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

mtr, 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

mtr. 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 arabino- galactan (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 lipo- mann (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

mtr), 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

mtr 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...
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-B-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 (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 pEMPY6. The lacZ-embC-B-hyg cassette from the marker cassette vector pGOAL19 (25) was then inserted to replace the integrated pEMTY25 vector with pRG603 and pRG643 was carried into pGEM T Easy. pRG603 was constructed by amplifying the embA gene and sequence verified: p3790 (Rv3789-Rv3790), forward primer M. tuberculosis 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., pEMPY4, 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 replace the integrated embC gene with an unmarked complete deletion of the embC gene. One SCO strain was generated with each deletion vector and cloned as Scal or SmaI fragments into L5-based integrating vector pSM128 (10) of the promoterless lacZ gene and sequence verified: p3790 (Rv3789-Rv3790), forward primer CCC AGT TAC GTG GGA GCA CCT AAC CAC CAC TGT and reverse primer CCC AGT TAC GTG GGA GCA CCT AAC CAC CAC TGT and reverse primer CCC AGT TAC GTG GGA GCA CCT AAC CAC CAC TGT and reverse primer CCC AGT TAC GTG GGA GCA CCT AAC CAC CAC TGT and reverse primer CCC AGT TAC GTG GGA GCA CCT AAC CAC CAC TGT and reverse primer CCC AGT TAC GTG GGA GCA CCT AAC CAC CAC TGT and reverse primer CCC AGT TAC GTG GGA GCA CCT AAC CAC CAC TGT and reverse primer CCC AGT TAC GTG GGA GCA CCT AAC CAC CAC TGT and reverse primer CCC AGT TAC GTG GGA GCA CCT AAC CAC CAC TGT and reverse primer CCC AGT TAC GTG GGA GCA CCT AAC CAC CAC TGT and reverse primer CCC AGT TAC GTG GGA GCA CCT AAC CAC CAC TGT and reverse primer CCC AGT TAC GTG GGA GCA CCT AAC CAC CAC TGT and reverse primer CCC AGT TAC GTG GGA GCA CCT AAC CAC CAC TGT. 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., pEMPY4, 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

Extraction and analysis of LM and LAM. M. smegmatis was grown in Lenco 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 10% acrylamide gel, followed by periodic acid-Schiff staining (8, 28).
constructed a merodiploid strain carrying a second functional copy of \textit{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 \textit{embC} is indeed essential in \textit{M. tuberculosis} under normal growth conditions.

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

We cloned EmbC\textsubscript{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 \textit{M. smegmatis} \textit{embC} mutant and looked at complementation of LAM biosynthesis (11). As a positive control, we used pVV16 carrying \textit{M. smegmatis} \textit{embC} (pMSembC). LM and LAM were extracted from these strains and analyzed (Fig. 2). We confirmed that the \textit{embC} mutant did not produce any LAM. The strain complemented with EmbC\textsubscript{Msm} produced a larger LAM than the wild type, as previously noted (4). \textit{M. smegmatis} \textit{ΔembC} complemented with either vector expressing EmbC\textsubscript{Mtb} was able to synthesize LAM, confirming that EmbC\textsubscript{Mtb} is an arabinosyltransferase with activity similar to that of its ortholog in \textit{M. smegmatis}. Interestingly, the size of LAM in the complemented strains was dependent on the level of expression of \textit{embC}, with a larger LAM being produced in the strain expressing EmbC\textsubscript{Mtb} to a higher level (pMTembC). In addition, the strain complemented by EmbC\textsubscript{Msm} produced a larger LAM than the one complemented by EmbC\textsubscript{Mtb}, even when it was expressed from the same promoter.

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

![FIG. 1. Demonstration of the essentiality of \textit{embC} in \textit{M. tuberculosis}.](image)

(A) The genetic organization of the wild-type \\textit{embC} region is shown. BamHI sites are indicated (BHI); 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 \textit{embC}).

![FIG. 2. Analysis of LAM/LM from \textit{M. smegmatis} mutants.](image)

LAM/LM was extracted from \textit{M. smegmatis} and analyzed by sodium dodecyl sulfate-polycrylamide gel electrophoresis. (A) Wild-type strain. (B) Δ\textit{embC} strain with pVV16. (C) Δ\textit{embC} strain with pMSembC (P\textsubscript{hsp60}-EmbC\textsubscript{Msm}). (D) Δ\textit{embC} strain with pEMPTY25 (P\textsubscript{ag85a}-EmbC\textsubscript{Mtb}). (E) Δ\textit{embC} strain with pMTembC (P\textsubscript{hsp60}-EmbC\textsubscript{Mtb}). M\textsubscript{Mw}, molecular mass marker (masses are in kilodaltons).
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 (embCMtb) with pRG643 (embCmSm) in the strain carrying the chromosomal deletion (embCA) was achieved at a high efficiency of 1.5 × 10^3 transformants/µg DNA, comparable to switching with the control vector (embCMtb), 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).

**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 than 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 AhA (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 during stationary phase, where embC is down-regulated in hypoxia, when cell division ceases, reflecting a lack of requirement for novel cell wall biosynthesis.

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REFERENCES


