The Virulence-associated Two-component PhoP-PhoR System Controls the Biosynthesis of Polyketide-derived Lipids in Mycobacterium tuberculosis

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Two-component regulatory signal transduction systems are important elements of the adaptive response of prokaryotes to a variety of environmental stimuli. Disruption of PhoP-PhoR in Mycobacterium tuberculosis dramatically attenuates virulence, implying that this system directly and/or indirectly coordinates the expression of important virulence factors whose identity remains to be established. Interestingly, in knocking out the PhoP-PhoR two-component system of M. tuberculosis Mt103, dramatic changes in the colonial morphology, cording properties, and reactivity of the mutant strain to the basic dye neutral red, all intrinsic properties of tubercle bacilli known to correlate with virulence, were noted. Because deficiencies in the ability of the mutant to form serpentine cords and stain with the dye are likely the results of alterations of its cell envelope composition, we undertook to analyze the lipid content of phoP and phoP-phoR mutants constructed in two different strains of M. tuberculosis. Our results indicate that PhoP coordinates and positively regulates the synthesis of methyl-branched fatty acid-containing acyltrehaloses known to be restricted to pathogenic species of the M. tuberculosis complex, namely diacyltrehaloses, polycytylthrehaloses, and sulfolipids. Evidence is also provided that PhoP but not PhoR is required for the production of these lipids. This work represents an important step toward the functional characterization of PhoP-PhoR and the understanding of complex lipid synthesis in M. tuberculosis.

Mycobacterium tuberculosis, the causative agent of tuberculosis in humans, is one of the leading causes of mortality due to a single infectious agent (1). In the tubercle bacilli as in other prokaryotes, two-component signal transduction systems are important elements of the adaptive response to a variety of stimuli (2). So far, of the 11 paired two-component systems, 5 unpaired response regulators and 2 unpaired protein sensors that M. tuberculosis possesses, the two-component system PhoP-PhoR is the one whose disruption was shown to affect the most dramatically the ability of M. tuberculosis to replicate in cellular and animal models (3). Interestingly, PhoP shows high similarity to the PhoP response regulator of Salmonella enterica serovar typhimurium, which senses Mg2+ starvation and controls the expression of at least 40 genes, among which some encoding important virulence determinants (4). Further supporting the concept that PhoP is important for virulence and transmission of tubercle bacilli, a multidrug-resistant strain of Mycobacterium bovis (strain B) responsible for large tuberculosis outbreaks in Spain was found to carry an IS6110 insertion in the promoter region of phoP causing a strong up-regulation of the expression of this gene (5). To date, the stimuli sensed by the sensor histidine kinase PhoR and the genes controlled by the DNA-binding response regulator PhoP are not known. The identification of the environmental signals regulating PhoP-PhoR and the characterization of the PhoP regulon would provide important information about the stresses encountered by the tubercle bacilli in vivo and allow the discovery of novel virulence factors.

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EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Media—Escherichia coli XLI-blue was maintained in Luria Bertani (LB) broth (pH 7.5) (BD Biosciences). M. tuberculosis 1237 (a clinical isolate belonging to the W Beijing family) and Mt103 (10) were grown in Middlebrook 7H11 medium supplemented with ADC (0.2% dextrose, 0.5% bovine serum albumin fraction V, 0.085% NaCl, 0.0003% beef catalase) (Difco) and 0.005% Tween 80 or on solid Middlebrook 7H10 medium supplemented with OADC (0.005% oleic acid, 0.2% dextrose, 0.5% bovine serum albumin fraction V, 0.085% NaCl, 0.0003% beef catalase) (Difco). The antibiotics, kanamycin (25 μg ml−1) and hygromycin (50 μg ml−1) were added when appropriate.

Construction of phoP and phoP-phoR Mutants of M. tuberculosis Mt103 and M. tuberculosis 1237—The plasmids used to disrupt phoP or both the phoP and phoR genes in M. tuberculosis were constructed as follows. For the disruption of phoP and phoR, a 3,804-bp DNA fragment carrying both genes was PCR-amplified from genomic DNA using phoP polymerase (Stratagene) and primers PhoP5′ (5′-aatctagatcaagcatcagcc-3′) and PhoR3′ (5′-aartcgggtacctgagcagcggctt-3′). A 774-bp EcoRV-BspEI restriction fragment encompassing part of the phoP and phoR coding sequences was released from this PCR product and replaced with an hygromycin resistance cassette from Streptomyces hygroscopicus. The disrupted allele phoPR::hyg was then introduced into the suicide vector described above. Mutants were obtained following a two-step homologous recombination strategy as described (11).

Preparation and Analysis of Lipids and Fatty Acids

Bacterial Strains and Culture Media—Escherichia coli XLI-blue was maintained in Luria Bertani (LB) broth (pH 7.5) (BD Biosciences). M. tuberculosis 1237 (a clinical isolate belonging to the W Beijing family) and Mt103 (10) were grown in Middlebrook 7H11 medium supplemented with ADC (0.2% dextrose, 0.5% bovine serum albumin fraction V, 0.085% NaCl, 0.0003% beef catalase) (Difco) and 0.005% Tween 80 or on solid Middlebrook 7H10 medium supplemented with OADC (0.005% oleic acid, 0.2% dextrose, 0.5% bovine serum albumin fraction V, 0.085% NaCl, 0.0003% beef catalase) (Difco). The antibiotics, kanamycin (25 μg ml−1) and hygromycin (50 μg ml−1) were added when appropriate.

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Preparation and Analysis of Lipids and Fatty Acids—Radiolabeling of whole M. tuberculosis cells with [1,2-14C]acetic acid (specific activity, 113 Ci mol−1,

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As observed in M. tuberculosis Mt103 earlier (3), disruption of phosphoenolpyruvate:phosphoribosyltransferase in wild-type M. tuberculosis Beijing 1237 dramatically impaired the ability of this strain to grow inside human THP-1 monocyte-derived macrophages. While the number of intracellular wild-type colony forming units increased ~52-fold after 7 days of infection, the number of mutant colony forming units increased only about 10-fold during the same period (data not shown). Normal intracellular growth was restored in the mutant upon complementation with wild-type copies of the phoP and phoR genes carried by pJUZ1K (data not shown).

phoP and phoR PhoR Mutants of M. tuberculosis Are Deficient in the Production of Multiple Methyl-branched Fatty Acid-containing Acyltrehaloses—
We next undertook to compare the cell envelope composition of the phoP and phoR-phoR mutants to that of their wild-type parental strains. Lipid extracts

from bacterial cells and culture filtrates were examined by TLC following metabolic labeling of all classes of lipids with [1,2-14C]acetate and that of methyl-branched fatty acids with [1-13C]propionate. In all cases, mutants were found to contain the same amounts of extractable lipids as their wild-type parents. Analyses of [1-14C]propionate-derived lipids from bacterial cells revealed that all of the mutants clearly lacked two families of methyl-branched fatty acids.

FIGURE 1. Cytochemical staining of phoP and phoP-phoR mutants of M. tuberculosis with neutral red. Neutral red staining was performed as described (38) on wild-type M. tuberculosis 1237, 1237ΔphoP-hyg, 1237ΔphoR-hyg, and on the complemented mutant strains 1237ΔphoP-phoR-hyg/pSU5K and 1237ΔphoP-hyg/pJUZ1K. Similar results were obtained with the phoP mutants of Mt103.

FIGURE 2. Lipid content of the wild-type, mutant, and complemented mutant strains of M. tuberculosis and effect of overexpressing phoP in wild-type M. tuberculosis 1237. Autoradiograms of thin-layer chromatograms of lipids derived from M. tuberculosis 1237. Autoradiograms of thin-layer chromatograms of lipids derived from

A

B

C

7H10 Middlebrook agar plates (data not shown). Auramine staining of cultures grown to late stationary phase in liquid broth devoid of Tween 80 also revealed that all of the mutants exhibited the same cording defect (3) (data not shown). Finally, staining of the phoP and phoP-phoR mutants with neutral red, a classical cytochemical test used to differentiate virulent from attenuated M. tuberculosis strains on 7H10 Middlebrook agar plates (data not shown). Auramine staining also revealed that all of the mutants exhibited the same cording defect (3) (data not shown). Finally, staining of the phoP and phoP-phoR mutants with neutral red, a classical cytochemical test used to differentiate virulent from attenuated M. tuberculosis strains. Lipid extracts

of [1-14C]propionate and [1-14C]acetate acid are shown. A, for the analysis of DAT and SL, [1-14C]propionate-derived total lipids (30,000 total cpm) were subjected to TLC with chloroform:methanol:water (90:10:1, v:v:v) as the solvent. B, PAT were analyzed by loading 40,000 cpm of [1-14C]propionate-derived total lipids on TLC plates and developing the plates three times in petroleum ether (60/80 °C) :acetone (92:8, v/v) in the first direction and once in toluene:acetonitrile (95:5, v/v) in the second direction. C, for the analysis of trehalose monomycolates (TMM) and trehalose dimycolates (TDM), [1-14C]acetate-derived total lipids (20,000 total cpm) were subjected to TLC with chloroform:methanol:water (90:10:1, v:v:v) as the solvent. Only the PAT, TMM, and TDM profiles of the 1237 mutant strains are shown. Similar profiles were obtained with the Mt103 mutants. 1237ΔphoP-hyg/pSU5K, phoP-phoR mutant of M. tuberculosis 1237 complemented with the phoP gene; 1237ΔphoP-hyg/pJUZ1K, phoP-phoR mutant of M. tuberculosis 1237 complemented with the phoP and phoR genes.

RESULTS
Morphological and Cytochemical Traits of phoP and phoP-phoR Deletion Mutants of M. tuberculosis—Dramatic changes in the morphological characteristics of a phoP allelic exchange mutant of M. tuberculosis Mt103 have been reported (3) with Mt103 phoP::km bacilli aligning along their long axes in parallel arrays instead of forming the typical braided bundles (or cords) of wild-type Mt103 and pathogenic M. tuberculosis strains in general (6). Because differences in cording properties are likely the result of changes in cell surface composition (7–9), which is known to vary among clinical isolates of M. tuberculosis (7, 15–17), we first sought to disrupt the PhoP-PhoR two-component system in another clinical isolate of M. tuberculosis harboring a slightly different cell envelope composition than Mt103. Isogenic mutants of 1237 (a clinical isolate belonging to the W-Beijing family), in which the phoP or both phoP and phoR genes were disrupted by deletion of an internal fragment and insertion of an hygromycin resistance cassette, were constructed by homologous recombination. Following the same strategy, a phoP-phoR and a novel phoP mutant of Mt103 were also constructed to ensure that these mutants carried exactly the same mutations as their 1237 counterparts. Recombination at the phoP or phoP-phoR loci was confirmed in each mutant by Southern hybridization (data not shown).

As reported earlier for Mt103 phoP::km (3), all of the newly constructed phoP and phoP-phoR mutants formed smaller colonies than their wild-type parental strains on 7H10 Middlebrook agar plates (data not shown). Auramine staining of cultures grown to late stationary phase in liquid broth devoid of Tween 80 also revealed that all of the mutants exhibited the same cording defect (3) (data not shown). Finally, staining of the phoP and phoP-phoR mutants with neutral red, a classical cytochemical test used to differentiate virulent from attenuated isolates of M. tuberculosis (18), also revealed that all of the mutant strains had lost the ability to fix the dye (Fig. 1).

As observed in M. tuberculosis Mt103 earlier (3), disruption of phosphoenolpyruvate:phosphoribosyltransferase in wild-type M. tuberculosis Beijing 1237 dramatically impaired the ability of this strain to grow inside human THP-1 monocyte-derived macrophages. While the number of intracellular wild-type colony forming units increased ~52-fold after 7 days of infection, the number of mutant colony forming units increased only about 10-fold during the same period (data not shown). Normal intracellular growth was restored in the mutant upon complementation with wild-type copies of the phoP and phoR genes carried by pJUZ1K (data not shown).

The abbreviations used are: MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; DAT, diacyltrehaloses; PAT, polycyaltrehaloses; SL, sulfolipids; TMM, trehalose monomycolates; TDM, trehalose dimycolates; GC, gas chromatography; pks, polyketide synthase.

We next undertook to compare the cell envelope composition of the phoP and phoP-phoR mutants to that of their wild-type parental strains. Lipid extracts

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MP Biomedicals Inc.) and [1-14C]propionate (specific activity, 56.7 Ci mol⁻¹, MP Biomedicals Inc.), preparation of mycolic acids and extraction of total cellular and exocellular lipids from cold or radiolabeled bacterial cultures were performed as described (13). Lipids and fatty acid methyl esters were analyzed by TLC on silica gel 60-precoated plates F254 (E. Merck, Darmstadt, Germany) in various solvent systems (see the legend to Fig. 2). Radiolabeled lipids and fatty acids were visualized by exposure of TLC to Kodak BioMax MR films at −70 °C. Analysis of mycostes by MALDI-TOF mass spectrometry was performed as described previously (14).
Addition, sulfolipid (SL) production (22, 23) was abolished in 1237ΔphoP:hyg and 1237ΔphoPR:hyg (Figs. 2A and 3). *M. tuberculosis* Mt103 being naturally devoid of this latter family of acyltrehaloses, this phenotype was only visible in the 1237 background. All of the lipids found to be absent from the mutant cells were also absent from their culture filtrates indicating that their absence from bacterial cells was not the result of secretion into the culture medium (data not shown). Analyses of labeled lipids in a variety of solvent systems revealed no other qualitative or quantitative differences between the wild-type and the mutant strains. Likewise, a combination of MALDI-TOF mass spectrometry and TLC analyses revealed that all of the *M. tuberculosis* mutants produced the same types and amounts of mycolates as their parent strains (data not shown). Finally, gas chromatography (GC) analysis of short chain (up to C26) fatty acid methyl esters from total lipids showed that the mutants generate all of the classes of fatty acids produced by the wild-type strains (data not shown).

Since the non-mycoloylated trehalose esters, DAT, PAT, and SL, mainly contain methyl-branched fatty acids such as mycolipenic acids, mycosanoic acids, and (hydroxy)phthioceranic acids (Fig. 3), further analyses were performed comparing wild-type and mutant strains for the presence of these fatty acids. To this aim, fatty acid methyl esters and acetylated fatty acid methyl esters were prepared from the non-radiolabeled and [1-14C]propionate-derived lipids of each of the strains described in this study and analyzed by GC and TLC, respectively. Although the autoradiograms of the TLC plates showed bands of significantly lower intensity in the mutants as compared with the wild-type strains, it was not possible to accurately compare the long-chain methyl-branched fatty acid composition of the different strains due to the co-migration of the fatty acids of interest with other [1-14C]propionate-derived compounds (mycocerosic acids in particular) (data not shown). Unfortunately, the low quantities of SL, DAT, and PAT produced by the *M. tuberculosis* isolates 1237 and Mt103 also hampered the detection of the long-chain methyl-branched fatty acids esterifying these lipids by GC.

Interestingly, both the complementation of 1237ΔphoPR:hyg with the *phoP* and *phoR* genes carried by the multicopy vector pJUZ1K and with the *phoP* gene alone carried by pSO5K restored SL, PAT, and DAT production (Fig. 2, A and B), indicating that *phoP* but not *phoR* is required for the production of methyl-branched fatty acid-containing acyltrehaloses in *M. tuberculosis*. Moreover, the amounts of SL produced by 1237 (but not those of DAT and PAT) appeared to be directly correlated to the level of expression of *phoP* as overexpression of this gene in 1237 from the multicopy plasmid pSO5K resulted in a significant increase in the amounts of 14C incorporated into SL (Fig. 2A). Paralleling the recovery of acyltrehaloses synthesis, the ability of the complemented 1237 mutants to fix neutral red was restored (Fig. 1).

**DISCUSSION**

A previous study had reported that disruption of *phoP* slightly increased the ratio of monoacylated to triacylated lipoarabinomannan in *M. tuberculosis* Mt103, already pointing to the existence of a relationship between PhoP and lipid metabolism (24). In this study, we provided evidence that *phoP* is required for the synthesis of complex acyltrehaloses restricted to pathogenic species of the *M. tuberculosis* complex, namely DAT, PAT, and SL. Moreover, there seems to be a direct correlation between the level of expression of *phoP* and the amounts of some of these lipids as overexpression of *phoP* from a multicopy plasmid in strain 1237 resulted in a significant increase in the amounts of SL synthesized. These results suggest that the two-component regulator PhoP-PhoR up-regulates the expression of genes involved in the biosynthesis of SL.
DAT, and PAT. Considering that the synthesis of trehalose mono- and di- 
ymycobacteria is unaffected in the mutants (Fig. 2C), it seems unlikely that PhoP- 
PhoR exerts its control at the level of trehalose synthesis. It is more likely that it 
regulates the expression of the acyltransferase, polyketide synthase (pks) or 
pks-associated genes involved in the synthesis or transfer of the methyl- 
 branch fatty acyl substituents found in SL-DAT, and PAT. 

Interestingly, the histidine kinase encoded by phoD does not seem to be required for 
the synthesis of these lipids as complementation of Δ37ΔphoPΔphoRΔpyc with the 
phoD gene alone was sufficient to restore their synthesis. As already described in 
other two-component systems, phosphorylation might not be an absolute require- 
ment for the binding of PhoP to its DNA targets and might only serve to increase 
the affinity of the response regulator for certain promoters (25). Alternatively, as 
the case in Salmonella, it is possible that instead of phosphorylating PhoP, PhoR 
catalyzes the dephosphorylation of phospho-PhoP, thereby abolishing the trans- 
cription of PhoP-activated genes (4). In that case, the lack of PhoP expression in 
Δ37ΔphoPΔphoRΔpyc complemented with phoP would not abdul the produc- 
ction of acyltrehaloses. 

The abolition of the synthesis of DAT, PAT, and SL probably accounts, at least in part, for the decreased ability of the phoP mutants to form cords and to 
fix neutral red, morphological and cytochemical traits, which have been asso- 
ciated with virulence attenuation of tubercle bacilli since Dubos and Middle- 
brook’s seed studies in the 1940s. Indeed, trehalose esters are located at or near 
the surface of M. tuberculosis where their fatty acid chains have been proposed to 
interact with the mycolic acids of the cell wall core to provide a mycobacte- 
rial “outer membrane” system, while their sugar moiety interacts with the 
hydrophilic capsule (26). In good agreement with this model, we have found that 
the absence of some forms of DAT and PAT in a pks-3-4 mutant of M. tuberculosis H37Rv resulted in defects in capsule attachment causing the mutant to express a more hydrophobic surface (27). Consequently, to aggregate in 
liquid broth (27) and to interact differently with phagocytic and non-phago- 
cytes (13). More recently, in a search for the molecular determinants 
underlying the different cording properties of the M. tuberculosis H37Ra and 
H37Rv strains using the microarray technology, Gao et al. (28) identified two 
polyketide synthase-associated genes, fasD221 and papA1, among the 22 genes 
whose expression was consistently higher in the cord-forming strain H37Rv. 
The pks-3-4-activated gene fasD221 is expected to encode a fatty acid-activat- 
ing enzyme involved in DAT and PAT synthesis (27), while papA1, which is 
associated to pks2, is likely to encode an acyltransferase required for SL forma- 
tion (29). As the cording strain M. tuberculosis H37Rv is proficient in the 
synthesis of SL and mycolate-containing DAT and PAT, while the non- 
cordic M. tuberculosis H37Ra and phoP mutants are not (7, 15–16), it is 
tempting to speculate that SL, DAT, and PAT are, with mycolic acids (8–9), 
major determinants of cording in M. tuberculosis. 

If the association between cording and virulence has been well established (6), it is 
at present not as clear to what extent the lack of SL, DAT, and PAT accounts for 
the virulence attenuation and, perhaps, reduced transmissibility (5) of the 
phoP knock-out mutants. The fact that, within the Mycobacterium genus, SL, PAT, and 
DAT are relatively restricted to the virulent strains of the M. tuberculosis complex 
(15, 16), is consistent with the idea that this family of lipids serves important functions in 
the pathogenicity of tubercle bacilli, an assumption further supported by multiple 
in vitro and in vivo studies (for a review, see Refs. 13, 22, 23, 30 and references cited 
therein, and 31–34). Somewhat tempering the above assumption, our recent work has 
indicated that M. tuberculosis isogenic mutants specifically impaired in their 
ability to synthesize SL or PAT and DAT did not display any marked reduction of 
virulence in cellular and animal models (13, 30). Nevertheless, as the mutants 
analyzed in both studies retained the ability to synthesize some methyl-branched 
fatty acid-containing acyltrehaloses, one could not exclude that the remaining 
families of acyltrehaloses in the mutant compensated for the missing types. The 
fact that the production of all of these molecules is coordinately regulated by PhoP 
 actually tends to suggest that they act synergistically. Therefore, the effects of these 
lipids in virulence might only be clearly visible when the synthesis of all of them is 
abolished simultaneously. 

It is noteworthy that this is not the first report of a two-component system 
regulating the synthesis of polyketides or polyketide-derived products in pro- 
karyotes. In Streptomyces lividus and Streptomyces coelicolor, it was shown that 
phosphate control of antibiotic biosynthesis is mediated by the two-component 
PhoP-PhoR system (35, 36). It is interesting that actinomycetes have 
adopted similar strategies to regulate the production of polyketide-derived 
products, despite the structural and functional diversity of these molecules. 

Although much work remains to be done in identifying the PhoP regulon 
and understanding the virulence attenuation of phoP mutants, this work rep- 
resents an important step toward the functional characterization of PhoP and 
the understanding of complex lipid synthesis in M. tuberculosis. Identifying the 
specific signals sensed by the histidine kinase PhoR, whether Mg2+ as in Salmo- 
nella (4), phosphate as in Streptomyces (36), acetate as in E. coli (37), or otherwise, is now required to determine under which conditions and toward 
which aim(s) the production of methyl-branched fatty acids-containing acyl- 
trehaloses is regulated. Several approaches, including promoter fishing and 
proteomics, have been undertaken by our laboratories to complete our under- 
standing of this important two-component regulator. 

REFERENCES