Phosphatidylinositol (PI) is an abundant phospholipid in the cytoplasmic membrane of mycobacteria and the precursor for more complex glycolipids, such as the PI mannosides (PIMs) and lipoarabinomannan (LAM). To investigate whether the large steady-state pools of PI and apolar PIMs are required for mycobacterial growth, we have generated a *Mycobacterium smegmatis* inositol auxotroph by disruption of the *ino1* gene. The *ino1* mutant displayed wild-type growth rates and steady-state levels of PI, PIM, and LAM when grown in the presence of 1 mM inositol. The non-dividing *ino1* mutant was highly resistant to inositol starvation, reflecting the slow turnover of inositol lipids in this stage. In contrast, dilution of growing or stationary-phase *ino1* mutant in inositol-free medium resulted in the rapid depletion of PI and apolar PIMs. Whereas depletion of these lipids was not associated with loss of viability, subsequent depletion of polar PIMs coincided with loss of major cell wall components and cell viability. Metabolic labeling experiments confirmed that the large pools of PI and apolar PIMs were used to sustain polar PIM and LAM biosynthesis during inositol limitation. They also showed that under non-limiting conditions, PI is catabolized via lyso-PI. These data suggest that large pools of PI and apolar PIMs are not essential for membrane integrity but are required to sustain polar PI biosynthesis, which is essential for mycobacterial growth.

*Mycobacterium tuberculosis*, the causative agent of tuberculosis, infects nearly one third of the world population and causes active disease in an estimated 16 million people worldwide (1). The distinctive cell wall of *M. tuberculosis* and other pathogenic mycobacteria (*M. leprae*, *M. avium-M. intracellulare complex*, and *M. ulcerans*) confers protection against a range of microbiocidal processes and many classes of antibiotics and undoubtedly contributes to the success of these organisms as pathogens. The mycobacterial cell wall contains a number of unusual features, including the presence of a highly structured peptidoglycan-arabinogalactan (AG) \(^1\)-mycolic acid macromolecule and a diverse array of glycolipids that form an asymmetric outer bilayer with the mycolic acids (2–4). Mycobacteria and other members of the Actinomycetales also differ from other eubacteria in synthesizing phosphatidylinositol (PI) and the biosynthetically related lipoglycans, PI mannosides (PIMs), lipomannan (LM), and lipoarabinomannan (LAM) (5–7). Whereas there is accumulating evidence that the PIMs and LM/LAM have potent immuno-modulatory activities that may be important for the pathogenesis of *M. tuberculosis* (7–10), the presence of structurally related PIMs and LAMs in saprophytic mycobacterial species suggests that these lipoglycans have a more fundamental role(s) in mycobacterial physiology. This conclusion is supported by the finding that both phosphatidylinositol synthase and Fima, the first mannoyltransferase in the PIM/LM/LAM pathway (Fig. 1), are essential for growth and viability of the saprophytic mycobacteria species *M. smegmatis* (11, 12). It is possible that the large steady-state pools of PI and apolar PIM species may be required for cell membrane integrity and/or other membrane functions. In this regard, it is notable that PI is a bilayer-forming phospholipid, whereas cardiolipin and phosphatidylethanolamine, the other major phospholipids in the mycobacterial membrane, can form non-bilayer structures (13). Alternatively, or in addition, PI and apolar PIMs could function as a large dynamic pool of precursors for polar PIMs and LM/LAM biosynthesis.

In order to investigate the potential role(s) of PI and PIMs as both precursors and end products, we have generated a *M. smegmatis ino1* mutant required very high levels of exogenous inositol for growth and was severely attenuated in macrophages and severe combined immunodeficient (SCID) mice (14). Surprisingly, cellular levels of inositol lipids did not decrease when the *M. tuberculosis* mutant was suspended in inositol-free medium, although this may have been due to the use of non-replicating cultures (14). In this study, we show that inositol starvation has little effect on the cellular levels of inositol lipids or the viability of the non-replicating *M. smegmatis ino1* mutant. In contrast, dilution of the *ino1* mutant in inositol-free medium resulted in the rapid depletion of PI and apolar PIMs and a slower depletion of polar PIMs. Depletion of polar PIMs coincided with loss of other cell wall components and cell viability. We have

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PI Function in Mycobacteria

Fig. 1. Inositol metabolism in mycobacteria. Inositol (Ino) can be scavenged from the environment or synthesized de novo from D-glucose-6-phosphate (Glc-6-P). Inositol can be incorporated into PI or the major intracellular thiol, myothiol (Myo). PI can accumulate to be a major membrane phosphopholipid or can be further modified with glycan chains to form the mono- and diacylated PIM species and the hypermannosylated lipoglycans, LM and LAM. Some of the enzymes identified in these pathways are shown in bold. Evidence that mono- and diacylated PIMs may be interconverted (dotted arrows) is provided in this study.

also identified a novel lipase-mediated catabolic pathway that is involved in regulating cellular levels of PI in both wild-type and mutant M. smegmatis. These findings provide new insights into the function and metabolism of PI and PI-containing glycolipids in mycobacteria.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—Escherichia coli XL-1 Blue MRF’ (Stratagene) was grown in LB medium. Wild-type M. smegmatis mc²155 (15) and the mutants derived from it were routinely grown on M9 agar (16) with 0.4% glucose and 1.5% agar supplemented with 1 mM inositol, except where stated. Mycobacteria used for all growth and biochemical studies were grown in M9 broth with 0.4% (w/v) glucose and 0.05% (v/v) Tween 80 and supplemented with inositol as required. Antibiotics, streptomycin (20 μg/ml), kanamycin (20 μg/ml), or ampicillin (100 μg/ml) was added to media as required. Growth and survival of the cultures were measured by counting colony-forming units (cfu) using solid M9 media containing 1 mM inositol as required. Growth of cultures was monitored by bio-}

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EXPERIMENTAL PROCEDURES
RESULTS

Characterization of the M. smegmatis ino1 Mutant—A gene with 87% amino acid identity to the M. tuberculosis ino1 gene was isolated from M. smegmatis. The M. smegmatis homologue was present in an essentially identical loci to the M. tuberculosis ino1 gene (29). Specifically, two ORFs of unknown function (Rv0048c and Rv0047c in M. tuberculosis) were located downstream of ino1, whereas another ORF of unknown function, Rv0045c, preceded it. These ORFs have 48–81% amino acid identity to M. smegmatis homologues. The protein encoded by M. smegmatis ino1 had 87% amino acid identity to M. tuberculosis IPS.

A M. smegmatis ino1 mutant was generated by targeted disruption of the ino1 gene encoding a putative IPS (Fig. 2A). Colonies of the ino1 mutant were only recovered from M9 agar containing inositol, supporting a role for this gene in inositol metabolism. Disruption of ino1 was confirmed by Southern hybridization (Fig. 2B) and measurement of IPS activity. Whereas wild-type M. smegmatis contained high levels of IPS activity (3.29 ± 0.77 units/mg protein), no IPS activity was detected in the ino1 mutant (<0.01 ± 0.03 unit/mg protein). Finally, complementation of the ino1 mutant with an intact copy of the M. smegmatis ino1 gene on a mycobacterial shuttle vector increased IPS activity to 0.15 ± 0.08 unit/mg protein and restored inositol prototrophy. These data indicate that ino1 encodes the only functional IPS in M. smegmatis and that the ino1 mutant is auxotrophic for inositol.

Effect of Inositol Starvation on the Non-replicating ino1 Mutant—Non-replicating stages of the ino1 mutant were highly resistant to inositol starvation. When stationary-phase cells were resuspended in inositol-free medium at high density, culture viability remained constant over 400 h (Fig. 3A). Analysis of the total lipid fraction showed that levels of polar PIMs (AcPIM6/AcPIM6) did not decrease over this period, whereas levels of apolar PIMs (AcPIM2/AcPIM2) decreased by ~60% (Fig. 3B). Remarkably, PI was not detectable in stationary-phase ino1 mutant, regardless of whether inositol was present in the medium or not (Fig. 3B). In contrast, wild-type M. smegmatis expresses high levels of PI during stationary growth (Fig. 3B). These data suggest that apolar PIMs are very stable metabolites that are turned over very slowly in non-dividing cells. They also show that in the absence of the endogenous pathway of inositol biosynthesis, stationary-phase M. smegmatis is unable to accumulate PI. A large steady-state pool of PI is thus not essential for the viability of non-dividing M. smegmatis.

Effect of Inositol Starvation on Replicating Stages of the ino1 Mutant—To examine whether PI or downstream products were essential for growth of M. smegmatis, stationary-phase ino1 cells were diluted into fresh medium containing 1000 μM inositol, 1 μM inositol, or no inositol. The growth kinetics of the ino1 mutant was indistinguishable from that of wild-type M. smegmatis in the presence of 1000 μM inositol (Fig. 4, A and B; data not shown). In contrast, biomass did not increase substantially (<0.5 log unit), and viability decreased to zero over 200 h when the ino1 mutant was diluted into inositol-free medium (Fig. 4, A and B). Interestingly, ino1 mutant cells remained intact for ~25 days after loss of viability, as shown by unchanged biomass (Fig. 4A). The ino1 mutant grew slowly in the presence of 1 μM inositol, and the viability of these cultures decreased dramatically after 150 h (Fig. 4, A and B).

Aliquots of the ino1 mutant grown in the presence of 1 or 1000 μM inositol were harvested at various time points to determine whether the loss of viability of cultures grown in 1 μM inositol was associated with the loss of specific inositol lipids. PI was detected in inositol-replete cultures during exponential growth phase but decreased to below the level of detection in stationary-phase cultures (Fig. 5A). In contrast, PI never accumulated to detectable levels in either exponential or stationary-phase ino1 cells grown in the presence of 1 μM inositol (Fig. 5A). These data extend the observation made on stationary-phase cells, suggesting that a large pool of PI is not required for either the viability or growth of M. smegmatis. Because wild-type mycobacteria express high levels of PI throughout growth (Fig. 3B), these data also suggest that the...
The ino1 mutant has a reduced capacity to take up inositol in stationary phase and/or an increased dependence on endogenously synthesized inositol for PI biosynthesis.

Unlike PI, cellular levels of PIM2 and PIM6 were expressed throughout growth when the ino1 mutant was grown in 1000 μM inositol, with the relative abundance of the diacylated species increasing with culture age (Fig. 5B). However, in the absence of 1 μM inositol, levels of apolar PIM species decreased to below the level of detection within 78 h. Levels of polar PIMs also decreased, but at a slower rate (Fig. 5B). Surprisingly, LAM levels in inositol-deprived ino1 mutant decreased to ~20% of the level found in the ino1 mutant grown in 1000 μM inositol, but then they remained constant for the remainder of the experiment (Fig. 5C). The initial decrease in LAM levels may reflect the dilution of this component as a result of ongoing cell division (Fig. 4). The fact that apolar and polar PIMs persisted for longer than LAM suggested that most of the PI was redistributed into PIM biosynthesis rather than LAM biosynthesis under inositol-limiting conditions.

To investigate whether the loss of polar PIMs was associated with loss of other cell wall components, the ino1 mutant was grown in 1000 or 1 μM inositol, and cells were harvested at the beginning of stationary phase. As shown in Fig. 5, D and E, components of both the outer lipid layer (GPLs) and inner peptidoglycan-AG-mycolic acid complex (AG) were reduced by >90% in inositol-limited cultures. In contrast, cellular levels of cytoplasmic membrane phospholipids, phosphatidylethanolamine, and cardiolipin were not decreased after inositol starvation (Fig. 5A). These data suggest that the loss of polar PIMs has little effect on the composition of the cytoplasmic membrane but is associated with the catastrophic loss of major cell wall components and cell viability.

Relationship between PI and PIM in Actively Dividing Mycobacteria—The effect of inositol starvation on actively dividing mycobacteria was monitored by cultivating the ino1 mutant in 1000 μM inositol and then transferring cells in mid-exponential phase to fresh medium containing either 1000 μM inositol or no inositol at the same cell density. M. smegmatis wild type and the ino1 mutant in 1000 μM inositol medium continued to multiply for 15 h before entering stationary phase (Fig. 6A). Whereas levels of PI decreased by 50% when the ino1 mutant
reached stationary phase, cellular levels of other phospholipids and total PIM remained constant throughout exponential and stationary growth (Fig. 7, A and C). Interestingly, levels of diacylated PIM2 and PIM6 in the ino1 mutant (Fig. 7C) and wild type (data not shown) invariably increased at 5 h and subsequently decreased, suggesting that the inositol acylation reaction is reversible. In the absence of exogenous inositol, the actively dividing ino1 mutant underwent 3–4 cell divisions before biomass plateaued after ~15 h and viability decreased after 26 h (Fig. 6B). Inositol starvation led to the rapid decrease in PI (depleted within 3 h) and apolar PIMs (depleted within 5 h) (Fig. 7, B and D) but to a small increase in AcPIM6 levels over 5 h (Fig. 7, B and D). Levels of LM/LAM remained constant during this period (data not shown). These data support the notion that the major cellular pools of PI and PIM2 can be used to sustain polar PIM synthesis during nutrient starvation. They also suggest that dividing cells are more resistant to inositol starvation than stationary-phase cells, by virtue of containing a larger steady-state pool of PI.

Catabolism of PI during Inositol Starvation—The analyses described above suggest that the large cellular pools of PI and PIM2 are dynamic and rapidly channeled into polar PIM/LM/LAM and/or are catabolized during inositol starvation. To investigate which of these processes predominates under inositol-replete or -limiting conditions, M. smegmatis wild type and the ino1 mutant were pulse-labeled with myo-[3H]inositol and then resuspended in fresh medium containing 1000 μM inositol or no inositol. In wild-type M. smegmatis, label was initially incorporated into polar PIM/LM/LAM and/or catabolized during inositol starvation. To investigate which of these processes predominates under inositol-replete or -limiting conditions, M. smegmatis wild type and the ino1 mutant were pulse-labeled with myo-[3H]inositol and then resuspended in fresh medium containing 1000 μM inositol or no inositol. In wild-type M. smegmatis, label was initially incorporated into PIM and subsequently chased into AcPIM2, LAM, and AcPIM6 (Fig. 8, A–D). The efficiency of labeling of the latter species was increased when the chase was performed in inositol-free medium, which appeared to coincide with reduced turnover of labeled PI (Fig. 8, A and C). Regardless of the chase conditions, total incorporation of myo-[3H]inositol into PIM and LAM fractions never exceeded 48–56% of the label initially incorporated into the PI fraction (Fig. 8, B and D). These data suggest that the majority of the PI may be hydrolyzed rather than incorporated into PIMs and LAM, and the rate of hydrolysis appears to be reduced when exogenous inositol levels are low. To investigate whether PI turnover and the channeling of PI into PIM biosynthesis is regulated by inositol availability, identical labeled experiments were performed with the ino1 mutant. As expected, inositol phospholipids were labeled more efficiently in the ino1 mutant because exogenous label was not being diluted by de novo synthesized inositol (Fig. 8, E and F).

As observed in wild-type cells, labeled PI was rapidly taken over when the ino1 mutant was suspended in high-inositol medium (Fig. 8, E and F). Approximately 42% of the label in the PI fraction was initially chased into AcPIM2 and subsequently chased into AcPIM6. A novel species with a slower HPTLC mobility than PI or AcPIM2 was also observed at the end of the pulse and throughout the chase (Fig. 8E). This species was susceptible to PI-specific phospholipase C digestion and mild base hydrolysis and comigrated with authentic lyso-PI (data not shown). This species was also detected in wild-type cells, when fluorographs were developed for longer periods (data not shown). When the ino1 mutant pulse-labeled with [3H]inositol was chased in inositol-free medium, there was a marked increase in the extent to which labeled PI was chased into AcPIM2 and AcPIM6 (73%) and a reduction in the rate at which label was chased out of the lyso-PI species (Fig. 8, G and H). These analyses demonstrate that the entire pool of newly synthesized PI in both the wild type and ino1 mutant can be utilized for PIM and LM/LAM synthesis or catabolized via lyso-PI. The catabolism of PI is dramatically reduced when inositol is limiting, presumably reflecting the channeling of limiting PI precursors into PIM and LM/LAM synthesis.
were developed in solvent 1, and lipids were detected by fluorography.

Cells were harvested at the indicated time points, and 1 mutant were pulse-labeled with inositol-replete and -limiting conditions. Cells remain viable without PI, whereas polar PIMs may be important for cell wall biogenesis and apolar PIMs are primarily metabolic precursors that can be used to sustain polar PIM synthesis when mycobacteria are subjected to nutrient limitation. It remains unclear whether the loss of viability of inositol-starved cells is due to loss of polar PIMs, loss of other cell wall components, and cell death. A major function of the large cellular pool of PI is thus to provide a dynamic pool of precursors for polar PIM and LM/LAM biosynthesis. We also show that the steady-state levels of PI may be regulated by the action of one or more PI-specific lipases.

The growth phenotype of the M. smegmatis ino1 mutant is similar to that of the recently generated M. tuberculosis ino1 mutant, which was unable to survive in macrophages or highly susceptible SCID mice (14). Both mutants required exogenous inositol for growth but survived for extended periods of time without inositol while in stationary growth phase (this study and Ref. 14). The resistance of stationary-phase cultures to inositol starvation appears to reflect the lower requirement for new membrane and cell wall components in non-dividing stages and is consistent with the finding that the rate of PIM synthesis is dramatically down-regulated in stationary-phase M. smegmatis. Interestingly, the growth of the M. smegmatis ino1 mutant was the same as that of wild-type cells in the presence of 1 mM inositol, whereas the M. tuberculosis ino1 mutant required much higher levels of inositol (70 mM) for normal growth (14). M. tuberculosis may be less efficient at scavenging inositol from the environment than the saprophytic M. smegmatis and/or have a higher dependence on inositol generated via the d-glucose-6-phosphate pathway.

A dramatically different response was observed when either stationary-phase or mid-exponential phase ino1 mutant cells were diluted into inositol-free medium. Under these conditions, the steady-state levels of PI and apolar PIMs decreased rapidly. Metabolic labeling experiments indicated that the decrease in PI and apolar PIMs was mainly due to the conversion of these lipids into polar PIMs. Significantly, PI levels decreased below the level of detection, while inositol-starved cells were still in mid-exponential growth. Taken together with the observation that stationary-phase ino1 mutant cells lacking detectable PI remain viable for extended periods, these data suggest that large steady-state pools of PI and apolar PIMs are not essential for either the viability or growth of M. smegmatis. In contrast, the cellular levels of polar PIM species (i.e. AcPIM6 and Ac2PIM6) decreased slowly during inositol limitation, and loss of these glycolipids was associated with the catastrophic loss of other cell wall components (AG, outer layer glycolipids) and cell viability. Interestingly, cellular levels of LM/LAM decreased more rapidly than polar PIMs during initial stages of inositol limitation but then stabilized at ~20% of wild-type levels when cells stopped dividing. The decrease in LM/LAM may thus represent the dilution of these molecules during cell division.

DISCUSSION

We have generated a M. smegmatis inositol auxotroph by disrupting the ino1 gene and examined the consequences of inositol starvation on the synthesis and turnover of inositol lipids and cell viability. Our analyses show that the PI pool of M. smegmatis is very dynamic and rapidly depleted under inositol-limiting conditions. Cells remain viable without PI, although PI depletion eventually leads to depletion of polar PIMs, loss of other cell wall components, and cell death.

2 Y. S. Morita, H. Billman-Jacobe, and M. J. McConville, unpublished data.
3 S. Kovacevic, D. Anderson, J. Patterson, Y. Morita, R. Haites, R. Coppell, M. J. McConville, and H. Billman-Jacobe, manuscript in preparation.
and cell viability, these functions may also be fulfilled by smaller PIM species.

The phospholipids, PI, phosphatidylethanolamine, and cardiolipin, are thought to be restricted to the cytoplasmic membrane (2). In contrast, it has been proposed that the PIMs may be located in the cytoplasmic membrane and/or transported to the cell wall or extracellular space (31, 32). The finding that the entire pool of Ac/Ac$_2$PIM2 can be utilized as precursors for polar PIM synthesis suggests that these glycolipids are not transported to the outer layer of the cell wall but remain within the cytoplasmic membrane and/or cell fractions that contain enzymes involved in polar PIM biosynthesis. At least one of these enzymes, PimC, a putative mannosyltransferase that catalyzes the conversion of AcPIM2 to AcPIM3, appears to be located in the cytoplasmic membrane, based on the finding that it utilizes the cytoplasmic mannose donor, GDP-mannose, and lacks a recognizable signal sequence (33). We have also obtained evidence that the polyprenl-phosphate-mannose-dependent mannosyl-transfereases involved in the synthesis of polar PIMs are also located in the cytoplasmic membrane (25).

Collectively, these data suggest that the major pools of apolar PIMs remain in the cytoplasmic membrane.

The finding that PI was rapidly and constitutively catalyzed in M. smegmatis was unexpected. PI catabolism was observed in both M. smegmatis wild type and the inol1 mutant and appeared to involve the generation of a lyso-PI species. Mycobacteria express a number of phospholipase activities, although the endogenous or exogenous targets of these activities are not well defined (34–36). The identification of lyso-PI species implies the involvement of a PI-specific PLA$_2$, although phospholipase C or D activities could also be involved and would not be detected in these analyses. Interestingly, PI catabolism was reduced when cells were suspended in inositol-free media, suggesting that the activity of the lipases can be metabolized in wild type and the inol1 strain (2). In contrast, it has been proposed that the PIMs may be located in the cytoplasmic membrane and/or transported to the outer layer of the cell wall but remain within the cytoplasmic membrane and/or cell fractions that contain enzymes involved in polar PIM biosynthesis. At least one of these enzymes, PimC, a putative mannosyltransferase that catalyzes the conversion of AcPIM2 to AcPIM3, appears to be located in the cytoplasmic membrane, based on the finding that it utilizes the cytoplasmic mannose donor, GDP-mannose, and lacks a recognizable signal sequence (33). We have also obtained evidence that the polyprenl-phosphate-mannose-dependent mannosyl-transfereases involved in the synthesis of polar PIMs are also located in the cytoplasmic membrane (25).

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Function of Phosphatidylinositol in Mycobacteria
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