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Attenuation and Vaccine Potential of *aroQ* Mutants of *Corynebacterium pseudotuberculosis*

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***Corynebacterium pseudotuberculosis*, a gram-positive intracellular bacterial pathogen, is the etiological agent of the disease caseous lymphadenitis (CLA) in both sheep and goats. Attenuated mutants of *C. pseudotuberculosis* have the potential to act as novel live veterinary vaccine vectors. We have cloned and sequenced the *aroB* and *aroQ* genes from *C. pseudotuberculosis* C231. By allelic exchange, *aroQ* mutants of both C231, designated CS100, and a *pld* mutant strain TB521, designated CS200, were constructed. Infection of BALB/c mice indicated that introduction of the *aroQ* mutation into C231 and TB521 attenuated both strains. In sublethally infected BALB/c mice, both CS100 and CS200 were cleared from spleens and livers by day 8 postinfection. The in vivo persistence of these strains was increased when the intact *aroQ* gene was supplied on a plasmid in *trans*. Mice infected with TB521 harbored bacteria in organs at least till day 8 postinfection without ill effect. When used as a vaccine, only the maximum tolerated dose of CS100 had the capacity to protect mice from homologous challenge. Vaccination with TB521 also elicited protective immunity, and this was associated with gamma interferon (IFN-γ) production from splenocytes stimulated 7 days postvaccination. The role of IFN-γ in controlling primary infections with *C. pseudotuberculosis* was examined in mice deficient for the IFN-γ receptor (IFN-γR$^{-/-}$ mice). IFN-γR$^{-/-}$ mice cleared an infection with CS100 but were significantly more susceptible than control littermates to infection with C231 or TB521. These studies support an important role for IFN-γ in control of primary *C. pseudotuberculosis* infections and indicate that *aroQ* mutants remain attenuated even in immunocompromised animals. This is the first report of an *aroQ* mutant of a bacterial pathogen, and the results may have implications for the construction of aromatic mutants of *Mycobacterium tuberculosis* for use as vaccines.**

Corynebacterium pseudotuberculosis, a gram-positive facultative intracellular pathogen, is the etiological agent of caseous lymphadenitis (CLA) in sheep and goats. CLA is a chronic disease typically characterized by necrotizing inflammation of one or more superficial lymph nodes. In sheep, CLA results in reduced wool production and meat losses due to carcass condemnation (31). In Australia, it is one of the most prevalent diseases of sheep, with economic losses in the order of $20 million per year (30). While the pathogenic process employed by *C. pseudotuberculosis* in causing CLA in sheep and goats is not well defined, at least two major virulence determinants have been identified. One of these is the toxic lipid cell wall, which may mediate the bacterium's resistance to killing by phagocytic cells (10). The other identified virulence determinant is a sphingomyelin-degrading phospholipase D (Pld) exotoxin (22). Pld is thought to mediate dissemination of the pathogen within the host by increasing local vascular permeability (3). A role for Pld in the virulence of *C. pseudotuberculosis* was confirmed when an isogenic *pld* mutant was constructed and shown to be unable to cause CLA. Importantly, sheep immunized with a *pld* mutant were protected from subsequent challenge with the wild-type parental strain (13). This Δ*pld* mutant holds promise as a veterinary vaccine vector, since it is capable of eliciting immune responses to coexpressed antigens in vaccinated sheep (14). There is, however, accumulating evidence to suggest that the type of mutation used to attenuate a vaccine vector can have a critical influence on the vector's ability to elicit an immune response to a carried foreign antigen (23). This most probably reflects the altered in vivo growth rate or persistence of the pathogen and coincident altered interaction with the immune system.

Several different bacterial pathogens have been attenuated by stable introduction of mutations in the aromatic amino acid biosynthetic pathway. Aromatic-dependent mutants of the following pathogens have been shown to be attenuated and capable of stimulating protective immunity in different animal models: *Salmonella typhimurium* (17), *Salmonella typhi* (26), *Salmonella cholerasuis* (27) *Shigella flexneri* (43), *Bordetella pertussis* (36), *Pasteurella multocida* (18), *Bacillus anthracis* (20), *Aeromonas salmonicida* (42), *Yersinia enterocolitica* (4), and *Yersinia pestis* (29). The reduced virulence of these bacterial strains is likely to be due to their requirement for *p*-aminobenzoic acid, a precursor of folic acid and a compound which is not synthesized by chordates. Since bacteria are unable to take up exogenous folate and the availability of *p*-aminobenzoic acid is limited in vertebrate tissues, the growth of *aro* mutants in vivo is severely restricted.

Here we report the cloning of the *aroB* and *aroQ* genes from *C. pseudotuberculosis* and their similarity with the corresponding genes from *Mycobacterium tuberculosis*. An *aroQ* mutant of *C. pseudotuberculosis* was constructed by allelic exchange, and experiments were conducted to test its efficacy as a vaccine in a murine model of infection. We believe this to be the first rationally attenuated prechorismate *aro* mutant of a gram-positive bacterium. The construction of an aromatic mutant of

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TABLE 1. Bacterial strains and plasmids used in this present study

Strain or plasmid	Relevant characteristics	Reference or source
C. pseudotuberculosis		
C231	Wild type	13
TB521	*pld* mutant of C231; generated by in vitro site-directed mutagenesis of *pld* sequence encoding Pld active site (His20→Ser20), then allelic exchange with wild-type gene in C231	41 and unpublished data
CS100	C231 *aroQ*::*erm*	This study
CS200	TB521 *aroQ*::*erm*	This study
E. coli		
AB2829	K-12 *aroA* mutant	A. J. Pittard (32)
AB2826	K-12 *aroB* mutant	A. J. Pittard (32)
AB2830	K-12 *aroC* mutant	A. J. Pittard (32)
AB1360	K-12 *aroD* mutant	A. J. Pittard (32)
BRD728	Lambda lysogen	Gift from G. Dougan
JM101	Cloning host	
DH5α	Cloning host	
Plasmids		
pHC79	Cosmid cloning vector	16
pBluescript	Cloning vector	Stratagene, La Jolla, Calif.
pEP-2	*E. coli-C. pseudotuberculosis* shuttle vector	34
pUC4K	Source of *Kan* cassette	Pharmacia, Piscataway, N.J.
pBTB24	Source of *erm* cassette	15
pCS1	*aroB*/*aroD* complementing cosmid	This study
pCS2	3-kb *Bam*HI fragment from pCS1 containing *aroB* and *aroQ* genes	This study
pCS3	3-kb *Bam*HI fragment from pCS2 blunt-end ligated to *Pvu*II-digested pBluescript	This study
pCS4	*kan* gene blunt-end ligated to *Sca*I-digested pCS3	This study
pCS5	*kan* gene blunt-end ligated to *Sca*I-digested pBluescript	This study
pCS6	*erm* gene blunt-end ligated to *Eco*RI-digested pCS4	This study
pCS7	*erm* gene blunt-end ligated to *Pvu*II-digested pCS5	This study
pCS8	3-kb *Bam*HI fragment from pCS2 in pEP-2	This study

C. pseudotuberculosis will facilitate an immunobiological comparison with the previously constructed Δ*pld* mutant with respect to efficacy as a CLA vaccine and also as a vaccine vector.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The properties of the bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were routinely grown in Luria-Bertani (LB) broth or agar supplemented with ampicillin (50 μg/ml), kanamycin (50 μg/ml), or erythromycin (200 μg/ml) when appropriate. M9 minimal medium (37) containing ampicillin and essential nonaromatic amino acids was used to select for cosmid clones which complemented the *E. coli* aromatic mutants. *C. pseudotuberculosis* strains were cultured in brain heart infusion (BHI); (Oxoid, Basingstoke, Hampshire, England) broth or agar for 2 days at 37°C. When appropriate, the medium was supplemented with erythromycin (150 ng/ml) or kanamycin (50 μg/ml).

DNA manipulation and analyses. Plasmid DNA preparation, restriction enzyme digests, DNA hybridization, ligations, and transformations were performed by using standard techniques (37). DNA fragments were purified from agarose gels by using Geneclean (Bio 101 Inc., Vista, Calif.). DNA probes were prepared by nick translation using [α-^{32}P]dATP (Amersham International, Buckinghamshire, England) according to the manufacturer's instructions. Similarly, DNA probes were stripped from Hybond-N (Amersham) membranes according to the manufacturer's instructions. DNA sequencing was performed by the dideoxy-chain termination method, using fluorescein-labeled dideoxynucleotides. The DNA sequence was analyzed with an Applied Biosystems 373A DNA sequencer (Perkin-Elmer, Melbourne, Victoria, Australia). Specific synthetic oligonucleotides and commercial (Promega Corp., Madison, Wis.) universal and reverse oligonucleotides were used in sequencing. C231 genomic DNA for construction of the cosmid library was a kind gift from Catherine Pogson (CSIRO Division of Animal Health). Genomic DNA from *C. pseudotuberculosis* was isolated by the whole-cell lysate technique (44).

Construction of a cosmid genomic library. C231 genomic DNA was partially digested with *Sau*3A and fractionated on a 10 to 40% sucrose gradient. DNA fragments in the 35- to 50-kb range were ligated to the cosmid vector pHC79, digested with *Bam*HI, and dephosphorylated with calf intestinal alkaline phosphatase. Aliquots of the ligation were packaged in vitro into bacteriophage lambda, using a commercial packaging kit (Boehringer GmbH, Mannheim, Germany).

Packaged cosmid clones were transduced into *E. coli* BRD728, which harbors a defective bacteriophage lambda lysogen stably maintained at 30°C. Cells were prepared for transduction according to the method supplied with the packaging kit. The library was amplified essentially as described by Jacobs et al. (21) and stored under chloroform at 4°C.

Complementation analysis. Repackaged recombinant cosmid molecules were transduced into cells of the particular *E. coli* aromatic auxotroph, prepared as described above for bacteriophage lambda infection, at a multiplicity of infection of 0.1. After a 30-min absorption period, 1 ml of LB broth was added and the cells were incubated at 37°C for 45 min to allow expression of antibiotic resistance genes. Cells were then washed twice with saline before being plated onto M9 minimal medium containing ampicillin.

Construction of aromatic mutants by allelic exchange. A suicide plasmid construct which could mediate allelic exchange and the generation of an *aroQ* mutant of *C. pseudotuberculosis* was constructed. The 3-kb *Bam*HI fragment in pCS2 was excised with *Bam*HI, made blunt-ended by using the Klenow fragment of DNA polymerase, and ligated to *Pvu*II-digested pBluescript KS$^+$ to produce pCS3. The *Eco*RI site 324 bp downstream from the putative ATG of the *aroQ* open reading frame was now unique in pCS3. The unique *Sca*I site located in the ampicillin resistance gene in pBluescript was then used. A *Hin*cII fragment containing the kanamycin resistance cassette from pUC4K was blunt-end ligated to *Sca*I-digested pCS3 and also to *Sca*I-digested pBluescript, generating pCS4 and pCS5, respectively. Finally, the *aroQ* gene was insertionally inactivated by blunt-end ligation of an erythromycin resistance cassette from pBTB24 to the blunt-ended (Klenow fragment) unique *Eco*RI site within the *aroQ* open reading frame in pCS4 to generate pCS6. The same erythromycin resistance cassette was also ligated to *Pvu*II-digested pCS5 to generate pCS7. Thus, pCS7 is identical to pCS6 but lacks the 3-kb *Bam*HI fragment harboring the *aroB* and -*Q* genes. pCS7 was used as a control to screen for the frequency of illegitimate recombination of plasmid sequences with the *C. pseudotuberculosis* chromosome. *C. pseudotuberculosis* C231 and pTB521 were electroporated with 5 μg of the appropriate plasmid construct (pCS6 or pCS7). Following electroporation, putative *aroQ* mutants were selected on BHI plates containing 150 ng of erythromycin per ml for 4 days at 37°C. Erythromycin-resistant colonies were presumed to result from a recombination event. Bacteria which had undergone an allelic exchange event whereby the entire plasmid had been integrated (merodiploid) would also be kanamycin resistant. Erythromycin-resistant colonies were patched onto BHI agar containing kanamycin. Erythromycin-resistant, kanamycin-sensitive colonies were subsequently analyzed by Southern hybridization for an allelic replacement event at the *aroQ* locus.

LD_{50} experiments and in vivo growth. BALB/c mice were pedigree bred and maintained in the Department of Microbiology, University of Melbourne, Melbourne, Parkville, Australia. For 50% lethal dose (LD_{50}) experiments, groups of five sex- and age-matched mice were infected intraperitoneally with serial 10-fold dilutions of *C. pseudotuberculosis* in saline. The infecting dose was calculated retrospectively by viable count on BHI agar. Mice were killed by cervical dislocation when moribund. The LD_{50} value was calculated by the method of Reed and Muench (35) at the end of 8 weeks. The kinetics of bacterial growth in vivo was evaluated by sacrificing groups of four mice at regular time intervals postinfection. Spleens and livers were homogenized in a blender (Stomacher 80) Seward Medical, London, England), and the bacterial load was enumerated by viable count on BHI agar.

129/Sv/Ev mice of either sex homozygous for a disrupted gamma interferon receptor (IFN-γR) gene (IFN-$\gamma R^{-/-}$) and for null mutation (IFN-$\gamma R^{+/+}$) were produced as described previously (19) and bred at the John Curtin School of Medical Research, Australian National University, Canberra, Australia.

Immunization and wild-type challenge. Groups of 12 BALB/c mice were immunized intraperitoneally with 10-fold serial doses of CS100, TB521, or heat-killed bacteria. The viable count of the immunizing inoculum was determined retrospectively. Bacteria were heat killed by incubation at 60°C for 30 min. Verification of killing was determined by viable count. All mice, including naive controls, were challenged intraperitoneally 21 days postvaccination with an infectious dose of the wild-type strain. Groups of challenged mice were sacrificed on days 7 and 14 postchallenge, and organs were removed for bacterial culture.

Cytokine induction and IFN-γ ELISA. Spleens from immunized mice were removed aseptically, and single-cell suspensions were prepared by passage through wire sieves. Erythrocytes were removed by treatment with 0.017 M Tris-ammonium chloride, washed twice, and suspended in RPMI (CSL Ltd., Melbourne, Victoria, Australia) containing 10% fetal bovine serum, 2 mM L-glutamine, 1 mM pyruvate, 5×10^{-5} M β-mercaptoethanol, penicillin (100 U/ml), and streptomycin (50 μg/ml). Bulk splenocytes were seeded at 5×10^{6}/ml in 0.5 ml in 48-well tissue culture plates (Costar, Cambridge, Mass.). Cells were stimulated with 5 μg of soluble *C. pseudotuberculosis* cell lysate and incubated for 48 h before supernatant fluids were harvested. Control wells were stimulated with concanavalin A (5 μg/ml; Sigma, St. Louis, Mo.) or medium alone. Supernatant fluids were used in a IFN-γ cytokine enzyme-linked immunosorbent assay (ELISA) (CSL Ltd.) which was performed according to the manufacturer's instructions. The limit of detection in the assay was 2 IU/ml.

Statistics. The mean number of challenge bacteria recovered from immunized mice was compared to the number recovered from unvaccinated mice by using ordinary one-way analysis of variance with Dunett's analysis. The unpaired Student *t* test was used for comparison of bacterial counts from IFN-$\gamma R^{-/-}$ mice with control mice.

Nucleotide sequence accession number. The complete nucleotide sequence of the *C. pseudotuberculosis* C231 *aroB* and *aroQ* genes has been lodged in GenBank under accession no. U88628.

RESULTS

Cloning of genes from the prechorismate aromatic amino acid biosynthetic pathway. Initial attempts to clone the *C. pseudotuberculosis aroA* gene by shotgun cloning 2- to 4-kb *Sau*3A-digested genomic DNA fragments and identification of recombinants which could complement the *aroA E. coli* mutant AB2829 proved unsuccessful. To increase the likelihood of obtaining a representative genomic library, a cosmid library was constructed in the vector pHC79. The library was amplified in the lambda lysogen BRD728 by incubation at 37°C, and the lysate containing a cosmid bearing defective phage particles was used to independently transduce each of the prechorismate *E. coli aro* mutants AB2829, AB2826, AB2830, and AB1360. Complementation of growth on minimal medium was achieved only for the *aroB* and *aroD E. coli* mutants, AB2826 and AB1360, respectively. Restriction enzyme digests of complementing cosmids isolated from both mutants suggested the presence of many shared restriction fragments. Indeed, all analyzed cosmids that complemented AB2826 could also complement AB1360.

One such cosmid (pCS1) was subcloned to a 3-kb *Bam*HI fragment in pBluescript KS^{+}. The resultant construct, pCS2, retained the ability to complement growth of both AB2826 and AB1360. Further subcloning and sequencing of pCS2 identified two open reading frames. BLAST analysis of the DNA and predicted amino acid sequences of the two open reading frames indicated most significant amino acid identity with the *aroB* and *aroQ* gene products from *M. tuberculosis*. These shikimate pathway genes encode the enzymes dehydroquinate synthase and a type II 3-dehydroquinase, respectively. On the basis of sequence identities, these genes from *C. pseudotuberculosis* were designated *aroB* and *aroQ*. The deduced amino acid sequences of the *aroB* and *aroQ* genes from *C. pseudotuberculosis* were aligned with the amino acid sequences of other *aroB* and *aroQ* genes by using the CLUSTAL multiple alignment program (Australian Genomic Information Service). The deduced amino acid sequence of the *aroB* gene displayed significant identity with *aroB*-encoded enzymes from other bacterial species (Fig. 1A). The deduced amino acid sequence of the *aroQ* gene, while displaying significant identity with the *M. tuberculosis aroQ* gene product, also displayed identity with catabolic enzymes involved in quinic acid catabolism in fungi (Fig. 1B).

Construction of *C. pseudotuberculosis aroQ*::*erm*. The observation that ColE1-based plasmids such as pBluescript KS^{+} cannot replicate in *C. pseudotuberculosis* facilitated the construction of a suicide targeting vector designed to mutate the *aroQ* gene. The construction of pCS6 (targeting vector) and pCS7 (control plasmid) is outlined in the Materials and Methods. Importantly, pCS6 could not complement the growth of *aroD E. coli* mutant AB1360 on minimal media. This result suggested that the product of the cloned *aroQ* gene was now no longer functional. Electroporation of pCS6, but not the control plasmid pCS7, into *C. pseudotuberculosis* C231 and TB521 yielded erythromycin-resistant colonies of various sizes after 4 days of incubation at 37°C. These colonies were presumed to have resulted from a recombination event. Erythromycin-resistant colonies were then patched onto BHI agar containing kanamycin. Of the erythromycin-resistant colonies generated, approximately 5% were kanamycin sensitive.

Unlike the situation with many gram-negative pathogens, the lack of a defined minimal medium for culture of *C. pseudotuberculosis* prevented screening putative *aroQ* mutants for aromatic amino acid auxotrophy. As a means of verifying allelic exchange, chromosomal DNAs from putative mutants and the wild-type strain were analyzed by Southern hybridization with probes specific for *aroB* and -*Q* and the erythromycin resistance gene. The strategy used to construct and analyze these mutants is represented schematically in Fig. 2A.

As predicted, a 3.8-kb *Bgl*II fragment in representative *aroQ* mutants of C231 and TB521 hybridized to the 2-kb *Bgl*II *aroB*,*Q*-specific probe (Fig. 2B). The gel shift of 1.8 kb in the mutants relative to the band in the wild-type parental strain corresponds to the size of the erythromycin resistance cassette inserted into the *aroQ* gene. After stripping of the *aroB*,*Q*-specific probe, an identically sized 3.8-kb *Bgl*II fragment in DNA extracted from the putative mutants hybridized to an erythromycin resistance gene-specific probe (Fig. 2C). With this probe, there was no hybridization to DNA extracted from the wild-type strain. Labeled pBluescript DNA did not hybridize to DNA from either the wild-type strain or the representative mutants (data not shown). The results indicate legitimate allelic exchange at the *aroB* and -*Q* loci, resulting in the construction of an *aroQ* mutant of C231, designated CS100, and also of TB521, designated CS200.

In vitro growth characteristics. Initial observations on the growth characteristics of CS100 and CS200 suggested that they grew more slowly in vitro than their parental counterparts. Importantly, however, the growth rate could be restored by providing the cloned *aroQ* gene on a plasmid in *trans* (Fig. 3). These results suggested that the reduced growth rate displayed by the *aroQ* mutants was not a pleiotropic effect caused by introduction of the *aroQ* mutation but rather more likely re-

A

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C. pseudo.       ------MQTIEVNGASPYEVTIGHNLFKDVAKSMSQLG--ANQAAIITQPVMG-ETAKKL
M. tb.           MTDIGAPVTVQVAVDPPYPVVIGTGLLDELEDLLADR----HKVAVVHQPGLA-ETAEEI
B. subt.         -----MKTLHVQTASSSYPVFIGQGIRKKACELLTSLNRPLTRIMFVTDEEVDRLYGDEM
E coli           -----MERIVVTLGERSYPITIASGLFNEPASFLPLKS--GEQVMLVTNETLAPLYLDKV
                                  * . *  ..       .        .   .    .       .

C. pseudo.       VGAIEALGKEATIITVPDAEDGKNLNVAGDCWDVLGRKAFGRKDVIISLGGGAVTDLAGF
M. tb.           RKRLAGKGVDAHRIEIPDAEAGKDLPVVGFIWEVLGRIGIGRKNALVSLGGGAATDVAGF
B. subt.         LHLLQEK-WPVKKVTVPSGEQAKSMDMYTKLQSEAIRFHMDRSSCIIAFGGGVVGDLAGF
E coli.          RGVLEQAGVNVDSVILPDGEQYKSLAVLDTVFTALLQKPHGRDTTLVALGGGVVGDLTGF
                    .         .. *  *  * . .         .    *   ... ***   *..**

C. pseudo.       VAACWMRGIAVIHVPTTLLSMVDAAVGGKTGINTSAGKNLVGAFHEPSGVFIDLDMIATL
M. tb            AAATWLRGVSIVHLPTTLLGMVDAAVGGKTGINTDAGKNLVGAFHQPLAVLVDLATLQTL
B. subt          VAATFMRGIDFIQMPTTLLAH-DSAVGGKVAVNHPLGKNLIGAFYQPKAVLYDTDFLRSL
E coli           AAASYQRGVRFIQVPTTLLSQVDSSVGGKTAVNHPLGKNMIGAFYQPASVVVDLDCLKTL
                  **.  **.  ...*****   *..****  .*   ***..*** .*  *  *   . .*

C. pseudo.       PDREKISGSAEIIKTGFIADTKILSLYEEDPEACFN--GRILAELIGRSVAAKARVVASD
M. tb            PRDEMICGMAEVVKAGFIADPVILDLIEADPQAALDPAGDVLPELIRRAITVKAEVVAAD
B. subt          PEKELRSGMAEVIKHAFIYDRAFLEEL-LNIHSLRDITNDQLNDMIFKGISIKASVVQQD
E coli           PPRELASGLAEVIKYGIILDGAFFNWLEENLDALLRLDGPAMAYCIRRCCELKAEVVAAD
                 *  *  .* **..*   * *            .     .  .   * .    ** **  *

C. pseudo.       LREAGQREILNYGHTFGHAVELKEKY-EWRHGNAVSVGMMFVAALARNRGLITDELYLRH
M. tb            EKESELREILNYGHTLGHAIERRERY-RWRHGAAVSVGLVFAAELARLAGRLDDATAQRH
B. subt          EKEEGIRAYLNFGHTLGHAVEAEYGYGQITHGDAVALGMQFALYISEKT-VGCEMDRKRL
E coli           ERETGLRALLNLGHTFGHAIEAEMGYGNWLHGEAVAAGMVMAARTSERLGQFSSAETQRI
                  .*   *  ** *** ***.*    *    ** **. *.      .            *

C. pseudo.       KNILSSVGLPTTYP-EGHFAELYQAMLRDKKNRDDRIRFVALIGAGKTIRIEDA--DRAE
M. tb            RTILSSLGLPVSYD-PDALPQLLEIMAGDKKTRAGVLRFVVLDGLAKPGRMVGP--DPGL
B. subt          VSWLKSLGYPSQIRKETETSVLLNRMMNDKKTRGGKIQFIVLNELGKVADHTFSRNELES
E coli           ITLLKRAGLPVNGPREMSAQAYLPHMLRDKKVLAGEMRLIILPLAIGKSEVRSGV--SHEL
                    *   * *               *  ***     .. .      *

C. pseudo.       LIAAYETLNKGGV
M. tb            LVTAYAGVCAP--
B. subt          WLNKWRLEETS--
E coli           VLNAIADCQSA--
                  .
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B

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C.pseudo.        ----MNILVLNGPNLDRLGKRQPEIYGRTTLADVEKLLVKRADALGVTVIVKQSNYEGEL
M.tubercul.      -MSELIVNVINGPNLGRLGRREPAVYGGTTHDELVALIEREAAELGLKAVVRQSDSEAQL
A.nidulans.      --MEKSILLINGPNLNLLGTREPHIYGSTTLSDVEESSKGHAASLGASLQTFQSNHEGAI
N.crassa.        MASPRHILLINGPNLNLLGTREPQIYGSTTLHDIEQASQTLASSLGLRLTTFQSNHEGAI
                       . ..*****  ** *.* .** **  ..       *  **      **  *  .

C.pseudo.        IDWVHEAADAG-------------------------WPVIINPGGLTHTSVSLRDALAEI
M.tubercul.      LDWIHQAADAA-------------------------EPVILNAGGLTHTSVALRDACAEL
A.nidulans.      VDRIHAARGNT-------------------------DAIIINPGAYTHTSVAIRDALLGV
N.crassa.        IDRIHQAAGFVPSPPSPSPSSAATTTEAGLGPGDKVSAIIINPGAYTHTSIGIRDALLGT
                 .* .* *                               .*.* *  ****. .***

C.pseudo.        HDGAAFVEVHISNIHAREEFRHHSFLSPIARGVIAGLGVMGYELALEYLVLNSHSZ----
M.tubercul.      S--APLIEVHISNVHAREEFRRHSILSPIATGVIVGLGIQGYLLALRYLAEHVGT-----
A.nidulans.      E--IPFIELHVSNVHAREPFRHHSYFSDKASGIIVGLGVYGYKVAVEHVALNFKPLEKKA
N.crassa.        G--IPFVEVHVSNVHAREAFRHHSYLSDKAVAVICGLGPFGYSAALDFLGRHMKF-----
                       .*.*.**.**** **.**  *  *  .* ***  **  *.  .  .

C.pseudo.        --
M.tubercul.      --
A.nidulans.      AL
N.crassa.        --
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FIG. 1. Amino acid comparisons of *aroB* and *aroQ* gene products. (A) Comparison of deduced amino acid sequences of *aroB*-encoded dehydroquinate synthase enzymes from *C. pseudotuberculosis* (*C. pseudo.), M. tuberculosis (M. tb.), E. coli*, and *B. subtilis* (B. subt.). (B) Comparison of deduced amino acid sequences of *aroQ*-encoded 3-dehydroquinase enzymes from *C. pseudotuberculosis* and *M. tuberculosis* (M. tubercul.) in optimal alignment with the catabolic 3-dehydroquinases from *Aspergillus nidulans* and *Neurospora crassa*. The alignment was made by using the CLUSTAL DNA alignment program made available through the Australian Genomic Information Service. In each alignment, residues that are identical between species are indicated by asterisks. Conservative substitutions are indicated by dots.

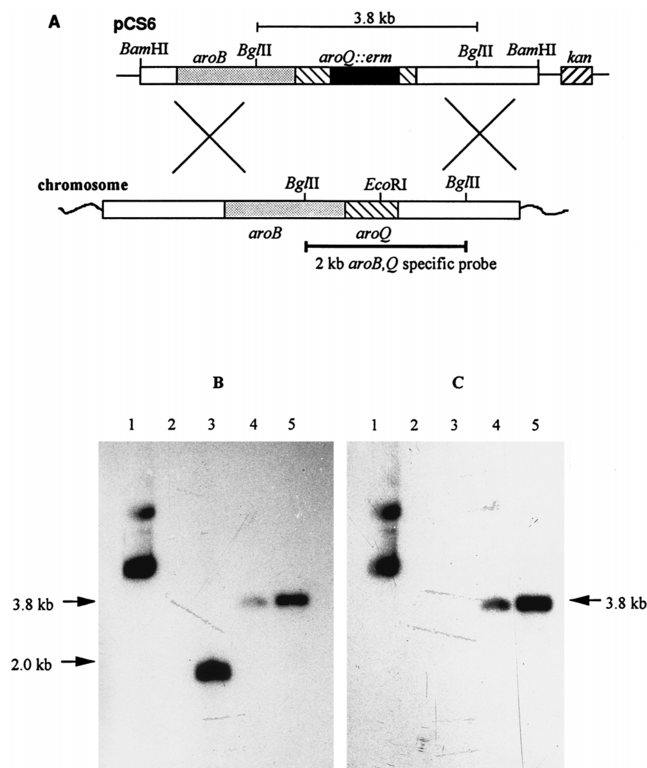

FIG. 2. The construction of *aroQ* mutants of *C. pseudotuberculosis*. (A) Schematic diagram illustrating the strategy used to generate *aroQ* mutants and derive probes for screening recombinants. (B and C) Results of Southern hybridization of chromosomal DNA extracted from C231, CS100, and CS200, digested with *Bgl*II, and probed with (B) an α-^{32}P-labeled 2-kb *Bgl*II *aroB,Q*-specific gene probe (B) and an α-^{32}P-labeled erythromycin resistance gene. Lanes: 1, uncut pCS6; 2, empty lane; 3, C231; 4, CS100; 5, CS200.

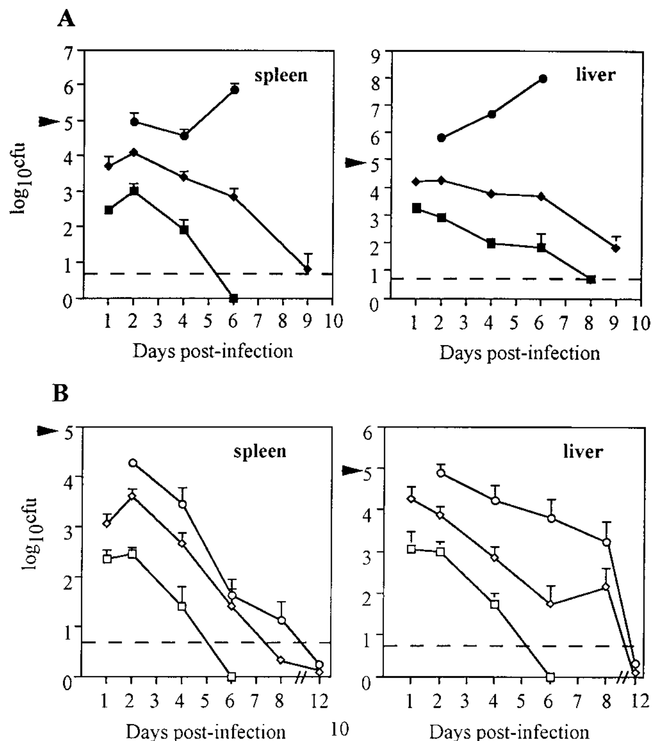

FIG. 4. *aroQ* mutants fail to persist in vivo. (A) In vivo growth of C231 (●), CS100 (■), and CS100(pCS8) (◆) in spleens and livers of BALB/c mice after intraperitoneal injection of 10^5 bacteria of each strain. (B) In vivo growth of TB521 (○), CS200 (□), and CS200(pCS8) (◇) in spleens and livers of BALB/c mice after intraperitoneal injection of 10^5 bacteria of each strain. In all cases, each point represents the mean of four mice plus the standard deviation. The detection limit (dashed line) was 5 CFU/organ.

flects the inability of *C. pseudotuberculosis* to scavenge one or more key aromatic metabolites from the growth medium.

aroQ **mutants of** ***C. pseudotuberculosis*** **are attenuated in a mouse model of infection.** Introduction of the *aroQ* mutation into *C. pseudotuberculosis* C231 increased the LD_{50} for BALB/c mice by ~3 logs (increase in the $\log_{10} LD_{50}$ value from 2.5 to 5.3). In contrast, introduction of the *aroQ* mutation into

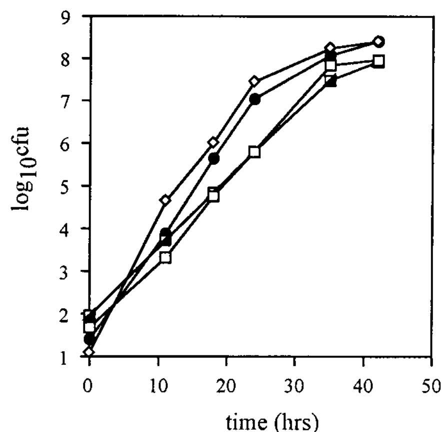

FIG. 3. In vitro growth rates of C231 (●), CS100 (□), CS100(pEP-2) (), and CS100(pCS8) (◇) in BHI broth. The results are representative of two separate experiments.

TB521 attenuated this strain by only a further log relative to the attenuation already caused by mutation of *pld* (increase in the $\log_{10} LD_{50}$ value from 5.3 to 6.3). Mice which succumbed to infection with high doses of either *aroQ* mutant died within 24 to 48 h. However, death appeared to be associated with direct toxicity and not bacteremia, since, as determined by bacterial culture of organs, there was no significant expansion of bacterial numbers in vivo. These experiments identified the maximum tolerated dose for BALB/c mice as ~10^6 CFU.

Persistence of ***aro*** **mutants and parental counterparts in vivo.** To better understand the basis for the observed attenuation, we examined the degree of in vivo persistence of CS100, CS200, and their parental strains in sublethally infected mice (Fig. 4A and B). The results demonstrated that introduction of an *aroQ* mutation severely restricted the in vivo growth of the mutants compared with their parental counterparts. Bacteria harboring an *aroQ* mutation, regardless of parental background, could not be cultured from spleens and livers of infected mice beyond 8 days postinfection. In contrast, mice infected with wild-type strain C231 harbored a significant bacterial burden in spleens and livers and were moribund by day 6 postinfection (Fig. 4A). Mice infected with TB521 harbored bacteria in spleens and livers, albeit in reduced numbers, until at least day 8 postinfection (Fig. 4B). These mice did not display any clinical symptoms. When the *aroQ* mutants were complemented in *trans* with an intact *aroQ* gene, the in vivo persistence of both CS100 and CS200 was increased but remained less than that of either parental strain.

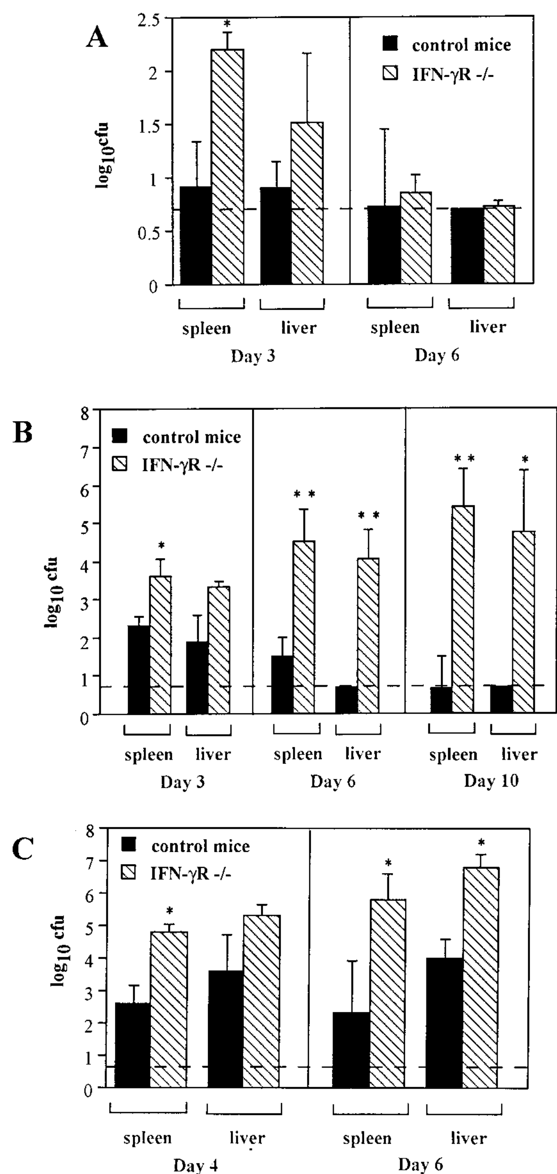

FIG. 5. In vivo growth of CS100 (A), TB521 (B), and C231 (C) in IFN-γR$^{-/-}$ and homozygous control mice. Mice were intraperitoneally infected with 5×10^4 CFU of CS100, 10^4 CFU of TB521, and 10^4 CFU of C231. Each bar represents the mean organ count from four mice plus the standard deviation. The detection limit (dashed line) was 5 CFU/organ. * denotes $P < 0.01$, and ** denotes $P < 0.001$, (Student's *t* test) compared to homozygous control mice.

***aroQ* mutants are attenuated in IFN-γR$^{-/-}$ mice.** A requirement of live attenuated vaccines is that they should be safe even when used in immunocompromised hosts. In relation to this, the importance of the cytokine IFN-γ in controlling growth of both intracellular bacteria and viruses is well documented. We have used gene knockout mice which lack the receptor for IFN-γ to determine whether this cytokine has a role in mediating clearance of *C. pseudotuberculosis* in vivo. At the infectious dose given, the overall kinetics of infection with CS100 in IFN-γR$^{-/-}$ mice was not greatly different from that in homozygous control mice (Fig. 5A). This result suggests that IFN-γ plays only a minor role in mediating clearance of an aromatic mutant of *C. pseudotuberculosis.*

In direct contrast, IFN-γR$^{-/-}$ mice infected with either the *pld* mutant TB521 (Fig. 5B) or the wild-type strain, C231 (Fig. 5C) were highly susceptible to infection compared to homozygous control mice. As a control for the phenotype of these mice, animals were also infected with an *S. typhimurium* Δ*aroA* Δ*aroD* mutant (BRD509); in accordance with published results (12), IFN-γR$^{-/-}$ mice were more susceptible to infection than control mice (data not shown).

Protection against *C. pseudotuberculosis* wild-type challenge. Groups of six BALB/c mice were immunized intraperitoneally with graded sublethal doses of either CS100, TB521, or heat-killed CS100. All mice, including naive controls, were subsequently challenged 21 days postvaccination with an infectious dose (5×10^3 CFU) of the wild-type strain. Groups of challenged mice were sacrificed on days 7 and 14 postchallenge, and organs were removed for bacterial culture of the wild-type strain (Fig. 6). Protection was assessed by comparison of the bacterial load in naive mice to that in immunized mice. At 14 days postchallenge, the absence of challenge bacteria in the organs of mice immunized with 5×10^5 or 5×10^4 CFU of TB521 indicated the development of protective immunity in all of these animals. Similarly, mice immunized with the maximum tolerated dose of CS100 (8×10^5 CFU) harbored significantly fewer challenge bacteria at both days 7 and 14 postchallenge compared to naive animals. In contrast, mice which received 8×10^4 CFU of CS100, 1×10^6 CFU of heat-killed bacteria, or no vaccine were not protected from challenge and harbored significant bacterial burdens in both the spleen and liver at days 7 and 14 postinfection.

Induction of IFN-γ by vaccine strains. At day 7 postvaccination, IFN-γ could be detected in supernatant fluids of stimulated splenocytes derived from mice vaccinated with 8×10^5 CFU of CS100 or either dose of TB521. Thus, there was a qualitative correlation between detectable IFN-γ production

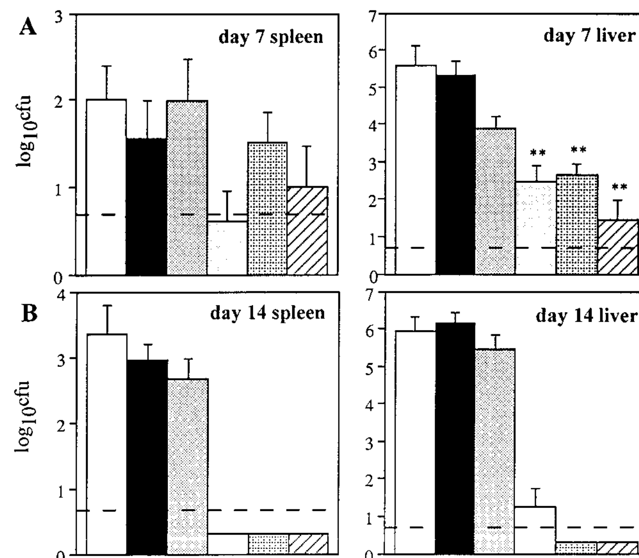

FIG. 6. Capacity of attenuated mutants of *C. pseudotuberculosis* to protect mice from a lethal homologous challenge. Groups of six mice were not vaccinated (▭), or vaccinated intraperitoneally at day 0 with 10^6 heat-killed CS100 (▬), 8×10^4 CFU of CS100 (▭), 8×10^5 CFU of CS100 (▭), 5×10^4 CFU of TB521 (▭), or 5×10^5 CFU of TB521 (▨). by the same route at day 21 with 5×10^3 CFU of the wild-type strain. Groups of mice were sacrificed, and organs were removed for bacterial culture of the challenge strain on day 7 (A) and day 14 (B) postchallenge. Each bar represents the mean organ count from spleens and livers of six mice plus the standard deviation. The detection limit (dashed line) was 5 CFU/organ. ** denotes $P < 0.01$ (analysis of variance) compared to unvaccinated mice.

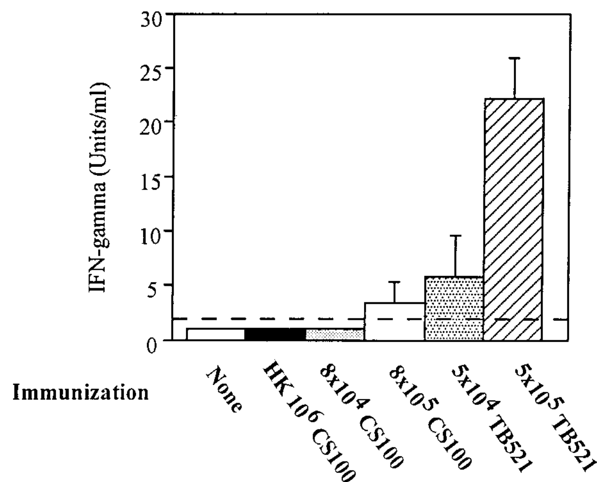

FIG. 7. IFN-γ detection in supernatant fluids of cultured mouse splenocytes isolated and pooled from vaccinated mice. Splenocytes were prepared 7 days postvaccination and stimulated with 5 μg of *C. pseudotuberculosis* soluble antigen. The detection limit of the cytokine ELISA was 2 IU/ml (dashed line).

by antigen-stimulated splenocytes derived from vaccinated mice and subsequent protection from challenge (Fig. 7). IFN-γ was not detected in supernatant fluids from stimulated splenocytes at day 14 postvaccination.

DISCUSSION

In this report, we describe the construction of *C. pseudotuberculosis* strains having insertion mutations in the *aroQ* gene and subsequent preliminary in vivo characterization. While *aro* mutants of other gram-positive bacteria have been constructed by transposon mutagenesis (1, 20), this is, to our knowledge, the first report of a rationally attenuated *aro* mutant of a gram-positive bacterial pathogen generated through allelic exchange.

Historically, *aro* genes from pathogenic bacteria have been cloned by complementation of growth of defined *E. coli aro* mutants on minimal media. This complementation approach has been highly successful in obtaining genes encoding aromatic biosynthetic enzymes from gram-negative bacterial pathogens. Using this same approach, we were able to identify cosmids of *C. pseudotuberculosis* DNA which complemented *aroB* and *aroD E. coli* mutants but not cosmids which complemented *aroA* or *aroC* mutants. This may reflect the potentially nonrepresentative nature of the cosmid library. Alternatively, it may reflect a significant degree of divergence between the *E. coli aroA*- and *aroC*-encoded enzymes and the aromatic pathway enzymes from *C. pseudotuberculosis.*

The sequential genetic arrangement of *aroB* and *aroQ* in *C. pseudotuberculosis*, as established in this study, corresponds to the order in which homologous genes are found in the taxonomically related *M. tuberculosis* (7). Moreover, the significant identity at the deduced amino acid level between the products of the *C. pseudotuberculosis* housekeeping genes *aroB*, *aroQ*, and *recA* (33), the *groEL* and *dnaK* gene products (unpublished data), and the corresponding gene products from *M. tuberculosis* confirms their relatedness at the molecular level.

Despite the amino acid sequence relatedness of the *C. pseudotuberculosis aroQ*-encoded 3-dehydroquinase enzyme to fungal catabolic enzymes from *Neurospora crassa* (8) and *Aspergillus nidulans* (11), there are several lines of reasoning in support of its involvement in the *C. pseudotuberculosis* aromatic amino acid biosynthetic pathway. First, of the analyzed recombinant cosmids and subclones capable of complementing growth of the *aroD E. coli* mutant on minimal media, virtually all displayed identical restriction enzyme digest patterns, suggesting that there is only one gene encoding a 3-dehydroquinase enzyme in *C. pseudotuberculosis*. Second, the close genetic linkage of the *aroQ* gene to the *aroB* gene, which, in *E. coli*, encodes an enzyme catalyzing the preceding step of the aromatic biosynthetic pathway, similarly suggests a biosynthetic function for the *aroQ* gene product. Third, there is evidence that *aroQ* homologs from other procaryotes, including the closely related *M. tuberculosis*, do not encode enzymes with catabolic activity (7, 25).

Random recombination of transformed DNA, as occurs in slow-growing mycobacteria, does not appear to be an impediment to the construction of mutants of *C. pseudotuberculosis* by allelic exchange. The construction of *aroQ* mutants of *C. pseudotuberculosis* was, however, problematic, since such mutants unexpectedly displayed a reduced growth rate in vitro on complex media. Importantly, however, the in vitro growth rate could be restored to wild-type levels by introduction of the intact *aroQ* gene in trans. The *aroQ* mutants generated in this study were significantly attenuated in a murine model of infection. *C. pseudotuberculosis aroQ* mutants CS100 and CS200 were cleared from livers and spleens of intraperitoneally infected mice by day 8 postinfection. While complementation in *trans* with an intact *aroQ* gene increased their in vivo persistence, it did not fully restore virulence to wild-type levels. This may, in part, have been due to plasmid segregation in vivo. Mice infected with a lethal dose of either *aroQ* mutant died within 48 h; there was not a significant expansion of bacterial numbers in either the spleen or liver, which suggested that toxicity was the cause of death.

The in vivo growth kinetics of *C. pseudotuberculosis aroQ* mutants in BALB/C mice contrasts with the behavior of *S. typhimurium aroA* mutants, which can persist in vivo for several weeks after intravenous infection (28). However, it would appear that *S. typhimurium aroA* mutants are exceptional with respect to their in vivo persistence, since *aroA* mutants of *B. pertussis* (36), *A. salmonicida* (42), *P. multocida* (18), and *Y. enterocolitica* (4) are also rapidly cleared from major organs of experimentally infected animals. Indeed, the capacity of aromatic mutants of *S. typhimurium* to kill IFN-$\gamma R^{-/-}$ mice suggests that aromatic metabolites are not limiting in vivo and that bacterial clearance is at least partially dependent on the host's immune response. That *aroQ* mutants of *C. pseudotuberculosis* were attenuated in IFN-$\gamma R^{-/-}$ mice suggests, not surprisingly, fundamental differences in bacterial physiology and pathogenesis between *S. typhimurium* and *C. pseudotuberculosis.*

Given the relatedness of *C. pseudotuberculosis* to *M. tuberculosis*, it is interesting to speculate on the likely phenotype of *aro* mutants of *M. tuberculosis*. The prechorismate genes *aroA*, *aroB*, and *aroQ* have been cloned from *M. tuberculosis* and represent suitable targets for mutagenesis. In light of our findings, we predict that should rational *aro* mutants of *M. tuberculosis* be constructed, they will be highly attenuated. By using cosmid DNA to mediate site-specific allelic exchange, the construction of defined mutants of *M. tuberculosis* strains now appears possible (2).

The immune mechanisms mediating clearance of CS100 in vivo remain undefined. While IFN-$\gamma R^{-/-}$ mice have the capacity to mount a Th1-type T-cell response, macrophage activation is largely abrogated (19, 38, 39). Since IFN-$\gamma R^{-/-}$ mice adequately controlled infections with CS100, bacterial clearance of CS100 may not be critically dependent on IFN-γ-activated macrophages. The absence of activated macrophages

does not, however, preclude the induction of acquired immunity via the two primary sources of IFN-γ, T lymphocytes and NK cells. Importantly, from a vaccine vector point of view, the results suggest that infection with CS100 is largely self-limiting, even in immunocompromised animals. On the other hand, IFN-γR$^{-/-}$ mice were highly susceptible to primary infections with either *C. pseudotuberculosis* C231 or the *pld* mutant TB521 compared to control mice. Thus, control of these primary *C. pseudotuberculosis* infections, as with infections caused by other intracellular bacterial pathogens, is significantly dependent on the bacteriocidal capacity of activated macrophages to contain and destroy the pathogen. Concordantly, Hard (9) has shown a role for T lymphocytes and activated macrophages in suppression of *C. pseudotuberculosis* growth and lesion development in a murine model.

Despite the lower level of in vivo persistence of CS100 than of TB521, this strain retained the capacity to elicit a protective immune response in BALB/c mice. The induction of protective immunity was dose dependent, however, since mice immunized with 8×10^4 CFU of CS100 were not protected from challenge. In these experiments, the induction of protective immunity by TB521 was not dose dependent, since mice immunized with either 5×10^5 or 5×10^4 CFU of TB521 were completely protected. Thus, we hypothesize that the enhanced capacity of TB521 to elicit a protective immune response can be attributed to its greater in vivo persistence. As a consequence, this strain may have an increased capacity to stimulate IFN-γ-producing T cells and/or NK cells. Indeed, appropriate T-cell stimulation is considered an essential requirement for acquired resistance against most intracellular pathogens (24). The observation that mice immunized with 10^6 heat-killed *C. pseudotuberculosis* were not protected from challenge supports the contention that a live vaccine is better able to stimulate the appropriate protective immune response. Indeed, with one exception (40), the use of killed bacterial preparations as vaccines against intracellular bacterial pathogens has historically been relatively unsuccessful (5, 6).

The role of the humoral immune response in mediating acquired resistance to *C. pseudotuberculosis* challenge remains incompletely defined. At the time of challenge, antibodies to *C. pseudotuberculosis* whole-cell lysate could not be detected in any CS100-vaccinated mice (data not shown), despite the immune status of these mice. This finding suggests that the presence of pathogen-specific circulating antibodies is not essential for protection against *C. pseudotuberculosis* challenge in a mouse model.

The use of live, rationally attenuated bacterial pathogens as vaccines, and as vaccine vectors, represents an attractive means of relatively safe, cheap, long-lasting, and efficacious vaccination, particularly within a veterinary context. We envisage that an *aroQ* mutant of *C. pseudotuberculosis*, as described here, represents a potential live vaccine and/or vaccine vector in sheep, since significantly more bacteria (up to 10^{10}) can be administered than in mice. Studies addressing these issues are currently in progress.

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