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The Critical Role of *embC* in *Mycobacterium tuberculosis*▽

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Arabinan polymers are major components of the cell wall in *Mycobacterium tuberculosis* and are involved in maintaining its structure, as well as playing a role in host-pathogen interactions. In particular, lipoarabinomannan (LAM) has multiple immunomodulatory effects. In the nonpathogenic species *Mycobacterium smegmatis*, EmbC has been identified as a key arabinosyltransferase involved in the incorporation of arabinose into LAM, and an *embC* mutant is viable but lacks LAM. In contrast, we demonstrate here that in *M. tuberculosis*, *embC* is an essential gene under normal growth conditions, suggesting a more crucial role for LAM in the pathogenic mycobacteria. *M. tuberculosis* EmbC has an activity similar to that of *M. smegmatis* EmbC, since we were able to complement an *embC* mutant of *M. smegmatis* with $embC_{Mtb}$, confirming that it encodes a functional arabinosyltransferase. In addition, we observed that the size of LAM produced in *M. smegmatis* was dependent on the level of expression of $embC_{Mtb}$. Northern analysis revealed that *embC* is expressed as part of a polycistronic message encompassing *embC* and three upstream genes. The promoter region for this transcript was identified and found to be up-regulated in stationary phase but down-regulated during hypoxia-induced nonreplicating persistence. In conclusion, we have identified one of the key genes involved in LAM biosynthesis in *M. tuberculosis* and confirmed its essential role in this species.

Mycobacteria remain the causative agents of devastating infections. The increasing appearance of multiple and extremely drug-resistant strains poses further threats and underscores the need for novel therapeutic agents. The mycobacterial cell wall contains a number of carbohydrate residues or glycans in the form of unique species-specific glycolipids and lipoglycans, several of which play important roles in the physiology and virulence of these bacteria. Thus, the specific pathways leading to their synthesis are of interest for drug development. Of the 50 or so proposed *Mycobacterium tuberculosis* glycosyltransferases, approximately 20 have been functionally characterized and, for the most part, are involved in or associated with cell wall biosynthesis (3).

The cell wall of the mycobacteria has some characteristic features of the gram-positive bacteria, in particular, the presence of a complex arabinogalactan (AG) heteropolysaccharide which is covalently attached to the peptidoglycan (17). However, in *Mycobacterium* and related genera, the nonreducing ends of the AG are the attachment sites for the ester-linked mycolic acids forming the mycolyl AG-peptidoglycan complex (18). In *M. tuberculosis*, several characteristic lipids are found interspersed within this layer that contribute to host-pathogen interactions; these include a major component of the mycobacterial cell wall lipoarabinomannan (LAM), as well as lipomannan (LM), and the phthiocerol-containing lipids (5, 6). LAM is a key factor in many aspects of the interaction between *Mycobacterium* species and host cells (7, 34, 36). Mannose-capped LAM produced by *M. tuberculosis* is involved in the modulation of macrophage and dendritic cell activation and is therefore able to control the host inflammatory response (12, 15, 22).

Arabinans are common constituents of both AG and LAM and dominate the structure of the mycobacterial cell wall; consequently, they have important structural and pathogenic implications (5). Previous work with *Mycobacterium smegmatis* has demonstrated that the Emb proteins (EmbA, EmbB, and EmbC) are required for the biosynthesis of the arabinan components of AG and LAM (11, 37). The three Emb homologs located adjacently on the chromosomes of both *M. tuberculosis* and *M. smegmatis* have 65% identity at the amino acid level and belong to glycosyltransferase superfamily C (4, 14). *embA* and *embB* are cotranscribed in *M. tuberculosis* (2). If their exact biochemical functions remain unknown, it appears that both EmbA and EmbB are dedicated to the biosynthesis of the arabinan portion of AG (11), whereas EmbC is involved in LAM biosynthesis (37), at least in *M. smegmatis*.

EmbC is involved in LAM biosynthesis in *M. smegmatis*, where disruption of *embC* leads to a loss of LAM production, while LM synthesis is unaffected (37). EmbC is a membrane protein with 13-transmembrane helices in the N-terminal domain coupled to an extracytoplasmic domain involved in arabinan chain extension during LAM biosynthesis (4, 33). Previous studies have focused on *embC* of *M. smegmatis*. In order to determine the function of *M. tuberculosis* EmbC ($EmbC_{Mtb}$), we attempted to construct a deletion mutant by gene replacement. We demonstrate here that *embC* is essential in *M. tuberculosis* under normal culture conditions. $EmbC_{Mtb}$ is a functional arabinosyltransferase, since it is able to restore LAM production in an *M. smegmatis embC* mutant. We demonstrate that *embC* is expressed as part of a polycistronic mRNA transcript together with three genes upstream, from a promoter region located upstream of Rv3790. We analyze the expression

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of the promoter region and show that it is induced during late stationary phase but is down-regulated in a hypoxia-induced nonreplicating state.

MATERIALS AND METHODS

Culture. *M. tuberculosis* was grown in Middlebrook liquid medium (7H9-OADC) containing 4.7 g liter^{-1} Middlebrook 7H9 plus 10% (vol/vol) OADC (oleic acid, albumin, dextrose, catalase) supplement (Becton Dickinson) and 0.05% (wt/vol) Tween 80 or in Middlebrook solid medium (7H10-OADC) containing 19 g liter^{-1} Middlebrook 7H10 plus 10% (vol/vol) OADC supplement. Dubos medium (Becton Dickinson) supplemented with 10% (vol/vol) Dubos medium albumin (Becton Dickinson) was used for hypoxic cultures. Aerobic liquid cultures of *M. tuberculosis* were grown statically in 10-ml cultures. Hypoxic cultures were performed with 17 ml medium in 20-mm glass tubes with slow stirring (50 rpm) and a starting optical density at 570 nm of 0.004. We used kanamycin at 20 μg ml^{-1}, hygromycin at 100 μg ml^{-1}, streptomycin at 20 μg ml^{-1}, gentamicin at 10 μg ml^{-1}, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) at 50 μg ml^{-1}, and sucrose at 5% (wt/vol). *M. smegmatis* was cultivated in Lemco medium (9).

Construction of *embC* deletion vectors. The upstream and downstream flanking regions of *embC* were amplified with primer pairs embC5 (CAA GCT TCA TCG GAT CCA CCA CCT G) plus embC6 (CGG TAC CCA CGG AGG TAG ATG GTA G) and embC7 (GGG TAC CGA TCT GAA CCT AGG AAC G) plus embC8 (GGC GGC CGC GCA AGC ACC GAT GTA TAC) and cloned into pGEMT-Easy (Promega). Restriction sites (underlined) were engineered into the primers. The two fragments were excised as HindIII-KpnI and NotI-KpnI fragments and cloned into p2NIL (25) to make pEMPTY4. The *lacZ-sacB-hyg* cassette from the marker cassette vector pGOAL19 (25) was excised as a PacI fragment and inserted into pEMPTY4 to make the final deletion delivery vector, pEMPTY6. A second deletion vector was constructed by inverse PCR on pEMPTY4 with primer pair RG embC-1 (CCA TCA CGC GCT CTC CTG C) plus RG embC-2 (CCT TAA CCG CGT CGC CTA C); the *lacZ-sacB-hyg* cassette from the marker cassette vector pGOAL19 (25) was then inserted to make the final deletion delivery vector, pRG76.

Attempts to construct *embC* deletion strains. Plasmids pEMPTY6 and pRG76 were pretreated with UV to promote homologous recombination and electroporated into *M. tuberculosis* (13). Single crossovers (SCOs) were isolated on hygromycin, kanamycin, and X-Gal and checked by Southern blotting. One SCO of each was streaked onto solid media without antibiotics to allow the second crossover to occur. Double crossovers (DCOs) were isolated on sucrose–X-Gal plates. White colonies were patch tested for kanamycin and hygromycin sensitivity to confirm vector loss. A PCR screen with primers EMBC9 (CCA AGC TTC GCC GCT ACA CGG TGG) and EMBC11 (CTG GGT GAT GTT GCC GTC) was used to distinguish between wild-type (3.6 kb) and deletion (0.3 kb) DCOs.

Construction of an *embC* merodiploid strain. The complementation vector pEMPTY25 was constructed by amplifying the *embC* gene from *M. tuberculosis* with primers Empathy1 (TTA ATT AAG TTT CGT CGT CGA GGA CAT T) and Empathy7 (TTA ATT AAC AAC CTG TGG CTT CTT CTC C) and subcloning it into pAPA3 (L5 integrating vector with the Ag85a promoter) (24) as a PacI fragment (sites underlined) in the correct orientation for expression from the Ag85a promoter. pEMPTY25 was transformed into SCO strains to generate a merodiploid strain. Generation of DCOs was carried out as before, but with the inclusion of gentamicin. Sucrose-resistant white colonies were screened by PCR. Del-int DCO strains (one *del*eted copy and one *int*egrated copy of *embC*) were isolated and confirmed by Southern analysis.

Switching experiments with *M. tuberculosis embC* delinquent strain. Integrating vectors carrying *embC* from *M. tuberculosis* and *M. smegmatis* with hygromycin markers were created. pRG603 was constructed by replacing the Gm-Int fragment in pEMPTY25 with the Hyg-Int HindIII cassette from pUC-Hyg-Int (16). *embC* of *M. smegmatis* was excised as an NdeI-HindIII fragment from pVEwt (4), blunt ended, and cloned into the PacI site of pAPA3 (carrying the mycobacterial Ag85a promoter) (24) to obtain pRG642. The final plasmid, pRG643, was constructed by replacing the Gm-Int fragment in pRG642 with the Hyg-Int HindIII cassette from pUC-Hyg-Int (16). The switching experiment to replace the integrated pEMTY25 vector with pRG603 and pRG643 was carried out as previously described (27). pRG603 and pRG643 were electroporated into the *M. tuberculosis embC* delinquent strain, and transformants were isolated by hygromycin selection (for the incoming vector). Transformants were patch tested for gentamicin resistance.

Extraction and analysis of LM and LAM. *M. smegmatis* was grown in Lemco broth containing 100 μg ml^{-1} hygromycin and 20 μg ml^{-1} kanamycin. After 24 h of culture, cells were harvested, resuspended in 400 μl phenol-water at 1:1, and incubated at 80°C for 2 h. A 100-μl volume of chloroform was added, and 10 μl of the aqueous phase was analyzed with a denaturing nonreducing 16% acrylamide gel, followed by periodic acid-Schiff staining (8, 28).

Analysis of promoter activity. The intergenic regions between adjacent genes spanning the sequence from Rv3789 to *embC* (Rv3793) of *M. tuberculosis* were amplified with the following primer pairs and cloned as ScaI or SmaI fragments into L5-based integrating vector pSM128 (10) upstream of the promoterless *lacZ* gene and sequence verified: p3790 (Rv3789-Rv3790), forward primer CCC AG TACT GTC GGA CTC AAC CAC CTC TG and reverse primer CCC AGTACT GTA GCT CCC ACG CTC AAC AT; p3791 (Rv3790-Rv3791), forward primer CAT CCC GGG CTG GAA CAT TCT G and reverse primer G CCC GGG CTC GGA GGT GCC ACC GAG C; Rv3791-Rv3792, forward primer C CCC GGG CGA ACT TCG TCT AC and reverse primer CA CCT CCC GGG CGC GAG CAG; p3792 (Rv3792-*embC*), forward primer CCC AGT ACT GTT CGC CGC TAC ACG GTG G and reverse primer CCC AGT ACT GTC TTG CCG GTG TTC TGC GAT CC; p3793 (*embA-embB*), forward primer CCC AGT ACT CGT TGT CGA GGA GGG CGT G and reverse primer CCC AGT ACT CAT GGC GCG CAC CAC GTC G.

Plasmids were electroporated into *M. tuberculosis*, and streptomycin-resistant transformants were isolated. Three independent transformants for each were selected for promoter activity determinations. Cell extracts were prepared (26), and β-galactosidase assays were performed as previously described (20). Hypoxic cultures were performed with 17 ml medium in 20-mm glass tubes with slow stirring (50 rpm) and a starting optical density at 570 nm of 0.004. Assays were performed in NRP2 phase (14 days old). Oxidative stress was generated by adding 10 mM hydrogen peroxide to the medium and omitting catalase and albumin. Exposure to ethambutol and ofloxacin was performed for 1 h with 1 μg/ml drug.

Isolation of RNA and identification of the *embC* transcript. Total RNA was isolated as previously described (2). For Northern analysis, 12 μg total RNA from *M. tuberculosis* grown in 7H9 medium was separated by gel electrophoresis with a 1% agarose gel and transferred to a positively charged nylon membrane (Amersham Hybond-N+). The *embC* probe (probe A) was a 325-bp purified PCR fragment amplified with primers Mut test f (CGT CGG GGC CAA CAC CTC CGA CGA C) and Mut test r (CCG AAG GCC GTT GTC CAG CGG). The Rv3790 probe (probe B) was a 1,300-bp purified PCR fragment amplified with N3790f (CGG AGC GAA CCT TTG AAA TTC G) and N3790r (GAC CCG AGG CTT GAA TAA CGC). Labeling and detection were carried out with the AlkPhos Direct kit (Amersham) according to the manufacturer's instructions.

RESULTS

Essentiality of *embC* in *M. tuberculosis*. The role of EmbC in the biosynthesis of LAM in *M. smegmatis* was previously elucidated by the construction of an *embC* mutant (11). However, predictions based on saturating transposon mutagenesis suggested that *embC* might be an essential gene in *M. tuberculosis* (29). Our previous work had demonstrated that another arabinosyltransferase, EmbA, is essential in *M. tuberculosis* (2) but not in *M. smegmatis* (11), confirming major differences between the two species. Therefore, we decided to determine whether we could construct an *embC* deletion mutant of *M. tuberculosis*.

Two deletion vectors were constructed, i.e., pEMPTY4, carrying an unmarked partial deletion of the *embC* gene, and pRG76, carrying an unmarked complete deletion of the *embC* gene. Each of these was used in a two-step homologous recombination procedure to attempt to generate DCO *embC* deletion strains. One SCO strain was generated with each plasmid; DCOs were isolated from these strains and screened by PCR for the presence of either the wild-type or the deletion allele. We screened 100 DCO strains for each deletion vector; all 200 strains carried the wild-type allele, strongly suggesting that the gene is essential. In order to demonstrate this, we

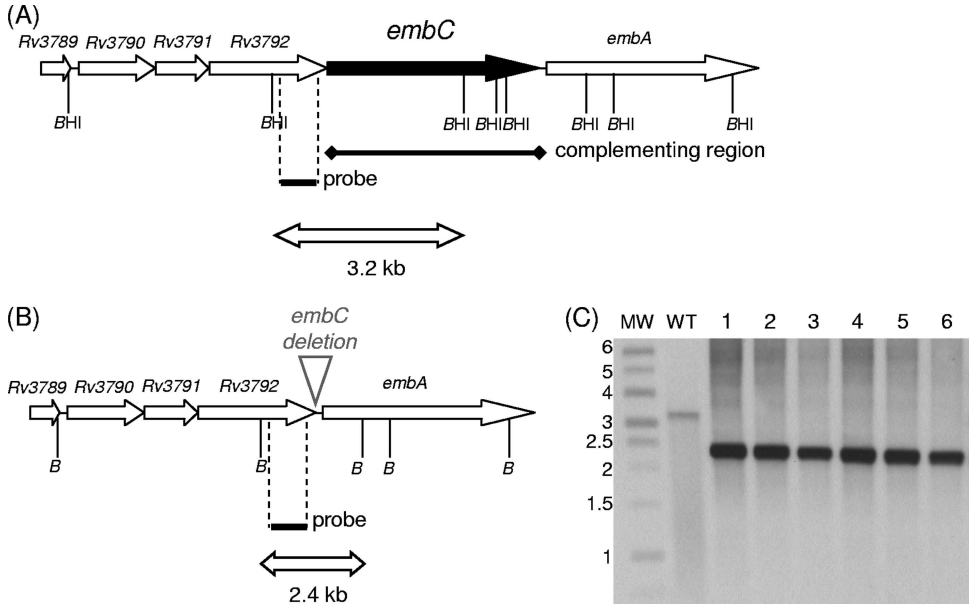

FIG. 1. Demonstration of the essentiality of *embC* in *M. tuberculosis*. (A) The genetic organization of the wild-type *embC* region is shown. BamHI sites are indicated (*B*HI); the probe used for Southern analysis is shown as a solid bar. The region present in the complementing vector is indicated. (B) Map of the deletion. (C) Southern analysis of deletion DCOs isolated in the merodiploid background. Genomic DNA was digested with BamHI and hybridized to the probe. Lane MW, molecular mass marker (sizes are in kilobase pairs). Lane WT, wild-type genomic DNA. Lanes 1 to 6, genomic DNAs from Del-int strains (deletion DCOs with integrated *embC*).

constructed a merodiploid strain carrying a second functional copy of *embC* under the control of the constitutive Ag85a promoter (with plasmid pEMPTY25) in the SCO carrying the complete deletion vector (pRG76). In this background, we were able to isolate both wild-type and deletion DCOs; 10/24 DCOs had the deletion allele. The genotypes of six DCO strains were confirmed by Southern blotting (Fig. 1). Thus, we have confirmed that *embC* is indeed essential in *M. tuberculosis* under normal growth conditions.

***M. tuberculosis embC* encodes an arabinosyltransferase involved in LAM biosynthesis.** *M. tuberculosis* EmbC was previously identified as an arabinosyltransferase on the basis of sequence similarity to *M. smegmatis embC* (4, 37). We tested the ability of $EmbC_{Mtb}$ to complement the arabinosyltransferase activity of $EmbC_{Msm}$ by using the previously constructed *embC* disruption strain of *M. smegmatis* (11). In this strain, lack of EmbC activity results in an inability to synthesize LAM.

We cloned $EmbC_{Mtb}$ into two expression vectors under the control of mycobacterial promoters of differing strengths, i.e., pVV16, a multicopy extrachromosomal vector with the strong Hsp60 promoter (pMTembC), and pAPA3, an L5 mycobacteriophage-derived integrating vector with the weaker Ag85a promoter (pEMPTY25). We transformed each plasmid into the *M. smegmatis embC* mutant and looked at complementation of LAM biosynthesis (11). As a positive control, we used pVV16 carrying *M. smegmatis embC* (pMSembC). LM and LAM were extracted from these strains and analyzed (Fig. 2). We confirmed that the *embC* mutant did not produce any LAM. The strain complemented with $EmbC_{Msm}$ produced a larger LAM than the wild type, as previously noted (4). *M. smegmatis* Δ*embC* complemented with either vector expressing $EmbC_{Mtb}$ was able to synthesize LAM, confirming that $EmbC_{Mtb}$ is an arabinosyltransferase with activity similar to that of its ortholog in *M. smegmatis*. Interestingly, the size of LAM in the complemented strains was dependent on the level of expression of *embC*, with a larger LAM being produced in the strain expressing $EmbC_{Mtb}$ to a higher level (pMTembC). In addition, the strain complemented by $EmbC_{Msm}$ produced a larger LAM than the one complemented by $EmbC_{Mtb}$, even when it was expressed from the same promoter.

Functional complementation of *M. tuberculosis* EmbC by *M. smegmatis* EmbC. *embC* is essential in *M. tuberculosis*, unlike in *M. smegmatis*. However, *M. tuberculosis embC* can complement the LAM^- phenotype of the *M. smegmatis embC* mutant. Although there is 74% amino acid identity between the EmbC proteins from *M. tuberculosis* and *M. smegmatis*, it is possible that the *M. tuberculosis* protein could have additional functions

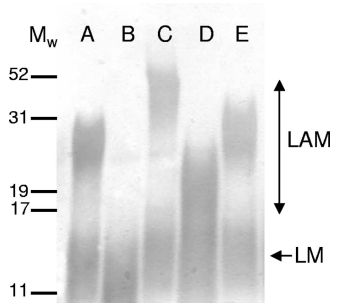

FIG. 2. Analysis of LAM/LM from *M. smegmatis* mutants. LAM/LM was extracted from *M. smegmatis* and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. (A) Wild-type strain. (B) Δ*embC* strain with pVV16. (C) Δ*embC* strain with pMSembC (P_{hsp60}-$EmbC_{Msm}$). (D) Δ*embC* strain with pEMPTY25 (P_{Ag85a}-$EmbC_{Mtb}$). (E) Δ*embC* strain with pMTembC (P_{hsp60}-$EmbC_{Mtb}$). M_W, molecular mass marker (masses are in kilodaltons).

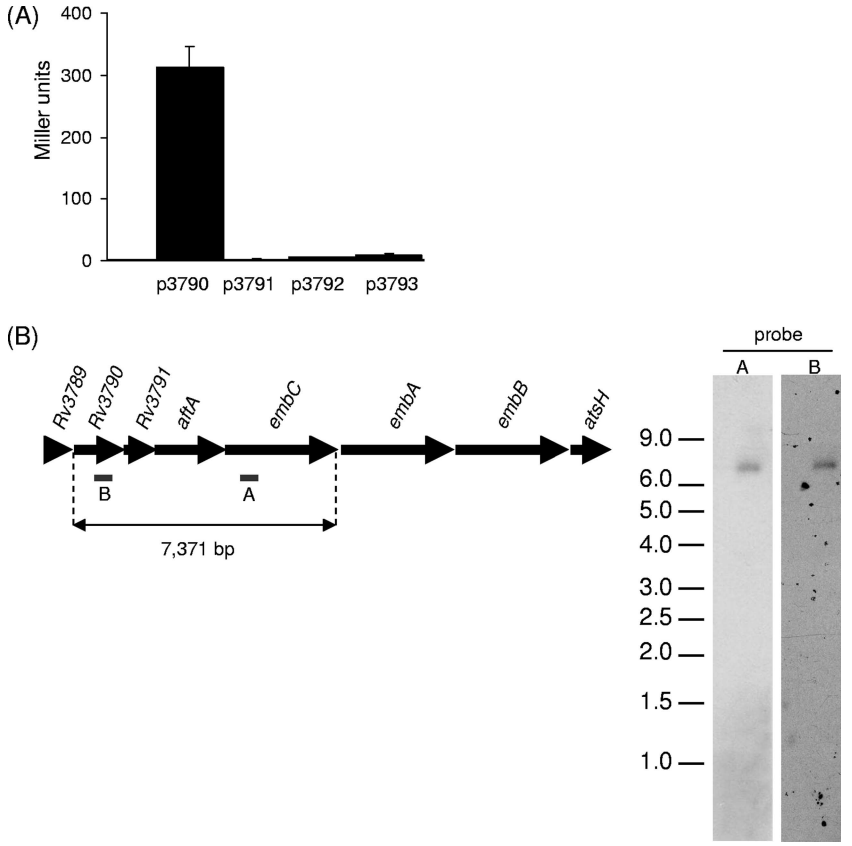

FIG. 3. Identification of the promoter for *embC*. (A) Promoter activities of p3791, p3792, and p3793 in *M. tuberculosis*. The regions upstream of Rv3790, Rv3791, Rv3792, and *embC* were cloned into the pSM128 reporter vector. β-Galactosidase activity from 10-ml static cultures was determined after 10 days. Results are the mean ± standard deviation of three individual transformants, each assayed in duplicate and are given in nanomoles of *O*-nitrophenyl-β-D-galactopyranoside produced per minute per milligram of total protein. (B) Northern blot analysis. Twelve micrograms of total RNA from *M. tuberculosis* grown in 7H9 medium was separated on a 1% agarose gel and transferred to a positively charged nylon membrane. The blot was hybridized to the two probes indicated, probe A for the *embC* transcript and probe B for the Rv3790 transcript.

not found in the *M. smegmatis* protein. In order to address this, we determined whether *M. smegmatis embC* could functionally complement the *M. tuberculosis embC* deletion. We used gene switching to replace the resident integrated vector carrying *M. tuberculosis embC* (pEMPTY25) with an alternate integrating vector carrying *M. smegmatis embC* (pRG643) in the Del-int strain. Gene switching is based on the high-efficiency replacement of resident L5-based integrating vectors in *M. tuberculosis* with incoming vectors carrying alternative selection markers (27). Replacement of pEMPTY25 ($embC_{Mtb}$) with pRG643 ($embC_{Msm}$) in the strain carrying the chromosomal deletion (*embC*Δ) was achieved at a high efficiency of 1.5×10^3 transformants/μg DNA, comparable to switching with the control vector ($embC_{Mtb}$), with an efficiency of 2.7×10^3 transformants/μg DNA. We confirmed that both plasmids carried functional copies of *embC*, as assessed by the complementation of the *M. smegmatis embC* mutant (data not shown). Thus, we confirmed that we were able to generate a strain of *M. tuberculosis* whose only functional copy of *embC* was derived from *M. smegmatis* and that the *M. smegmatis* gene was able to complement the function of the *M. tuberculosis* gene.

Identification of the promoter region for *embC*. The genomic organization of *embC* in *M. tuberculosis* is shown in Fig. 1. Previous studies suggested that *embC* could be expressed from a promoter located immediately upstream (11) or from a polycistronic message encompassing the *embCAB* region (35). In order to identify the promoter for *embC*, we looked at the expression of *M. tuberculosis embC* in its native host. The genetic organization and spacing suggest that *embC* is part of an operon with the upstream genes Rv3790, Rv3791, and Rv3792, since there are no intergenic regions. *embC* is likely to be the last gene in the operon, since there is an intergenic region of 86 bp downstream of *embC* containing a functional promoter (2).

In order to identify the promoter of *embC*, we cloned the regions upstream of *embC*, Rv3790, Rv3791, and Rv3792 into pSM128, creating transcriptional fusions with the β-galactosidase gene (10), and assayed promoter activity in *M. tuberculosis*. The region preceding Rv3790 had a strong promoter activity during exponential phase (312 ± 35 Miller units) (Fig. 3A). None of the other regions had significant activity, even after drug (ethambutol or ofloxacin) treatment (data not shown). This strongly suggests that *embC* is part of an operon starting with Rv3790.

In order to determine if *embC* was expressed as part of a polycistronic message, we conducted a Northern blot assay with two different probes, one to *embC* and one to Rv3790 (Fig. 3B). A single transcript was identified with the *embC*

probe with an approximate size of 7.4 kb. This corresponds to the length of a transcript spanning the sequence from Rv3790 to *embC*. No other, smaller, transcripts were identified. A single transcript of the same size was detected with the Rv3790 probe, confirming that both genes are present on the same transcript. Thus, *embC* is expressed from a single promoter located upstream of Rv3790. Reverse transcription-PCR on the junctions of each gene pair confirmed that a polycistronic message was present (data not shown). Thus, *embC* is transcribed independently from *embA* and *embB* but is part of a polycistronic message with Rv3790, Rv3791, and Rv3792.

Activity of P_{embC}. We looked at the expression of *embC*, as assessed by promoter activity from P_{3790} (P_{embC}) during different growth phases to determine if there was any regulation. We measured P_{embC} activity in *M. tuberculosis* over 158 days in liquid and solid media. P_{embC} was more active in cells grown on solid medium than in cells grown in liquid medium. In both media, significant induction occurred after extended growth periods. In the liquid medium, promoter activity was constant during the first 40 days but was increased about threefold to reach 800 Miller units. Promoter activity steadily increased on the solid medium, peaking at 1,600 Miller units before slowly decreasing to 1,200 Miller units (Fig. 4A).

Since we had seen that the size of LAM in *M. smegmatis* was dependent on the expression level of *embC* (Fig. 2), we predicted that the induction of the promoter driving *embC* in stationary phase could result in an increase in the size of LAM in *M. tuberculosis*. Thus, we analyzed LM and LAM profiles during extended growth of *M. tuberculosis* in liquid medium. However, we did not see any significant increase in the size of LAM or the LM/LAM ratio (Fig. 4B).

We also looked at P_{embC} activity under conditions of stress exposure, antibiotic treatment, and nonreplicating persistence. P_{embC} was clearly down-regulated (10-fold) in the hypoxia-induced nonreplicating state (Fig. 4C). P_{embC} activity was also assayed in response to oxidative stress generated by hydrogen peroxide exposure in *M. tuberculosis*. No change in promoter activity was seen (Fig. 4C). Previous work suggested that *embC* is up-regulated in response to ethambutol treatment (23). We assayed P_{embC} activity in *M. tuberculosis* cultivated with ethambutol and ofloxacin (at 0.5 times the MIC). No induction of the promoter activity was seen in response to ethambutol or ofloxacin treatment, revealing that expression of *embC* is not controlled in response to these drugs (Fig. 4C).

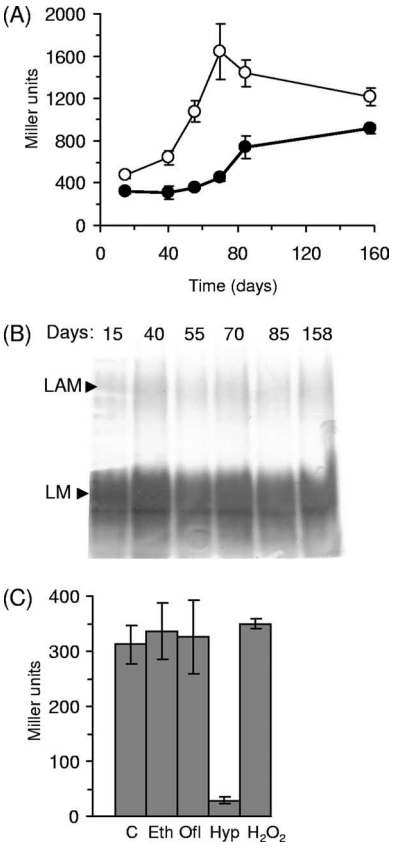

FIG. 4. P_{embC} activity and LAM production in *M. tuberculosis*. (A) Promoter activity assays. *M. tuberculosis* transformants carrying P_{embC} were grown in 10-ml static cultures (filled symbols) or on 7H10-OADC plates (open symbols), and β-galactosidase activity was measured. Results are the mean ± standard deviation of three individual transformants assayed in duplicate and are given in nanomoles of *O*-nitrophenyl-β-D-galactopyranoside produced per minute per milligram of total protein. (B) LM/LAM profile during growth. LM and LAM were extracted from liquid cultures after 15, 40, 55, 70, 85, and 158 days of static culture and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. (C) Response of P_{embC} to stress conditions and drug treatment. P_{embC} activity was measured in *M. tuberculosis* after exposure to ethambutol (Eth; 0.5 μg ml^{-1}), ofloxacin (Ofl; 0.2 μg ml^{-1}), or hydrogen peroxide (H_2O_2; 10 mM for 30 min) or after 2 weeks under hypoxic conditions (Hyp). Results are the mean ± standard deviation of three individual transformants assayed in duplicate and are given in nanomoles of *O*-nitrophenyl-β-D-galactopyranoside produced per minute per milligram of total protein. C, control.

DISCUSSION

We have demonstrated that *embC* is an essential gene in the pathogenic species *M. tuberculosis*, in contrast to its dispensability in the nonpathogenic species *M. smegmatis*. *M. smegmatis* EmbC was able to complement the function of *M. tuberculosis* EmbC. This suggests that the essentiality of *embC* comes from a more crucial role for LAM in the biology of the pathogenic species rather that an additional, uncharacterized role for *M. tuberculosis* EmbC. A large number of studies have shown that LAM is a potent immune modulator which affects many processes, including phagocytosis, cytokine induction, and dendritic cell activity. However, most of these functions have been determined with the isolated LAM molecule in vitro, and the role of LAM in the context of the whole organism and in vivo settings is much more restricted. We demonstrate here, for the first time, that LAM plays a critical role in the physiology of the bacterium itself. To date, the functionality of LAM in the bacterial cell has not been determined. Aside from maintaining structural integrity, it could also be involved in defense against stress, such as reactive oxygen intermediates. The complementation of the LAM^- phenotype of the *M. smegmatis embC* mutant by *embC* from *M. tuberculosis* confirms that it has a similar arabinosyltransferase activity. In addition, the essentiality of *embC*, together with its level of expression, makes it a potentially interesting drug target, especially since the Emb proteins appear to be unique to the *Actinomycetales* and are not found in eukaryotes.

Our results demonstrate that *embC* is transcribed as part of

a polycistronic mRNA in *M. tuberculosis*. We have previously shown that *embA* and *embB* are coexpressed on a transcript of a different size, further reinforcing our conclusion that *embCAB* is not a bona fide operon (2). It is cotranscribed with Rv3790 and Rv3791, both of which are involved in the biosynthesis of the arabinose donor decaprenylarabinose (19), and AftA (Rv3792), which attaches the first arabinose unit from the decaprenylarabinose carrier to AG (1). Thus, this operon is dedicated to the biosynthesis of the cell wall arabinans for both AG and LAM. Our data also confirm our previous observation that *embC* is not cotranscribed with *embA* and *embB* (2), allowing for differential expression. In this light, it is interesting that no up-regulation of the *embAB* promoter was seen in stationary phase, in contrast to that seen with the *embC* promoter, possibly reflecting a differential requirement for AG. Both promoters are down-regulated in hypoxia, when cell division ceases, reflecting a lack of requirement for novel cell wall biosynthesis.

It is interesting that the *embC* promoter region is up-regulated in late stationary phase in aerobic culture but turned off in a hypoxia-induced nonreplicating state. In contrast to previous work measuring mRNA (23), we saw no induction of *embC* in response to ethambutol as assessed by promoter activity. It is possible that mRNA stability is affected under these conditions, and an increase in expression of the protein cannot be ruled out. However, the previously reported up-regulation was only 1.96-fold for *embC* and 1.33-fold for *embB*, indicating that only minor changes were seen, despite the use of a sensitive real-time PCR technique (23). Regulation of *embC* expression has also been seen in *M. smegmatis* (32), where it can be controlled by the *M. tuberculosis* regulatory protein EmbR. EmbR itself is a substrate of multiple serine/threonine kinases (*pknA*, *pknB*, and *pknH*) and a phosphatase (31) and could form part of a complex network of control. However, care needs to be taken when interpreting data from *M. smegmatis*, since the promoter activity of P_{embC} is significantly different in this species than in its native host (data not shown).

Our results and previous work (4) suggest that the overexpression of *embC* results in the production of larger LAM species, at least in *M. smegmatis*. However, the analysis of LAM during stationary phase, where *embC* was up-regulated, did not reveal any increase in the size (or amount) of LAM compared to exponential-phase cells of *M. tuberculosis*. This was surprising, but we cannot exclude the possibility that there is a higher turnover of LAM under these conditions. There are reports of arabinomannan (AM) in culture supernatants of *M. tuberculosis* (21, 30); AM has a structure very similar to that of LAM and could represent a processed form of LAM which is secreted into the supernatant. However, we were unable to detect LAM or AM in the supernatant, making it unlikely that it accumulates under these conditions. Alternatively, it may be that overexpression of *embC* does not lead to increased LAM in *M. tuberculosis* and that this phenomenon is specific to *M. smegmatis*.

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