INFECTION AND IMMUNITY, Mar. 2005, p. 1898–1902 Vol. 73, No. 3
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.3.1898–1902.2005

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

Priska Peirs,[1] Philippe Lefèvre,[1] Samira Boarbi,[1] Xiao-Ming Wang,[1] Olivier Denis,[1] Martine Braibant,[1] Kevin Pethe,[2] Camille Locht,[2] Kris Huygen,[1] and Jean Content[1]*

Pasteur Institute of Brussels, Brussels, Belgium,[1] and INSERM U629, Pasteur Institute-Lille, Lille, France[2]

Received 5 July 2004/Returned for modification 5 August 2004/Accepted 2 October 2004

By measuring phosphate uptake by *Mycobacterium tuberculosis* strains with the *pstS1* and *pstS2* genes genetically inactivated, we showed that these *pstS* genes encode high-affinity phosphate binding proteins. In a mouse infection model, both mutants were attenuated in virulence, suggesting that *M. tuberculosis* encounters limiting phosphate concentrations during its intracellular life span.

As inorganic phosphate is an essential but often limiting nutrient in the environment, its import in bacteria is important and can be accomplished through the phosphate-specific transporter (Pst) (7, 8, 19, 25, 28, 31). Pst is a membrane-associated complex that belongs to the superfamily of ABC transporters (1, 6, 15). In *Escherichia coli* (12, 30) and other procaryotes (26), it is composed of four distinct subunits encoded by the *pstS*, *pstA*, *pstC*, and *pstB* genes arranged in an operon. PstS is the periplasmic phosphate binding protein, PstA and PstC are integral inner membrane proteins, and the PstB subunit provides energy for transport through ATP hydrolysis. Interestingly, in *Mycobacterium tuberculosis*, three putative *pst* operons have been identified (7, 8, 10), which probably constitutes a subtle biochemical adaptation of this microorganism for its growth and survival under different phosphate-limiting conditions during its infectious cycle (19). It has been shown that PstS1 from *M. tuberculosis* is able to bind phosphate with an affinity similar to that of PstS from *E. coli* (9, 29) and that the production of the different PstS proteins is induced under phosphate starvation in *M. tuberculosis* (3, 19).

To further investigate the importance of the *pstS1* and *pstS2* genes for the phosphate uptake and virulence of *M. tuberculosis*, we created *M. tuberculosis pstS1* and *pstS2* knockout strains using genes isolated from an *M. tuberculosis* H37Rv cosmid library. A kanamycin resistance cassette (the *aph* gene from pYUB53) (18) was cloned into *pstS1* and *pstS2*, yielding *pstS1*::*aph* and *pstS2*::*aph*, respectively. These genes and the *xylE* gene (from pXYL4 carrying the *xylE* colored marker gene from *Pseudomonas putida*) were cloned into pPR27; transformed into *M. tuberculosis* H37Rv, where the knockout mutants were selected by a two-step counterselection strategy (24); and further analyzed by Southern hybridization (Fig. 1A) and immunoblot analysis (Fig. 1B). Anti-PstS1-reactive material was lacking in the *pstS1* knockout mutant but present in the parental strain and in the *pstS2* knockout mutant. Conversely, anti-PstS2-reactive material was lacking in the *pstS2* knockout mutant but present in the other strains. In this *pstS2* knockout mutant, we observed that the expression of the *pknD* (*mbk*) gene (22, 23), located downstream of the *pstS2* gene, is also abolished (data not shown). The PstS3 subunit is present in all strains (Fig. 1B). The different strains exhibited similar apparent growth rates in Middlebrook 7H9 albumin-dextrose-catalase (ADC) liquid medium in a 14-day experiment, suggesting that the two PstS proteins are not essential for growth in this phosphate-rich medium (25 mM P_i).

To assess the involvement of PstS1 and PstS2 in phosphate uptake, the different strains were grown in Middlebrook 7H9 ADC medium to an optical density at 600 nm of 0.3. The cells were then washed in 7H9 ADC medium without phosphate (4) and further cultivated in this medium for 24 h at 37°C to induce maximal phosphate uptake by the high-affinity Pst system (11). The bacteria were then washed twice in the uptake buffer [50 mM Tris-HCl (pH 6.9), 15 mM KCl, 10 mM $(NH_4)_2SO_4$, and 1 mM $MgSO_4$] and incubated in the uptake buffer supplemented with 0.5, 2, 5, 10, or 25 μM P_i and $^{33}P_i$ (25 nM; 10 μCi/ml). The rate of uptake of orthophosphate was measured as described previously (8). At 0.5 μM P_i, the rates of phosphate uptake by the *pstS1* and *pstS2* knockout mutants were reduced compared to that of the wild-type (Fig. 2A). The reduced phosphate uptake by the *pstS2* knockout strain is due to the absence of the PstS2 protein and not to the absence of the PknD protein kinase, since the rate of phosphate uptake by a *pknD* knockout mutant is not reduced compared to that of the parental strain (results not shown). These results indicate that PstS1 and PstS2 are involved in phosphate uptake from this medium. Increasing the phosphate concentration resulted in less pronounced differences in phosphate uptake between the parental and mutant strains (Fig. 2B, C, and D). At 25 μM P_i, no difference in phosphate uptake was observed among the three strains (Fig. 2E), suggesting that PstS1 and PstS2 can substitute for each other and/or that phosphate uptake may be mediated by PstS3 or the putative Pit transporter (14, 27, 32).

PstS1 and PstS2 may contribute to the intracellular survival of *M. tuberculosis*, since both PstS1 and PstS2 appear to be

* Corresponding author. Mailing address: Pasteur Institute of Brussels, Engelandstraat 642, B-1180-Brussels, Belgium. Phone: 32-2-3733416. Fax: 32-2-3733291. E-mail: jcontent@pasteur.be.

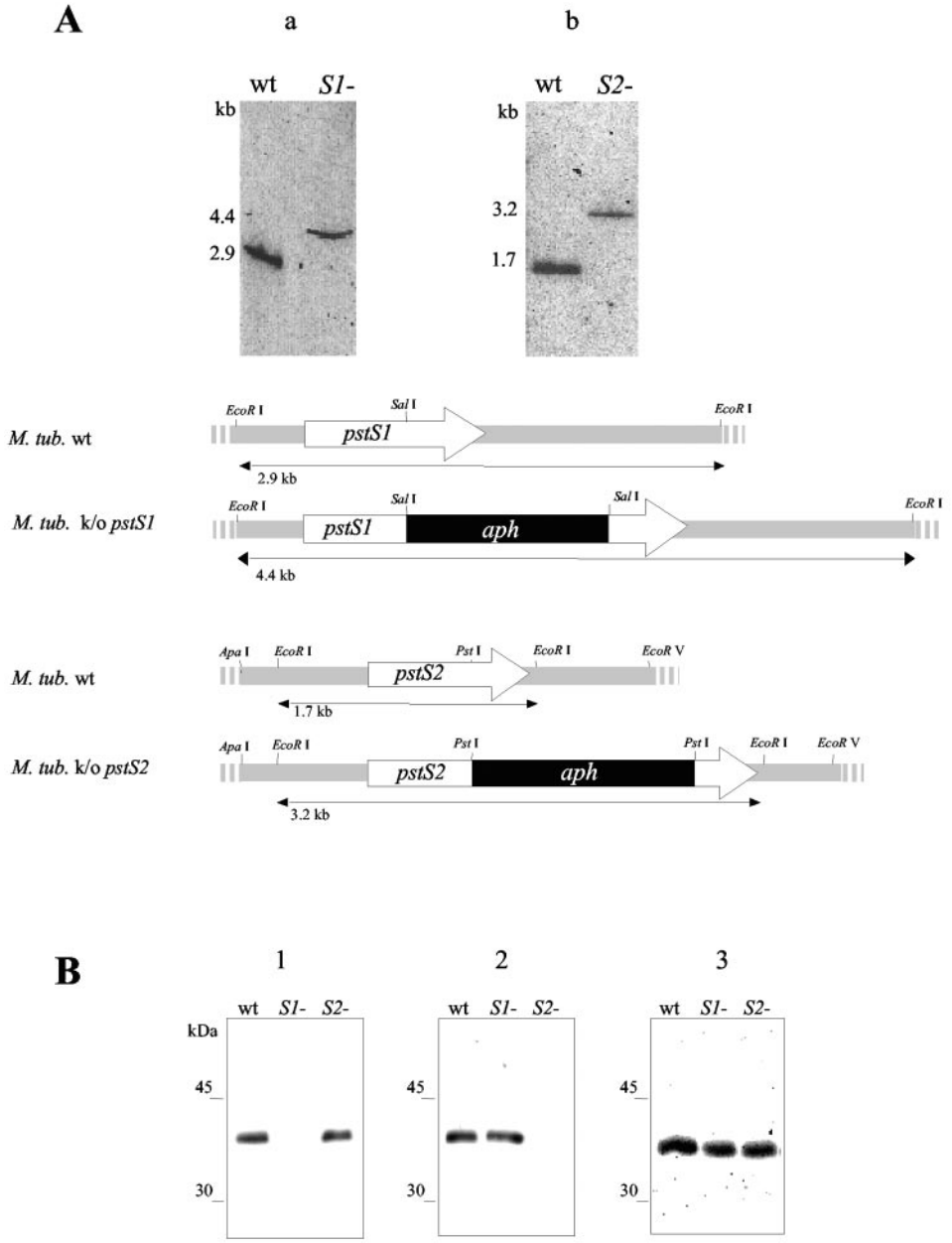

FIG. 1. (A) Southern blot analysis of the *M. tuberculosis* (*M. tub.*) H37Rv *pstS1* and *pstS2* knockout (k/o) mutants. Genomic DNA was digested with EcoRI, subjected to electrophoresis, blotted onto membranes, and probed with the *pstS1* (an NaeI-SalI fragment of the *pstS1*gene) (a) or the *pstS2* (a SacI-PstI fragment of the *pstS2* gene) (b) probe. The probes were labeled with [α-^{32}P]dCTP using the Megaprime random-primed labeling kit (Amersham). The hybridization and washing protocols were carried out under high-stringency conditions as described previously (5). The sizes of the hybridizing bands, indicated on the left, were determined from the migration distance of the DNA molecular marker Smartladder (Eurogentec). The arrows depict the lengths and transcriptional orientations of the *pstS1* and *pstS2* genes. The black boxes represent the *aph* gene, and the hatched boxes show the *pstS1* and *pstS2* gene flanking regions used for allelic exchange. Only the relevant restriction sites are indicated. wt, wild type. (B) Immunoblot analysis of the lysates of the *pstS1* and *pstS2* knockout mutants and the wild-type *M. tuberculosis* strain. Total cell extracts of the wild-type and the *pstS1* (*S1*$^-$) and *pstS2* (*S2*$^-$) knockout mutant strains were probed with anti-PstS1 (HBT12) (2, 8) (blot 1), anti-PstS2 (2A1-2) (17, 19) (blot 2), and anti-PstS3 (2F-8) (7, 17) (blot 3), and goat alkaline phosphatase-conjugated anti-mouse immunoglobulin G (Sigma). Bound antibodies were detected using BCIP (5-bromo-4-chloro-3-indolylphosphate)–nitroblue tetrazolium visualization solution (Promega). The electrophoretic mobilities of the rainbow-colored protein molecular mass markers (Amersham Pharmacia Biotech) as observed on the blots are indicated on the left.

involved in phosphate uptake from media with low phosphate concentrations. This concentration is similar to what has been found within macrophages infected with *Salmonella enterica* serovar Typhimurium (20). Therefore, mouse peritoneal macrophages were infected with the three *M. tuberculosis* strains, and we observed that both *pstS* knockout mutant strains showed significantly reduced multiplication within the macrophages compared to the parental strain (results not shown).

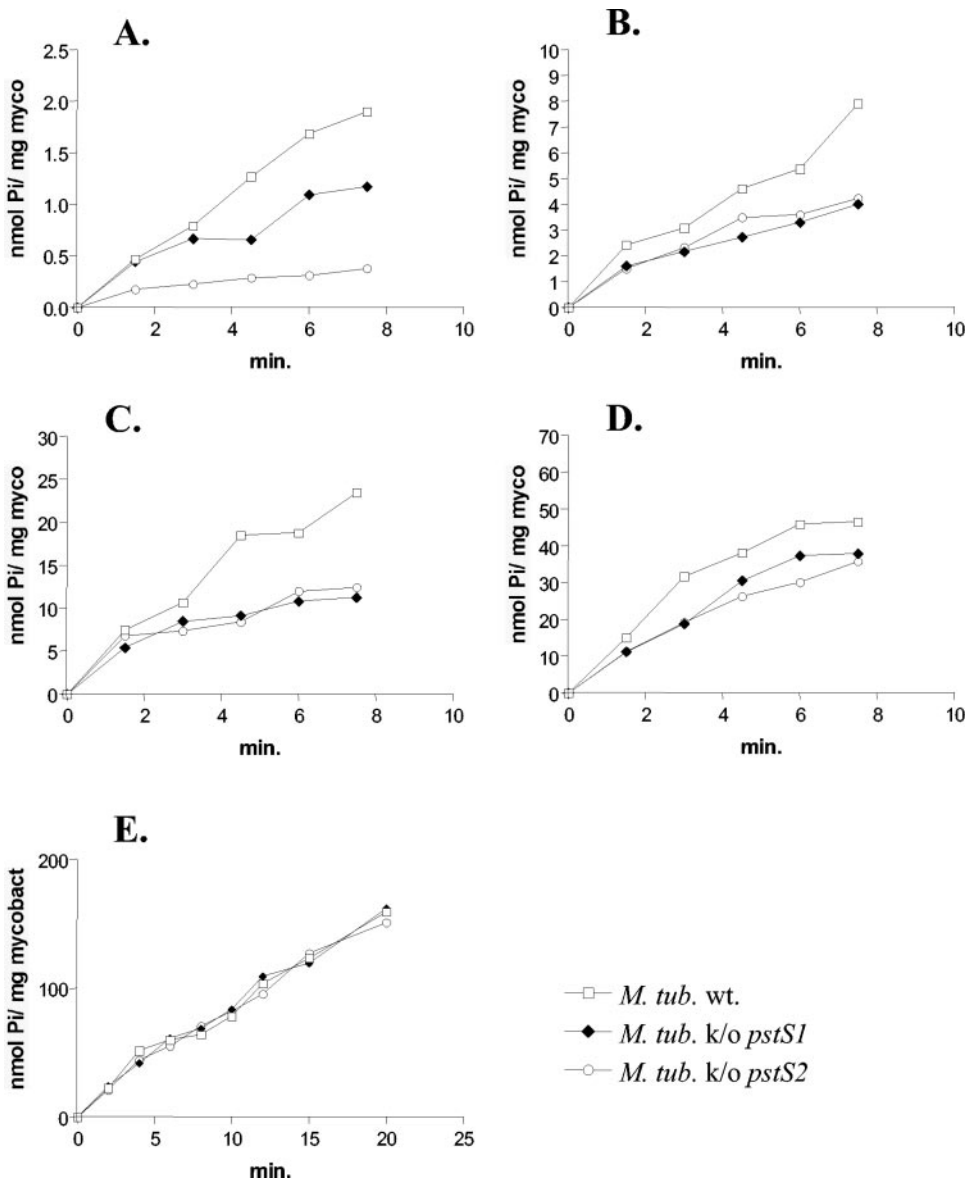

FIG. 2. Phosphate uptake rates of the *pstS1* and *pstS2* knockout (k/o) mutant and the wild-type *M. tuberculosis* (*M. tub*. wt.) strains. The uptake rate of orthophosphate (10 mCi/mmol; Amersham-Pharmacia) (expressed in nanomoles of P_i per milligram of mycobacterial protein extract) of the parental wild type and the *pstS1* and *pstS2* mutant derivatives of *M. tuberculosis* H37Rv were measured at 0.5 (A), 2 (B), 5 (C), 10 (D), and 25 (E) μM P_i.

To further investigate the roles of the two PstS proteins in tuberculosis virulence, we used an in vivo infection model. BALB/c and C57BL/6 mice were infected intravenously with either the mutant or wild-type strain, and growth in lungs and spleens was monitored over time (Fig. 3). In both mouse strains, the *pstS1* and *pstS2* mutants were attenuated (10- to 30-fold lower CFU numbers). In the spleen (Fig. 3B and D), this reduction was observed throughout the entire 3 and 5 months in the BALB/c and C57BL/6 mice, respectively. However, in the lungs (Fig. 3A and C), attenuation was strong for the first 3 months, but in the C57BL/6 mice, the CFU numbers of both mutant strains started to increase at later time points. The observed effect on the multiplication of the *pstS2* knockout mutant strain is most probably due to the inactivation of the *pstS2* gene and not to the disruption of the *pknD* gene, since in mice, a *pknD* knockout mutant does not seem to be attenuated compared to the parental strain (preliminary results).

The reduced multiplication of the two *pstS* mutants observed in infected macrophages and mice suggests that PstS1 and PstS2 are functional in vivo during infection and cannot be replaced by each other, by PstS3, by the putative Pit transporter, or by any other phosphate transporter. In addition, our results suggest that during intracellular growth, *M. tuberculosis* encounters low phosphate concentrations. *M. tuberculosis* preferentially resides within macrophages; little is known about the biochemical environment in the phagosomes harboring *M. tuberculosis* (21), and restrictions in phosphate availability for *M.*

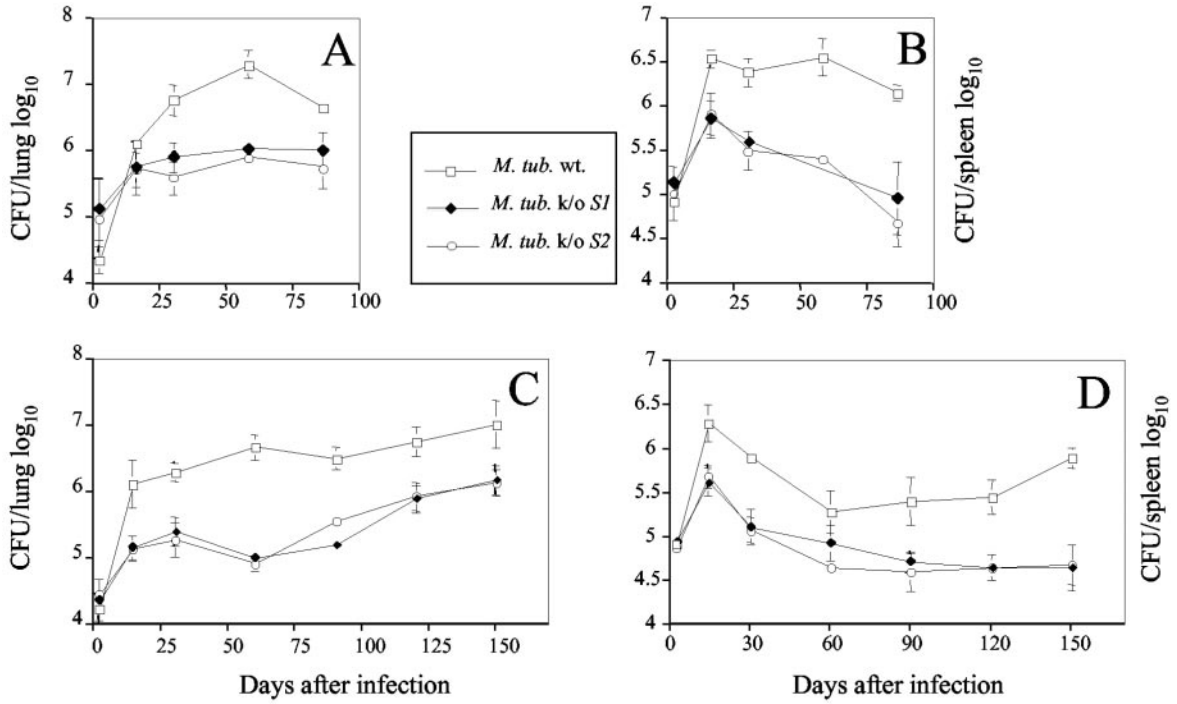

FIG. 3. Growth of *pstS1* (*M. tub S1*$^-$) and *pstS2* (*M. tub S2*$^-$) knockout mutant and wild-type (*M. tub* wt.) *M. tuberculosis* strains in lungs (A and C) and spleens (B and D) of infected mice. The bacteria were grown as a surface pellicle on synthetic Sauton medium for 14 days at 37°C and then harvested and homogenized by ball mill as previously described (16). The *M. tuberculosis* H37Rv wild-type and *pstS1* and *pstS2* knockout mutant strains were used to infect BALB/c (A and B) and C57BL/6 (C and D) mice intravenously with 2×10^5 CFU from the different *M. tuberculosis* H37Rv strains. At the indicated time points, the spleen and lungs from individual mice were homogenized in phosphate-buffered saline, and serial threefold dilutions were plated in duplicate onto Middlebrook 7H11 oleic acid-albumin-dextrose-catalase medium and incubated at 37°C for 3 to 4 weeks. The bacteria were then counted visually, and the numbers of CFU per organ were determined. The results represent the mean $\log_{10}$ values ± standard deviations of at least four mice per group. The mice (3 to 4 months old at the time of infection) were bred in the animal facilities of the Pasteur Institute of Brussels from breeding pairs obtained from Bantin and Kingman (Grimston, United Kingdom).

tuberculosis have not been shown in vivo. Our results suggest that low phosphate concentrations in intracellular vacuoles of phagocytic cells may stimulate bacteria to differentially express genes so as to survive and replicate within the host.

The *M. tuberculosis* complex is unusual in having three phosphate binding proteins and four membrane-spanning proteins organized in three operons. There is only one *pstB* gene encoding an ATP-binding subunit from the transporter in these operons, but another gene, called *phoT*, located 130 kb from *pstB* on the chromosome also encodes ATP-binding protein from the transporter. In fact, this protein has even higher homology to PstB in some other prokaryotes (http://genolist.pasteur.fr/TubercuList) than does PstB of *M. tuberculosis*. Sequencing of the *Mycobacterium bovis* genome has revealed that in this member of the *M. tuberculosis* complex, the *pstB* gene is frameshifted (13). It has been shown that PhoT is necessary for growth at low phosphate concentrations (11) and that PhoT is a virulence gene, since an *M. bovis phoT* knockout strain was significantly less virulent than its parental strain in different animal models (11). These results, together with our observation that the phosphate concentration is restricted to the intracellular vacuoles of phagocytic cells, lead to the hypothesis that the high-affinity phosphate-specific transporters are virulence factors of *M. tuberculosis* and *M. bovis*.

We thank B. Giquel for the plasmids pPR27 and pXyl4, Douglas B. Young (Imperial College, London, United Kingdom) for *M. tuberculosis* H37Rv containing pSMT1, and W. R. Jacobs for the *M. tuberculosis* H37Rv cosmid library. The excellent technical assistance of Fabienne Jurion and Kamiel Palfliet is gratefully acknowledged.

This work was supported by grant G.0266.00 from the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen, by EEC (TB Vaccine Cluster QLK2-CT-1999-01093), by the Brussels Hoofdstedelijk Gewest, by the Damiaanaktie Belgium, and by grants from "Les Amis de L'Institut Pasteur de Bruxelles (ASLB)-De Vrienden van het Instituut Pasteur van Brussel (VZW)."

REFERENCES

1. **Ames, G. F.-L.** 1993. Bacterial periplasmic permeases as model systems for the superfamily of traffic ATPases, including the multidrug resistance protein and the cystic fibrosis transmembrane conductance regulator. Int. Rev. Cytol. **137:**1–35.
2. **Andersen, A. B., and E. B. Hansen.** 1989. Structure and mapping of antigenic domains of protein antigen b, a 38,000-molecular-weight protein of *Mycobacterium tuberculosis*. Infect. Immun. **57:**2481–2488.
3. **Andersen, A. B., L. Ljungqvist, and M. Olsen.** 1990. Evidence that protein antigen b of *Mycobacterium tuberculosis* is involved in phosphate metabolism. J. Gen. Microbiol. **136:**477–480.
4. **Braibant, M., and J. Content.** 2001. The cell surface associated phosphatase activity of *Mycobacterium bovis* BCG is not regulated by environmental inorganic phosphate. FEMS Microbiool. Lett. **195:**121–126.
5. **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.
6. **Braibant, M., P. Gilot, and J. Content.** 2000. The ATP binding cassette

(ABC) transport systems of *Mycobacterium tuberculosis*. FEMS Microbiol. Rev. **24:**449–467.
7. **Braibant, M., P. Lefèvre, L. De Wit, J. Ooms, P. Peirs, K. Huygen, R. Wattiez, and J. Content.** 1996. Identification of a second *Mycobacterium tuberculosis* gene cluster encoding proteins of an ABC phosphate transporter. FEBS Lett. **394:**206–212.
8. **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.
9. **Chang, Z., A. Choudhary, R. Lathigra, and F. A. Quiocho.** 1994. The immunodominant 38-kDa lipoprotein of *M. tuberculosis* is a phosphate binding protein. J. Biol. Chem. **269:**1956–1958.
10. **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, B. G. Barrell, et al.** 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature **393:**537–544.
11. **Collins, D. M., R. P. Kawakami, B. M. Buddle, B. J. Wards, and G. W. de Lisle.** 2003. Different susceptibility of two animal species infected with isogenic mutants of *Mycobacterium bovis* identifies *phoT* as having roles in tuberculosis virulence and phosphate transport. Microbiology **149:**3203–3212.
12. **Cox, G. B., H. Rosenberg, J. A. Downie, and S. Silver.** 1981. Genetic analysis of mutants affected in the Pst inorganic phosphate transport system. J. Bacteriol. **148:**1–9.
13. **Garnier, T., K. Eiglmeier, J. C. Camus, N. Medina, H. Mansoor, M. Pryor, S. Duthoy, S. Grondin, C. Lacroix, C. Monsempe, S. Simon, B. Harris, R. Atkin, J. Doggett, R. Mayes, L. Keating, P. R. Wheeler, J. Parkhill, B. G. Barrell, S. T. Cole, S. V. Gordon, and R. G. Hewinson.** 2003. The complete genome sequence of *Mycobacterium bovis*. Proc. Natl. Acad. Sci. USA **100:**7877–7882.
14. **Harris, R. M., D. C. Webb, S. M. Howitt, and G. B. Cox.** 2001. Characterization of PitA and PitB from *Escherichia coli*. J. Bacteriol. **183:**5008–5014.
15. **Higgins, C. F.** 1992. ABC transporters: from microorganisms to man. Annu. Rev. Cell Biol. **8:**67–113.
16. **Huygen, K., D. Abramowicz, P. Vandenbussche, F. Jacobs, J. De-Bruyn, A. Kentos, A. Drowart, J. P. Van-Vooren, and M. Goldman.** 1992. Spleen cell cytokine secretion in *Mycobacterium bovis* BCG-infected mice. Infect. Immun. **60:**2880–2886.
17. **Huygen, K., A. Drowart, M. Harboe, R. ten-Berg, J. Cogniaux, and J. P. Van-Vooren.** 1993. Influence of genes from the major histocompatibility complex on the antibody repertoire against culture filtrate antigens in mice infected with live *Mycobacterium bovis* BCG. Infect. Immun. **61:**2687–2693.
18. **Kalpana, G. V., B. R. Bloom, and W. Jacobs, Jr.** 1991. Insertional mutagenesis and illegitimate recombination in mycobacteria. Proc. Natl. Acad. Sci. USA **88:**5433–5437.
19. **Lefèvre, P., M. Braibant, L. De Wit, M. Kalai, D. Röeper, J. Grötzinger, 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. **Lucas, R. L., and C. A. Lee.** 2000. Unravelling the mysteries of virulence gene regulation in *Salmonella typhimurium*. Mol. Microbiol. **36:**1024–1033.
21. **McKinney, J. D., and J. E. Gomez.** 2003. Life on the inside for *Mycobacterium tuberculosis*. Nat. Med. **9:**1356–1357.
22. **Peirs, P., L. De Wit, M. Braibant, K. Huygen, and J. Content.** 1997. A serin/threonine protein kinase from *Mycobacterium tuberculosis*. Eur. J. Biochem. **244:**604–612.
23. **Peirs, P., B. Parmentier, L. De Wit, and J. Content.** 2000. The Mycobacterium bovis homologous protein of the Mycobacterium tuberculosis serine/threonine protein kinase Mbk (PknD) is truncated. FEMS Microbiol. Lett. **188:**135–139.
24. **Pelicic, V., M. Jackson, J. M. Reyrat, W. R. Jacobs, Jr., B. Gicquel, and C. Guilhot.** 1997. Efficient allelic exchange and transposon mutagenesis in *Mycobacterium tuberculosis*. Proc. Natl. Acad. Sci. USA **94:**10955–10960.
25. **Qi, Y., Y. Kobayashi, and 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.
26. **Quentin, Y., G. Fichant, and F. Denizot.** 1999. Inventory, assembly and analysis of *Bacillus subtilis* ABC transport systems. J. Mol. Biol. **287:**467–484.
27. **Rosenberg, H., R. G. Gerdes, and K. Chegwidden.** 1977. Two systems for the uptake of phosphate in *Escherichia coli*. J. Bacteriol. **131:**505–511.
28. **Surin, B. P., H. Rosenberg, and G. B. Cox.** 1985. Phosphate-specific transport system of *Escherichia coli*: nucleotide sequence and gene-polypeptide relationships. J. Bacteriol. **161:**189–198.
29. **Vyas, N. K., M. N. Vyas, and F. A. Quiocho.** 2003. Crystal structure of *M. tuberculosis* ABC phosphate transport receptor: specificity and charge compensation dominated by ion-dipole interactions. Structure **11:**765–774.
30. **Wanner, B. L.** 1993. Gene regulation by phosphate in enteric bacteria. J. Cell Biochem. **51:**47–54.
31. **Wanner, B. L.** 1996. Signal transduction in the control of phosphate-regulated genes of *Escherichia coli*. Kidney Int. **49:**964–967.
32. **Willsky, G. R., and M. H. Malamy.** 1980. Characterization of two genetically separable inorganic phosphate transport systems in *Escherichia coli*. J. Bacteriol. **144:**356–365.

Editor: F. C. Fang