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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 adaptative 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 in *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 coordinately 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, polyacyltrehaloses, 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 bacillus as in other prokaryotes, two-component signal transduction systems are important elements of the adaptative 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 Mg^{2+} 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 transmissibility of tubercle bacilli, a multidrug-resistant strain of *Mycobacterium bovis* (strain B) responsible for large tuberculosis outbreaks in Spain was found to carry an IS*6110* 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 bacillus *in vivo* and allow the discovery of novel virulence factors.

In constructing a *phoP* mutant of *M. tuberculosis* (clinical isolate Mt103), it was noted that the mutant was smaller in size and different from the wild-type strain in its cording properties (3). Given the relationship that exists between cording properties and virulence on the one hand (6) and between the cording of tubercle bacilli and their lipid composition on the other hand (7–9), we sought to compare the cell envelope composition of *M. tuberculosis phoP* and *phoP-phoR* mutants to those of their wild-type parental strains as a first step toward the identification of the PhoP regulon.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Media—Escherichia coli XL1-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 7H9 medium supplemented with ADC (0.2% dextrose, 0.5% bovine serum albumin fraction V, 0.085% NaCl, 0.0003% beef catalase) (Difco) and 0.05% 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 *Pfu* polymerase (Stratagene) and primers PhoPF (5′-aatctagatcaagcatcagcc-3′) and PhoRR (5′-aatctagacgagtttgacggc-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 a non-replicative plasmid harboring the counter-selectable marker *sacB* and the colored marker *xylE* (11). For the disruption of the only *phoP* gene, a 2,057-bp DNA fragment carrying the entire *phoP* was PCR-amplified using *Pfu* polymerase and primers PhoPF (described above) and PhoPR (5′-aatctagacccgaacgtagaa-3′). An EcoRV-BclI restriction fragment internal to the *phoP* gene was cut out and replaced by an hygromycin resistance cassette, and the disrupted allele *phoP*::*hyg* was then introduced into the suicide vector described above. Mutants were obtained following a two-step homologous recombination strategy as described (11).

Complementation of the phoP and phoP-phoR Mutants—The kanamycin resistance cassette from pUC4K (Amersham Biosciences) was cloned into the PstI restriction site of pSO5 (a mycobacterial replicative plasmid carrying the entire coding sequence of *phoP* and 1 kb of its promoter region) (3) to yield pSO5K, the vector used to complement 1237Δ*phoPR*::*hyg*. A 3.8-kb DNA fragment carrying the *phoP-phoR* genes from *M. tuberculosis* H37Rv was PCR-amplified using the couple of primers phoPFtb (5′-aatctagatcaagcatcagccgaggtac-3′) and phoRRtb (5′-aatctagacgagtttgacggcggcttta-3′), cut with XbaI, and cloned into the unique XbaI site of pNBV1 (12) to yield pJUZ1. The kanamycin resistance cassette from pUC4K was then cloned into the PstI restriction site of pJUZ1 to yield pJUZ1K, the vector used to complement 1237Δ*phoPR*::*hyg*.

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

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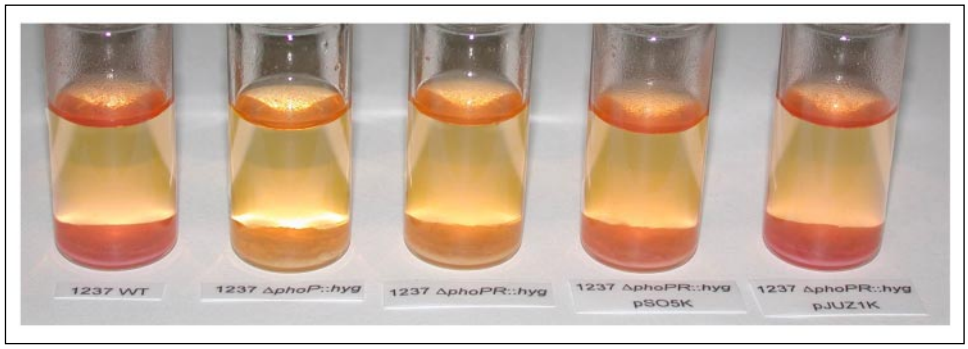

FIGURE 1. **Cytochemical staining of *phoP* and *phoP-phoR* mutants of *M. tuberculosis* with neutral red.** Neutral red staining was performed as described (38) on wild-type *M. tuberculosis* 1237, 1237Δ*phoP::hyg*, 1237Δ*phoP-phoR::hyg*, and on the complemented mutant strains 1237Δ*phoP-phoR::hyg*/pSO5K and 1237Δ*phoP-phoR::hyg*/pJ UZ 1K. Similar results were obtained with the *phoP* mutants of Mt103.

MP Biomedicals Inc.) and [1-^{14}C]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 F_{254} (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 mycolates by MALDI-TOF[4] mass spectrometry was performed as described previously (14).

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 or avirulent 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 *phoP-phoR* in *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 phoP-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 *phoP-phoR* mutants to that of their wild-type parental strains. Lipid extracts

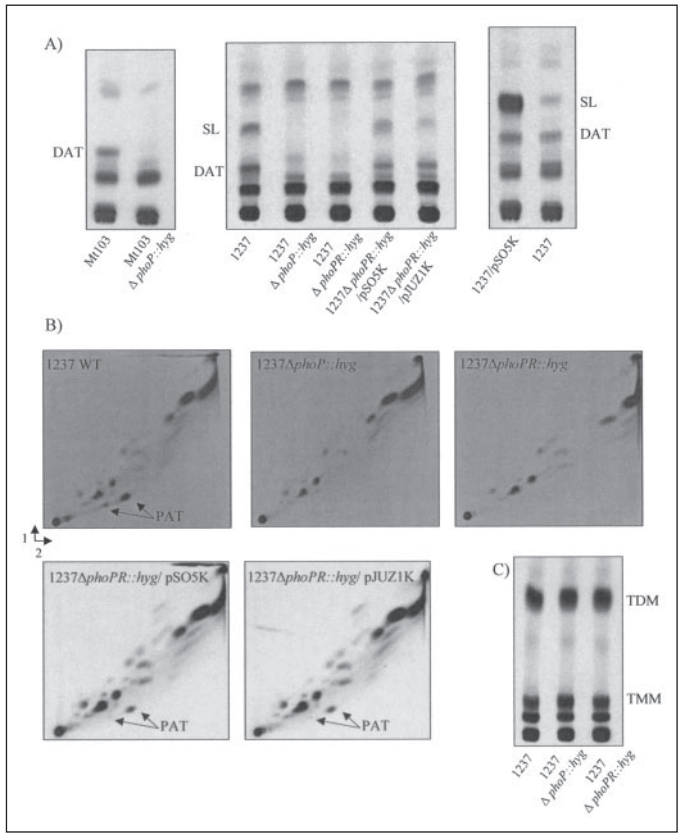

FIGURE 2. **Lipid content of the wild-type, mutant, and complemented mutant strains of *M. tuberculosis* and effect of overexpressing *phoP* in wild-type *M. tuberculosis* 1237.** Autoradiograms of thin-layer chromatograms of lipids derived from [1-^{14}C]propionate and [1,2-^{14}C]acetic acid are shown. *A*, for the analysis of DAT and SL, [1-^{14}C]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-^{14}C]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:acetone (95:5, v:v) in the second direction. *C*, for the analysis of trehalose monomycolates (*TMM*) and trehalose dimycolates (*TDM*), [1,2-^{14}C]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 mutants are shown. Similar profiles were obtained with the Mt103 mutants. 1237Δ*phoPR::hyg*/pSO5K, *phoP-phoR* mutant of *M. tuberculosis* 1237 complemented with the *phoP* gene; 1237Δ*phoPR::hyg*/pJUZ1K, *phoP-phoR* mutant of *M. tuberculosis* 1237 complemented with the *phoP* and *phoR* genes.

from bacterial cells and culture filtrates were examined by TLC following metabolic labeling of all classes of lipids with [1,2-^{14}C]acetate and that of methyl-branched fatty acids with [1-^{14}C]propionate. In all cases, mutants were found to contain the same amounts of extractable lipids as their wild-type parents. Analyses of [1-^{14}C]propionate-derived lipids from bacterial cells revealed that all of the mutants clearly lacked two families of methyl-branched fatty acid-containing acyltrehaloses, namely 2,3-di-*O*-acyltrehaloses (DAT) (19, 20) and polyacyltrehaloses (PAT) (16, 21) (Figs. 2, *A* and *B*, and 3). In

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

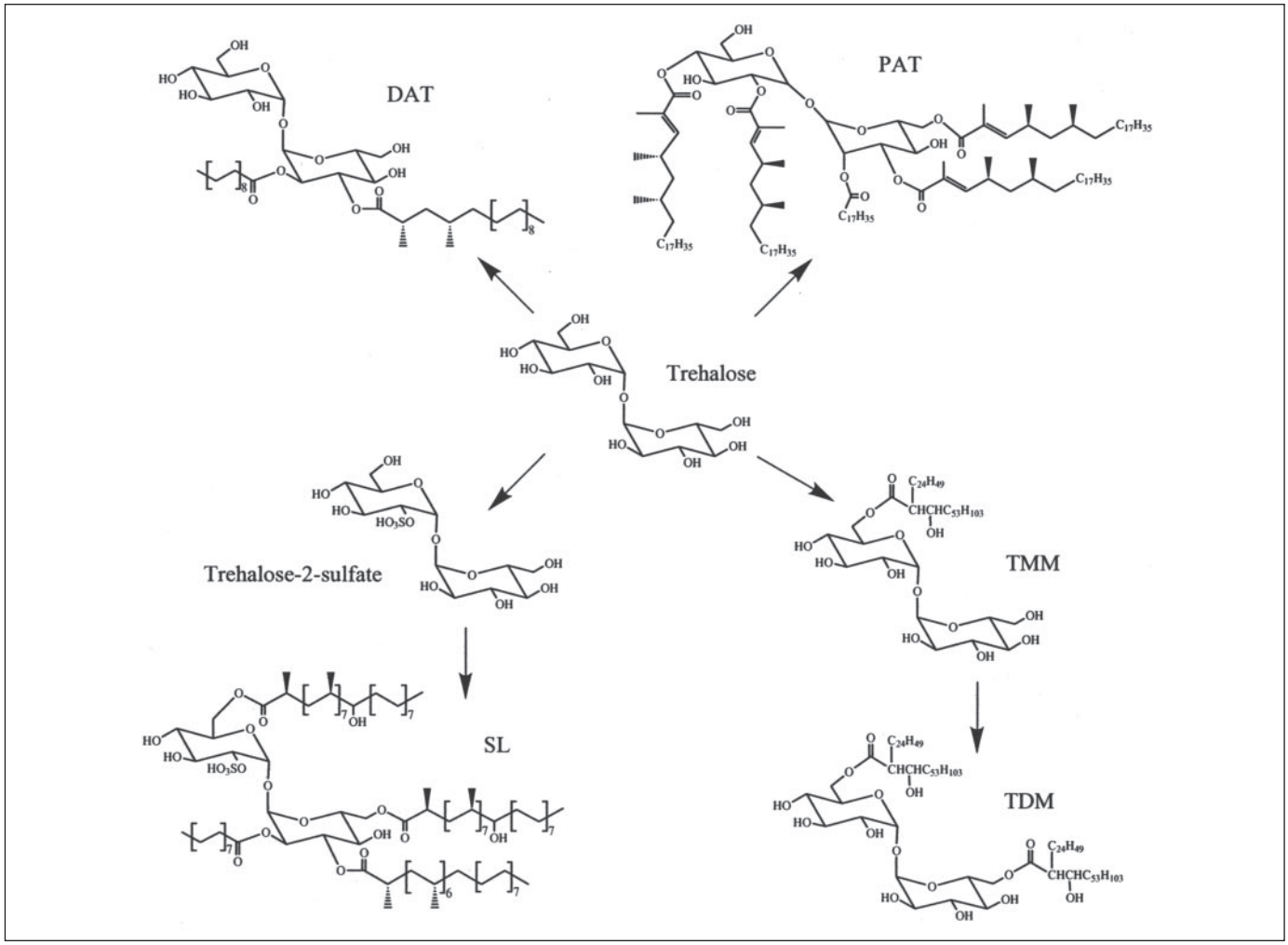

FIGURE 3. **Structures of some trehalose-derived molecules from *M. tuberculosis*.** The major sulfolipid SL-I is represented. In SL-I, trehalose is sulfated at the 2 position and esterified with palmitic acid and the multimethyl-branched phthioceranic and hydroxyphthioceranic acids. In DAT, trehalose is esterified with stearic acid and the multimethyl-branched mycosanoic acid. In PAT, trehalose is esterified with stearic acid and the multimethyl-branched mycolipenic acids. In TMM and TDM, trehalose is esterified with mycolic acids.

addition, sulfolipid (SL) production (22, 23) was abolished in 1237Δ*phoP*::*hyg* and 1237Δ*phoPR*::*hyg* (Figs. 2*A* 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 C_{26}) 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-^{14}C]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-^{14}C]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 1237Δ*phoPR*::*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 ^{14}C incorporated into SL (Fig. 2*A*). 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 dimycolates is unaffected in the mutants (Fig. 2*C*), 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-branched fatty acyl substituents found in SL, DAT, and PAT.

Interestingly, the histidine kinase encoded by *phoR* does not seem to be required for the synthesis of these lipids as complementation of 1237Δ*phoPR::hyg* with the *phoP* gene alone was sufficient to restore their synthesis. As already described in other two-component systems, phosphorylation might not be an absolute requirement 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 is the case in *Salmonella*, it is possible that instead of phosphorylating PhoP, PhoR catalyzes the dephosphorylation of phospho-PhoP, thereby abolishing the transcription of PhoP-activated genes (4). In that case, the lack of PhoR expression in 1237Δ*phoP-phoR::hyg* complemented with *phoP* would not abolish the production of acyltrehaloses.

The abolition of the synthesis of PAT, DAT, 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 associated with virulence attenuation of tubercle bacilli since Dubos and Middlebrook'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 mycobacterial "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 *pks3-4* mutant of *M. tuberculosis* H37Rv resulted in defects in capsule attachment causing the mutant to expose a more hydrophobic surface and, consequently, to aggregate in liquid broth (27) and to interact differently with phagocytic and non-phagocytic cells (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, *fadD21* and *papA1*, among the 22 genes whose expression was consistently higher in the cord-forming strain H37Rv. The *pks3-4*-associated gene *fadD21* is expected to encode a fatty acid-activating enzyme involved in DAT and PAT synthesis (27), while *papA1*, which is associated to *pks2*, is likely to encode an acyltransferase required for SL formation (29). As the cording strain *M. tuberculosis* H37Rv is proficient in the synthesis of SL and mycolipenate-containing DAT and PAT, while the non-cording *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, 23) strongly suggests 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 prokaryotes. In *Streptomyces lividans* 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 represents 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 Mg^{2+} as in *Salmonella* (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 acyltrehaloses is regulated. Several approaches, including promoter fishing and proteomics, have been undertaken by our laboratories to complete our understanding of this important two-component regulator.

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

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