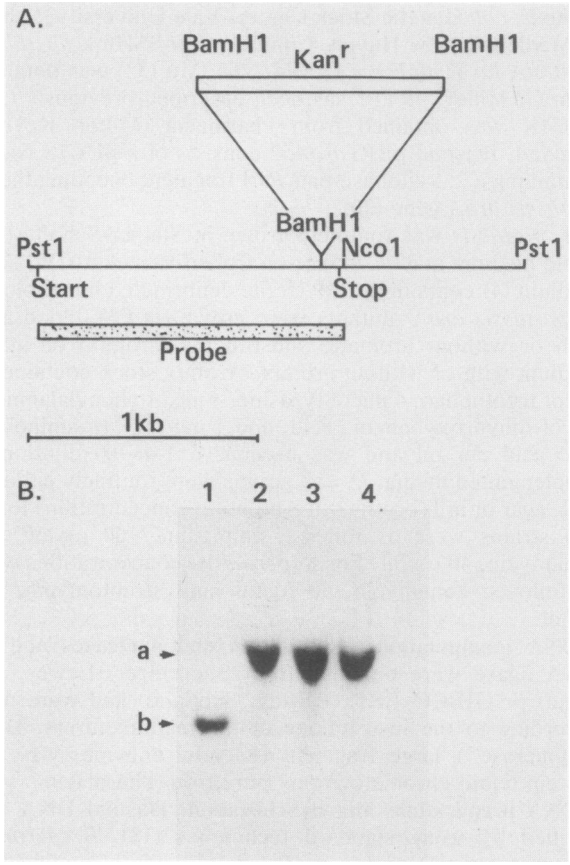

FIG. 1. Physical map of the *aroA* gene of *B. pertussis* and Southern blot of the *aroA* gene of wild-type and mutant strains. (A) Map of the 2.2-kilobase *Pst*I fragment of *B. pertussis* DNA carried on the plasmid pBPT*aroA*52 coding for the *aroA* gene. The positions of the start and stop codons of the gene and the site of insertion of the kanamycin resistance cassette of pUC4K in the mutated gene are indicated (see text for details). The speckled bar below the map defines the DNA probe used in Southern blotting to identify *aroA* gene sequences. The probe did not contain the kanamycin cassette. (B) Southern blot of genomic DNA from *B. pertussis* 2992FS and three independent isolates of 2992FS *aroA*. Lanes: 1, 2992FS; 2, 2992FS *aroA*/2; 3, 2992S *aroA*/4; 4, 2992FS *aroA*/6. Chromosomal DNA was digested with *Nco*I and probed with the *Pst*I-*Nco*I fragment shown in panel A. The arrows labeled a and b denote the restriction fragments hybridizing to the probe in the mutant and wild-type strains, respectively. kb, Kilobase.

Km^r and Sm^r *B. pertussis* transconjugants were purified by being replated on CW medium with or without aromix. Purified colonies grew very slowly or not at all on medium without aromix but grew at a rate similar to that of wild-type *B. pertussis* on medium containing aromix, giving rise to colonies which were similar in size to the parent strains. Some of these colonies were subcultured on CW blood agar plates and, after 72 h, the growth was washed off and inoculated into 200 ml of SS medium in a 500-ml conical flask and incubated at 37°C in an orbital shaker. These isolates could grow in the SS medium containing aromix but not in nonsupplemented medium.

After some matings with BP1 as a recipient, some small

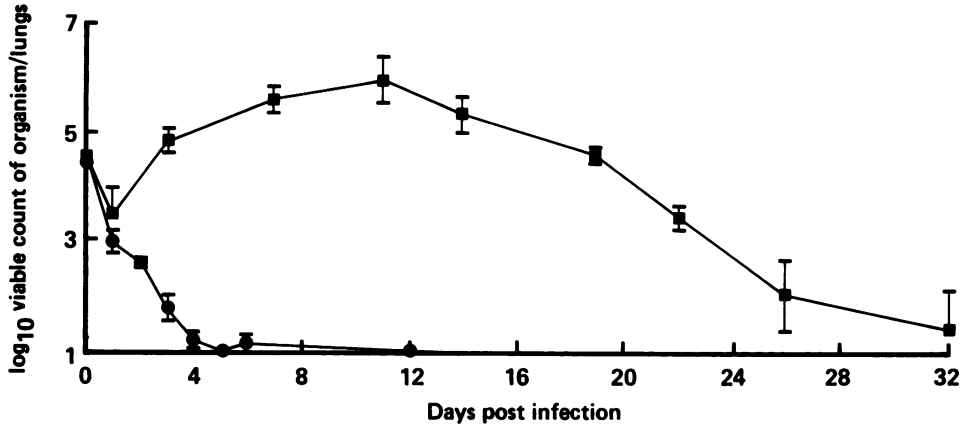

FIG. 2. Colonization of lungs of NIH/S mice after they were exposed to aerosols of CN2992FS (■) or CN2992FS *aroA* (●). Each point represents the geometric mean plus or minus two standard errors of the mean for four mice.

colonies which grew on aromix-supplemented CW blood agar but failed to grow on unsupplemented CW blood agar were isolated. These colonies would not grow in SS medium with or without aromix and were not studied further. A number of individual transconjugants which displayed the expected *aroA* phenotype were stored, and one mutant derived from BP1 and one mutant derived from CN2992FS were used in further studies.

Phenotypic properties of *B. pertussis aroA* mutants. Transconjugants capable of growth in aromix-supplemented SS medium mutiplied at a rate comparable to that of the parental strains on subculturing. However, they differed from the parental strains in that they produced a brownish discoloration of the SS medium which was similar in hue to the brownish pigment produced in SS medium during growth of *Bordetella parapertussis*. Preliminary nuclear magnetic resonance analysis showed the presence of unidentified aromatic components in the spent medium of *B. pertussis aroA* mutants (B. Sweatman, Physical Sciences, Wellcome Research Laboratories, unpublished results). To date, this pigment has not been characterized further.

The mutants produced adenylate cyclase, pertussis toxin, FHA, and 69-kilodalton outer membrane protein in amounts comparable to those produced by the parent strains. To assess the production of protective cell components, the *aroA* mutants were compared with the parent strains for their abilities to act as killed whole-cell vaccines in mice against intracerebral challenge with *B. pertussis* 18323. Whole-cell vaccines prepared from three *aroA* mutants were highly protective. There was no statistical difference between the protection afforded by the killed *aroA* vaccines and that of the British pertussis reference vaccine 66/84 (data not shown).

Genotypic properties of *B. pertussis aroA* mutants. The site of insertion of the kanamycin cassette in the genome of BP1 *aroA* and CN2992FS *aroA* was checked by Southern blotting. Total chromosomal DNA was prepared from parental and mutant strains and digested with *Nco*I, and the fragments were transferred to nylon membranes after being electrophoretically separated. Membranes were probed with a radioactively labeled preparation of the 1.4-kilobase *Pst*I-*Nco*I DNA fragment from pBPT*aroA*52. This fragment contains the entire *aroA* coding sequence plus 54 base pairs upstream of the translation initiation codon and 13 base pairs downstream of the stop codon. The results are shown for CN2992FS and CN2992FS *aroA* (Fig. 1). The probe hybridized to a 3.5-kilobase *Nco*I fragment in the parental strain and a 4.8-kilobase *Nco*I fragment in the *aroA* mutant. The increased size of the fragment corresponds to the insertion of the kanamycin resistance gene (Fig. 1). This was confirmed by using digestion with other restriction endonucleases (results not shown). BP1 *aroA* produced an identical hybridization pattern.

Survival of *B. pertussis aroA* mutants in vivo. The *B. pertussis* parent strains and *aroA* mutants were assessed for their abilities to colonize the lungs of mice. Adult outbred NIH/S mice were exposed to an aerosol generated from a suspension of between 2×10^9 and 4×10^9 CFU of *B. pertussis* bacteria per ml. This reproducibly led to seeding of the mouse lungs with 10^3 to 10^4 *B. pertussis* organisms. Survival of viable bacteria was followed by performing viable counts on homogenates of lungs removed from groups of four mice at intervals after the mice were exposed to aerosol. The results obtained with CN2992FS and CN2992FS *aroA* are shown in Fig. 2. BP1 and BP1 *aroA* behaved in a similar manner (results not shown). The parental strain, after an initial drop in numbers during the first day, multiplied to reach 10^5 to 10^6 CFU in the lung 11 days postchallenge (Fig. 2). Thereafter, the counts decreased gradually, but *B. pertussis* could still be detected in the lungs 32 days after infection. CN2992FS *aroA* did not efficiently colonize the mouse lung. The numbers of CN2992FS *aroA* bacteria present decreased daily after aerosol presentation until bacteria were cleared from the lungs between days 5 and 8 (Fig. 2). The *aroA* mutants are clearly highly attenuated for colonization of the lungs relative to the wild-type parent strains.

Vaccination of mice with *B. pertussis aroA*. To determine whether *B. pertussis aroA* strains could function as effective live respiratory vaccines, mice were immunized with CN2992FS *aroA* by exposure to a bacterial aerosol. The immunized mice and age-matched controls were then challenged 3 to 4 weeks later (when the mice were approximately 7 to 8 weeks old) with a nonlethal dose of the parental wild-type strain. Viable counts were performed on the lungs to determine whether immunization affected colonization or clearance of the wild-type strain. A single dose of 10^3 to 10^4 CFU of live CN2992F *aroA* affected the course of infection with wild-type CN2992FS in the lungs of immunized mice compared with that in controls, but the difference was not pronounced. The counts were lower in the lungs of immunized mice, and the organism was cleared faster (data not

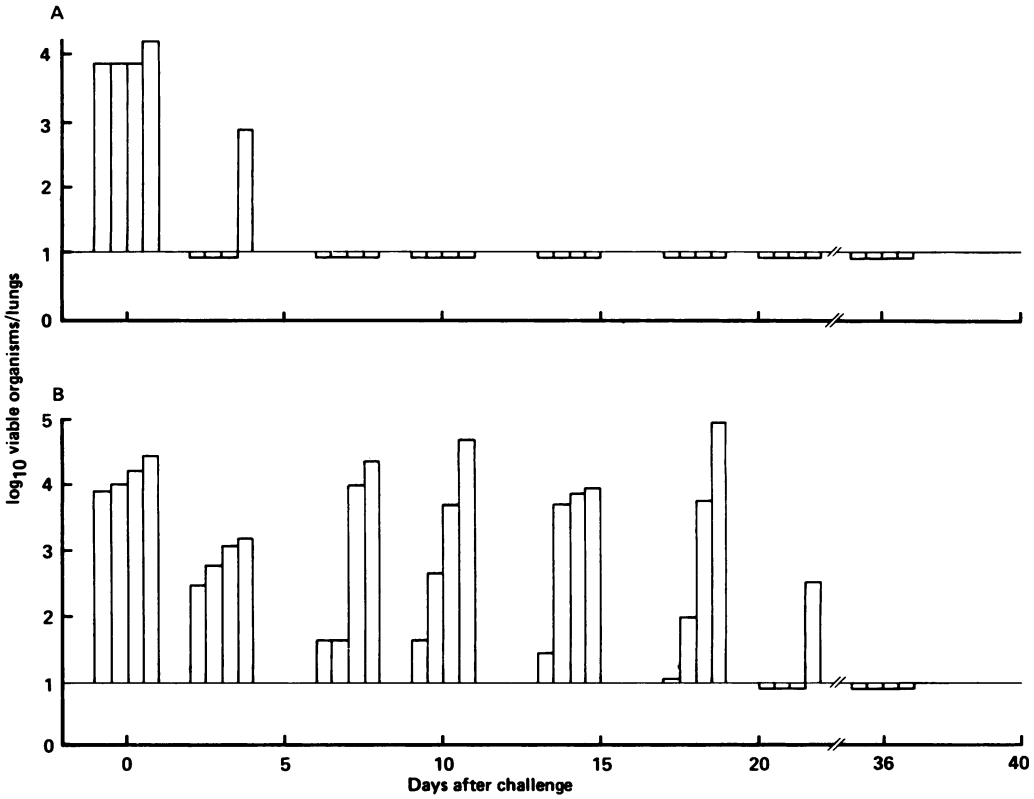

FIG. 3. Effect of immunization of NIH/S mice with CN2992FS *aroA* on lung colonization by CN2992FS. Mice were given three doses of CN2992FS *aroA* by aerosol (see text for details). Immunized (A) and control (B) mice were challenged with CN2992FS. Each bar represents the lung counts for an individual mouse. The horizontal line represents the level of detection of bacteria in our system; bars below this line indicate that there were less than 10 organisms in the lungs of the mice.

shown). To enhance the protection observed, mice were given multiple doses of the attenuated strain. Mice were immunized three times with intervals of 3 days between the first and second doses and 4 days between the second and third doses. The means of the doses received, calculated by using groups of four mice, were 2.6×10^3 CFU in the first dose, 8.6×10^3 CFU in the second dose, and 1.2×10^4 CFU in the third dose. The CN2992FS *aroA* strain was not detected in the lungs of mice 11 days after the final inoculation. The immunized and control mice were challenged with CN2992FS 24 days after the third dose was administered.

The immunized mice cleared the challenge strain rapidly. At 3 days postchallenge, only one of the four mice examined had *B. pertussis* present in its lungs, and all subsequent mice examined were free from *B. pertussis* (Fig. 3). The control mice remained colonized for at least 21 days after being exposed to CN2992FS. It can also be seen from the control group that CN2992FS did not grow to levels as high as those in younger mice and was cleared earlier than it was in younger mice. Aerosol immunization with three doses of a *B. pertussis aroA* mutant clearly prevents subsequent normal colonization of the lungs of mice by wild-type organisms.

Serum antibody response to *B. pertussis*. Sera were collected from aerosol-immunized and control mice at intervals after respiratory challenge with 2992FS and assayed for *B. pertussis* antibodies by ELISA, using whole *B. pertussis* as antigen. A low level of anti-*B. pertussis* antibodies was detected in the sera of immunized, but not control, mice 10 days before challenge (Fig. 4). Anti-pertussis immunoglobulins appeared rapidly in the sera of immunized mice and reached high levels by the second week after challenge (Fig. 4). The anti-*B. pertussis* antibodies remained high for several weeks and then decreased, returning to low levels by day 46 postchallenge (Fig. 4). The rise in pertussis-specific serum antibodies in immunized mice correlates with the disappearance of the challenge organism from the lungs. In contrast, significant levels of anti-pertussis antibodies were only detected in the sera of control mice 46 days after challenge, which is 25 days after *B. pertussis* was last isolated from their lungs (Fig. 4).

The predominant class of serum anti-*B. pertussis* immunoglobulins, in both vaccinated and control mice, was IgG, but significant rises in pertussis-specific IgA and IgM were detected in both groups of mice.

DISCUSSION

In this report we describe the construction of *B. pertussis* strains with an insertion mutation in the *aroA* gene and the preliminary characterization of the mutants in vivo, using aerosol challenge of mice. Although *B. pertussis* is a fastidious organism, the *aroA* mutants grew well in laboratory medium supplemented with aromix. However, in the mouse challenge systems employed, the *aroA* strains were much less efficient at colonizing the lungs than were the wild-type parental strains. *B. pertussis aroA* strains are therefore attenuated for growth in vivo in our mouse lung colonization

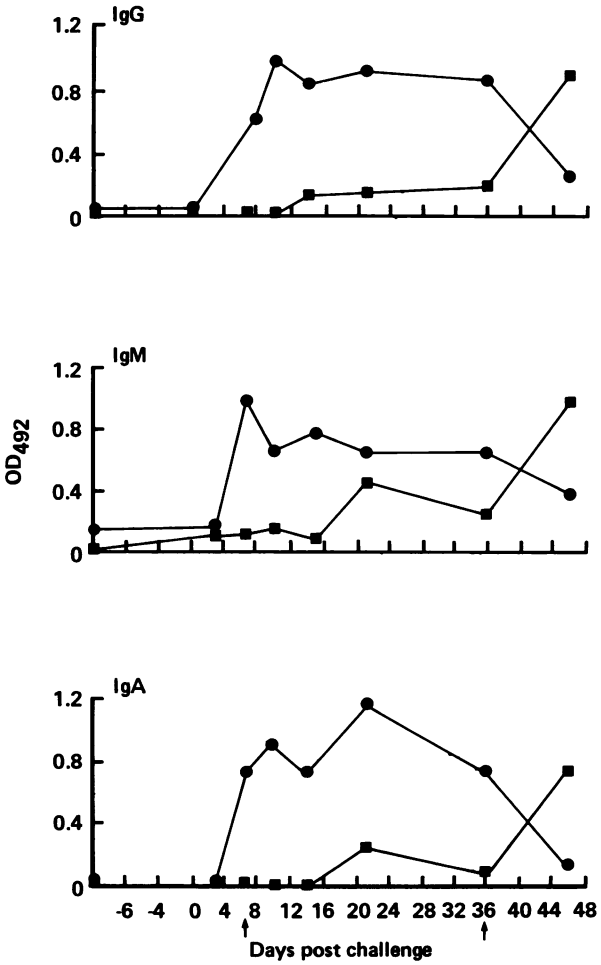

FIG. 4. Serum anti-*B. pertussis* immunoglobulin response of aerosol-immunized (●) and control (■) mice after aerosol challenge. Sera were tested by ELISA, using whole *B. pertussis* as antigen. Each point represents a pool of serum from four sacrificed mice per group. The arrows indicate when *B. pertussis* was no longer detectable in the lungs of immunized (day 7) and control (day 36) mice. The sera were diluted to test for particular immunoglobulin classes as follows: IgG, 1 in 1,250; IgM, 1 in 100; IgA, 1 in 500.

model. Weiss described the isolation of a *B. pertussis* lysine auxotroph after mutagenesis with Tn*5*, but this strain retained virulence in an infant mouse model involving pulmonary infection (A. A. Weiss, Abstr. N.I.A.I.D. Workshop on Pertussis, Hamilton, Mont., 1988). We know of no other work in which defined *B. pertussis* auxotrophs were tested for virulence. Although the *B. pertussis aroA* mutants grew poorly in vivo, they could still produce important virulence factors such as pertussis toxin, adenylate cyclase, FHA, and 69-kilodalton outer membrane protein. Therefore, attenuation is probably due to starvation in vivo for an essential aromatic metabolite rather than indirect effects on the expression of virulence factors. This is supported by the fact that the *B. pertussis aroA* strains described here are protective when used to immunize mice before intracerebral challenge with the virulent *B. pertussis* 18323. Thus, important protective antigens are still expressed by these strains when grown in vitro. We have found some phenotypic differences between wild-type *B. pertussis* strains and *B. pertussis aroA* mutants in addition to those expected for *aroA* mutants. After *B. pertussis aroA* strains grew, the spent medium often contained a brown pigment of unknown composition which is not produced by the wild-type strains. At present, the composition of this pigment is unknown, although it is similar in hue to pigment produced by some strains of *B. parapertussis* grown under similar conditions.

The *B. pertussis aroA* mutants were rapidly cleared from the lungs of infected mice. This contrasts with the behavior of *Salmonella typhimurium aroA* mutants, which can persist for several weeks in the livers and spleens of mice after they are orally infected (13, 19). This could be a reflection of the animal infection systems used or differences in physiological properties between *Salmonella* and *Bordetella* species. *Salmonella* spp. can enter and replicate within macrophages and eucaryotic cells, whereas *B. pertussis* is predominantly an extracellular pathogen (2, 8, 17). The location of the pathogen within the host could obviously have a major effect on the persistence of the infection.

Administration of *B. pertussis aroA* mutants by aerosol to mice reduced the ability of a subsequent exposure to the wild-type strain to establish an infection. Several groups of workers have reported that mice convalescing from a sublethal *B. pertussis* respiratory infection are immune to subsequent infection (1, 5, 25). Only one group attempted to measure circulating antibodies to *B. pertussis* in the sera of immunized mice after challenge (1) and, in contrast to our findings, they could not detect *B. pertussis* antibodies 21 days postchallenge. This discrepancy may have arisen because we used whole *B. pertussis* cells as coating antigen in ELISA, whereas Alonso et al (1) used two purified antigens, PTX and FHA.

Our results suggest that exposure of mice to aerosol infection by the *B. pertussis aroA* strains primes their immune system, since immunized animals show a rapid anti-*B. pertussis* humoral antibody response after exposure to wild-type challenge, unlike unvaccinated control animals. The rise in *B. pertussis* antibodies in the sera of immunized mice correlates with the disappearance of *B. pertussis* from the lungs. Serum antibodies may play a major role in the clearance of *B. pertussis* from the murine lung, because numerous studies have demonstrated that passive or active immunization of mice by the intraperitoneal route is effective in protecting mice from pulmonary infection with *B. pertussis* (1, 6, 24, 29). However, the importance of the local secretory immune system in controlling lung infections should not be overlooked.

The results described above and those of others show that serum anti-*B. pertussis* immunoglobulins only rise to significant levels in mice recovering from sublethal respiratory infection some weeks after the infection has been terminated. This indicates that local factors are responsible for clearing bacteria from the lungs of nonimmune mice. Geller and Pittman (11) detected *B. pertussis*-specific immunoglobulins, predominantly IgA, in the tracheobronchial washings of intranasally infected mice 15 days after infection. The only change in the serum immunoglobulin profile was a change in serum IgA (the specificity of which was not determined) 30 days after the initiation of infection. We are currently investigating the local immune response in the murine lung after administration of *B. pertussis aroA*.

Preliminary studies using convalescent sera of immunized and nonimmunized mice suggest that the antibody response is directed against several polypeptides (our unpublished results). The availability of purified *B. pertussis* antigens should make characterization of humoral and cellular immune responses to *B. pertussis* simpler.

The mouse aerosol challenge system is an unsatisfactory model for human whooping cough. We have never observed mouse-to-mouse lung infection with *B. pertussis*, although this is common with *Bordetella bronchiseptica*, which causes serious infections in a variety of animal species. We have recently constructed *B. bronchiseptica aroA* mutants, using methods similar to those described in this report. These will be evaluated in the appropriate model systems to see whether other *Bordetella* species, better adapted to survival in their hosts, can be attenuated by using *aroA* mutations.

Salmonella aroA mutants have been used to deliver heterologous antigens to the mammalian immune system. The heterologous antigens have been derived from bacteria (3, 19), viruses (37), and parasites (30). After the attenuated *Salmonella* species expressing the heterologous antigen have been delivered orally, both systemic and secretory antibody responses have been detected. The attenuated *B. pertussis aroA* strains described in this report could be used to deliver non-*Bordetella* antigens to the respiratory tract mucosal surface. We are currently investigating this possibility.

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