Identification of Novel Virulence Determinants in *Mycobacterium paratuberculosis* by Screening a Library of Insertional Mutants†

Sung Jae Shin,¹ Chia-wei Wu,¹ Howard Steinberg,² and Adel M. Talaat^{1*}

Departments of Animal Health and Biomedical Sciences¹ and Pathobiological Sciences,²
University of Wisconsin—Madison, 1656 Linden Drive, Madison, Wisconsin 53706-1581

Received 27 October 2005/Returned for modification 21 February 2006/Accepted 6 April 2006

Johne's disease, caused by *Mycobacterium paratuberculosis* infection, is a worldwide problem for the dairy industry and has a possible involvement in Crohn's disease in humans. To identify virulence determinants of this economically important pathogen, a library of 5,060 transposon mutants was constructed using Tn5367 insertion mutagenesis, followed by large-scale sequencing to identify disrupted genes. In this report, 1,150 mutants were analyzed and 970 unique insertion sites were identified. Sequence analysis of the disrupted genes indicated that the insertion of Tn5367 was more prevalent in genomic regions with G+C content (50.5 to 60.5%) lower than the average G+C content (69.3%) of the rest of the genome. Phenotypic screening of the library identified disruptions of genes involved in iron, tryptophan, or mycolic acid metabolic pathways that displayed unique growth characteristics. Bioinformatic analysis of disrupted genes identified a list of potential virulence determinants for further testing with animals. Mouse infection studies showed a significant decrease in tissue colonization by mutants with a disruption in the *gcpE*, *pstA*, *kdpC*, *papA2*, *impA*, *umaA1*, or *fabG2_2* gene. Attenuation phenotypes were tissue specific (e.g., for the *umaA1* mutant) as well as time specific (e.g., for the *impA* mutant), suggesting that those genes may be involved in different virulence mechanisms. The identified potential virulence determinants represent novel functional classes that could be necessary for mycobacterial survival during infection and could provide suitable targets for vaccine and drug development against Johne's and Crohn's diseases.

Mycobacterium paratuberculosis causes Johne's disease (paratuberculosis), characterized by chronic granulomatous enteritis, in dairy cattle, with an estimated loss of \$220 million per year in the United States alone (22). Worldwide, the prevalence of the disease can range from 3 to 4% of herds in regions with low incidence (such as England) (6) to high levels of 50% of herds in some areas within the United States (such as Wisconsin and Alabama) (8, 16). In humans, *M. paratuberculosis* bacilli have been found in examined tissues from Crohn's disease patients, suggesting a possible role for *M. paratuberculosis* in developing Crohn's disease (7). Unfortunately, the mechanisms of virulence that control *M. paratuberculosis* persistence during infection are poorly understood and the key steps for developing paratuberculosis remain elusive. For example, mechanisms responsible for invasion and persistence of *M. paratuberculosis* inside the intestine remain undefined at the molecular level (42). Both live and dead bacilli are observed in subepithelial macrophages after uptake, most likely in M cells covering the ileum (24). Once inside the macrophages, *M. paratuberculosis* survives and proliferates inside the phagosomes by using unknown mechanisms (41). In one report examining intestinal invasion, it was suggested that the 35-kDa membrane protein antigen could play a possible

role in bacillus invasion of the epithelial cells (2). The main goal of our research is to identify virulence factors that contribute to the pathogenicity of *M. paratuberculosis*. This information could identify candidates for vaccine design or chemotherapies that can protect against Johne's and Crohn's diseases.

The complete genome sequence of *M. paratuberculosis* (21) is currently available and will help to rapidly screen *M. paratuberculosis* for novel vaccine and drug targets. The current challenge is to identify those targets that are essential for survival of the bacterium during infection. Recently, random transposon mutagenesis-based protocols have been employed successfully for functional analysis of a large number of genes in *M. tuberculosis* (23, 30, 32) as well as in *M. paratuberculosis* (5, 13). Screening of the *M. tuberculosis* library identified novel genes involved in virulence, such as Rv1290c and *pepD* (23). When *M. paratuberculosis* was used as a target for mutagenesis, the libraries were screened to identify auxotrophs or genes responsible for survival under in vitro conditions (5, 13), which led to the identification of six auxotrophs and two genes responsible for cell wall biosynthesis. However, the libraries were not screened for virulence determinants. We devised a simple approach to screen a mutant library of the virulent strain of *M. paratuberculosis* by identifying the precise insertion sites in disrupted genes. Mutants were selected based on bioinformatic analysis for additional virulence studies in animals. The important advantage of this approach is its simplicity to directly identify disrupted genes and test their contributions to the bacterium survival inside the host. So far, we characterized 1,150 mutants by a junction site sequencing protocol and bioin-

* Corresponding author. Mailing address: Department of Animal Health and Biomedical Sciences, University of Wisconsin—Madison, 1656 Linden Drive, Madison, WI 53706-1581. Phone: (608) 262-2861. Fax: (608) 262-7420. E-mail: atalaat@wisc.edu.

† Supplemental material for this article may be found at <http://iai.asm.org/>.

formatic analysis. Finally, we used the mouse model of paratuberculosis (38, 39) to examine the persistence of 11 mutants in BALB/c tissues compared to the persistence of the wild-type strain of *M. paratuberculosis*. The described approach identified seven putative virulence determinants that could play a role in the pathogenesis of *M. paratuberculosis*. The role of each identified gene in mycobacterial pathogenesis and its impact on a control strategy for Johne's disease are also discussed.

MATERIALS AND METHODS

Animals. BALB/c mice at 3 to 4 weeks of age were purchased from Harlan (Indianapolis, IN) and housed in a pathogen-free environment at the University of Wisconsin—Madison for at least 2 weeks before infection. Food and water were provided ad libitum, and all mice were cared for according to the guidelines of the Institutional Animal Care and Use Committee. Groups of BALB/c mice ($n = 20$) were infected with *M. paratuberculosis* strains by intraperitoneal injection as outlined before (38, 39). For animal infection, *M. paratuberculosis* strains were cultured to mid-log phase (optical density at 600 nm [OD₆₀₀] of 1.0) and centrifuged at 5,000 rpm for 10 min to harvest mycobacterial cells. Bacterial pellets were resuspended in equal volumes of phosphate-buffered saline buffer and used for animal inoculation with a dilution that allowed the delivery of 10⁷ CFU/mouse. An aliquot of the inoculum was plated on Middlebrook 7H10 agar media for colony counting. Infected mice ($n = 6$ to 8) were sacrificed at 3, 6, and 12 weeks postinfection, and their livers, spleens, and intestines were collected for both histological and bacteriological examinations (36). Tissue sections collected for histopathology were preserved in 10% neutral buffered formalin before being embedded in paraffin, cut into 4- to 5- μ m sections, and stained with hematoxylin and eosin or the Ziehl-Neelsen method for acid-fast staining. Two independent pathologists examined tissue sections from infected animals at 3, 6, and 12 weeks postinfection. The severity of the inflammatory response was ranked using a score of 0 to 5 based on lesion size and number per field. Tissues with more than three fields containing multiple and large-sized lesions were given a score of 5.

Bacterial strains, cultures, and vectors. *Mycobacterium avium* subsp. *paratuberculosis* strain ATCC 19698 (*M. paratuberculosis*) was obtained from Mike Collins (University of Wisconsin—Madison) and used for constructing the mutant library. This strain was grown at 37°C in Middlebrook 7H9 broth enriched with 10% albumin dextrose complex, 0.5% glycerol, 0.05% Tween 80, and 2 μ g/ml of mycobactin J (Allied Monitor, IN). The temperature-sensitive, conditionally replicating phasmid (pAE94) used to deliver the transposon Tn5367 was obtained from the Bill Jacobs laboratory (Albert Einstein College of Medicine) and propagated in *Mycobacterium smegmatis* mc²155 at 30°C as described previously (3). Tn5367 is an IS1096-derived insertion element containing a kanamycin resistance gene as a selectable marker. After phage transduction, mutants were selected on Middlebrook 7H10 medium plates supplemented with 30 μ g/ml of kanamycin. *Escherichia coli* DH5 α cells used for cloning purposes were grown on Luria-Bertani (LB) agar or broth supplemented with 100 μ g/ml ampicillin. For some of the mutants, the plasmid vector pGEM-T Easy (Promega, Madison, WI) was used for TA cloning of the PCR products before sequencing.

Construction of a transposon mutant library. The phasmid pAE94 was used to deliver Tn5367 to mycobacterial cells by use of a protocol established earlier for *M. tuberculosis* (3). For each transduction, 10 ml of *M. paratuberculosis* culture was grown to 2×10^8 CFU/ml (OD₆₀₀ of 0.6 to 0.8), centrifuged, resuspended in 2.5 ml of MP buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 2 mM CaCl₂), and incubated with 10¹⁰ PFU of pAE94 at the nonpermissive temperature (37°C) for 2 h in a shaking incubator to inhibit the possible lytic or lysogenic cycle of the phage (3). Adsorption stop buffer (20 mM sodium citrate and 0.2% Tween 80) was added to prevent further phage infections, and this mixture was immediately plated on 7H10 agar supplemented with 30 μ g/ml of kanamycin and incubated at 37°C for 6 weeks. Kanamycin-resistant colonies ($n = 5,060$) were inoculated into 2 ml of 7H9 broth supplemented with kanamycin in 96-deep-well plates for additional analysis.

Southern blot analysis. To examine the characteristics of Tn5367 transposition in the *M. paratuberculosis* genome, 10 randomly selected mutants were analyzed by Southern blotting using a standard protocol (37). Briefly, kanamycin-resistant *M. paratuberculosis* single colonies were grown separately in 10 ml of 7H9 broth for 10 days at 37°C before genomic DNA extraction and digestion (2 to 3 μ g) with BamHI restriction enzyme (Promega) (35). Digested DNA fragments from both mutant and wild-type strains were electrophoresed on a 1% agarose gel and transferred to a nylon membrane (Perkin Elmer, CA), using an alkaline

TABLE 1. Oligonucleotides used in this study

Primer name	Oligonucleotide sequence (5' to 3')	Purpose
AMT31	TGC AGC AAC GCC AGG TCC ACA CT	First-round amplification
AMT38	GTA ATA CGA CTC ACT ATA GGG CNN NNC ATG	First-round amplification
AMT32	CTC TTG CTC TTC CGC TTC TTC TCC	Second-round amplification
AMT39	TAA TAC GAC TCA CTA TAG GG	Second-round amplification
AMT152 SP6	TTG CTC TTC CGC TTC TTC T TAT TTA GGT GAC ACT ATA G	Direct sequencing Sequencing from pGEM-T

transfer protocol as recommended by the manufacturer. A 1.3-kb DNA fragment from the kanamycin resistance gene was radiolabeled with [α -³²P]dCTP by use of a random prime labeling kit (Promega) in accordance with the manufacturer's direction. The radiolabeled probe was hybridized to the nylon membrane at 65°C for 12 to 16 h in a shaking water bath before the membrane was washed and exposed to X-ray film (Kodak, Rochester, NY). The film was developed to visualize the hybridization signals (37).

Sequencing of the transposon insertion site. To determine the exact transposon insertion site within the *M. paratuberculosis* genome, a protocol for sequencing randomly primed PCR products was adopted from previous work on *M. tuberculosis* (23), with slight modifications. For PCR amplification, the genomic DNA of each mutant was extracted from individual cultures by boiling the cultures for 10 min and centrifugation at 10,000 $\times g$ for 1 min, and 10 μ l of the supernatant was used for a standard PCR. For the first round of PCR, a transposon-specific primer (AMT31) and a degenerate primer (AMT38) (Table 1) were used to amplify the chromosomal sequence flanking the transposon insertion site (Fig. 1A). PCR was carried out in a total volume of 25 μ l in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM MgCl₂, 0.01% (wt/vol) bovine serum albumin, 0.2 mM deoxynucleoside triphosphates, 0.1 μ M of primer AMT31, 1.0 μ M of primer AMT38, and 0.75 U of *Taq* polymerase (Promega). First-round amplification was performed with an initial denaturing step at 94°C for 5 min, followed by 40 cycles of denaturing at 94°C for 1 min, annealing at 50°C for 30 s, and extension at 72°C for 90 s, with a final extension step at 72°C for 7 min. Only 1 μ l of the first-round amplification was then used as the template for the second-round PCR (nested PCR), done using a nested primer (AMT32) derived from Tn5367 and the T7 primer (AMT39) present within the degenerative primer sequence (Table 1). Reactions were carried out in a total volume of 50 μ l in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM deoxynucleoside triphosphates, 0.5 μ M primers, 5% (vol/vol) dimethyl sulfoxide, and 0.75 U of *Taq* polymerase (Promega). The amplification was performed with a denaturing step at 95°C for 5 min followed by 35 thermocycles (94°C for 30 s, 57°C for 30 s, and 72°C for 1 min), with a final extension step at 72°C for 10 min. For almost two-thirds of the sequenced mutants, the AMT152 primer present in Tn5367 was used to directly sequence gel-purified amplicons (Table 1). The product of the second amplification was gel purified (Wizard SV gel extraction kit; Promega) and cloned into pGEM-T Easy vector for plasmid miniprep preparation followed by sequencing. Inserts in pGEM-T Easy vector were confirmed by EcoRI (Promega) restriction digestion, and the sequencing was carried out using the SP6 primer (Table 1).

To identify the precise transposon insertion site in the *M. paratuberculosis* genome, the transposon sequence was trimmed from the cloning vector sequences and a BLASTN search was used against the *M. paratuberculosis* K-10 complete genome sequence (21). Sequences with at least 100 bp of alignment to the *M. paratuberculosis* genome were further analyzed. Sequences without any transposon sequence were not analyzed to avoid using amplicons generated by nonspecific primer binding and amplification.

Statistical analysis. All bacterial counts from mouse organs were statistically analyzed using the Excel program (Microsoft, Seattle, WA). All counts were expressed as means $\pm$ standard deviations. Differences in counts between groups were analyzed with a Student *t* test for paired samples. Differences were considered to be significant if a *P* value of <0.05 was obtained when the CFU counts of the mutant strains were compared to that of the wild-type strain.

FIG. 1. Transposon mutagenesis of *M. paratuberculosis*. (A) Schematic representation of the transposon Tn5367 used for insertion mutagenesis of *M. paratuberculosis*. Thin arrows indicate the schematic design for primers used for PCR and sequence analysis. 1° PCR, first-round PCR; 2° PCR, second-round PCR; IR, inverted repeat; Kan^r, kanamycin resistance gene; *tnpA* and *tnpR*, transposase-encoding genes. (B) Distribution of 1,128 transposon insertion sites on the genome of *M. paratuberculosis* K-10 (21), indicated by long bars on the outermost circle. The inner two circles of short bars show predicted genes transcribed in sense and antisense directions.

characterized for *M. tuberculosis* (46), and *trpE2* encodes an isochorismate anthranilate synthase that could be involved in the synthesis of water-soluble siderophores (9, 31) or tryptophan biosynthesis (19). Because *M. paratuberculosis* does not produce siderophores (9), it is possible that the dark color of the $\Delta trpE2$ mutant is a product of accumulation of anthranilate precursors.

To examine the uniqueness of Tn5367 integrations throughout the *M. paratuberculosis* genome, we performed Southern blot analysis with genomic DNA extracted from 10 randomly chosen kanamycin-resistant colonies. Each of the 10 mutants tested by this method showed a single hybridization band, and these bands were of different sizes, demonstrating that a single transposon insertion occurred in different chromosomal locations. Three mutants analyzed by Southern blotting showed similar fragment sizes (data not shown), indicating possible clone redundancy. However, sequence analysis of these mutants identified three different insertion sites in MAP3752, MAP4327c, and MAP3733c genes. Based on the Southern analysis of the mutants, we concluded that Tn5367 integrated only once into the genome of *M. paratuberculosis*. More information about the randomness of Tn5367 can be gleaned from the sequence analysis described below.

Identification of the transposition sites in *M. paratuberculosis* mutants. Among the library of 5,060 mutants, the first 1,150 were analyzed using a high-throughput sequencing protocol employing a randomly primed PCR protocol that was successful in characterizing a library of *M. tuberculosis* mutants (23). The generated sequences were used to search the *M. paratuberculosis* K-10 complete genome with a BLASTN algorithm to identify the insertion site in each mutant. Unique insertion sites ($n = 970$) were identified, and almost two-thirds of the insertions occurred in predicted open reading frames (ORFs), while the rest of the insertions occurred in the intergenic re-

RESULTS

Generation of *M. paratuberculosis* mutant library. A genome-wide random-insertion mutant library was generated for *M. paratuberculosis* ATCC 19698 by using the temperature-sensitive mycobacteriophage phAE94 (3). One transduction reaction of 10^9 mycobacterial cells with phAE94 yielded all of the kanamycin-resistant colonies used throughout this study. A total of 5,060 kanamycin resistance colonies were patched onto 96-well plates and stored at -80°C until further analysis was performed. Colonies with variant morphology were not easily noticeable compared to the variations usually seen with members of the *M. avium* complex (17). Nonetheless, when some colonies were grown in 7H9 broth for further analysis, brownish to dark-brown colors were observed for cultures of two strains that were found to have disruptions in either the *umaA1* or the *trpE2* gene (see Fig. S1 in the supplemental material). The discoloration suggested the formation of incomplete secreted products or accumulation of bacterial by-products. The *umaA1* gene encodes a mycolic acid synthase that has been

TABLE 2. Percentages and numbers of unique insertions in a library of 5,060 mutants generated using Tn5367 and *M. paratuberculosis*

Insertion site	No. of insertion sites	No. of unique insertions	% Unique ^a
ORF	714	640	89.6
Intergenic region	436	330	75.7
Total	1,150	970	84.3

^a Indicates the percentage of insertions in unique sites within ORFs or intergenic regions.

gions ($n = 330$) (Table 2). Among the 970 unique insertions within ORFs, only 288 of the predicted mycobacterial ORFs were disrupted at least once by the transposition of Tn5367, indicating that more than one insertion occurred at different locations within the same gene. In fact, 10.4% of the disrupted ORFs showed more than one insertion per ORF, indicating the presence of “hot spots” for the transposition of Tn5367. Compared to the insertions in ORFs, a high rate of multiple insertions (24.3%) was observed when the intergenic regions were examined. Generally, the overall characteristics of the *M. paratuberculosis* library of mutants were similar to those of the *M. tuberculosis* library (23).

More scrutiny of the DNA sequences in both coding and intergenic regions revealed that the regions most susceptible to transposon insertions are those with G+C contents ranging from 50.5 to 60.5%, which are considerably lower than the average G+C content of the whole *M. paratuberculosis* genome (69.3%) (see Table S1 in the supplemental material). Analysis of the flanking regions of the Tn5367 site of insertion in genes with a high frequency of transposition ($n \geq 4$) identified areas of AT or TA repeats [e.g., TTT(T/A), AA(A/T), or TAA] as the most predominant sequences. Additionally, several mutants showed insertions into ORFs that have multiple copies in the genome (e.g., gene families or paralogous genes). A total of 22 mutants were present in this group and were excluded from further analysis until more experiments can be performed to identify the exact disrupted gene copy. An additional 17 mutants were excluded from further analysis because of the lack of a transposon-specific region in the obtained sequence, implying possible nonspecific amplifications from the *M. paratuberculosis* genome. To illustrate the randomness of the Tn5367 transposition in the *M. paratuberculosis* genome, the gene positions of all sequenced mutants were mapped to the genome sequence of *M. paratuberculosis* K-10 (21). As shown in Fig. 1B, the transposon insertions were evenly distributed throughout the genome. Overall, 1,128 mutants underwent the second level of bioinformatic analysis.

Bioinformatic analysis of disrupted genes. A total of 288 genes represented by 970 mutants were identified as disrupted from the initial screening of the transposon mutant library constructed in *M. paratuberculosis*. Examining the potential functional contribution of each disrupted gene will better characterize their roles in infection. With the help of the Clusters of Orthologous Groups website (<http://www.ncbi.nlm.nih.gov/COG/>) (40), disrupted genes were placed into functional categories (Table 3). Six genes did not have a match in the COG functional category of *M. paratuberculosis* and were conse-

TABLE 3. List of functional categories of 288 disrupted genes identified in this study

Functional category	No. (%) of genes in <i>M. paratuberculosis</i> K-10 genome	No. (%) ^a of disrupted genes in mutant library
Translation	154 (3.5)	6 (3.9)
RNA processing and modification	1 (0.02)	0 (0.0)
Transcription	262 (6.0)	8 (3.1)
Replication, recombination, and repair	179 (4.1)	13 (7.3)
Chromatin structure and dynamics	1 (0.02)	0 (0.0)
Cell cycle control, mitosis, and meiosis	34 (0.8)	3 (8.8)
Defense mechanisms	46 (1.1)	5 (10.9)
Signal transduction mechanisms	112 (2.6)	6 (5.4)
Cell wall/membrane biogenesis	132 (3.0)	12 (9.1)
Cell motility	10 (0.2)	0 (0.0)
Intracellular trafficking and secretion	20 (0.5)	0 (0.0)
Posttranslational modification, protein turnover, and chaperones	102 (2.3)	5 (4.9)
Energy production and conversion	277 (6.4)	10 (3.6)
Carbohydrate transport and metabolism	187 (4.3)	18 (9.6)
Amino acid transport and metabolism	246 (5.7)	16 (6.5)
Nucleotide transport and metabolism	67 (1.5)	2 (3.0)
Coenzyme transport and metabolism	126 (2.9)	3 (2.4)
Lipid transport and metabolism	326 (7.5)	20 (6.1)
Inorganic ion transport and metabolism	174 (4.0)	9 (5.2)
Secondary-metabolite biosynthesis, transport, and catabolism	357 (8.2)	26 (7.3)
General function prediction only	375 (8.6)	30 (8.0)
Unknown function	248 (5.7)	16 (6.5)

^a The percentage is relative to the number of genes in the category in the *M.*

quently analyzed using the *M. tuberculosis* functional category (<http://genolist.pasteur.fr/TubercuList/>). The predictive functions of these genes include different cellular processes such as lipid metabolism (*desA1*), cell wall biosynthesis (*mmpS4*), and several possible lipoproteins (*lppP*, *lpqJ*, and *lpqN*), including a member of the proline-rich proteins (PE) family (PE6). Interestingly, two gene groups (bacterial defense mechanisms and cell cycling) were overrepresented in the mutant library (10.9 and 8.8%, respectively) compared to their percentages in the genome. The preference of integrations into these two groups could be attributed to the lower G+C percentage and the tendency of Tn5367 to integrate into low-G+C regions. As expected, for most functional groups, the percentages of disrupted genes ranged between 2.5 and 10%, in agreement with their percentages within the genome of *M. paratuberculosis*.

To further analyze the expected phenotypes of the disrupted

TABLE 4. Bioinformatic analysis of disrupted genes of the mutants tested in the mouse model of paratuberculosis

Gene	Insertion site % ^a	Molecular function	Role ^b	Orthologue ^c
<i>mmpL10</i>	18.6	Conserved membrane transport protein	N	Rv1183
<i>fprA</i>	56.5	Adrenodoxin oxidoreductase	N	Rv3106
<i>papA2</i>	12.1	Conserved polyketide synthase-associated protein	N	Rv3820c
<i>gcpE</i>	56.8	Isoprenoid biosynthesis	E	Rv2868c
<i>papA3_1</i>	65.2	Probable conserved polyketide synthase-associated protein	E	Rv1182
<i>kdpC</i>	45.1	Probable potassium-transporting ATPase	N	Rv1031
<i>umaA1</i>	63.5	Possible mycolic acid synthase	N	Rv0469
<i>pstA</i>	3.8	Nonribosomal binding peptide synthetase	N	Rv0101
<i>fabG2_2</i>	70.1	Putative oxidoreductase activity	E	Rv1350
<i>trpE2</i>	81.2	Probable anthranilate synthase component I	E	Rv2386c
<i>impA</i>	52.0	Probable inositol monophosphatase	Unknown	Rv1604

^a Insertion site % indicates the distance of the Tn5367 insertion from the start codon of the gene.

^b Designations of genes as essential (E) or nonessential (N) were based on transposon mutagenesis of *M. tuberculosis* (33).

^c Orthologues to the genome of *M. tuberculosis*.

genes, we examined the flanking sequences to examine any possible polar effect on gene expression due to operon structure. By use of the operon prediction algorithm (OPERON) (11), approximately 124 (43.0%) of the disrupted ORFs were identified as members of 113 putative operons (see Table S2 in the supplemental material), indicating that possible phenotypes may be related to the disruption of functions performed by the whole operons and not just the disrupted genes. A total of 52 (41.9%) disrupted genes were within the last gene of an operon and therefore were unlikely to affect expression of other genes of the operon. On the other hand, 40 and 32 disrupted genes, respectively, were within the first or middle genes of an operon (32.3 and 25.8%, respectively). Accordingly, it is expected that in almost half of the mutants the observed phenotype could be attributed to the disruption of an entire operon rather than a single gene. Interestingly, a total of 23 Tn5367 insertions were present in several genes of the same 12 operons, suggesting the preference of transpositions throughout these sequences. For example, in the *kdp* operon (encoding putative potassium translocating proteins), four genes out of the five genes constituting this operon were disrupted.

Organ colonization of *M. paratuberculosis* mutants. To identify novel genes involved in *M. paratuberculosis* invasion, colonization, and persistence, we employed the mouse model of paratuberculosis to characterize a selected list of transposon mutants gleaned from the bioinformatic analysis. Target genes were selected if their functional information in mycobacterial growth was known, especially genes involved in cellular processes important for mycobacterial survival during infection or homologues to other known virulence factors (Table 4). The list was also designed to encompass mutations over a broad range of metabolic pathways to determine whether any particular pathway could play an essential role for *M. paratuberculosis* persistence. Among the 11 mutant genes tested in mice, four genes (*gcpE*, *kdpC*, *umaA1*, and *impA*) were predicted to be members of operons. Only *umaA1* is the last gene of its operon and may not disrupt the expression of other genes in that operon (see the supplemental material). Alternatively, the *gcpE*, *impA*, and *kdpC* genes were shown to be in the middle of their respective operons and could have a polar effect on the expression of downstream genes.

Before animal infection, the growth curves of all mutants

grown in Middlebrook 7H9 broth supplemented with kanamycin were shown to be similar to that of the parent strain (data not shown). However, most mutants reached an OD₆₀₀ of 1.0 at 35 days, compared to 25 days for ATCC 19698 (parent strain). For mouse infection, it is noteworthy to mention here that colonization levels of the spleen did not show a significant change between mutants and the wild type (data not shown), while those of the liver and intestine were variable. In the parental infection model of *M. paratuberculosis* employed here, it is possible that bacterial colonization of the liver affected further colonization of the spleen. Therefore, liver and intestine were the most informative organs. For samples collected at 3 weeks postinfection, only the strains with a disruption in the *gcpE* or *kdpC* gene displayed colonization levels significantly ($P < 0.05$) lower than that of the parent strain (Fig. 2). The decline in CFU counts was maintained to the end of the experiments, especially in the intestine, the primary target of *M. paratuberculosis*. At 6 weeks postinfection, additional mutants (e.g., the Δ *papA2* and Δ *pstA* mutants) showed significant reduction in the intestinal colonization levels. At 12 weeks postinfection, the Δ *umaA1*, Δ *fabG2_2*, and Δ *impA* mutants displayed significant ($P < 0.05$) decline in intestinal colonization (Fig. 2). Finally, the Δ *mmpL10*, Δ *fprA*, Δ *papA3_1*, and Δ *trpE2* mutants showed a 10-fold reduction in mycobacterial levels at least in one examined organ by 12 weeks postinfection; however, this reduction was not statistically significant ($P > 0.05$). The colonization trend of these mutants may indicate involvement in later stages of paratuberculosis that were not examined in this study.

Histopathology of mice infected with transposon mutants.

All animal groups infected with mutants or with the parent strain displayed a granulomatous inflammatory reaction consistent with infection with *M. paratuberculosis* by use of the mouse model of paratuberculosis (25, 39). Liver sections were most reflective for paratuberculosis, where a typical granulomatous response, consisting of aggregation of macrophages with admixed lymphocytes occasionally surrounded by a thin layer of fibrous connective tissues, was found. Animals infected with strain ATCC 19698 and some mutants, such as the Δ *mmpL10* mutant, showed apparent granuloma formation in liver sections (Fig. 3). Both the size and number of granulomatous inflammatory foci or granulomas increased over time, indicating the progression of the disease (see Fig. S2 in the

FIG. 2. Colonization levels of two classes of *M. paratuberculosis* mutants to mouse organs. Groups of mice were infected via intraperitoneal injection (10^7 to 10^8 CFU/mouse) with the wild-type strain (ATCC 19698) or one of the 11 examined mutants listed in Table 4. Colonization levels of class I mutants (A and B) and class II mutants (C and D) in livers and intestines of mice are shown at different times postinfection. Bars represent the standard errors calculated from the means of colony counts estimated from organs of six to eight mice.

supplemental material). It is noteworthy to mention here that during the early time of infection (3- and 6-week samples) most mutants displayed only lymphocytic inflammatory responses, while the granulomatous inflammation and formation of granulomas were observed only at the late time point (12-week samples). Additionally, the severity of inflammation reached level 3 (out of 5) at 12 weeks postinfection for mice infected with ATCC 19869, while in the group infected with mutants such as the $\Delta gcpE$ and $\Delta kdpC$ mutants, the granulomatous responses ranged between levels 1 and 2. Interestingly, the granulomatous response in mice infected with $\Delta mmpL10$ was more extensive (larger in size and surrounded by fibrous tissues) than the response formed in mice infected with ATCC 19698 (Fig. 3). On the other hand, infection with some mutants (e.g., the $\Delta gcpE$ and $\Delta impA$ mutants) induced relatively minor lesions at 3 weeks postinfection and remained at this level as time progressed, while infection with others (the $\Delta pap3_1$ and $\Delta fabG2_2$ mutants) began with mild lesions and progressively increased in severity over time. Finally, infection with another group of mutants (the $\Delta fprA$ and $\Delta kdpC$ mutants) began with a level of response similar to that of the parent strain and continued to display the same type of lesions until the end of the sampling time. The overall inflammatory response in livers

of infected mice is summarized in Fig. S2 in the supplemental material.

Generally, by combining the histopathology and colonization data, we were able to assess the overall virulence of the examined mutants and classify the disrupted genes into two major classes. In class I (colonization mutants), the disrupted *gcpE*, *pstA*, *kdpC*, *papA2*, and *umaA1* genes generated mutants that were unable to efficiently colonize the mouse organs (particularly intestine and liver); therefore, a modest level of lesions was generated and colonization levels were significantly lower than that of the wild type. For one mutant of class I (the $\Delta papA2$ mutant), the levels of bacterial colonization were not reduced in livers up to 6 weeks postinfection. No characteristic pathology of this subgroup could be delineated from that of other groups, since only liver sections were reflective of the paratuberculosis by use of the mouse model employed in this study. With class II mutants (persistence mutants), levels of colonization were slightly reduced in the first 6 weeks and then reduced significantly at a later time (e.g., the *fabG2_2* and *impA* mutants). The lesions formed in animals infected with class II mutants showed a pattern of progression similar to that of lesions formed in animals infected with the parent strain. Overall, with most attenuated mutants, there was an inverse

FIG. 3. Histopathology of mice infected with *M. paratuberculosis* strains. Only liver sections stained with hematoxylin and eosin from mice sacrificed at 12 weeks are shown here. Arrows point to granulomatous inflammatory responses. Representative sections from livers of mice infected with ATCC 19698 (wild type) (A), the $\Delta mmpL10$ mutant (hypervirulent strain) (B), or the $\Delta gcpE$ mutant (attenuated strain) (C). Bar, 50.0 μ m.

relationship between inflammatory scores and mycobacterial colonization levels of mutants for samples collected at 12 weeks postinfection. It is possible that the decline of *M. paratuberculosis* levels at 12 weeks postinfection could be attributed

to the initiation of a strong immune response, represented by an increase in granuloma formation. Alternatively, the inability of mutants to survive peritoneal macrophages following mouse inoculation could be the reason for the inability of the identified attenuation mutants to colonize mouse organs.

DISCUSSION

A better understanding of the virulence mechanisms and pathogenesis of *M. paratuberculosis* is required to develop more-effective vaccines and chemotherapies directed against animal and human infections with *M. paratuberculosis*. In this report, we employed the transposable element Tn5367 to generate a mutant library of *M. paratuberculosis* that was used to identify novel virulence factors. We began our efforts to characterize the generated library by sequence and bioinformatic analyses of 20% of the mutants. More insertions were found in AT-rich sites, which is consistent with previous reports identifying favorable target sites for Tn5367 (13, 23). Although the transposition of Tn5367 was not completely random, we were able to construct a library where unique insertions were obtained in 84.3% of the 1,150 mutants examined so far. Previously, two *M. paratuberculosis* transposon libraries were generated and screened for mutants unable to grow in defined broth media (5, 13). Our approach is based on the large-scale identification of disrupted genes and the use of bioinformatics to select mutants that could be characterized in animals. Employing such an approach, we were able to identify seven novel virulence determinants out of 11 mutants investigated in a mouse model of paratuberculosis. Compared to similar protocols established for identifying virulence genes, such as signature-tagged mutagenesis (15), the approach employed here is simple and uses a small number of animals. For the attenuated mutants identified by animal screening, in-frame deletions and complementation studies are needed to confirm that their phenotype is due to the gene disruption and not caused by the polar effects on downstream genes.

Both the bacteriological and histological analyses employed in this study were able to classify attenuated mutants into two classes. Class I mutants, including the $\Delta gcpE$, $\Delta pstA$, $\Delta umaA1$, $\Delta papA2$, and $\Delta kdpC$ mutants, had impaired organ colonization with low inflammatory scores (colonization mutants). The *gcpE* gene encodes a product that controls a terminal step of isoprenoid biosynthesis via the mevalonate-independent 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway (14). The *gcpE* gene is considered an essential gene in *E. coli* (1), and its role in *M. paratuberculosis* survival during infection needs to be further investigated. Because of its conserved nature and divergence from a mammalian counterpart, *gcpE* is considered a suitable target for drug development (18); hence, understanding its role in *M. paratuberculosis* pathogenesis is worthy of further investigation. Another mutant in this class, the $\Delta pstA$ mutant, encodes a nonribosomal peptide synthetase in *M. tuberculosis* with a role in glycopeptidolipid synthesis. The glycopeptidolipids are a class of species-specific mycobacterial lipids and major constituents of the cell envelopes of many nontuberculous mycobacteria, such as *M. smegmatis* (4), as well. The $\Delta pstA$ mutant in *M. smegmatis* showed an altered surface property and also was internalized by macrophages more rapidly than the parent strain. Consequently, the reduc-

tion in colonization level of the $\Delta pstA$ mutant could result from the inability of the mutant to survive in the macrophage environment once it crosses the intestinal barrier. On the other hand, the *kdpC* gene encodes an inducible high-affinity potassium uptake system (12). Recently, two groups reported that the genes involved in potassium uptake highly increased in expression during *M. tuberculosis* infection of human macrophage (10) and that potassium concentration also increased in the microenvironment of the macrophage (44). Accordingly, it is possible that a defect in the potassium shuttling mechanism due to the disruption of *kdpC* resulted in this attenuation phenotype.

Other mutants belonging to class I include those with disruptions in the *umaA1* or *papA2* gene (the $\Delta umaA1$ and $\Delta papA2$ mutants). Interestingly, the *umaA1* gene encodes a mycolic acid methyltransferase involved in cell wall biosynthesis that, when it was disrupted in *M. tuberculosis*, showed a hypervirulence phenotype in mice (23). However, in our hands, disruption of *umaA1* resulted in lower colonization levels in all organs examined at 6 weeks postinfection and beyond. It is possible that *umaA1* plays a different role in *M. paratuberculosis* than in *M. tuberculosis*. The last member of this class is the $\Delta papA2$ mutant. *papA2* is a member of the polyketide synthase-associated protein (Pap) family of highly conserved genes. Members of the Pap family encode virulence-enhancing lipids (28). We demonstrated here that a disruption in a member of this family could contribute to mycobacterial attenuation. Another mutant (the $\Delta papA3_1$ mutant) of the Pap family was also investigated with mice; however, it showed a CFU count significantly higher than that of the $\Delta papA2$ mutant, reflecting the different roles played by genes belonging to the same family.

As for class II mutants, both the $\Delta impA$ and $\Delta fabG2_2$ mutants had lower colonization levels in mouse tissues beyond 6 weeks postinfection than did the wild-type strain, implying a role in mycobacterial entry to the persistent stage of paratuberculosis (persistence mutants). The *impA* gene encodes an inositol monophosphatase protein with an activity which has been shown to contribute to cell wall permeability in *M. smegmatis* (27) and to play a role in the synthesis of phosphatidyl-inositol dimannoside (29). On the other hand, the *fabG2_2* gene (disrupted in the $\Delta fabG2_2$ mutant) encodes a putative oxidoreductase activity (21). It is possible that functions encoded by the two genes could play a role in bacterial defense during entry to the persistence stage of paratuberculosis. Clearly, more analysis of the roles of *impA* and *fabG2_2* is needed to uncover the nature of host-pathogen interactions at persistence stages of infection. Also, it will be interesting to profile the immunological responses following immunization with any of the colonization mutants in a ruminant model for Johne's disease. Such analysis could identify novel vaccine candidates suitable to combat Johne's disease.

An earlier report using comparative genomic hybridization to analyze the content of the *M. paratuberculosis* genome identified large regions that could explain its differential virulence compared to that of the closely related *M. avium* subsp. *avium* (45). In this report, large-scale sequencing of a mutant library allowed us to rapidly identify a list of potential virulence factors specific for *M. paratuberculosis*. Because this study served as a screening tool for a large number of mutants, the amena-

ble mouse model (26, 39, 43) was successfully used. Nonetheless, further testing of the attenuated mutants in the calf or goat model of Johne's disease (20, 34) is warranted. It is also possible that using an oral route of mouse infection or examining tissues at times later than 12 weeks postinfection could reveal different aspects of virulence that were not elucidated in this study. These variations on the animal model used to screen mutants could be employed to further dissect the roles of identified genes in *M. paratuberculosis* virulence. Further analysis of the roles of the identified list of potential virulence factors in *M. paratuberculosis* survival during infection will improve our understanding of the molecular pathogenesis of Johne's disease and will help us to design a better strategy for controlling the infection. Such an approach of direct identification of disrupted genes followed by bioinformatic analysis to select mutants for animal testing was successfully applied to *M. paratuberculosis* and can be adapted to other bacterial systems.

ACKNOWLEDGMENTS

We acknowledge Gireesh Rajashekara and Christine Tavano for reading the manuscript.

Research in the AMT laboratory is supported by the Animal Formula Fund (WIS04794) and the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service (grant no. WIS04823 and Johne's Disease Integrated Program, 2004-35605-14243). S.J.S. was partially supported by the Korean government (MOEHRD, Basic Research Promotion Fund, KRF-M01-2004-000-10072-0).

REFERENCES

- Altincicek, B., A.-K. Kollas, S. Sanderbrand, J. Wiesner, M. Hintz, E. Beck, and H. Jomaa. 2001. GcpE is involved in the 2-C-methyl-D-erythritol 4-phosphate pathway of isoprenoid biosynthesis in *Escherichia coli*. *J. Bacteriol.* **183**:2411–2416.
- Bannantine, J. P., J. F. J. Huntley, E. Miltner, J. R. Stabel, and L. E. Bermudez. 2003. The *Mycobacterium avium* subsp. *paratuberculosis* 35 kDa protein plays a role in invasion of bovine epithelial cells. *Microbiology* **149**:2061–2069.
- Bardarov, S., J. Kriakov, C. Carriere, S. W. Yu, C. Vaamonde, R. A. McAdam, B. R. Bloom, G. F. Hatfull, and W. R. Jacobs, Jr. 1997. Conditionally replicating mycobacteriophages: a system for transposon delivery to *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. USA* **94**:10961–10966.
- Billman-Jacobe, H., M. J. McConville, R. E. Haines, S. Kovacevic, and R. L. Coppel. 1999. Identification of a peptide synthetase involved in the biosynthesis of glycopeptidolipids of *Mycobacterium smegmatis*. *Mol. Microbiol.* **33**:1244–1253.
- Cavaignac, S. M., S. J. White, G. W. De Lisle, and D. M. Collins. 2000. Construction and screening of *Mycobacterium paratuberculosis* insertional mutant libraries. *Arch. Microbiol.* **173**:229–231.
- Cetinkaya, B., K. Egan, D. A. Harbour, and K. L. Morgan. 1996. An abattoir-based study of the prevalence of subclinical Johne's disease in adult cattle in south west England. *Epidemiol. Infect.* **116**:373–379.
- Chamberlin, W., D. Y. Graham, K. Hulten, H. M. T. El-Zimaity, M. R. Schwartz, I. Shafran, and F. A. K. El-Zaatari. 2001. *Mycobacterium avium* subsp. *paratuberculosis* as one cause of Crohn's disease. *Aliment. Pharmacol. Ther.* **15**:337–346.
- Collins, M. T., D. C. Sockett, W. J. Goodger, T. A. Conrad, C. B. Thomas, and D. J. Carr. 1994. Herd prevalence and geographic distribution of, and risk factors for, bovine paratuberculosis in Wisconsin. *J. Am. Vet. Med. Assoc.* **204**:636–641.
- De Voss, J. J., K. Rutter, B. G. Schroeder, and C. E. Barry. 1999. Iron acquisition and metabolism by mycobacteria. *J. Bacteriol.* **181**:4443–4451.
- Dubnau, E., and I. Smith. 2003. *Mycobacterium tuberculosis* gene expression in macrophages. *Microbes Infect.* **5**:629–637.
- Ermolaeva, M. D., H. G. Khalak, O. White, H. O. Smith, and S. L. Salzberg. 2000. Prediction of transcription terminators in bacterial genomes. *J. Mol. Biol.* **301**:27–33.
- Gassel, M., and K. Altendorf. 2001. Analysis of KdpC of the K⁺-transporting KdpFABC complex of *Escherichia coli*. *Eur. J. Biochem.* **268**:1772–1781.
- Harris, N. B., Z. Y. Feng, X. F. Lie, S. L. G. Cirillo, J. D. Cirillo, and R. G. Barletta. 1999. Development of a transposon mutagenesis system for *Mycobacterium avium* subsp. *paratuberculosis*. *FEMS Microbiol. Lett.* **175**:21–26.
- Hecht, S., W. Eisenreich, P. Adam, S. Amslinger, K. Kis, A. Bacher, D.

- Arigoni, and F. Rohdich. 2001. Studies on the nonmevalonate pathway to terpenes: the role of the GcpE (IspG) protein. *Proc. Natl. Acad. Sci. USA* **98**:14837–14842.
15. Hensel, M., J. E. Shea, C. Gleeson, M. D. Jones, E. Dalton, and D. W. Holden. 1995. Simultaneous identification of bacterial virulence genes by negative selection. *Science* **269**:400–403.
 16. Hill, B. B., M. West, and K. V. Brock. 2003. An estimated prevalence of Johne's disease in a subpopulation of Alabama beef cattle. *J. Vet. Diagn. Invest.* **15**:21–25.
 17. Kansal, R. G., R. Gomezflores, and R. T. Mehta. 1998. Change in colony morphology influences the virulence as well as the biochemical properties of the *Mycobacterium avium* complex. *Microb. Pathog.* **25**:203–214.
 18. Kemp, L. E., C. S. Bond, and W. N. Hunter. 2002. Structure of 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase: an essential enzyme for isoprenoid biosynthesis and target for antimicrobial drug development. *Proc. Natl. Acad. Sci. USA* **99**:6591–6596.
 19. Knochel, T., A. Ivens, G. Hester, A. Gonzalez, R. Bauerle, M. Wilmanns, K. Kirschner, and J. N. Jansonius. 1999. The crystal structure of anthranilate synthase from *Sulfolobus solfataricus*: functional implications. *Proc. Natl. Acad. Sci. USA* **96**:9479–9484.
 20. Koo, H. C., Y. H. Park, M. J. Hamilton, G. M. Barrington, C. J. Davies, J. B. Kim, J. L. Dahl, W. R. Waters, and W. C. Davis. 2004. Analysis of the immune response to *Mycobacterium avium* subsp. *paratuberculosis* in experimentally infected calves. *Infect. Immun.* **72**:6870–6883.
 21. Li, L., J. P. Bannantine, Q. Zhang, A. Amonsin, B. J. May, D. Alt, N. Banerji, S. Kanjilal, and V. Kapur. 2005. The complete genome sequence of *Mycobacterium avium* subspecies *paratuberculosis*. *Proc. Natl. Acad. Sci. USA* **102**:12344–12349.
 22. Linnabary, R. D., G. L. Meerdink, M. T. Collins, J. R. Stabel, R. W. Sweeney, M. K. Washington, and S. J. Wells. 2001. Johne's disease in cattle. *Counc. Agric. Sci. Technol.* **17**:1–10.
 23. McAdam, R. A., S. Quan, D. A. Smith, S. Bardarov, J. C. Betts, F. C. Cook, E. U. Hooker, A. P. Lewis, P. Woollard, M. J. Everett, P. T. Lukey, G. J. Bancroft, W. R. Jacobs, and K. Duncan. 2002. Characterization of a *Mycobacterium tuberculosis* H37Rv transposon library reveals insertions in 351 ORFs and mutants with altered virulence. *Microbiology* **148**:2975–2986.
 24. Momotani, E., D. L. Whipple, A. B. Thiermann, and N. F. Cheville. 1988. Role of M cells and macrophages in the entrance of *Mycobacterium paratuberculosis* into domes of ileal Peyer's patches in calves. *Vet. Pathol.* **25**:131–137.
 25. Mutwiri, G. K., U. Kosecka, M. Benjamin, S. Rosendal, M. Perdue, and D. G. Butler. 2001. *Mycobacterium avium* subspecies *paratuberculosis* triggers intestinal pathophysiologic changes in beige/scid mice. *Comp. Med.* **51**:538–544.
 26. Mutwiri, G. K., S. Rosendal, U. Kosecka, J. A. Yager, M. Perdue, D. Snider, and D. G. Butler. 2002. Adoptive transfer of BALB/c mouse splenocytes reduces lesion severity and induces intestinal pathophysiologic changes in the *Mycobacterium avium* subspecies *paratuberculosis* beige/scid mouse model. *Comp. Med.* **52**:332–341.
 27. Nigou, J., and G. S. Besra. 2002. Characterization and regulation of inositol monophosphatase activity in *Mycobacterium smegmatis*. *Biochem. J.* **361**:385–390.
 28. Onwueme, K. C., J. A. Ferreras, J. Buglino, C. D. Lima, and L. E. N. Quadri. 2004. Mycobacterial polyketide-associated proteins are acyltransferases: proof of principle with *Mycobacterium tuberculosis* PapA5. *Proc. Natl. Acad. Sci. USA* **101**:4608–4613.
 29. Parish, T., J. Liu, H. Nikaido, and N. G. Stoker. 1997. A *Mycobacterium smegmatis* mutant with a defective inositol monophosphate phosphatase gene homolog has altered cell envelope permeability. *J. Bacteriol.* **179**:7827–7833.
 30. Pethe, K., D. L. Swenson, S. Alonso, J. Anderson, C. Wang, and D. G. Russell. 2004. Isolation of *Mycobacterium tuberculosis* mutants defective in the arrest of phagosome maturation. *Proc. Natl. Acad. Sci. USA* **101**:13642–13647.
 31. Quadri, L. E., J. Sello, T. A. Keating, P. H. Weinreb, and C. T. Walsh. 1998. Identification of a *Mycobacterium tuberculosis* gene cluster encoding the biosynthetic enzymes for assembly of the virulence-conferring siderophore mycobactin. *Chem. Biol.* **5**:631–645.
 32. Sassetti, C. M., D. H. Boyd, and E. J. Rubin. 2003. Genes required for mycobacterial growth defined by high density mutagenesis. *Mol. Microbiol.* **48**:77–84.
 33. Sassetti, C. M., D. H. Boyd, and E. J. Rubin. 2001. Comprehensive identification of conditionally essential genes in mycobacteria. *Proc. Natl. Acad. Sci. USA* **98**:12712–12717.
 34. Storset, A. K., H. J. Hasvold, M. Valheim, H. Brun-Hansen, G. Berntsen, S. K. Whist, B. Djonje, C. M. Press, G. Holstad, and H. J. S. Larsen. 2001. Subclinical paratuberculosis in goats following experimental infection. An immunological and microbiological study. *Vet. Immunol. Immunopathol.* **80**:271–287.
 35. Talaat, A. M., R. Reimschuessel, and M. Trucksis. 1997. Identification of mycobacteria infecting fish to the species level using polymerase chain reaction and restriction enzyme analysis. *Vet. Microbiol.* **58**:229–237.
 36. Talaat, A. M., R. Reimschuessel, S. S. Wasserman, and M. Trucksis. 1998. Goldfish, *Carassius auratus*, a novel animal model for the study of *Mycobacterium marinum* pathogenesis. *Infect. Immun.* **66**:2938–2942.
 37. Talaat, A. M., and M. Trucksis. 2000. Transformation and transposition of the genome of *Mycobacterium marinum*. *Am. J. Vet. Res.* **61**:125–128.
 38. Tanaka, S., S. Itoharu, M. Sato, T. Taniguchi, and Y. Yokomizo. 2000. Reduced formation of granulomata in gamma(delta) T cell knockout BALB/c mice inoculated with *Mycobacterium avium* subsp. *paratuberculosis*. *Vet. Pathol.* **37**:415–421.
 39. Tanaka, S., M. Sato, T. Taniguchi, and Y. Yokomizo. 1994. Histopathological and morphometrical comparison of granulomatous lesions in BALB/c and C3H/HeJ mice inoculated with *Mycobacterium paratuberculosis*. *J. Comp. Pathol.* **110**:381–388.
 40. Tatusov, R. L., E. V. Koonin, and D. J. Lipman. 1997. A genomic perspective on protein families. *Science* **278**:631–637.
 41. Tooker, B. C., J. L. Burton, and P. M. Coussens. 2002. Survival tactics of *M. paratuberculosis* in bovine macrophage cells. *Vet. Immun. Immunopathol.* **87**:429–437.
 42. Valentin-Weigand, P., and R. Goethe. 1999. Pathogenesis of *Mycobacterium avium* subspecies *paratuberculosis* infections in ruminants: still more questions than answers. *Microbes Infect.* **1**:1121–1127.
 43. Veazey, R. S., H. W. Taylor, D. W. Horohov, J. L. Krahenbuhl, J. L. I. Oliver, and T. G. I. Snider. 1995. Histopathology of C57BL/6 mice inoculated orally with *Mycobacterium paratuberculosis*. *J. Comp. Pathol.* **113**:75–80.
 44. Wagner, D., J. Maser, B. Lai, Z. H. Cai, C. E. Barry, K. H. Z. Bentrup, D. G. Russell, and L. E. Bermudez. 2005. Elemental analysis of *Mycobacterium avium*-, *Mycobacterium tuberculosis*-, and *Mycobacterium smegmatis*-containing phagosomes indicates pathogen-induced microenvironments within the host cell's endosomal system. *J. Immunol.* **174**:1491–1500.
 45. Wu, C.-W., J. Glasner, M. T. Collins, S. Naser, and A. M. Talaat. 2006. Whole-genome plasticity among *Mycobacterium avium* subspecies: insights from comparative genomic hybridizations. *J. Bacteriol.* **188**:711–723.
 46. Yuan, Y., R. E. Lee, G. S. Besra, J. T. Belisle, and C. E. Barry. 1995. Identification of a gene involved in the biosynthesis of cyclopropanated mycolic acids in *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. USA* **92**:6630–6634.