JOURNAL OF BACTERIOLOGY, Jan. 2009, p. 625–631 Vol. 191, No. 2
0021-9193/09/$08.00+0 doi:10.1128/JB.00932-08

Acid-Susceptible Mutants of *Mycobacterium tuberculosis* Share Hypersusceptibility to Cell Wall and Oxidative Stress and to the Host Environment[▽]

Omar H. Vandal, Julia A. Roberts, Toshiko Odaira, Dirk Schnappinger, Carl F. Nathan, and Sabine Ehrt*

Department of Microbiology and Immunology, Weill Cornell Medical College, New York, New York 10065

Received 8 July 2008/Accepted 5 November 2008

Mycobacterium tuberculosis **can persist in macrophage phagosomes that acidify to a pH of ~4.5 after activation of the macrophage with gamma interferon. How the bacterium resists the low pH of the acidified phagosome is incompletely understood. A screen of 10,100 *M. tuberculosis* transposon mutants for mutants hypersensitive to pH 4.5 led to the discovery of 21 genes whose disruption attenuated survival of *M. tuberculosis* at a low pH (41). Here, we show that acid-sensitive *M. tuberculosis* mutants with transposon insertions in Rv2136c, Rv2224c, *ponA2*, and *lysX* were hypersensitive to antibiotics, sodium dodecyl sulfate, heat shock, and reactive oxygen and nitrogen intermediates, indicating that acid resistance can be associated with protection against other forms of stress. The Rv2136c mutant was impaired in intrabacterial pH homeostasis and unable to maintain a neutral intrabacterial pH in activated macrophages. The Rv2136c, Rv2224c, and *ponA2* mutants were attenuated in mice, with the Rv2136c mutant displaying the most severe level of attenuation. Pathways utilized by *M. tuberculosis* for acid resistance and intrabacterial pH maintenance are potential targets for chemotherapy.**

Mycobacterium tuberculosis is an intracellular pathogen that encounters acidic environments during the course of infection. *M. tuberculosis* is able to arrest fusion of phagosomes with acidic lysosomes so that they reside in a mildly acidic compartment with a pH of ~6.2 in nonactivated macrophages (1, 26, 38). After activation of macrophages with gamma interferon (IFN-γ), phagosomes fuse with lysosomes, and the *M. tuberculosis* bacterium-containing compartment acidifies to a pH of ~4.5 (26, 35, 36, 42). *M. tuberculosis* is able to maintain its intrabacterial pH (pH_{IB}) and survive in activated macrophages and thus possesses resistance against the acidity of the phagolysosome (41).

Mechanisms of survival at a low pH have been extensively explored in many enteric pathogens, which must resist the harsh acidity of the stomach (pHs 2 to 3) in order to establish infection (16, 27). Exposure of *Escherichia coli* and *Salmonella enterica* to mildly acidic conditions protects the bacteria against a more extreme acid challenge. Induction of this acid tolerance response is believed to be important for virulence. In comparison, little is known about acid resistance in *M. tuberculosis*, and its role in virulence needs to be better understood. An *M. tuberculosis* mutant lacking OmpAtb, a pH-dependent porin (29), was sensitive to low pH in vitro and attenuated in macrophages and in mice (31). *M. tuberculosis* requires MgtC, a putative magnesium transporter, for growth at an acidic pH when magnesium concentrations are limited (4). This suggests that import of magnesium may be required at low pH; however, it has been shown that *Salmonella*'s MgtC does not function as an Mg^{2+} transporter (20), and the transport function of *M. tuberculosis*'s MgtC remains to be examined. The *M. tuberculosis mgtC* mutant is attenuated in macrophages and mice (4). These reports suggest that resistance to low pH is required for virulence; however, the mutants may also be sensitive to other components of the host immune response, and this may contribute to their attenuation in vivo.

Recently, we identified 21 *M. tuberculosis* transposon mutants that are hypersensitive to pH 4.5 (41). These mutants were attenuated in acidified 7H9 growth medium containing Tween 80. When Tween 80 was replaced with the detergent tyloxapol, only 5 of the 21 mutants retained sensitivity to acid. It is possible either that Tween 80 increased the permeability of the *M. tuberculosis* cell envelope to protons or that oleic acid, which can be hydrolyzed from Tween 80, became mycobactericidal at low pH. The five *M. tuberculosis* mutants that were hypersensitive to pH 4.5 in 7H9 medium irrespective of the detergent utilized contained transposon insertions in the genes Rv3671c, Rv2136c, Rv2224c, *ponA2*, and *lysX* (41). Only two of the 21 acid-sensitive mutants, the Rv3671c and Rv2136c mutants, were hypersensitive to acidified phosphate-citrate buffer (pH 4.5). All five mutants grew normally in vitro in 7H9 growth medium at pH 6.6 (41). The Rv3671c mutant was unable to maintain a neutral pH_{IB} in vitro and in activated macrophages and was attenuated in vivo, whereas the *lysX* mutant was able to maintain its pH_{IB} and was fully virulent (41). Here, we characterize the Rv2136c, Rv2224c, *ponA2*, and *lysX* acid-sensitive mutants for additional defects in vitro and assess the virulence of the Rv2136c, Rv2224c, and *ponA2* mutants in mice.

MATERIALS AND METHODS

Strains and media. *M. tuberculosis* transposon mutants were isolated in a screen for acid-sensitive mutants described previously (41). *M. tuberculosis* strains were grown at 37°C in a humidified incubator with 5% CO_2 in Middlebrook 7H9 medium (Difco) containing 0.2% glycerol, 0.5% bovine serum albu-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Weill Cornell Medical College, 1300 York Avenue, A-275A, Box 62, New York, NY 10065. Phone: (212) 746-2994. Fax: (212) 746-8587. E-mail: sae2004@med.cornell.edu.

▽ Published ahead of print on 14 November 2008.

min, 0.2% dextrose, 0.085% NaCl, and 0.05% Tween 80 or in Middlebrook 7H10 medium or 7H11 agar (Difco) containing 10% oleic acid-albumin-dextrose-catalase (Becton Dickinson). The Rv2224c mutant showed slightly reduced growth on 7H11 agar and was cultured on 7H10 medium.

Antibiotic susceptibility assays. *M. tuberculosis* strains were grown to early log phase and diluted to an optical density at 580 nm of 0.01 in enriched 7H9 medium containing Tween 80. Bacteria were then exposed to twofold dilutions of erythromycin, rifampin, chloramphenicol, ethambutol, isoniazid, and streptomycin (Sigma-Aldrich). The MIC was recorded as the minimum concentration at which no growth was observed after 2 to 3 weeks.

Measurement of sensitivity to SDS. *M. tuberculosis* strains were grown to early log phase, a 10-fold dilution series was made from optical densities at 580 nm of 0.01 to 0.0001, and 5 μl was spotted onto 7H10 or 7H11 agar plates containing 10% oleic acid-albumin-dextrose-catalase with or without 0.01% sodium dodecyl sulfate (SDS).

Measurement of sensitivity to heat, lysozyme, hydrogen peroxide, and nitric oxide. Early-log-phase cultures were centrifuged at 3,000 × *g* for 8 min and washed with enriched 7H9 medium containing 0.02% tyloxapol at a pH of 7.0. They were then centrifuged at 120 × *g* for 10 min to remove clumps. Single-cell suspensions were adjusted to ~5 × 10^6 CFU/ml in enriched 7H9 medium containing 0.02% tyloxapol at a pH of 7.0. The bacteria were incubated at 45°C for 24 h to measure sensitivity to heat, at 37°C for 2 h with 2,500 μg/ml lysozyme, or at 37°C for 2 or 4 h with 5 mM H_2O_2 (Sigma-Aldrich). To measure sensitivity to nitric oxide, the bacteria were incubated at 37°C with diethylenetriamine NONOate (DETA-NO) (Cayman Chemicals) for 3 days. DETA-NO (200 μM) was added every 24 h on days 0, 1, and 2. Numbers of CFU were determined by plating serial dilutions onto 7H10 or 7H11 agar plates. Percent survival was calculated by dividing the number of output CFU by the mean number of input CFU and then multiplying by 100.

Complementation. Rv2224c including the 300-bp region upstream of the start codon was cloned on an integrative vector conferring streptomycin resistance. The Rv2224c mutant was transformed by electroporation, and transformants were selected with 20 μg/ml streptomycin.

pH_{IB} measurements. Measurements were performed as previously described (41). Ratiometric pH-sensitive green fluorescent protein (GFP) (28) was cloned downstream of the mycobacterial promoter P_{smyc} and transformed into *M. tuberculosis* strains. Bone marrow-derived mouse macrophages (BMDMs) from 8- to 10-week-old C57BL/6 mice were differentiated in Dulbecco modified Eagle medium (GibcoBRL) supplemented with 20% L-cell conditioned medium, 10% fetal bovine serum, 0.58 g/liter L-glutamine, 1 mM pyruvate, and 10 mM HEPES, providing a nearly pure macrophage population as assessed by morphology and cell surface staining of CD14, F4/80, FcγRII/III, and MHC II molecules, the last one after IFN-γ activation. BMDMs were seeded at 1.5 × 10^5 cells in glass bottom no. 1.5 thickness poly-D-lysine-coated 35-mm culture dishes (MatTek). BMDMs were infected with single-cell suspensions of *M. tuberculosis* strains at a multiplicity of infection of 2:1 (2 *M. tuberculosis* bacteria per 1 macrophage) for 2 h, after which extracellular bacteria were removed by washing the plates twice with phosphate-buffered saline (PBS). For microscopy, cells were washed twice with PBS and placed in Dulbecco modified Eagle medium without phenol red (GibcoBRL), supplemented with 1% fetal bovine serum, 0.58 g/liter L-glutamine, 1 mM Na-pyruvate, and 10 mM HEPES. Microscopy was performed using a Leica DMIRB inverted fluorescence microscope fitted with a 63× objective, 1.4-numerical-aperture lens and Chroma Technology Corp pH-sensitive GFP filter set (exciters D410/30× and D470/20×, beamsplitter 500dcxr, emitter 535/50m). Image acquisition and analysis were performed using a Photometrics CoolSnap HQ digital camera and MetaMorph v.6.2r6 image analysis software (Universal Imaging Corporation, Downingtown, PA). All images within an experiment were acquired and analyzed under identical conditions. For display in histograms, average bacterial-group (1 to 5 bacteria) ratio intensities were determined. The pH_{IB} was derived by interpolating the 410/470 ratios on a standard curve.

Mouse infections. C57BL/6 8-week-old female mice (Jackson Laboratories) were infected by aerosol using a Middlebrook inhalation exposure system (Glas-Col) and early-log-phase *M. tuberculosis* cultures as single-cell suspensions in PBS to deliver ~100 to 200 bacilli per mouse or more where stated. Serial dilutions of organ homogenates from four or five mice per data point were plated onto 7H10 or 7H11 agar plates to quantify CFUs. The upper left lobes of infected lungs were fixed in 10% buffered formalin. Procedures involving mice were reviewed and approved by the Institutional Animal Care and Use Committee of Weill Cornell Medical College.

Statistics. The unpaired, two-tailed *t* test was used to assess the statistical significance of the comparison of experimental groups using GraphPad Prism software (http://www.graphpad.com).

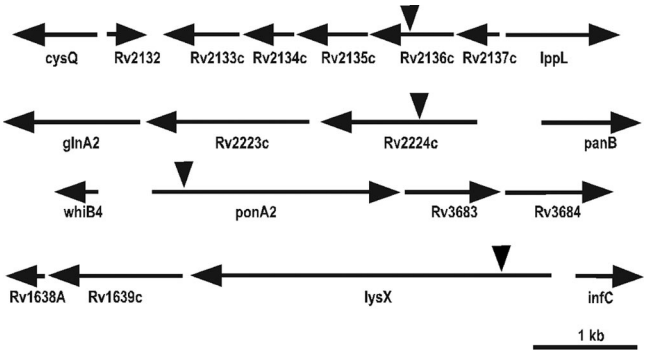

FIG. 1. Genomic organization of acid-sensitive mutants. Genes are depicted as arrows, and transposon insertion sites are indicated by triangles. Transposon insertions are at the following nucleotides (nt) within the respective gene: Rv2136c, nt 751; Rv2224c, nt 670; *ponA2*, nt 306; and *lysX*, nt 462.

RESULTS

Sensitivity of mutants to antibiotics, SDS, heat shock, and lysozyme. Figure 1 shows the genomic organization and transposon insertion sites of the acid-sensitive mutants. In the screen, we isolated six independent mutants with transposon insertions in *ponA2* and four mutants with transposon insertions in *lysX* (41). We focused on the mutants with the most N-terminal transposon insertions for phenotypic characterizations. We first examined the acid-sensitive *M. tuberculosis* mutants for defects associated with their cell wall. A cell wall permeability defect may result in enhanced penetration of protons into the cytosol of these mutants and hence make them susceptible to acid. We used hypersensitivity to lipophilic antibiotics as a measure of a cell wall permeability defect, based on the observation that the *Mycobacterium marinum kasB* mutant exhibits increased cell wall permeability and also enhanced susceptibility to lipophilic antibiotics (18). Previously, the Rv3671c and *lysX* mutants were shown to be hypersensitive to lipophilic antibiotics (41). Compared to wild-type *M. tuberculosis*, the Rv2136c, Rv2224c, and *ponA2* mutants were hypersensitive to the lipophilic antibiotics erythromycin (2- to 32-fold-lower MICs) and rifampin (2- to 8-fold-lower MICs) and were either not more sensitive or at most had twofold-lower MICs for the lipophilic antibiotic chloramphenicol and the nonlipophilic antibiotics ethambutol, isoniazid and streptomycin (Table 1). Of the three mutants tested, the Rv2136c mutant was as sensitive to both lipophilic and nonlipophilic antibiotics as an *M. tuberculosis erp* transposon mutant (Rv3810). Erp is believed to be required for the maintenance of mycobacterial cell wall integrity, and the *M. marinum erp* mutant is also hypersensitive to lipophilic antibiotics (8, 10).

The Rv2136c and Rv2224c mutants were also hypersensitive to the cell wall-perturbing detergent SDS, whereas the *ponA2* and *lysX* mutants grew like wild-type *M. tuberculosis* in the presence of SDS (Fig. 2A and B). The Rv2224c mutant also grew more slowly than wild-type *M. tuberculosis* on 7H11 agar plates in the absence of SDS (Fig. 2B). Complementation of the Rv2224c mutant with a single copy of the Rv2224c wild type restored growth of the mutant on agar (Fig. 2B), sensitivity to SDS (Fig. 2B), and its survival at pH 4.5 (not shown).

The Rv2136c and *ponA2* mutants were killed 11-fold and

TABLE 1. Sensitivity of mutants to antibiotics

Strain	MIC (μg/ml)[a]					
	Erythr	Rifampin	Chloramp	Etham	INH	Strep
H37Rv	1,280	0.0120	8	1.2	0.05	1.0
Rv2136c mutant	40	0.0015	4	1.2	0.05	0.5
Rv2224c mutant	160	0.0030	8	0.6	0.05	0.5
ponA2 mutant	640	0.0060	8	1.2	0.05	0.5
erp mutant	40	0.0015	4	1.2	0.05	0.5

[a] Erythromycin (Erythr), rifampin, and chloramphenicol (Chloramp) represent lipophilic antibiotics, and ethambutol (Etham), isoniazid (INH), and streptomycin (Strep) are nonlipophilic antibiotics. The MIC is the minimum concentration at which no growth was observed after 2 to 3 weeks. Data are representative of the results for three independent experiments.

sevenfold more, respectively, than wild-type *M. tuberculosis*, after exposure to heat (Fig. 3). Sensitivity to heat may be due to an impaired heat shock response or to an aberrant peptidoglycan layer, as *E. coli* mutants with defects in peptidoglycan biosynthesis have been reported to be thermosensitive (9, 14, 39). Compared to wild-type *M. tuberculosis*, only the *ponA2* mutant was killed sixfold more after exposure to lysozyme, whereas the Rv2136c, Rv2224c, and *lysX* mutants were not hypersensitive to this enzyme (Fig. 4).

Despite sensitivity to cell wall stresses, none of the mutants displayed an evident cording or colony morphology defect, and individual cells were morphologically similar to wild-type *M. tuberculosis* when visualized by scanning or transmission electron microscopy (not shown).

Sensitivity of mutants to oxidative and nitrosative stress. Macrophages produce both reactive oxygen and nitrogen intermediates (ROIs and RNIs, respectively) as defenses against microbial pathogens. RNIs and ROIs become more potent at an acidic pH (22, 37). To examine for increased sensitivity to oxidative and nitrosative stress independently of the acid sensitivity, the mutants were exposed to H_2O_2 and the nitric oxide donor DETA-NO at a pH of 7. After exposure to H_2O_2, the Rv2136c, Rv2224c, and *ponA2* mutants were killed 19-, 13-, and 11-fold more, respectively, than wild-type *M. tuberculosis*. The *lysX* mutant survived at a rate similar to that of wild-type *M. tuberculosis* in the presence of H_2O_2 (Fig. 5A). After exposure to DETA-NO, the Rv2136c and Rv2224c mutants were killed eight- and sevenfold more, respectively, than was wild-type *M. tuberculosis*, whereas the titers of *lysX* and *ponA2* mutants were not significantly lower than that of wild-type *M. tuberculosis* (Fig. 5B). The complemented Rv2224c mutant also survived at a rate similar to that of wild-type *M. tuberculosis* after exposure to H_2O_2 and DETA-NO (Fig. 5C).

Measurement of pH_{IB}. In our previous study, we demonstrated that the Rv3671c and Rv2136c mutants were unable to maintain their pH_{IB} in buffer at pH 4.5, whereas the Rv2224c, *lysX*, and *ponA2* mutants had no marked defect compared to wild-type *M. tuberculosis* (41). Here, we tested the Rv2136c mutant for its ability to maintain its pH_{IB} in macrophages by transforming the strains with pH-sensitive ratiometric GFP (28). Like the Rv3671c mutant, the Rv2136c mutant was unable to maintain its pH_{IB} in IFN-γ-activated macrophages but displayed no defect in pH_{IB} maintenance in nonactivated mac-

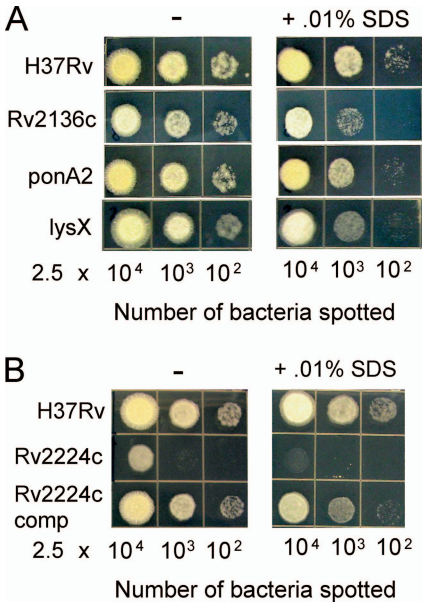

FIG. 2. Sensitivity of mutants to SDS. Indicated numbers of *M. tuberculosis* wild-type (H37Rv) or mutant bacteria were spotted onto 7H11 (A) or 7H10 (B) agar with (+) or without (−) 0.01% SDS. Growth was visualized 14 days after spotting. Data are representative of the results for three independent experiments.

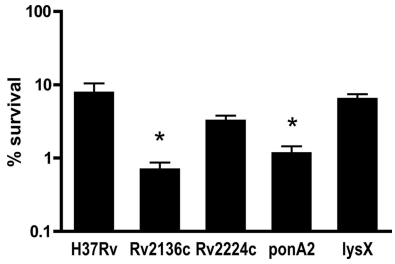

FIG. 3. Sensitivity of mutants to heat. Survival of *M. tuberculosis* wild-type (H37Rv) and mutant bacteria after incubation for 24 h at 45°C. Bacterial input was 0.5×10^7 to 1×10^7 CFU/ml. Raw data were normalized to the input CFU for each strain. Data are means ± standard errors of the results for two independent experiments, each performed with triplicate cultures. Statistically significant differences in survival relative to that of H37Rv are indicated (*, $P < 0.05$).

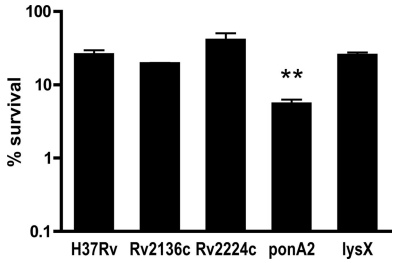

FIG. 4. Sensitivity of mutants to lysozyme. Survival of *M. tuberculosis* wild-type (H37Rv) and mutant bacteria after incubation with 2,500 μg/ml of lysozyme for 24 h. Bacterial input was 0.5×10^7 to 1×10^7 CFU/ml. Raw data were normalized to the number of input CFU of each strain. Data are means ± standard deviations from triplicate cultures. Statistically significant differences in survival relative to that of H37Rv are indicated by asterisks (**, $P < 0.01$).

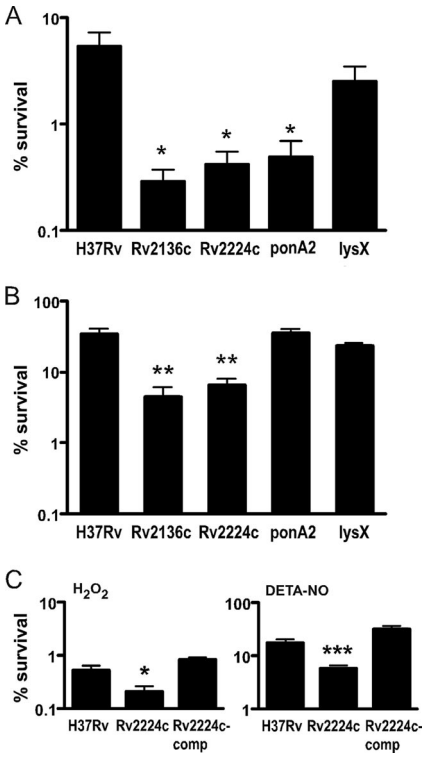

FIG. 5. Sensitivity of mutants to hydrogen peroxide and nitric oxide. Survival of *M. tuberculosis* wild-type (H37Rv) and mutant bacteria after incubation with 5 mM H_2O_2 for 2 h (A) or 200 μM DETA-NO added every 24 h for 3 days (B). Bacterial input was 0.5×10^7 to 1×10^7 CFU/ml. Raw data were normalized to the number of input CFU of each strain. Data are means ± standard errors of the results for two independent experiments, each performed with triplicate cultures. (C) Survival of wild-type *M. tuberculosis* (H37Rv), the Rv2224c mutant, and the complemented mutant (Rv2224c-comp) after incubation with 5 mM H_2O_2 for 4 h or 200 μM DETA-NO for 3 days. Bacterial input was 0.1×10^7 to 1×10^7 CFU/ml. Raw data were normalized to the number of input CFU of each strain. Statistically significant differences in survival relative to that of H37Rv are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$).

rophages (Fig. 6A and B). In nonactivated macrophages, the majority of both wild-type *M. tuberculosis* and Rv2136c mutant bacteria had pH_{IB} levels of 6.76 to 7.25 (Fig. 6A). In IFN-γ-activated macrophages, wild-type *M. tuberculosis* maintained its pH_{IB} between 6.76 and 7.25, whereas 61% of Rv2136c mutant bacteria were at a pH_{IB} of <6.75 (Fig. 6B).

Growth and survival in mice. Finally we measured growth and survival of the mutants in mice after aerosol infection. The Rv2136c mutant was severely attenuated in mouse lungs and spleens compared to wild-type *M. tuberculosis* (Fig. 7A and B). Growth during the acute phase of infection between days 1 and 21 was severely impaired, and after day 21, the mutant was cleared such that no bacteria were detected in lungs and spleens at day 56 of infection (Fig. 7A and B). The Rv2136c mutant also induced markedly reduced gross pathology in mouse lungs (Fig. 7C). The *ponA2* mutant displayed milder attenuation (Fig. 7A, B, and C). At day 56 postinfection, approximately 16- and 10-fold-fewer *ponA2* mutant bacteria than wild-type *M. tuberculosis* bacteria were found in lungs and spleens, respectively. The Rv2224c mutant was also attenuated in mice, but only by threefold in lungs at day 56 and day 150 (Fig. 7D and E). Complementation of the Rv2224c mutant with a single copy of the wild-type allele restored this defect in virulence. The complemented Rv2224c strain grew to higher titers than did wild-type *M. tuberculosis*; the mechanism that caused increased replication remains to be determined.

DISCUSSION

M. tuberculosis can reside in phagosomes that fuse with lysosomes and acidify to a pH of 4.5 in activated macrophages (21, 34). Because *M. tuberculosis* survives in the acidic phagolysosomes of activated macrophage, we sought to identify genes required by *M. tuberculosis* to resist low pH. In a previous study, we screened 10,100 *M. tuberculosis* transposon mutants and isolated 21 mutants that were hypersensitive to a pH of 4.5. The majority of these mutants exhibited reduced survival at pH 4.5 in 7H9 medium only in the presence of Tween 80. However, five mutants with transposon insertions in Rv3671c, Rv2136c, *lysX*, Rv2224c, and *ponA2* were attenuated at pH 4.5 in 7H9 growth medium containing either the detergent Tween 80 or tyloxapol. In the present study, we report the further characterization of the Rv2136c, Rv2224c, *ponA2*, and *lysX* mutants.

The Rv2136c mutant was highly sensitive to lipophilic anti-

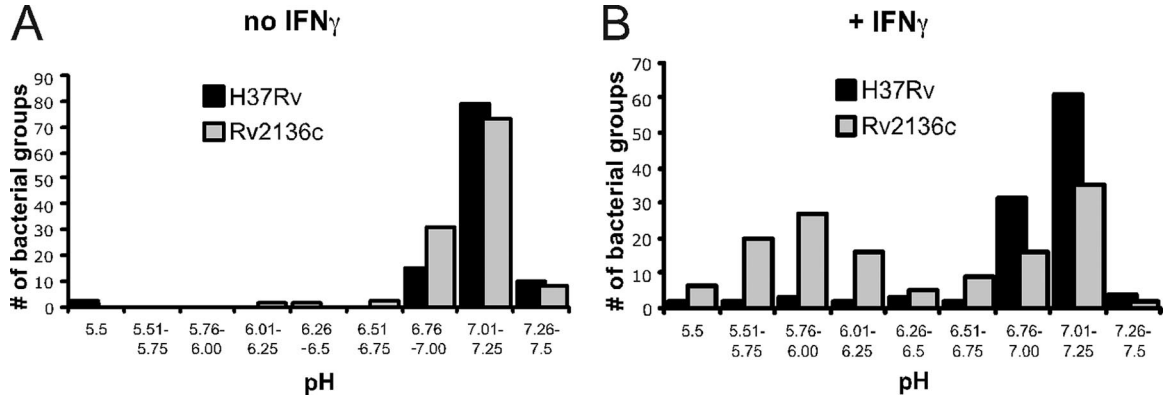

FIG. 6. The Rv2136c mutant fails to maintain neutral pH_{IB} in IFN-γ-activated macrophages. Number of wild-type *M. tuberculosis* (H37Rv) (black bars) and Rv2136c mutant (gray bars) bacterial groups plotted against their pH_{IB} in nonactivated (A) and IFN-γ-activated (B) macrophages at 24 h postinfection. Data are representative of the results for two independent experiments.

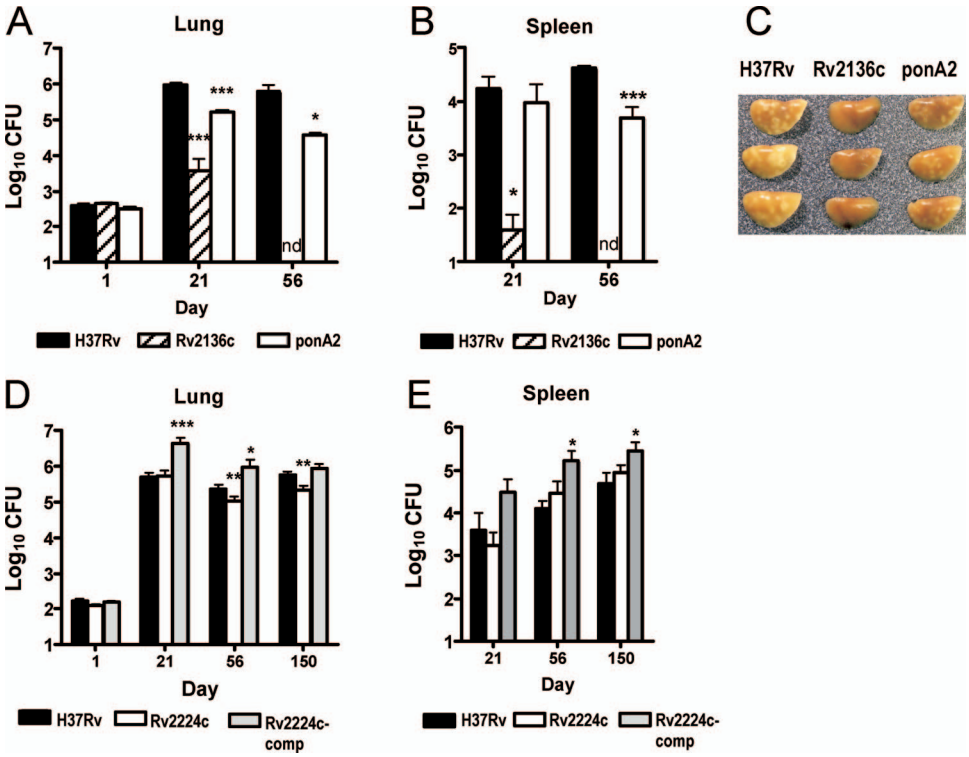

FIG. 7. Survival of mutants in mice. Lung (A) and spleen (B) bacterial loads from mice infected with wild-type *M. tuberculosis* (H37Rv, black bars), the Rv2136c mutant (hatched bars), and the *ponA2* mutant (white bars) at indicated time points. nd indicates that the number of CFU was below the limit of detection, which was 10 CFU/organ. Data are means ± standard deviations from four mice per time point and group and represent the results for two independent experiments. Statistically significant differences in the number of CFU relative to that of H37Rv are indicated by asterisks (*, $P < 0.05$; ***, $P < 0.0001$). (C) Gross pathology of lungs infected with wild-type *M. tuberculosis* (H37Rv) (left lane), the Rv2136c mutant (middle lane), or the *ponA2* mutant (right lane) at day 56 postinfection. Lung (D) and spleen (E) bacterial loads from mice infected with wild-type *M. tuberculosis* (H37Rv) (black bars), the Rv2224c mutant (blacks bars), or the Rv2224c complemented mutant (Rv2224c-comp) (gray bars) at indicated time points. Data are means ± standard deviations from five mice per time point and group and represent the results from two independent experiments. Statistically significant differences in the number of CFU relative to that of H37Rv are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$).

biotics, displaying the same degree of sensitivity as an *M. tuberculosis erp* mutant, and was also attenuated after exposure to SDS, heat, H_2O_2, or DETA-NO. In an earlier report, we observed that this mutant was unable to maintain its pH_{IB} in acid in vitro (41), and here, we show that it is also unable to maintain its pH_{IB} in IFN-γ-activated macrophages. The hypersensitivity of the Rv2136c mutant to low pH, oxidative and nitrosative stress, and a possible cell wall defect may explain its severe attenuation in vivo. The Rv2136c protein is a homolog of *Escherichia coli* BacA (7). BacA has now been named UppP because it is an undecaprenol pyrophosphate phosphatase (13) and not an undecaprenol kinase as was originally thought (5). Undecaprenol pyrophosphate serves as a lipid carrier and binds to disaccharide-pentapeptide peptidoglycan subunits to form lipid II. The subunits are transported across the lipid bilayer for polymerization into mature peptidoglycan. Undecaprenol pyrophosphate is then dephosphorylated by UppP and recycled for use in the pathway. These steps of peptidoglycan biosynthesis are considered to be attractive drug targets, and lipid II is targeted by at least four classes of natural product antibiotics (3). Deletion of BacA homologs in *Staphylococcus aureus* and *Streptococcus pneumoniae* attenuates the bacteria in the mouse model of infection (6). A *Mycobacterium smegmatis* mutant deficient in the Rv2136c homolog was also attenuated for virulence in a mouse model of smegma development (33). We were unable to complement the Rv2136c mutant with a single copy of the gene or the putative operon (Rv2133 to Rv2137). Rose et al. (33) were also unable to fully complement all phenotypes of the *M. smegmatis* Rv2136c mutant, suggesting that optimal expression of the single gene or operon is required for restoration of phenotypes. We confirmed that the Rv2136c mutant contains a single transposon insertion by Southern blotting (not shown), but it is possible that a secondary mutation on the chromosome may be causing the defects we observed. However, it is tempting to speculate that the aforementioned pathways of peptidoglycan biosynthesis are required for resistance to low pH because a *Streptococcus mutans* strain deficient in an undecaprenol kinase was also sensitive to acid (24, 43).

Flores and colleagues isolated a β-lactam-hypersensitive *M. smegmatis* mutant in *expA*, an Rv2224c homolog, that had swollen termini and showed increased sensitivity to lysozyme (15). It was proposed that ExpA is either directly or indirectly involved in cell wall remodeling and may regulate autolysis (15). In *M. tuberculosis*, Rv2224c protein is detected in the cell envelope (25), and its expression is upregulated during nutri-

ent starvation (2) and in THP-1 macrophage-like cells (12). In the absence of Rv2224c protein, *M. tuberculosis* elicited lower levels of proinflammatory cytokines and chemokines from macrophages and exhibited reduced growth in macrophages (32). Rv2224c protein was also shown to be important for *M. tuberculosis* virulence in mice (25, 32). Although Rv2224c protein is annotated as a putative protease, Lun and Bishai were unable to observe cleavage of protein substrates in vitro and demonstrated that Rv2224c protein possesses esterase activity (25). Rengarajan et al. observed reduced processing of the cell wall heat shock protein GroEL2 in the Rv2224c mutant, but whether GroEL2 is directly processed by Rv2224c protein remains to be examined (32). We found that the Rv2224c mutant was sensitive to antibiotics, SDS, heat, and oxidative and nitrosative stress and confirmed that the mutant is attenuated in vivo. The Rv2224c mutant displayed normal cell morphology by scanning and transmission electron microscopy and was not markedly susceptible to exposure to lysozyme.

PonA2 is predicted to have transglycosylase and transpeptidase activities, and it is believed to be involved in peptidoglycan biosynthesis (7). Flores et al. isolated an *M. tuberculosis* mutant with a transposon insertion in *ponA2* that was hypersusceptible to β-lactam antibiotics, suggesting that the mutant has a deficiency in its peptidoglycan layer (15). Mycobacteria have two types of peptide cross-links in their peptidoglycan, the 4→3 (diaminopimelic acid [DAP]-Ala) linkage and the more unusual 3→3 (DAP-DAP) linkage. It has been predicted that PonA2 may be involved in the formation of the 3→3 meso-diaminopimelate linkage (15, 19). The 3→3 linkage may be required during periods of slow growth, such as stationary phase, because the number of these cross bridges increases over 4→3 linkages in slow-growing *E. coli* and in *M. tuberculosis* during stationary phase (11, 23, 40). We found that although the *M. tuberculosis ponA2* mutant was not markedly sensitive to antibiotics or SDS, it was more sensitive to heat, H_2O_2, and NO than was wild-type *M. tuberculosis* and attenuated in mouse lungs and spleens.

M. tuberculosis possesses two genes, *lysS* and *lysX*, annotated as lysine tRNA synthetases, LysS and LysX (7). Unlike LysS, LysX contains a predicted N-terminal transmembrane domain. LysX may contribute to peptidoglycan peptide bridge formation, as has been shown for tRNA synthetases of other bacteria (30). Alternatively, a deficiency in *lysX* may lead to accumulation of lysine and feedback inhibition of the lysine biosynthetic pathway. As diaminopimelate is an intermediate in this pathway and also a key component of *M. tuberculosis*'s peptidoglycan pentapeptide bridge, a blockage in lysine biosynthesis may lead to peptidoglycan defects. Compared to the other mutants studied in this report, the *lysX* mutant showed only moderately increased sensitivity to SDS, heat, H_2O_2, and DETA-NO. The *lysX* mutant was, however, hypersensitive to the lipophilic antibiotics erythromycin and rifampin but was not attenuated in mice (41).

All of the acid-sensitive mutants studied in this report appear to have some defect in the function of their cell wall, as they were sensitive to certain lipophilic antibiotics and SDS. Three of the four mutants were also hypersensitive to oxidative or nitrosative stress at neutral pH. These data indicate that some mechanisms that confer resistance to acid also provide cross protection against other forms of extracellular stress and reinforce the important role of *M. tuberculosis*'s cell wall in protection against diverse stresses. In *Salmonella*, acid shock induces cross protection against a variety of other stresses, such as heat, oxidative stress, and osmotic stress (17). The Rv2136c mutant was unable to maintain a neutral pH_{IB} in acidic buffer in vitro (41) and in IFN-γ-activated macrophages and was severely attenuated in vivo. The same was observed for the Rv3671c mutant (41), suggesting that the inability to maintain a neutral pH_{IB} is associated with severe attenuation in mice. Phagolysosomal acid synergizes with various components of the host immune response, such as lysosomal hydrolases and reactive oxygen and nitrogen species. The *ponA2*, Rv2224c, and Rv2136c mutants may be attenuated during infection because they succumb to the low pH of the phagosome, cell wall damage, and/or nitrosative and oxidative defenses issued by the host. Thus, pathways utilized by *Mycobacterium tuberculosis* for acid resistance and pH_{IB} homeostasis are attractive chemotherapeutic targets.

ACKNOWLEDGMENTS

This work was funded by I.T. Hirschl Trust (S.E.) and NIH PO1 grant AI056293 (C.F.N.). The Department of Microbiology and Immunology acknowledges the support of the William Randolph Hearst Foundation.

We thank Lynda M. Pierini for assistance with intraphagosomal pH_{IB} measurements, Gero Miesenböck for providing ratiometric pH GFP, and Lee Cohen-Gould, Rebecca Smith, and Tony Labissiere at the Electron Microscopy and Histology Core Facilities at Weill Cornell Medical College and Hospital for Special Surgery for assistance with electron microscopy.

REFERENCES

1. **Armstrong, J. A., and P. D. Hart.** 1971. Response of cultured macrophages to *Mycobacterium tuberculosis*, with observations on fusion of lysosomes with phagosomes. J. Exp. Med. **134:**713–740.
2. **Betts, J. C., P. T. Lukey, L. C. Robb, R. A. McAdam, and K. Duncan.** 2002. Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling. Mol. Microbiol. **43:** 717–731.
3. **Breukink, E., and B. de Kruijff.** 2006. Lipid II as a target for antibiotics. Nat. Rev. Drug Discov. **5:**321–332.
4. **Buchmeier, N., A. Blanc-Potard, S. Ehrt, D. Piddington, L. Riley, and E. A. Groisman.** 2000. A parallel intraphagosomal survival strategy shared by *Mycobacterium tuberculosis* and *Salmonella enterica*. Mol. Microbiol. **35:**1375–1382.
5. **Cain, B. D., P. J. Norton, W. Eubanks, H. S. Nick, and C. M. Allen.** 1993. Amplification of the *bacA* gene confers bacitracin resistance to *Escherichia coli*. J. Bacteriol. **175:**3784–3789.
6. **Chalker, A. F., K. A. Ingraham, R. D. Lunsford, A. P. Bryant, J. Bryant, N. G. Wallis, J. P. Broskey, S. C. Pearson, and D. J. Holmes.** 2000. The *bacA* gene, which determines bacitracin susceptibility in *Streptococcus pneumoniae* and *Staphylococcus aureus*, is also required for virulence. Microbiology **146** (Pt. 7)**:**1547–1553.
7. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry III, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, A. Krogh, J. McLean, S. Moule, L. Murphy, K. Oliver, J. Osborne, M. A. Quail, M. A. Rajandream, J. Rogers, S. Rutter, K. Seeger, J. Skelton, R. Squares, S. Squares, J. E. Sulston, K. Taylor, S. Whitehead, and B. G. Barrell.** 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature **393:**537–544.
8. **Cosma, C. L., K. Klein, R. Kim, D. Beery, and L. Ramakrishnan.** 2006. *Mycobacterium marinum* Erp is a virulence determinant required for cell wall integrity and intracellular survival. Infect. Immun. **74:**3125–3133.
9. **Dai, D., and E. E. Ishiguro.** 1988. *murH*, a new genetic locus in *Escherichia coli* involved in cell wall peptidoglycan biosynthesis. J. Bacteriol. **170:**2197–2201.
10. **de Mendonca-Lima, L., M. Picardeau, C. Raynaud, J. Rauzier, Y. O. de la Salmoniere, L. Barker, F. Bigi, A. Cataldi, B. Gicquel, and J. M. Reyrat.** 2001. Erp, an extracellular protein family specific to mycobacteria. Microbiology **147:**2315–2320.

11. **Driehuis, F., and J. T. Wouters.** 1987. Effect of growth rate and cell shape on the peptidoglycan composition in *Escherichia coli*. J. Bacteriol. **169:**97–101.
12. **Dubnau, E., P. Fontan, R. Manganelli, S. Soares-Appel, and I. Smith.** 2002. *Mycobacterium tuberculosis* genes induced during infection of human macrophages. Infect. Immun. **70:**2787–2795.
13. **El Ghachi, M., A. Bouhss, D. Blanot, and D. Mengin-Lecreulx.** 2004. The *bacA* gene of *Escherichia coli* encodes an undecaprenyl pyrophosphate phosphatase activity. J. Biol. Chem. **279:**30106–30113.
14. **El Ghachi, M., A. Derbise, A. Bouhss, and D. Mengin-Lecreulx.** 2005. Identification of multiple genes encoding membrane proteins with undecaprenyl pyrophosphate phosphatase (UppP) activity in *Escherichia coli*. J. Biol. Chem. **280:**18689–18695.
15. **Flores, A. R., L. M. Parsons, and M. S. Pavelka, Jr.** 2005. Characterization of novel *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* mutants hypersusceptible to beta-lactam antibiotics. J. Bacteriol. **187**:1892–1900.
16. **Foster, J. W.** 1999. When protons attack: microbial strategies of acid adaptation. Curr. Opin. Microbiol. **2:**170–174.
17. **Foster, J. W., and M. P. Spector.** 1995. How *Salmonella* survive against the odds. Annu. Rev. Microbiol. **49:**145–174.
18. **Gao, L. Y., F. Laval, E. H. Lawson, R. K. Groger, A. Woodruff, J. H. Morisaki, J. S. Cox, M. Daffe, and E. J. Brown.** 2003. Requirement for *kasB* in *Mycobacterium* mycolic acid biosynthesis, cell wall impermeability and intracellular survival: implications for therapy. Mol. Microbiol. **49:**1547–1563.
19. **Goffin, C., and J. M. Ghuysen.** 2002. Biochemistry and comparative genomics of SxxK superfamily acyltransferases offer a clue to the mycobacterial paradox: presence of penicillin-susceptible target proteins versus lack of efficiency of penicillin as therapeutic agent. Microbiol. Mol. Biol. Rev. **66:** 702–738.
20. **Gunzel, D., L. M. Kucharski, D. G. Kehres, M. F. Romero, and M. E. Maguire.** 2006. The MgtC virulence factor of *Salmonella enterica* serovar Typhimurium activates Na^+,K^+-ATPase. J. Bacteriol. **188:**5586–5594.
21. **Huynh, K. K., and S. Grinstein.** 2007. Regulation of vacuolar pH and its modulation by some microbial species. Microbiol. Mol. Biol. Rev. **71:**452–462.
22. **Jackett, P. S., V. R. Aber, and D. B. Lowrie.** 1978. Virulence and resistance to superoxide, low pH and hydrogen peroxide among strains of *Mycobacterium tuberculosis*. J. Gen. Microbiol. **104:**37–45.
23. **Lavollay, M., M. Arthur, M. Fourgeaud, L. Dubost, A. Marie, N. Veziris, D. Blanot, L. Gutmann, and J. L. Mainardi.** 2008. The peptidoglycan of stationary-phase *Mycobacterium tuberculosis* predominantly contains cross-links generated by L,D-transpeptidation. J. Bacteriol. **190:**4360–4366.
24. **Lis, M., and H. K. Kuramitsu.** 2003. The stress-responsive *dgk* gene from *Streptococcus mutans* encodes a putative undecaprenol kinase activity. Infect. Immun. **71:**1938–1943.
25. **Lun, S., and W. R. Bishai.** 2007. Characterization of a novel cell wall-anchored protein with carboxyesterase activity required for virulence in mycobacterium tuberculosis. J. Biol. Chem. **282:**18348–18356.
26. **MacMicking, J. D., G. A. Taylor, and J. D. McKinney.** 2003. Immune control of tuberculosis by IFN-gamma-inducible LRG-47. Science **302:**654–659.
27. **Merrell, D. S., and A. Camilli.** 2002. Acid tolerance of gastrointestinal pathogens. Curr. Opin. Microbiol. **5:**51–55.
28. **Miesenbock, G., D. A. De Angelis, and J. E. Rothman.** 1998. Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. Nature **394:**192–195.
29. **Molle, V., N. Saint, S. Campagna, L. Kremer, E. Lea, P. Draper, and G. Molle.** 2006. pH-dependent pore-forming activity of OmpATb from *Mycobacterium tuberculosis* and characterization of the channel by peptidic dissection. Mol. Microbiol. **61:**826–837.
30. **Neihardt, F. C., J. Parker, and W. G. McKeever.** 1975. Function and regulation of aminoacyl-tRNA synthetases in prokaryotic and eukaryotic cells. Annu. Rev. Microbiol. **29:**215–250.
31. **Raynaud, C., K. G. Papavinasasundaram, R. A. Speight, B. Springer, P. Sander, E. C. Bottger, M. J. Colston, and P. Draper.** 2002. The functions of OmpATb, a pore-forming protein of *Mycobacterium tuberculosis*. Mol. Microbiol. **46:**191–201.
32. **Rengarajan, J., E. Murphy, A. Park, C. L. Krone, E. C. Hett, B. R. Bloom, L. H. Glimcher, and E. J. Rubin.** 2008. *Mycobacterium tuberculosis* Rv2224c modulates innate immune responses. Proc. Natl. Acad. Sci. USA **105:**264–269.
33. **Rose, L., S. H. Kaufmann, and S. Daugelat.** 2004. Involvement of *Mycobacterium smegmatis* undecaprenyl phosphokinase in biofilm and smegma formation. Microbes Infect. **6:**965–971.
34. **Russell, D. G.** 2001. Mycobacterium tuberculosis: here today, and here tomorrow. Nat. Rev. Mol. Cell Biol. **2:**569–577.
35. **Schaible, U. E., S. Sturgill-Koszycki, P. H. Schlesinger, and D. G. Russell.** 1998. Cytokine activation leads to acidification and increases maturation of *Mycobacterium avium*-containing phagosomes in murine macrophages. J. Immunol. **160:**1290–1296.
36. **Sibley, L. D., S. G. Franzblau, and J. L. Krahenbuhl.** 1987. Intracellular fate of *Mycobacterium leprae* in normal and activated mouse macrophages. Infect. Immun. **55:**680–685.
37. **Stuehr, D. J., and C. F. Nathan.** 1989. Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. J. Exp. Med. **169:**1543–1555.
38. **Sturgill-Koszycki, S., P. H. Schlesinger, P. Chakraborty, P. L. Haddix, H. L. Collins, A. K. Fok, R. D. Allen, S. L. Gluck, J. Heuser, and D. G. Russell.** 1994. Lack of acidification in *Mycobacterium* phagosomes produced by exclusion of the vesicular proton-ATPase. Science **263:**678–681.
39. **Suzuki, H., Y. Nishimura, and Y. Hirota.** 1978. On the process of cellular division in *Escherichia coli*: a series of mutants of *E. coli* altered in the penicillin-binding proteins. Proc. Natl. Acad. Sci. USA **75:**664–668.
40. **Tuomanen, E., and R. Cozens.** 1987. Changes in peptidoglycan composition and penicillin-binding proteins in slowly growing *Escherichia coli*. J. Bacteriol. **169:**5308–5310.
41. **Vandal, O. H., L. M. Pierini, D. Schnappinger, C. F. Nathan, and S. Ehrt.** 2008. A membrane protein preserves intrabacterial pH in intraphagosomal *Mycobacterium tuberculosis*. Nat. Med. **14:**849–854.
42. **Via, L. E., R. A. Fratti, M. McFalone, E. Pagan-Ramos, D. Deretic, and V. Deretic.** 1998. Effects of cytokines on mycobacterial phagosome maturation. J. Cell Sci. **111**(Pt. 7)**:**897–905.
43. **Yamashita, Y., T. Takehara, and H. K. Kuramitsu.** 1993. Molecular characterization of a *Streptococcus mutans* mutant altered in environmental stress responses. J. Bacteriol. **175:**6220–6228.