JOURNAL OF BACTERIOLOGY, Mar. 2007, p. 2435–2442 Vol. 189, No. 6
0021-9193/07/$08.00+0 doi:10.1128/JB.01600-06

Porins Are Required for Uptake of Phosphates by *Mycobacterium smegmatis*▽

Frank Wolschendorf,[1] Maysa Mahfoud,[2] and Michael Niederweis[1,2]*

Department of Microbiology, University of Alabama at Birmingham, 609 Bevill Biomedical Research Building, 845 19th Street South, Birmingham, Alabama 35294,[1] and Lehrstuhl für Mikrobiologie, Friedrich-Alexander-Universität Erlangen-Nürnberg, Staudtstr. 5, D-91058 Erlangen, Germany[2]

Received 13 October 2006/Accepted 21 December 2006

Phosphorus is an essential nutrient, but how phosphates cross the mycobacterial cell wall is unknown. Phosphatase activity in whole cells of *Mycobacterium smegmatis* was significantly lower than that in lysed cells, indicating that access to the substrate was restricted. The loss of the outer membrane (OM) porin MspA also reduced the phosphatase activity in whole cells compared to that in lysed cells. A similar result was obtained for *M. smegmatis* that overexpressed endogenous alkaline phosphatase, indicating that PhoA is not a surface protein, contrary to a previous report. The uptake of phosphate by a mutant lacking the porins MspA and MspC was twofold lower than that by wild-type *M. smegmatis*. Strikingly, the loss of these porins resulted in a severe growth defect of *M. smegmatis* on low-phosphate plates. We concluded that the OM of *M. smegmatis* represents a permeability barrier for phosphates and that Msp porins are the only OM channels for the diffusion of phosphate in *M. smegmatis*. However, phosphate diffusion through Msp pores is rather inefficient as shown by the 10-fold lower permeability of *M. smegmatis* for phosphate compared to that for glucose. This is likely due to the negative charges in the constriction zone of Msp porins. The phosphatase activity in whole cells of *Mycobacterium bovis* BCG was significantly less than that in lysed cells, indicating a similar uptake pathway for phosphates in slow-growing mycobacteria. However, porins that could mediate the diffusion of phosphates across the OM of *M. bovis* BCG and *Mycobacterium tuberculosis* are unknown.

Phosphorus is indispensable for the biosynthesis of nucleic acids and phospholipids and for the energy supply of any cell. Bacteria employ sophisticated transport mechanisms to acquire phosphorus-containing nutrients from the environment. In gram-negative bacteria, phosphates first need to cross the outer membrane (OM). To this end, *Escherichia coli* produces the two general porins, OmpF and OmpC, under conditions of phosphate excess. Under phosphate-limiting conditions, these porins are partially replaced by the pore protein PhoE (30), which preferentially allows the diffusion of anions (1), in contrast to the cation preference of OmpF and OmpC (29). Hence, the diffusion of phosphates through PhoE pores is more efficient and is the prevalent pathway for phosphates across the outer membrane under phosphate-limiting conditions (17). While inorganic phosphate is the preferred source of phosphorus, many bacteria can also take up organic phosphates and release phosphate by the action of periplasmic phosphatases such as PhoA. *E. coli* possesses four transport systems, Pst, Pit, GlpT, and UhpT, that translocate inorganic phosphate across the inner membrane (48). Part of the Pst system is the periplasmic protein PstS, which binds and transfers phosphate to the transmembrane components PstA and PstC. PstB hydrolyzes ATP and delivers energy for the translocation of phosphate across the inner membrane by PstA/PstC. Pst systems bind and transport phosphate with binding constants and apparent transport K_m values in the submicromolar range. These systems also exist in gram-positive bacteria (32) and in mycobacteria (5).

Mycobacteria are classified as gram-positive bacteria, but models of their cell walls include asymmetrical outer membranes that are covalently bound to an arabinogalactan-peptidoglycan copolymer (7, 25, 28). These models are consistent with the very efficient permeability barrier established by mycobacterial cell walls towards hydrophobic and hydrophilic compounds (7). The discovery of channel-forming outer membrane proteins in several mycobacteria lends further support to these models (26).

It was conclusively shown that MspA represents the major porin of *M. smegmatis* (43) and is required for the transport of glucose, serine, and hydrophilic β-lactam antibiotics (44, 45). However, the preference of MspA for cations (27) and the high density of negative charges in the constriction zone of MspA (14) appear to be adverse properties for efficient diffusion of phosphates. Thus, anions may cross the outer membrane of *M. smegmatis* via PhoE-like porins as in gram-negative bacteria. Importantly, the transport of phosphate across the inner membrane is essential for the growth of *Mycobacterium tuberculosis* in macrophages (34) and for the survival of mice (31, 38). However, how phosphate is transported across the outer membrane of *M. tuberculosis* is also unknown.

In this study, we used *phoA* as a reporter gene and analyzed the uptake of radiolabeled phosphate to examine the uptake of organic phosphate esters and inorganic phosphate by *M. smegmatis*. We show that the outer membrane represents a permeability barrier for phosphates in both assays, in contrast to previous claims that PhoA and PstS are cell surface proteins (18, 19). Importantly, we demonstrate that Msp porins are required for the

* Corresponding author. Mailing address: Department of Microbiology, University of Alabama at Birmingham, Mail Box 24, 609 Bevill Biomedical Research Building, 845 19th Street South, Birmingham, AL 35294. Phone: (205) 996-2711. Fax: (205) 934-9256. E-mail: mnieder@uab.edu.

▽ Published ahead of print on 5 January 2007.

TABLE 1. Bacterial strains and plasmids used in this study[a]

Strain or plasmid	Relevant genotype or description	Source or reference
Strains		
E. coli DH5α	*recA1 endA1 gyrA96 thi relA1 hsdR17*(r_K^- m_K^+) *supE44* ϕ80Δ*lacZ*Δ*M15* Δ*lacZYA-argF UE169*	36
M. smegmatis SMR5	mc^2155 derivative, not characterized, high transformation efficiency, Sm^r (*rpsL**)	37
M. smegmatis MN01	SMR5 derivative *mspA*::Gm^r	43
M. smegmatis ML10	SMR5 derivative Δ*mspA* Δ*mspC*	45
M. smegmatis ML40	mc^2155 derivative, *attB*::pML443, Hyg^r	This study
M. smegmatis ML41	ML10 derivative, *attB*::pML443, Hyg^r	This study
M. smegmatis ML42	ML10 derivative, *attB*::pML443, Δ*hyg*	This study
M. smegmatis ML43	MN01 derivative, *attB*::pML443, Hyg^r	This study
M. bovis BCG	Pasteur 35739	ATCC
M. bovis BCG ML44	*M. bovis* BCG derivative, *attB*::pML443	This study
Plasmids		
pMV361	*oriE aph attP int*	47
pMS2	PAL5000 origin, ColE1 origin, *hyg*	16
pMN402	PAL5000 origin, ColE1 origin, *hyg*, p_{hsp60}-*gfp*$^+$	40
pMN234	PAL5000 origin, pBR322 origin, *aph*, *rpsL*	46
pML102	PAL5000 origin, ColE1 origin, *aph*, *sacB*, *int*	This study
pML113	ColE1 origin, *bla*, *FRT-hyg-FRT*, *attP*	This study
pML118	ColE1 origin, *bla*, *FRT-hyg-FRT*, *attP*, p_{hsp60}-*gfp*$^+$	This study
pMN252	ColE1 origin, *bla*, *FRT-hyg-FRT*, *rpsL*	46
pML440	PAL5000 origin, ColE1 origin, *hyg*, p_{imyc}-*phoA*	This study
pML443	ColE1 origin, *bla*, *FRT-hyg-FRT*, *attP*, p_{imyc}-*phoA*	This study

[a] *, gene encoding a mutated ribosomal protein S12 (K43R) which confers streptomycin resistance (Sm^r).

efficient uptake of phosphates by *M. smegmatis* and for the growth of *M. smegmatis* under low-phosphate conditions.

MATERIALS AND METHODS

Chemicals and enzymes. Hygromycin B was purchased from Calbiochem. All other chemicals were purchased from Merck, Roth, or Sigma at the highest purities available. Enzymes for DNA restriction and modification were from New England Biolabs, MBI Fermentas, and Boehringer. Oligonucleotides were obtained from MWG Biotech.

Bacterial strains and growth conditions. *E. coli* DH5α was used for cloning experiments and routinely grown in Luria-Bertani broth at 37°C. *M. smegmatis* strains (Table 1) were grown at 37°C in Middlebrook 7H9 medium (Difco) supplemented with 0.2% glycerol and 0.05% Tween 80 or on Middlebrook 7H10 plates supplemented with 0.5% glycerol unless otherwise noted. *M. bovis* BCG (Strain Institute Pasteur) was grown in Middlebrook 7H9 medium or on Middlebrook 7H10 (Difco) plates supplemented with 0.2% glycerol, 0.05% Tween 80, and 10% OADC (oleic acid-albumin-dextrose-catalase; Remel) unless otherwise noted. When required, the antibiotics hygromycin (200 μg/ml for *E. coli*; 50 μg/ml for mycobacteria) and kanamycin (30 μg/ml for *E. coli*; 10 μg/ml for mycobacteria) were added.

Construction of plasmids. The *phoA* gene was amplified by PCR from chromosomal DNA of *M. smegmatis* by using the oligonucleotides phoA_Msfwd01 (5′-GATTACTTAATTAAGCATGCCAGAAAGGAGGTTAATATG CCTGT CAGTACCTATCTCAGAGCGACGGT-3′) and phoA_Msrev (5′-ATATAATT TTAAATGCGCTCCGGCACATCGCCAC-3′), introducing the restriction sites SphI and SwaI (underlined). The SphI-digested fragment was cloned into the plasmid pMS2 which was digested with SphI and EcoRV to give the replicative *phoA* expression vector pML440. To integrate the *phoA* expression cassette at the genomic *attB* site of mycobacteria, an integration plasmid, pML443, was constructed as followed. For the construction of the *attP* site, a 365-bp fragment was amplified from pMV361 (47) by PCR using the oligonucleotides attPbig_fwd (5′-GATTACCTGCAGATCCGCGACGTGCCAACTAG-3′) and attPbig_rev (5′-GATTAGCTCGAGAGCCAGATCAGGGATGCGTTG-3′), introducing the restriction sites PstI and XhoI (underlined). The digested fragment was cloned into pMN252 (46), which was cut with the same restriction endonucleases to give the proficient control vector pML113. The vector pMN402 (40) was digested with ClaI and PmeI, and the fragment was cloned into pML113 digested with the same restriction endonucleases to give pML118. To integrate the *phoA* expression cassette at the genomic *attB* site of mycobacteria, pML443 was constructed by cloning the SwaI/PmeI fragment of pML440 into the backbone of pML118 digested with PmeI and EcoRV.

Insertion of a *phoA* expression cassette into the chromosome of *M. smegmatis*. A two-plasmid system derived from mycobacteriophage L5 was used to integrate a *phoA* expression cassette at the *attB* site of the mycobacterial chromosome. The replicative vector pML102 carrying the L5 integrase gene (*int*) was transformed in *M. bovis* BCG and *M. smegmatis*. These cells were transformed with the nonreplicative vector pML443 containing the phage attachment site *attP* and the *phoA* expression cassette. Since the continued expression of L5 integrase can cause the excision of the integrated vector from the genome, which contributes to plasmid instability (42), cells were plated on 7H10 plates containing hygromycin and 10% sucrose to select for the insertion of pML443 and to counterselect against pML102. Single colonies were plated in parallel on 7H10 plates containing hygromycin and 7H10 plates containing kanamycin to confirm the loss of pML102. The integration of the plasmid resulted in clones resistant to hygromycin and sensitive to kanamycin. Integration into the *attB* site was confirmed by PCR using the primers attB2 (5′-ACAGGATTTGAACCTGCGGC-3′) and attL01 (5′-TCGCCACGTTCGCCCTAG-3′), while the primers attB1 (5′-ACG TGGCGGTCCCTACCG-3′) and attB2, which only give a PCR product if integration does not occur, were used as controls. The hygromycin resistance marker gene (*hyg*), flanked by FLP recombination target sites of pML443, was removed from the genome by using the Flp recombinase (46). Competent cells were transformed with the *flp* expression vector pMN234. After selection on 7H10 plates containing kanamycin, 4 ml of Middlebrook 7H9 medium with kanamycin was inoculated and incubated for 12 h to 20 h at 37°C on a roller drum. In order to get only single cells, the culture was filtrated using a 5.0-μm filter. Several dilutions of the filtrate were plated on 7H10 plates without any antibiotics. Then, single colonies were streaked in parallel on 7H10 plates with and without hygromycin to identify clones which lost the hygromycin resistance gene.

Phosphate-dependent growth and phosphatase activity of *M. smegmatis*. In order to examine the dependence of growth and *phoA* expression on the phosphate concentration, we home-prepared 7H10 medium containing 0.5, 10, and 25 mM inorganic phosphate by adding 1 M P_i buffer (1 M Na_2HPO_4, adjusted to pH 7 with 1 M KH_2PO_4). To detect PhoA activity on plates, 60 μg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 0.1% Tween 80 were also added. Malachite green was omitted from the self-made 7H10 medium to enhance the visibility of the blue color on the plates. The strains were streaked on Middlebrook 7H10 plates containing 0.1% Tween 80 and incubated at 37°C for 5 to 7 days. Cells were scraped off the plates and resuspended in home-made 7H9 medium containing 0.5 mM P_i buffer. The cell culture was filtered and adjusted to an optical density at 600 nm (OD_{600}) of 0.1. By using an inoculation loop, the cell suspension was streaked on the plates described above. The plates were scanned daily after the 3rd day by using a digital scanner (V700 Photo; Epson).

Pictures of single colonies were taken from the same plates by using a stereomicroscope (Stemi 2000-C; Zeiss) and a cooled digital color camera (AxioCam MRc; Zeiss).

Measurement of phosphatase activity of *M. smegmatis* and *M. bovis* BCG. Cells were grown on Middlebrook 7H10 medium. Tween 80 was added to a final concentration of 0.1% to improve the solubility of the cells in liquid medium. The cells were scraped from the plate and suspended in Middlebrook 7H9 medium. This culture was filtrated (pore size, 5 μm; Sartorius) to obtain single cells and diluted with the same medium to an OD_{600} of 0.5. By using an inoculation loop, the cell suspension was streaked on Middlebrook 7H10 medium containing 60 μg/ml BCIP. Tween 80 (0.1%) was also added to improve the homogeneity of the coloration of the colonies. The plates were put into a sealed plastic bag to keep them moist and incubated in the dark at 37°C. The color of the colonies was examined daily for a period of up to 10 days. Growth and PhoA activity were compared for only strains streaked on the same plate. Pictures of colonies were taken by using a stereomicroscope (Stemi 2000-C; Zeiss) and a cooled digital color camera (AxioCam MRc; Zeiss).

The PhoA activity of *M. smegmatis* and *M. bovis* BCG in liquid cultures was measured as described previously (6, 18) with small modifications. Unless otherwise noted, strains were grown in Middlebrook 7H9 medium to an OD_{600} of about 1.0. Cells were harvested by centrifugation at 3,000 × *g* for 10 min at 4°C and resuspended in an equal volume of ice-cold 1 M Tris-buffer (pH 8.0). The OD_{600} of the cell suspension was determined. Cells were kept on ice during all steps. A portion of each cell suspension was lysed by two 20-s pulses at 40 W applied by a sonifier (Branson) using a microtip. PhoA activity was measured in both whole and lysed cells by adding 0.15 ml of samples (V_s) to 1 ml of detection buffer (1 M Tris-HCl, pH 8.0, 4 mM *p*-nitrophenylphosphate [*p*NPP]). After 30 to 40 min of incubation at 37°C, 100 μl of 1 M K_2HPO_4 was added to stop the reaction. The sample was centrifuged in a microcentrifuge (Eppendorf) for 10 min at maximum speed. After centrifugation, 1 ml of the supernatant was used to determine the A_{405} by using a spectrophotometer (SmartSpec Plus; Bio-Rad). The PhoA activity of whole and lysed cells was calculated using the following formula (18): activity (units) = $(1{,}000 \times A_{405})/(t_i \times OD_{600} \times V_s)$, where t_i is the incubation time in minutes and V_s is the volume of sample.

Measurement of phosphate uptake by *M. smegmatis*. Phosphate uptake measurements were carried out as previously described for glucose (44), with some modifications. Cells were grown on Middlebrook 7H10 plates containing 0.1% Tween 80 and incubated for 5 to 7 days at 37°C. The cells were scraped off the plates, suspended in Middlebrook 7H9 medium, and filtered through a 5.0-μm-pore-size filter (Sartorius). The cell suspension was used to inoculate 100 ml of Middlebrook 7H9 medium to an OD_{600} of 0.05. The cells were harvested at an OD_{600} of 1.0 by centrifugation (1,250 × *g* at 4°C for 10 min), washed twice in uptake buffer [50 mM Tris-HCl (pH 6.9), 15 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.05% Tween 80], and resuspended in the same buffer. The cell suspension was kept for 15 min at 37°C before [^{32}P]phosphate and nonlabeled phosphate were mixed and added to the cell suspension to obtain final concentrations of 1, 2.5, 5, 10, and 20 μM. The mixtures were incubated at 37°C, and 1-ml samples were removed at the indicated times. The cells were filtered through a 0.45-μm-pore-size filter (Sartorius). The filters were washed with 0.1 M LiCl and water, and their radioactivity levels were measured in a liquid scintillation counter. All experiments were performed in triplicate. The uptake rate was expressed as nanomolars per milligram of cells. The Michaelis-Menten constant K_m, the maximal uptake velocity V_{max} for the overall transport, and a minimal estimate of the permeability coefficient were determined as described previously (15, 44).

Gel electrophoresis and Western blots. Protein samples were analyzed in denaturing polyacrylamide gels that were stained with Coomassie blue G250 or silver. All samples were mixed with loading buffer (150 mM Tris, 4% sodium dodecyl sulfate, 30% glycerol, 0.1% bromophenol blue, pH 7.5) and boiled in a water bath for 10 min before being loaded onto a denaturing 10% polyacrylamide gel. The protein gel was blotted (4 h at 150 mA in transfer buffer [10 mM $NaHCO_3$, 3 mM Na_2CO_3, 20% methanol]) onto a nitrocellulose membrane (Schleicher & Schuell). The presence of PhoA was detected by using a monoclonal mouse antibody against PhoA of *E. coli* and a secondary rabbit anti-mouse antibody coupled with horseradish peroxidase. Immunoblots were developed by using an ECL Plus kit according to the manufacturer's recommendations (Amersham).

RESULTS

The cell envelope represents a permeability barrier for PhoA substrates in *M. smegmatis*. Our first aim was to examine whether the enzymatic activity of the alkaline phosphatase PhoA can be used to analyze the uptake of organic phosphates across the OM of mycobacteria as was shown for *E. coli* (23). This requires that PhoA be localized in the periplasm of mycobacteria as in that of gram-negative bacteria (10). However, based on the association of PhoA with membranes in subcellular fractions, it was proposed that PhoA is a cell surface protein of *M. smegmatis* (18). In order to solve the puzzle of the subcellular localization of the *M. smegmatis* PhoA protein, which is encoded by two identical *phoA* genes, msmeg1000 and msmeg2294, we examined whether the phosphatase activity was limited by the accessibility to substrates in whole cells. To this end, *M. smegmatis* SMR5 was grown to end-log phase and the PhoA activity was measured for lysed and whole cells obtained from the same culture. Since the endogenous PhoA activity of *M. smegmatis* was very low, the difference between whole and lysed cells was small (Fig. 1A). To increase PhoA activity, the vector pML440 was constructed in which one of the *phoA* genes of *M. smegmatis* is transcribed from a constitutively active mycobacterial promoter, p_{imyc} (16, 22). The total PhoA activity in lysed *M. smegmatis* cells carrying pML440 was increased ninefold compared to that in the control vector pMS2 (Fig. 1A). Western blot experiments using an antiserum against PhoA of *E. coli* revealed a 55-kDa band, which was strongly enhanced in *M. smegmatis* containing pML440 (not shown), confirming that the increased PhoA activity was due to the enhanced expression of *phoA*. Importantly, PhoA activity in whole cells was twofold reduced compared to that in lysed cells, indicating that the substrate *p*NPP is not freely accessible for PhoA. This was also observed for wild-type (wt) *M. smegmatis* and the porin mutants as evidenced by a small but significantly increased level of PhoA activity in lysed cells compared to that in whole cells (Fig. 1A, inset). This rules out the possibility that the lower PhoA activity level in whole cells compared to that in cell lysates is caused by the overexpression and concomitant mislocation of PhoA. Hence, we concluded that the intact cell envelope of *M. smegmatis* represents a permeability barrier for the PhoA substrate *p*NPP.

Porins mediate the diffusion of PhoA substrates across the OM of *M. smegmatis*. The diffusion of the substrate *p*NPP across the OM is rate limiting for the PhoA activity in *E. coli* and is mediated mainly by the porins OmpF and OmpC when *E. coli* is grown in rich medium (24). To analyze whether porins of *M. smegmatis* play a role in the diffusion of *p*NPP, PhoA activities were determined for *M. smegmatis* MN01, which lacks the major porin MspA, and for ML10, which lacks MspA and MspC. The PhoA activities of lysed cells of the wild type and of both porin mutants transformed with pML440 were identical, demonstrating that the deletion of the porin genes did not interfere with *phoA* expression (Fig. 1A). By contrast, the PhoA activity in whole cells of the Δ*mspA* mutant MN01 was threefold less than that in wild-type cells. This is consistent with the threefold-reduced number of porin channels in the OM of MN01 (44). A further reduction of the PhoA activity in whole cells for the porin double-mutant ML10 was not observed. These results demonstrate that PhoA activity in *M. smegmatis* depends on the presence of porins in the OM of *M. smegmatis* and strongly support the conclusion that PhoA is localized in the periplasm of *M. smegmatis*.

To test whether any of these strains would produce PhoA in

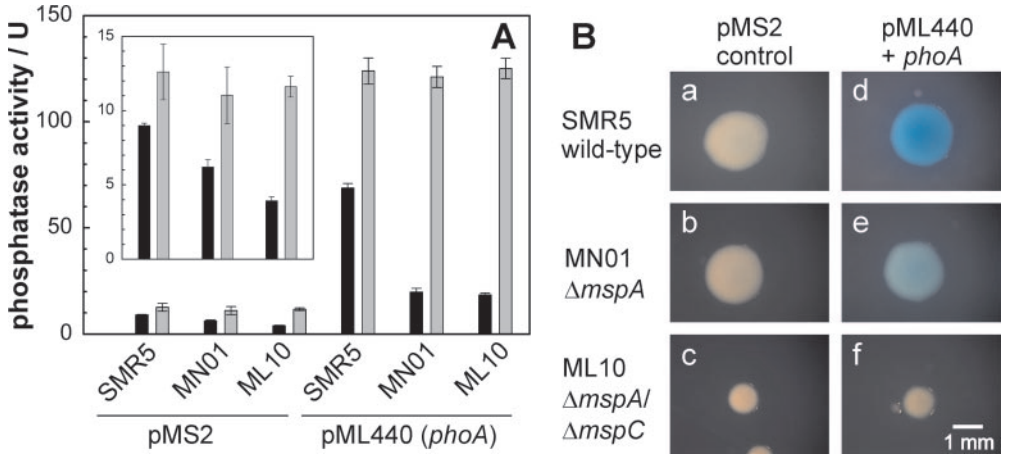

FIG. 1. Porin-dependent phosphatase activity of *M. smegmatis*. (A) The phosphatase activity of *M. smegmatis* grown in liquid culture was determined using *p*NPP. Black and gray bars indicate the phosphatase activity in whole and lysed cells, respectively. The inset shows the endogenous phosphatase activity in the parent strains SMR5, MN01, and ML10. Error bars indicate standard deviations. (B) Phosphatase activity of *M. smegmatis* on Middlebrook 7H10 agar plates containing Tween 80 and BCIP. After 5 days of incubation, pictures of colonies were taken with a Zeiss stereomicroscope Stemi 2000-C using the same magnification for all strains. In both experiments, the strains were SMR5 (wild-type) and the porin mutants MN01 (Δ*mspA*) and ML10 (Δ*mspA* Δ*mspC*), each of which was transformed with the control plasmid pMS2 (control) and the *phoA* expression plasmid pML440 (pML440+*phoA*), respectively. The blue color indicates the cleavage of BCIP by PhoA.

amounts sufficient for detection on plates, *M. smegmatis* wild type and the porin mutants (MN01 and ML10), carrying the vectors pMS2 and pML440, respectively, were streaked on 7H10 plates containing the chromogenic PhoA substrate BCIP. Colonies of *M. smegmatis* wild-type-carrying pMS2 appeared white, which was consistent with the very low intrinsic PhoA activity level, while colonies of *M. smegmatis* with pML440 were blue (Fig. 1B, panels a and d) as a result of increased PhoA expression. Importantly, the intensity of the blue color decreased from the wild type (Fig. 1B, panel d) over MN01 (panel e) to ML10 (panel f). The smaller sizes of the colonies of *M. smegmatis* ML10 were caused by the reduced growth rates due to the lower number of porins (44). Further incubation of the porin double-mutant ML10 for 2 days increased the sizes of the colonies to those of wt *M. smegmatis* but did not yield any blue color (not shown). Since the total PhoA activities were equal for all three strains (Fig. 1A), these results supported our previous conclusions that the uptake of BCIP across the OM is mediated by porins and that the PhoA activity is not surface associated in *M. smegmatis*. Furthermore, the fact that strains with high (*M. smegmatis* wild type) and low (MN01 and ML10) levels of porin activity are clearly distinguishable on plates containing BCIP showed that PhoA, in principle, is a suitable indicator of the uptake of phosphates across the OM and of the porin activity in *M. smegmatis*.

Porins are required for growth of *M. smegmatis* on low-phosphate medium. To examine whether porins play a role in the uptake of inorganic phosphate in vivo, the growth levels of *M. smegmatis* wild type and the porin double-mutant ML10 were examined on self-made 7H10 plates containing 0.5, 10, and 25 mM inorganic phosphate and BCIP. After 3 days of incubation, no growth of ML10 was observed on plates containing the lowest phosphate concentration (Fig. 2A, streaks 3 and 4) in contrast to that of the wild-type strain (Fig. 2A streaks 1 and 2). Even after 6 days of incubation, colonies of the ML10 strain were very small, indicating a severe growth defect of the ML10 strain at 0.5 mM inorganic phosphate (Fig. 2C, streaks 3 and 4). The *mspA* expression vector pMN016 fully restored the ability of *M. smegmatis* ML10 to grow on low-phosphate plates (Fig. 2A to D, streaks 5) and demonstrated that the loss of porins caused the growth defect. Surprisingly, a significantly lower level of growth of the porin double-mutant ML10 was also observed on plates containing 10 mM phosphate (not shown), indicating that the level of phosphate uptake is very low in this mutant and limits its growth rate at rather high phosphate concentrations. Taken together, these results show that Msp porins are required for the growth of *M. smegmatis* under low-phosphate conditions. These results also indicated that the uptake of inorganic phosphate across the OM of *M. smegmatis* depends on Msp porins.

Msp porins are required for uptake of inorganic phosphate by *M. smegmatis*. It was shown previously that diffusion through MspA and other Msp porins is the rate-limiting step for the uptake of glucose by *M. smegmatis* (43, 44). The reduced PhoA activity in whole cells of porin mutants (Fig. 1) and the limited growth of the porin mutant ML10 on plates with low-phosphate concentrations (Fig. 2) strongly indicated that Msp porins are used by *M. smegmatis* to enable the diffusion of organic and inorganic phosphates across the OM. To provide direct evidence for this conclusion, we examined the uptake of radioactive phosphate by wild-type *M. smegmatis* and the porin double-mutant ML10. For these experiments, strains were grown in Middlebrook 7H9 medium. The uptake rates were measured at a phosphate concentration of 20 μM because differences in diffusion through porins are more pronounced at solute concentrations in the micromolar range. *M. smegmatis* SMR5 (wt) took up phosphate with a rate of 0.06 nmol/min per mg cells. The porin double-mutant ML10 showed a 4.5-fold-lower uptake rate of 0.014 nmol/min per mg cells (Fig. 3A). The expression of *mspA* from the vector pMN016 increased the uptake rate of phosphate by *M. smegmatis* ML10 to 60% of the wild-type level (Fig. 3A). This demonstrated that the loss of porins in the ML10 strain reduced the ability of *M.*

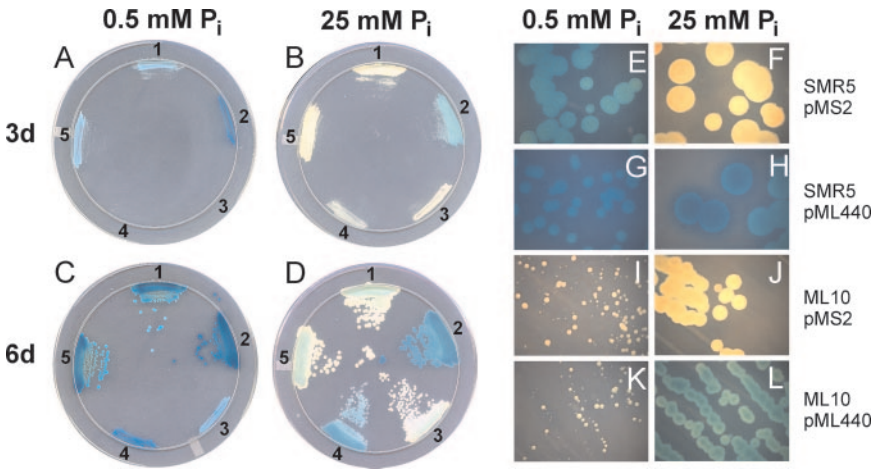

FIG. 2. Porin-dependent growth of *M. smegmatis* on low-phosphate plates. (A to D) *M. smegmatis* SMR5 (wt) and ML10 (Δ*mspA* Δ*mspC*) were streaked on self-made 7H10 agar plates containing different concentrations of phosphate and BCIP. The plates were incubated at 37°C for 6 days and were scanned (V700 Photo, Epson) each day. Scans of plates after 3 (A and C) and 6 days (B and D) of incubation are shown. The strains on the plates are as follows: streak 1, SMR5/pMS2 (control); streak 2, SMR5/pML440 (*phoA*); streak 3, ML10/pMS2; streak 4, ML10/pML440; and streak 5, ML10/pMN016 (*mspA*). (E to L) *M. smegmatis* SMR5 and ML10 were filtered through a 5-μm filter to obtain single cells and plated on self-made 7H10 agar plates containing different concentrations of phosphate and BCIP. Pictures of single colonies after 8 days of incubation at 37°C were taken at a 16-fold magnification using a Zeiss stereomicroscope Stemi 2000-C.

smegmatis to take up phosphate. We concluded that inorganic phosphate diffuses through the MspA and MspC porins into *M. smegmatis*.

Kinetic experiments with phosphate concentrations ranging from 1 μM to 20 μM and time points at 45, 75, 105, 135, 165, and 195 s were performed to determine the apparent permeability coefficients of wild-type *M. smegmatis* and Δ*mspA* Δ*mspC* mutant ML10 for phosphate. The data were fitted well by the Michaelis-Menten equation (Fig. 3B). Data analysis yielded V_{max} values of 0.4 and 0.2 nmol min^{-1} mg^{-1} and K_m values of 12.7 and 15.2 μM for wild-type *M. smegmatis* and the mutant ML10, respectively. The lower limit of the outer membrane permeability can be estimated by assuming that the phosphate transport across the inner membrane has a very high affinity. This has not been determined for *M. smegmatis* yet, but Pst systems are highly efficient (K_m) and such a system is present in *M. smegmatis* (2). Thus, the apparent permeability coefficients were calculated to 2.1×10^{-6} and 1×10^{-6} cm s^{-1} for wild-type *M. smegmatis* and the porin double-mutant ML10.

The cell envelope of *M. bovis* BCG represents a permeability barrier for PhoA substrates. The phosphatase activity in whole cells of *M. bovis* BCG was significantly reduced compared to that in lysed cells (Fig. 4, inset). This indicated that *M. bovis* BCG contains at least one endogenous cell-bound phosphatase which hydrolyzes *p*NPP. The expression of *phoA* of *M. smegmatis* from the *attB* site (strain ML44) or from the replicating plasmid pML440 increased the phosphatase activity level three- or sixfold, respectively. In both recombinant *M. bovis* BCG strains, the phosphatase activity in whole cells was approximately twofold reduced compared to that in the corresponding cell lysates (Fig. 4). Taken together, these results also demonstrated that the cell envelope of slow-growing mycobacteria represents a permeability barrier for phosphates. This

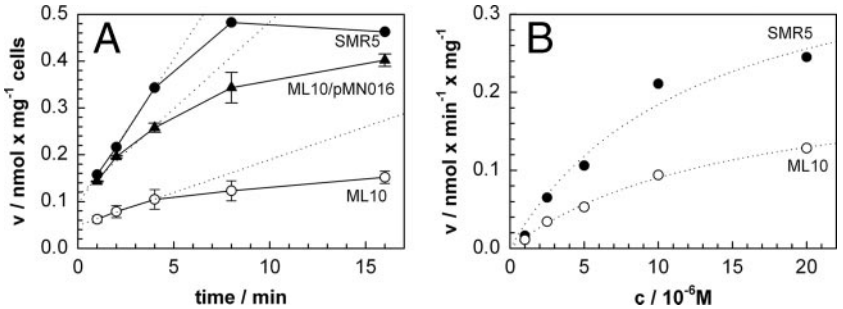

FIG. 3. Uptake of phosphate by porin mutants of *M. smegmatis*. (A) Accumulation of [^{32}P]phosphate by *M. smegmatis* SMR5 (wild-type) (black circles), the Δ*mspA* Δ*mspC* mutant ML10 (white circles), and ML10 complemented with the *mspA* expression plasmid pMN016 (triangles) was measured. The assay was performed at 37°C at a final phosphate concentration of 20 μM. The uptake rates were determined by a regression analysis of the first 4 min for each strain. The dotted lines represent regression lines. Error bars indicate standard deviations. (B) A series of phosphate uptake measurements was performed with phosphate concentrations ranging from 1 to 20 μM for *M. smegmatis* SMR5 (wild-type) and ML10 (Δ*mspA* Δ*mspC*). For each strain, the uptake rates at different phosphate concentrations were approximated by Michaelis-Menten functions that are shown as regression lines (dotted lines).

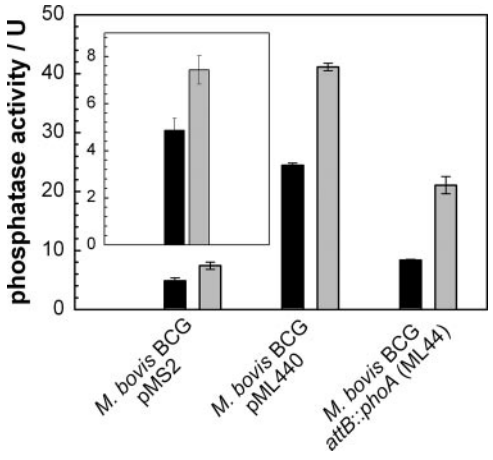

FIG. 4. Expression of *phoA* in *M. bovis* BCG. *M. bovis* BCG strains containing the control plasmid pMS2, the *phoA* expression plasmid pML440, or a *phoA* expression cassette integrated at the attachment site of phage L5 were grown in liquid culture. The phosphatase activity in these strains was determined by using *p*NPP. Black and gray bars indicate the phosphatase activity of whole and lysed cells, respectively. The inset shows the endogenous phosphatase activity of *M. bovis* BCG. Error bars indicate standard deviations.

was independent of the type of phosphatase exploited in this assay. Single colonies of *M. bovis* BCG expressing *phoA* from the plasmid pML440 at the *attB* site did not turn blue on plates containing BCIP (not shown). This was due to both a lower level of PhoA and a lower permeability for *p*NPP as derived from a comparison of cell lysates and whole cells of the recombinant strains of *M. bovis* BCG and *M. smegmatis* (Fig. 1A and 4).

Integration of a *phoA* expression cassette into the chromosome of *M. smegmatis*. Since porins that mediate the uptake of phosphates in *M. tuberculosis* and *M. bovis* BCG are unknown, we attempted to use a *phoA*-based screening system in *M. smegmatis* porin mutants to identify them. To this end, the plasmid pML443 carrying the p_{imyc}-*phoA* cassette was integrated into the *attB* sites of *M. smegmatis* and the porin mutants MN01 and ML10. The integration of the *phoA* expression cassette at the *attB* site increased the phosphatase activity in lysed cells threefold in all strains compared to that in their parent strains (Fig. 5A). The phosphatase activity in whole cells was significantly reduced compared to that in lysed cells both for the parent strains and for the strains with an integrated recombinant *phoA* expression cassette. Further, the phosphatase activity in whole cells decreased with a reduced number of Msp porins. These results are consistent with the previous observations that the OM of *M. smegmatis* represents a permeability barrier for *p*NPP and that the diffusion of *p*NPP is porin dependent. However, the colonies of the recombinant strains did not show a clearly detectable blue color on 7H10 agar plates with BCIP (Fig. 5B). We concluded that these *M. smegmatis* strains are not useful for screening for porins of *M. tuberculosis*.

DISCUSSION

Porins are required for the uptake of inorganic and organic phosphates by *M. smegmatis*. In this study, we demonstrated that the cell envelope of *M. smegmatis* represents a permeability barrier for phosphates and that access to both organic phosphates and inorganic phosphate in *M. smegmatis* is mediated by the porins MspA and MspC. Msp porins are integral pore-forming membrane proteins (14, 27) and are directly accessible on the cell surfaces of *M. smegmatis* by antibodies (14, 43). It follows that the membrane in which MspA is localized represents the permeability barrier for phosphates. These results are consistent with a model proposed by Minnikin (25) and substantial indirect evidence that the mycolic acids, together with a large variety of extractable lipids, form an unusual outer membrane in addition to the cytoplasmic membrane of mycobacteria (7, 8, 13, 21, 28). These results are similar to those obtained for *E. coli*: mutations of OmpF and OmpC porins reduced the OM permeability to *p*NPP by two- to sixfold and concomitantly reduced the phosphatase activity in whole cells (23).

The diffusion of phosphate through Msp porins is quite

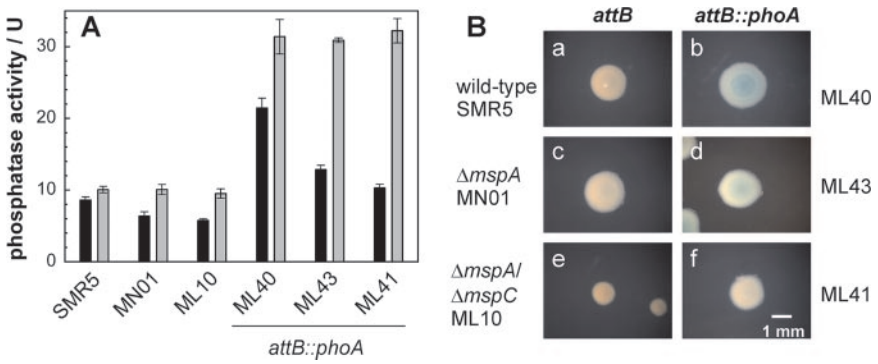

FIG. 5. Integration of *phoA* into the chromosomes of *M. smegmatis* wild-type and porin mutants. (A) The phosphatase activity of *M. smegmatis* grown in liquid culture was determined by using *p*NPP. Black and gray bars indicate the phosphatase activity of whole and lysed cells, respectively. Error bars indicate standard deviations. (B) Phosphatase activity of *M. smegmatis* on Middlebrook 7H10 agar plates containing Tween 80 and BCIP. After 5 days of incubation, pictures of colonies were taken with a Zeiss stereomicroscope Stemi 2000-C using the same magnification for all strains. In both experiments, the strains were SMR5 (wild-type) and the porin mutants MN01 (Δ*mspA*) and ML10 (Δ*mspA*/*mspC*) and derivatives with an *phoA* expression cassette integrated at the attachment site of the phage L5 (*attB*::*phoA*). The blue color indicates the cleavage of BCIP by PhoA.

inefficient compared to that of neutral solutes as shown by the 10-fold lower permeability coefficient of wild-type *M. smegmatis* for inorganic phosphate compared to that for glucose (44). This observation is not surprising considering the high density of negative charges in the constriction zones of MspA (14) and MspC, the two porins expressed by wild-type *M. smegmatis* (44). Furthermore, the fact that the deletion of the porins MspA and MspC reduced the OM permeability of ML10 for glucose by 75-fold (44) but only by 2-fold for phosphate compared to that for the isogenic parent strain indicated that the residual porins in ML10 allow a faster diffusion of phosphate than do MspA and MspC to partially compensate for the 15-fold lower number of Msp porins in ML10. We have shown that the expressions of the *mspB* and *mspD* genes, which are silent in wild-type *M. smegmatis*, are induced in ML10 (44). Relative to MspA, in the surface-exposed loop L9, MspB has only two amino acid exchanges (43), which are not likely to alter the solute specificity of this pore. However, the mutation D91G in MspD reduces the number of negative charges in the constriction zone and may increase the diffusion rate of negatively charged solutes relative to MspA.

Growth of *M. smegmatis* at low-phosphate concentrations depends on Msp porins. In order to efficiently scavenge phosphate at submillimolar concentrations, *E. coli* expresses the high-affinity phosphate transport system Pst and the outer-membrane porin PhoE, which preferentially allow the diffusion of anions across the OM (33). Under those conditions, PhoE completely compensates for the loss of the two general porins OmpF and OmpC in *E. coli* mutants (17). By contrast, the loss of porins MspA and MspC caused a severe growth defect in *M. smegmatis* on plates containing 0.5 mM phosphate. It is surprising that a twofold reduction of the overall permeability of the porin mutant ML10 for phosphate compared to that of the isogenic parent strain resulted in such a drastic growth defect. A 75-fold-lower level of permeability for glucose was required to produce a significant growth defect of the porin mutant ML10 on regular 7H9 plates containing 22 mM glycerol (44). This demonstrates that no other porin fully compensates for the loss of MspA and MspC in *M. smegmatis* under those conditions. In this regard, it was striking that the porin mutant already showed a significant growth defect at phosphate concentrations of 10 mM (not shown). Such high concentrations may be necessary to overcome the intrinsically slow diffusion of phosphate through the Msp porins.

Localization of phosphatases in mycobacteria. The reduced phosphatase activity in whole cells of the recombinant *M. smegmatis* strains that overexpressed the endogenous alkaline phosphatase (PhoA) to up to 90% of the total phosphatase activity may also be explained by the existence of the OM permeability barrier. However, it may be argued that this effect results from a (partial) mislocalization of PhoA in the cytoplasm due to overexpression and a putative overload of the export apparatus of *M. smegmatis*. PhoA of *M. smegmatis* shares almost 60% identical amino acids with PhoA of *Escherichia coli*, including the conserved residues of the active site and the four cysteines which form essential intrastrand disulfide bridges (41). The efficient formation of these disulfide bonds occurs in only the oxidative milieu of the periplasm of *E. coli*, explaining why PhoA is unable to fold into an active conformation if retained in the cytoplasm (11). Thus, it is likely that the export of *M. smegmatis* PhoA is also required for activity. This possibility argues against a contribution of putatively mislocated PhoA to the observed activity. In contrast to its *E. coli* homolog, PhoA of *M. smegmatis* has a lipoprotein signal sequence. Cell fractionation experiments of PhoA labeled with [^{14}C]acetate showed indeed that PhoA of *M. smegmatis* is a membrane-bound lipoprotein (18). Our results strongly suggest that PhoA is not a surface protein as was concluded from the association of PhoA with membranes (18) but rather localized in the periplasm of *M. smegmatis* as it is for *E. coli* (23).

Three lipoproteins with phosphatase activity and the secreted acid phosphatase SapM were identified in *M. bovis* BCG (3) and *M. tuberculosis* (35), respectively. Our observation that the cell envelope of *M. bovis* BCG establishes a permeability barrier for phosphates indicates that at least one phosphatase with significant activity is localized in the periplasm. Our experiments do not exclude the possibility that other phosphatases are either located on the surface or secreted. However, the conclusion that proteins are surface proteins based solely on membrane association (4) is premature as demonstrated for PhoA of *M. smegmatis*.

Phosphate uptake in slow-growing mycobacteria. Survival inside macrophages requires the adaptation of intracellular pathogens to the phagosomal environment. The transcriptional profiles of *M. tuberculosis* and *Salmonella enterica* in infected macrophages revealed that the proteins involved in inorganic phosphate transport are up-regulated (12, 39), indicating that phosphate levels inside phagosomes of macrophages are indeed limited. Consistent with this conclusion, genes encoding efficient phosphate transport systems were found to be essential for the survival of *M. tuberculosis* in macrophages and mice (34, 38). However, how inorganic or organic phosphates cross the OM of *M. tuberculosis* is unknown. Since the direct rate of diffusion of phosphates through model lipid membranes is extremely low (permeability coefficient of the monoanion = 5×10^{-12} cm/s [9]), it appears likely that slow-growing mycobacteria also use OM pore proteins for the uptake of phosphate. Indeed, the existence of a porin with anion specificity has been demonstrated (20). This porin still awaits discovery. A screening system based on complementation of the uptake defects for phosphates of a porin mutant of *M. smegmatis* as described in this study provides a tool to identify such a porin of *M. tuberculosis*. These experiments are currently being pursued in our laboratory.

ACKNOWLEDGMENTS

Preliminary sequence data for *Mycobacterium smegmatis* were obtained from the Institute for Genomic Research website at http://www.tigr.org.

This work was supported by grant AI063432 from the National Institutes of Health.

REFERENCES

1. **Bauer, K., M. Struyve, D. Bosch, R. Benz, and J. Tommassen.** 1989. One single lysine residue is responsible for the special interaction between polyphosphate and the outer membrane porin PhoE of *Escherichia coli*. J. Biol. Chem. **264:**16393–16398.
2. **Bhatt, K., S. K. Banerjee, and P. K. Chakraborti.** 2000. Evidence that phosphate specific transporter is amplified in a fluoroquinolone resistant *Mycobacterium smegmatis*. Eur. J. Biochem. **267:**4028–4032.
3. **Braibant, M., and J. Content.** 2001. The cell surface associated phosphatase activity of *Mycobacterium bovis* BCG is not regulated by environmental inorganic phosphate. FEMS Microbiol. Lett. **195:**121–126.

4. **Braibant, M., L. De Wit, P. Peirs, M. Kalai, J. Ooms, A. Drowart, K. Huygen, and J. Content.** 1994. Structure of the *Mycobacterium tuberculosis* antigen 88, a protein related to the *Escherichia coli* PstA periplasmic phosphate permease subunit. Infect. Immun. **62:**849–854.
5. **Braibant, M., P. Lefevre, L. de Wit, P. Peirs, J. Ooms, K. Huygen, A. B. Andersen, and J. Content.** 1996. A *Mycobacterium tuberculosis* gene cluster encoding proteins of a phosphate transporter homologous to the *Escherichia coli* Pst system. Gene **176:**171–176.
6. **Braunstein, M., T. I. Griffin, J. I. Kriakov, S. T. Friedman, N. D. Grindley, and W. R. Jacobs, Jr.** 2000. Identification of genes encoding exported *Mycobacterium tuberculosis* proteins using a Tn*552′phoA* in vitro transposition system. J. Bacteriol. **182:**2732–2740.
7. **Brennan, P. J., and H. Nikaido.** 1995. The envelope of mycobacteria. Annu. Rev. Biochem. **64:**29–63.
8. **Camacho, L. R., P. Constant, C. Raynaud, M. A. Laneelle, J. A. Triccas, B. Gicquel, M. Daffé, and C. Guilhot.** 2001. Analysis of the phthiocerol dimycocerosate locus of *Mycobacterium tuberculosis*. Evidence that this lipid is involved in the cell wall permeability barrier. J. Biol. Chem. **276:**19845–19854.
9. **Chakrabarti, A. C., and D. W. Deamer.** 1992. Permeability of lipid bilayers to amino acids and phosphate. Biochim. Biophys. Acta **1111:**171–177.
10. **Derman, A. I., and J. Beckwith.** 1991. *Escherichia coli* alkaline phosphatase fails to acquire disulfide bonds when retained in the cytoplasm. J. Bacteriol. **173:**7719–7722.
11. **Derman, A. I., W. A. Prinz, D. Belin, and J. Beckwith.** 1993. Mutations that allow disulfide bond formation in the cytoplasm of *Escherichia coli*. Science **262:**1744–1747.
12. **Eriksson, S., S. Lucchini, A. Thompson, M. Rhen, and J. C. Hinton.** 2003. Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. Mol. Microbiol. **47:**103–118.
13. **Etienne, G., C. Villeneuve, H. Billman-Jacobe, C. Astarie-Dequeker, M. A. Dupont, and M. Daffe.** 2002. The impact of the absence of glycopeptidolipids on the ultrastructure, cell surface and cell wall properties, and phagocytosis of *Mycobacterium smegmatis*. Microbiology **148:**3089–3100.
14. **Faller, M., M. Niederweis, and G. E. Schulz.** 2004. The structure of a mycobacterial outer-membrane channel. Science **303:**1189–1192.
15. **Jarlier, V., and H. Nikaido.** 1990. Permeability barrier to hydrophilic solutes in *Mycobacterium chelonei*. J. Bacteriol. **172:**1418–1423.
16. **Kaps, I., S. Ehrt, S. Seeber, D. Schnappinger, C. Martin, L. W. Riley, and M. Niederweis.** 2001. Energy transfer between fluorescent proteins using a co-expression system in *Mycobacterium smegmatis*. Gene **278:**115–124.
17. **Korteland, J., J. Tommassen, and B. Lugtenberg.** 1982. PhoE protein pore of the outer membrane of *Escherichia coli* K12 is a particularly efficient channel for organic and inorganic phosphate. Biochim. Biophys. Acta **690:**282–289.
18. **Kriakov, J., S. Lee, and W. R. Jacobs, Jr.** 2003. Identification of a regulated alkaline phosphatase, a cell surface-associated lipoprotein, in *Mycobacterium smegmatis*. J. Bacteriol. **185:**4983–4991.
19. **Lefèvre, P., M. Braibant, L. de Wit, M. Kalai, D. Roeper, J. Grotzinger, J. P. Delville, P. Peirs, J. Ooms, K. Huygen, and J. Content.** 1997. Three different putative phosphate transport receptors are encoded by the *Mycobacterium tuberculosis* genome and are present at the surface of *Mycobacterium bovis* BCG. J. Bacteriol. **179:**2900–2906.
20. **Lichtinger, T., B. Heym, E. Maier, H. Eichner, S. T. Cole, and R. Benz.** 1999. Evidence for a small anion-selective channel in the cell wall of *Mycobacterium bovis* BCG besides a wide cation-selective pore. FEBS Lett. **454:**349–355.
21. **Liu, J., E. Y. Rosenberg, and H. Nikaido.** 1995. Fluidity of the lipid domain of cell wall from *Mycobacterium chelonae*. Proc. Natl. Acad. Sci. USA **92:**11254–11258.
22. **Mailaender, C., N. Reiling, H. Engelhardt, S. Bossmann, S. Ehlers, and M. Niederweis.** 2004. The MspA porin promotes growth and increases antibiotic susceptibility of both *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis*. Microbiology **150:**853–864.
23. **Martinez, M. B., M. Flickinger, L. Higgins, T. Krick, and G. L. Nelsestuen.** 2001. Reduced outer membrane permeability of *Escherichia coli* O157:H7: suggested role of modified outer membrane porins and theoretical function in resistance to antimicrobial agents. Biochemistry **40:**11965–11974.
24. **Martinez, M. B., F. J. Schendel, M. C. Flickinger, and G. L. Nelsestuen.** 1992. Kinetic properties of enzyme populations in vivo: alkaline phosphatase of the *Escherichia coli* periplasm. Biochemistry **31:**11500–11509.
25. **Minnikin, D. E.** 1982. Lipids: complex lipids, their chemistry, biosynthesis and roles, p. 95–184. *In* C. Ratledge and J. Stanford (ed.), The biology of the mycobacteria: physiology, identification, and classification, vol. I. Academic Press, London, United Kingdom.
26. **Niederweis, M.** 2003. Mycobacterial porins—new channel proteins in unique outer membranes. Mol. Microbiol. **49:**1167–1177.
27. **Niederweis, M., S. Ehrt, C. Heinz, U. Klöcker, S. Karosi, K. M. Swiderek, L. W. Riley, and R. Benz.** 1999. Cloning of the *mspA* gene encoding a porin from *Mycobacterium smegmatis*. Mol. Microbiol. **33:**933–945.
28. **Nikaido, H., S. H. Kim, and E. Y. Rosenberg.** 1993. Physical organization of lipids in the cell wall of *Mycobacterium chelonae*. Mol. Microbiol. **8:**1025–1030.
29. **Nikaido, H., and E. Y. Rosenberg.** 1983. Porin channels in *Escherichia coli*: studies with liposomes reconstituted from purified proteins. J. Bacteriol. **153:**241–252.
30. **Overbeeke, N., and B. Lugtenberg.** 1980. Expression of outer membrane protein E of *Escherichia coli* K12 by phosphate limitation. FEBS Lett. **112:**229–232.
31. **Peirs, P., P. Lefevre, S. Boarbi, X. M. Wang, O. Denis, M. Braibant, K. Pethe, C. Locht, K. Huygen, and J. Content.** 2005. *Mycobacterium tuberculosis* with disruption in genes encoding the phosphate binding proteins PstS1 and PstS2 is deficient in phosphate uptake and demonstrates reduced in vivo virulence. Infect. Immun. **73:**1898–1902.
32. **Qi, Y., Y. Kobayashi, and F. M. Hulett.** 1997. The *pst* operon of *Bacillus subtilis* has a phosphate-regulated promoter and is involved in phosphate transport but not in regulation of the pho regulon. J. Bacteriol. **179:**2534–2539.
33. **Rao, N. N., and A. Torriani.** 1990. Molecular aspects of phosphate transport in *Escherichia coli*. Mol. Microbiol. **4:**1083–1090.
34. **Rengarajan, J., B. R. Bloom, and E. J. Rubin.** 2005. Genome-wide requirements for *Mycobacterium tuberculosis* adaptation and survival in macrophages. Proc. Natl. Acad. Sci. USA **102:**8327–8332.
35. **Saleh, M. T., and J. T. Belisle.** 2000. Secretion of an acid phosphatase (SapM) by *Mycobacterium tuberculosis* that is similar to eukaryotic acid phosphatases. J. Bacteriol. **182:**6850–6853.
36. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
37. **Sander, P., A. Meier, and E. C. Bottger.** 1996. Ribosomal drug resistance in mycobacteria. Res. Microbiol. **147:**59–67.
38. **Sassetti, C. M., and E. J. Rubin.** 2003. Genetic requirements for mycobacterial survival during infection. Proc. Natl. Acad. Sci. USA **100:**12989–12994.
39. **Schnappinger, D., S. Ehrt, M. I. Voskuil, Y. Liu, J. A. Mangan, I. M. Monahan, G. Dolganov, B. Efron, P. D. Butcher, C. Nathan, and G. K. Schoolnik.** 2003. Transcriptional adaptation of *Mycobacterium tuberculosis* within macrophages: insights into the phagosomal environment. J. Exp. Med. **198:**693–704.
40. **Scholz, O., A. Thiel, W. Hillen, and M. Niederweis.** 2000. Quantitative analysis of gene expression with an improved green fluorescent protein. Eur. J. Biochem. **267:**1565–1570.
41. **Sone, M., S. Kishigami, T. Yoshihisa, and K. Ito.** 1997. Roles of disulfide bonds in bacterial alkaline phosphatase. J. Biol. Chem. **272:**6174–6178.
42. **Springer, B., P. Sander, L. Sedlacek, K. Ellrott, and E. C. Böttger.** 2001. Instability and site-specific excision of integration-proficient mycobacteriophage L5 plasmids: development of stably maintained integrative vectors. Int. J. Med. Microbiol. **290:**669–675.
43. **Stahl, C., S. Kubetzko, I. Kaps, S. Seeber, H. Engelhardt, and M. Niederweis.** 2001. MspA provides the main hydrophilic pathway through the cell wall of *Mycobacterium smegmatis*. Mol. Microbiol. **40:**451–464. (Author's correction, **57:**1509.)
44. **Stephan, J., J. Bender, F. Wolschendorf, C. Hoffmann, E. Roth, C. Mailander, H. Engelhardt, and M. Niederweis.** 2005. The growth rate of *Mycobacterium smegmatis* depends on sufficient porin-mediated influx of nutrients. Mol. Microbiol. **58:**714–730.
45. **Stephan, J., C. Mailaender, G. Etienne, M. Daffe, and M. Niederweis.** 2004. Multidrug resistance of a porin deletion mutant of *Mycobacterium smegmatis*. Antimicrob. Agents Chemother. **48:**4163–4170.
46. **Stephan, J., V. Stemmer, and M. Niederweis.** 2004. Consecutive gene deletions in *Mycobacterium smegmatis* using the yeast FLP recombinase. Gene **343:**181–190.
47. **Stover, C. K., V. F. de la Cruz, T. R. Fuerst, J. E. Burlein, L. A. Benson, L. T. Bennett, G. P. Bansal, J. F. Young, M. H. Lee, G. F. Hatfull, S. B. Snapper, R. G. Barletta, W. R. Jacobs, and B. R. Bloom.** 1991. New use of BCG for recombinant vaccines. Nature **351:**456–460.
48. **van Veen, H. W.** 1997. Phosphate transport in prokaryotes: molecules, mediators and mechanisms. Antonie Leeuwenhoek **72:**299–315.