Identification of a Novel Arabinofuranosyltransferase AftB Involved in a Terminal Step of Cell Wall Arabinan Biosynthesis in Corynebacteriaceae, such as Corynebacterium glutamicum and Mycobacterium tuberculosis*

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Arabinofuranosyltransferase enzymes, such as EmbA, EmbB, and AftA, play pivotal roles in the biosynthesis of arabinogalactan, and the anti-tuberculosis agent ethambutol (EMB) targets arabinogalactan biosynthesis through inhibition of Mt-EmbA and Mt-EmbB. Herein, we describe the identification and characterization of a novel arabinofuranosyltransferase, now termed AftB (Rv3805c), which is essential in Mycobacterium tuberculosis. Deletion of its orthologue NCG2780 in the closely related species Corynebacterium glutamicum resulted in a viable mutant. Analysis of the cell wall-associated lipids from the deletion mutant revealed a decreased abundance of cell wall-bound mycolic acids, consistent with a partial loss of mycolylation sites. Subsequent glycosyl linkage analysis of arabinogalactan also revealed the complete absence of terminal β(1→2)-linked arabinofuranosyl residues. The deletion mutant biochemical phenotype was fully complemented by either Mt-AftB or Cg-AftB, but not with mutants of Mt-AftB, where the two adjacent aspartic acid residues, which have been suggested to be involved in glycosyltransferase activity, were replaced by alanine. In addition, the use of C. glutamicum and C. glutamicumΔaftB in an in vitro assay utilizing the sugar donor β-D-arabinofuranosyl-1-monophosphoryl-decaprenol together with the neoglycolipid acceptor α-D-araf-[(1→5)-α-D-araf-O-C9] as a substrate confirmed AftB as a terminal β(1→2) arabinofuranosyltransferase, which was also insensitive to EMB. Altogether, these studies have shed further light on the complexities of Corynebacteriaceae cell wall biosynthesis, and Mt-AftB represents a potential new drug target.

Mycobacterial diseases such as tuberculosis and leprosy still represent a severe public health problem (1). For instance, the recent emergence of multidrug-resistant tuberculosis strains and, more recently, extensively drug-resistant tuberculosis clinical isolates (2, 3) has prompted the need for new drugs and drug targets. The causative agent of these diseases, Mycobacterium tuberculosis and Mycobacterium leprae, respectively, are characterized by an intricate cell envelope (4–6). This characteristic mycobacterial cell envelope is composed of four macromolecules, lipoarabinomannan, mycolic acids, arabinogalactan (AG), and peptidoglycan (4–7). The galactan domain of AG is linked to peptidoglycan via a specialized “linker unit,” t-Rhap-(1→4)-α-D-GlcNAc, and its distal arabinan domain to mycolic acids, forming the mycolyl-arabinogalactan-peptidoglycan (mAGP) complex (4–6). The arabinan domain contains α(1→5), α(1→3), and β(1→2) arabinofuranosyl (Araf) linkages, arranged in several distinct structural motifs (5, 8, 9). The nonreducing arabinan termini of AG consists of t-Araf, 2-Araf, 5-Araf, and 3,5-Araf residues arranged into a characteristic terminal Araβ motif, with the 5-OH of the t-Araf and 2-Araf residues representing sites of mycolylation (6). The packing and ordering of mycolic acids within the mAGP and additional lipids within the outer envelope results in a highly impermeable barrier (10). It is interesting to note that several frontline anti-tubercular drugs, such as ethambutol (EMB) (11–13) and isoniazid (14, 15), target aspects of the biosynthesis of the mAGP complex.

Corynebacterium glutamicum has proven useful in the study of orthologous M. tuberculosis genes essential for viability (16, 17). This bacterium together with Corynebacterium diphtheriae and Corynebacterium jeikeium as well as M. tuberculosis and M. leprae and a number of other closely related species form the well defined taxon Corynebacteriaceae. The bacteria within this taxon share many characteristic cell wall features, such as AG and mycolic acids. In addition, the use of C. glutamicum together with its low number of paralogous genes (18) has proven useful in the study of the mAGP complex within this peculiar group of organisms (9). For instance, we recently identified a novel mycobacterial arabinofuranosyltransferase AftA using C. glutamicum due to the fact that it is largely tolerable
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with respect to the deletion of Cg-emb (9) and Cg-aftA (19), which are otherwise essential in M. tuberculosis.5

The structural basis of AG is now well defined (4, 5, 8); conversely, aspects of its biogenesis remained poorly resolved. The biosynthesis of AG involves the formation of a linear galactan chain with alternating β(1 → 5) and β(1 → 6)-d-galactofuranosyl (Galβ) residues of ~30 residues in length from the specialized "linker unit," L-Rhap→(1 → 4)-α-d-galactosamine (DPA) residues (18) of ~30 residues in length from the specialized "linker unit," L-Rhap→(1 → 4)-α-d-GlcNAc (20, 21). MALDI-TOF mass spectrometry (MS) analyzes of permethylated AG of C. glutamicum deleted of its single arabinofuranosyltransferase, Cg-emb, revealed that the 8th, 10th, and 12th Galβ residue possessed singular Araβ residues (9). These specific Araβ residues were recently shown to be transferred by a specialized arabinofuranosyltransferase AftA, whose gene in all Corynebacteriaceae analyzed to date is adjacent to the emb cluster (19). These initial Araβ residues "prime" the galactan backbone for further attachment of α(1 → 5)-linked Araβ residues. These reactions require the arabinofuranosyltransferase activities of Mt-EmbA and Mt-EmbB or Cg-Emb, which are also targets of EMB (9, 13, 22), to eventually result in mature AG. The Emb and AftA proteins utilize the specialized sugar donor, β-D-arabinofuranosyl-1-monophosphoryl-decaprenol (DPA) (23–25), and is a characteristic feature found only in Corynebacteriaceae (26–28). In addition, these proteins also revealed for the first time a clear domain organization of these glycosyltransferases (29). A recent topological analysis of Cg-Emb (30) together with a mutational study of Mt-EmbC (31) revealed for the first time a clear domain organization of these proteins, with the glycosyltransferase DDX signature evident in the extracellular loop that connects helices III-IV and the chain elongation "Pro-motif" in the extracellular loop connecting helices XIII-XIV (31).

It is interesting to note that the arabian domain of AG utilizes several different Araβ linkages, which suggests that additional arabinofuranosyltransferases must be required to form a fully matured AG. Moreover, initial Araβ residues at branching sites could require specialized arabinofuranosyltransferases as already observed for AftA (19), and it has to be considered that even further specialized arabinofuranosyltransferases might exist to incorporate Araβ into lipoarabinomannan. Clearly additional arabinofuranosyltransferases still remain to be identified in Corynebacteriaceae. Indeed, Liu and Mushegian (29) identified 15 members of the GT-C superfamily, representing candidates involved in the biosynthesis of cell wall related glycans in M. tuberculosis. We have continued our earlier studies (9, 16, 19) to identify genes required for the biosynthesis of the core structural elements of the mAGP complex in Corynebacteriaceae by studying mutants of C. glutamicum and the orthologous genes and enzymes of M. tuberculosis. Herein we present RV3805 as a new arabinofuranosyltransferase of the GT-C superfamily that is responsible for the transfer of Araβ residues from DPA to the arabian domain to form terminal β(1 → 2)-linked Araβ residues, which marks the "end point" for AG arabian biosynthesis before decoration with mycolic acids.

5 G. S. Besra, unpublished results.

**EXPERIMENTAL PROCEDURES**

**Strains and Culture Conditions—**M. tuberculosis H37Rv DNA was obtained from the Tuberculosis Research Material Contract (National Institutes of Health) at Colorado State University. C. glutamicum ATCC 13032 (the wild type strain, and referred for the remainder of the text as C. glutamicum) and Escherichia coli DH5α were grown in Luria-Bertani broth (LB, Difco) at 30 and 37°C, respectively. The mutants generated in this study were grown on complex brain heart infusion medium (32). Kanamycin and ampicillin were used at a concentration of 50 µg/ml. Samples for lipid analyzes were prepared by harvesting cells at an optical density of 10–15 followed by a saline wash and freeze drying.

**Construction of Plasmids and Strains—**The vectors made were pMSX-Cg-aftB (NCgl2780), pMSX-Mt-aftB (Rv3805c), and pK19mobsacBΔaftB, with the gene number of the M. tuberculosis and C. glutamicum aftB orthologue added in parentheses.

To express M. tuberculosis aftB in C. glutamicum, the primer pair GTATGACGATATGGTCCGGGTCACTTGTTGG (all primers in 5’-3’ direction) and ATGGCCCTCTACTGAGCATGATC (NCgl2780) were used to amplify the donor, and the gene number of the corresponding region in C. glutamicum aftB (NCgl2780), pMSX-Mt-aftB, with the primer number of the M. tuberculosis and C. glutamicum aftB orthologue added in parentheses.

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To construct the deletion vector pK19mobsacBΔaftB, construction of the vector was performed as described previously using two rounds of positive amplification and sequencing. The chromosomal deletion of Cg-aftB was performed as described previously using two rounds of positive selection (34), and its successful deletion was verified by use of different primer pairs. Plasmid pMSX-Mt-aftB and pMSX-Cg-aftB were introduced into C. glutamicum ΔaftB by electroporation with selection to kanamycin resistance (25 µg/ml).
Site-specific mutations were introduced in Mt-aftB using appropriate mutagenic primers and pMSX-Mt-aftB as the double-stranded template (QuikChange kit, Stratagene). After linear amplification of the newly synthesized strands and DpnI digestion of parental strands, plasmids pMSX-Mt-aftB-D29A and pMSX-Mt-aftB-D30A were generated carrying the mutations as indicated. All plasmids were verified by sequencing.

Protein Analysis—Recombinant C. glutamicum strains deleted of the chromosomal Cg-aftB copy but carrying either pMSX, pMSX-Mt-aftB, pMSX-Mt-aftB-D29A, or pMSX-Mt-aftB-D30A were each grown in LB up to an optical density of 4.

Cells were harvested by centrifugation, washed, and resuspended in 30 ml of 50 mM Tris-HCl (pH 7.4) buffer, containing 200 mM NaCl and 50 mM imidazole and disrupted by probe sonication. Centrifugation at 27,000 × g resulted in a clear supernatant, which was applied to a 1-ml HiTrapTM chelating chromatography system. The column was initially washed with 10 ml of the aforementioned buffer, and bound proteins were subsequently eluted with 2 ml of the same buffer but containing 500 mM imidazole. Eluted proteins were precipitated, dried, and resuspended in 10 ml of the aforementioned buffer, and SDS-PAGE was carried out on a 10% polyacrylamide gel, which was subsequently stained using 0.05% Coomassie G250 in 10% acetic acid to yield a highly purified cell wall preparation (5, 8, 9).

Extraction and Analysis of Cell Wall-associated and Cell Wall-bound Lipids—Cells (100 mg) were extracted by two consecutive extractions with 2 ml of CHCl3/CH3OH/H2O (10:10:3, v/v/v) for 3 h at 50 °C, and the resulting delipidated cells were stored for further use (as described below). Organic extracts were combined with 1.75 ml of CHCl3 and 0.75 ml H2O, mixed, and centrifuged. The lower organic phase was recovered, washed twice with 2 ml of CHCl3/CH3OH/H2O (3:47:48, v/v/v), dried, and resuspended in 200 μl of CHCl3/CH3OH/H2O (10:10:3, v/v/v). An aliquot (20 μl) was analyzed by thin layer chromatography (TLC) using silica gel plates (3535 silica gel 60F254, Merck) developed in CHCl3/CH3OH/H2O (60:16:2, v/v/v) and quantified using phosphorimaging after exposure to Kodak X-Omat film for 24 h. The bound [14C]corynomycolic acids from the delipidated extracts were released by base treatment and methylated as described above to afford [14C]CMAMEs. The [14C]CMAMEs were resuspended in 100 μl of CH3Cl2, and an aliquot (5 μl) was dried in a scintillation vial and then mixed with 10 ml of EcoScintA scintillation fluid (National Diagnostics, Atlanta, GA) and counted. Equal counts (25,000 cpm) of each sample were analyzed by TLC using silica gel plates (5735 silica gel 60F254, Merck) developed in CHCl3/CH3OH/H2O (60:16:2, v/v/v) and quantified using phosphorimaging after exposure to Kodak X-Omat film for 24 h. The bound [14C]corynomycolic acids were visualized using 0.05% Coomassie G250 in 10% acetic acid to yield a highly purified cell wall preparation (5, 8, 9).

Isolation of the mAGP Complex—The thawed cells were resuspended in phosphate-buffered saline containing 2% Triton X-100 (pH 7.2), disrupted by sonication, and centrifuged at 27,000 × g (5, 8, 9). The pelleted material was extracted 3 times with 2% SDS in phosphate-buffered saline at 95 °C for 1 h to remove associated proteins, successively washed with water, 80% (v/v) acetone in water, and acetone, and finally lyophilized to yield a highly purified cell wall preparation (5, 8, 9).

Glycosyl Composition and Linkage Analysis of Cell Walls by Alditol Acetates—Cell wall preparations were hydrolyzed using 2 m trifluoroacetic acid and reduced with NaBH4, and the resultant alditols per-O-acetylated were examined by gas chromatography (GC) (5, 8, 9). Cell wall preparations were per-O-methylated using dimethyl sulfvinyl carbanion (5, 8, 9). The per-O-methylated cell walls were hydrolyzed using 2 m trifluoroacetic acid, reduced with NaBH4, per-O-acetylated, and examined by GC/MS (5, 8, 9). Analysis of alditol acetate sugar derivatives was performed on a CE Instruments ThermoQuest Trace GC 2000. Samples were injected in the splitless mode. The column used was a DB225 (Supelco). The oven was programmed to hold at an isothermal temperature of 275 °C for a run time of 15 min (9). GC/MS was carried out on a Finnigan Polaris/GCQ PlusTM (9). The column used was a BPX5 (Supelco).

Arabinofuranosyltransferase Activity with Membrane Preparations of C. glutamicum, C. glutamicumΔaftB, and C. glutamicumΔaftB pMSX-Mt-aftB—Membranes were prepared as described previously (19, 24) and resuspended in 50 mM MOPS (pH 7.9) containing 5 mM β-mercaptoethanol and 10 mM MgCl2 (buffer A) to a final concentration of 15-10 mg/ml. The neoglycolipid acceptors α-D-Araf(1 → 5)-α-D-Araf-O-C8 (24, 35) (stored in C2H5OH) and DP[14C]A (25, 35) (stored in H2O).
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CHCl₃/CH₂OH, 2:1, v/v) were separated into aliquots into 1.5-ml Eppendorf tube to a final concentration of 2 mm and 200,000 cpm (90 μmol), respectively, and dried under nitrogen. The basic arabinofuranosyltransferase assay was carried out as described previously (24) with modifications. IgPal™ (Sigma-Aldrich) was added (0.1%, v/v) with the appropriate amounts of buffer A (final volume 80 μl). Tubes were sonicated for 15 min to resuspend lipid-linked substrates and then mixed with the remaining assay components, which included membrane protein (1 mg) from either C. glutamicum, C. glutamicumΔaftB, or C. glutamicumΔaftB pMSX-Mt-aftB, 1 mm ATP, 1 mm NADP, and in some cases EMB (0–1 mg/ml). Assays were incubated for 1 h at 37 °C and quenched by the addition of 533 μl of CHCl₃/CH₂OH (1:1, v/v). After mixing and centrifugation at 27,000 × g for 15 min at 4 °C, the supernatant was removed and dried under nitrogen. The residue was then resuspended in 700 μl of CH₃CH₂OH/H₂O (1:1, v/v) and loaded onto a 1-ml Sep-Pak strong anion exchange cartridge (Supelco) preequilibrated with CH₃CH₂OH/H₂O (1:1, v/v). The column was washed with 2 ml of CH₃CH₂OH/H₂O, and the eluate was collected, dried, and partitioned between the two phases arising from a mixture of n-butanol (3 ml) and water (3 ml). The resulting organic phase was recovered after centrifugation at 3500 × g, and the aqueous phase was then extracted twice with 3 ml of water-saturated n-butanol. The butanol phases were then combined and given an initial incubation at 37 °C with membranes (EMB was also added to in some cases EMB) and in some cases EMB (0–1 mg/ml). Assays were incubated for 1 h at 37 °C and then mixed with the remaining assay components. The remaining labeled material was subjected to TLC using silica gel plates (5735 silica gel 60F254, Merck) developed in CHCl₃:CH₂OH:H₂O:NH₄OH (65:25:3.6:0.5, v/v/v/v). TLC autoradiograms were obtained by exposing TLCs to Kodak BioMax MR film or HP-500 film (Kodak) and visualized under ultraviolet light (24). The plate was then re-developed in toluene to remove the reagent, and the bands were recovered from the plates by extraction with n-butanol. The butanol phases were washed with water saturated with n-butanol, and the dried products were subjected to 1H NMR, ES-MS and GC/MS as previously described (24).

RESULTS

Genome Comparison of the Rv3805c Locus—We recently identified AftA as a novel arabinofuranosyltransferase present in Corynebacteriaceae (19). Based on the fact that AftA is present in a highly conserved cell wall locus (19), we concentrated our studies to identify other cell wall related genes and subsequently identified Rv3805c (Fig. 1A), which is located in close proximity to the antigen 85 complex-encoding genes fbpA and fbpD (36). Furthermore, Rv3805c is likely to form an operon together with ubiA, which is required for prenyl transfer to 5-phosphoribose pyrophosphate to form decaprenylphosphoryl-5-phosphoribose before conversion to DPA (27, 28) and glfT, which is responsible for establishing the galactan backbone of AG (20, 21). The apparent fundamental function of aftB is indicated by the fact that the genome organization of this particular region is syntenic in Corynebacteriaceae, including all Mycobacterium and Corynebacterium species analyzed to date (see Fig. 1A), and also in Nocardia farcinica IFM 10152 and Rhodococcus sp. RHA1.

The gene product of Rv3805c, termed AftB, is predicted to form nine transmembrane (TM)-spanning helices in its N-terminal part, whereas a 237-amino acid C-terminal part is directed toward the periplasm (see Fig. 1C). Interestingly, AftB shows no obvious sequence similarity to the previously identified arabinofuranosyltransferases, such as Emb (9) and AftA (19), although the topology, with the C terminus directed toward the periplasmic side, is to some degree comparable. However, the similarity of the AftB proteins among each other is very high, even for the most distant pair, M. tuberculosis and D. pipheriae, exhibiting 33% identity over the entire length of the proteins. Even stronger conservation is found in the first periplasmic loop region (Fig. 1B), exhibiting a modified motif of the GT-C superfamily of glycosyltransferases consisting of two adjacent aspartic acid residues (29). Also, the periplasmic loop regions after helix V and VII are strongly conserved, which may play a role in presenting the nascent arabinose domain to the catalytic glycosyltransferase site. Taken together, the features of AftB and the locus where the gene is localized suggests that it represents a glycosyltransferase involved in AG biosynthesis.

Construction and Growth of C. glutamicum ΔaftB—In an attempt to delete aftB in C. glutamicum, the non-replicative plasmid pK19mobsacBΔaftB was constructed carrying sequences adjacent to Cg-aftB. The vector was introduced into C. glutamicum, and in several electroporation assays kanamycin-resistant clones were obtained, indicating integration of pK19mobsacBΔaftB into the genome by homologous recombination (Fig. 2A). The sacB gene enables for positive selection of a second homologous recombination event, which can result either in the original wild type genomic organization or in clones deleted of aftB (34). Forty-eight clones exhibiting the desired phenotype of vector-loss (KanR, SucR) were analyzed by PCR, and 21 of them were found to have Cg-aftB excised. These numbers indicate that the loss of Cg-aftB is apparently not a serious disadvantage for viability, in contrast with Cg-aftA, where deletion was rather difficult to obtain (19). As a result,
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A, the locus consists in M. tuberculosis (M. tub.) of aftB with the upstream-located ubiA gene product catalyzing prenylation of 5-phosphoribose pyrophosphate (26, 27) and the known galactofuranosyltransferase gftT (20, 21) and UDP-Galp mutase enzyme gft (45). Downstream of aftB the genes fbpA and fbpD are located which encode mycolyltransferases for decoration of the terminal arabinose residues with mycolic acids (6, 36). The organization of these genes is largely retained in a number of Corynebacteriaceae indicative for a basic functional unit. In N. farcinica (N. far.), a third paralogous mycolyltransferase is present, and in C. glutamicum (C. glu.) a transposon is inserted between the two mycolyltransferases. Orthologous genes are shaded accordingly. The M. tuberculosis region derived from NC_000962 extends from nucleotide 4,262,896 to 4,272,896. M. bov., Mycobacterium bovis; M. av. p., Mycobacterium avium paratuberculosis; M. lep., Mycobacterium leprae; R. spe., Rhodococcus sp. RHA1; C. eff., Corynebacterium efficiens; C. dip., Corynebacterium diphtheriae. B, partial sequence comparison of the first loop region of AftB. The conserved charged residues possibly involved in glycosyltransferase activity are shaded in gray, and the adjacent aspartate residues possibly directly involved in glycosyl transfer are in white on a black background (29). On top are the predicted structural properties of the peptide, with E indicating the β-sheet and H indicating the α-helix structure. The abbreviations are as above. C, topology of Mt-AftB based on dense alignment surface analysis (46). The membrane spanning helixes are given in roman numbers, and their aminoacyl residues are in arabic.

one clone was subsequently termed C. glutamicum ΔaftB and confirmed by PCR to have Cg-aftB deleted, whereas controls with C. glutamicum wild type and genes adjacent to Cg-aftB resulted in the expected amplification products (Fig. 2A).

Growth of wild type C. glutamicum and C. glutamicum ΔaftB were compared in brain heart infusion medium as well as salt medium CGXII (32). Both strains exhibited comparable growth rates, and the final cell densities reached were comparable (data not shown). Single colonies of the deletion mutant appeared less glossy. In streak-outs on brain heart infusion plates the surface of the deletion mutant appeared rough with a coarsely granular surface, as compared with wild type C. glutamicum (Fig. 2B). Taken together C. glutamicum ΔaftB possesses only a slight growth defect under the conditions assayed, indicating a degree of tolerance to the deletion of Cg-aftB. Complementation of C. glutamicum ΔaftB with either pMSX-Cg-aftB or pMSX-Mt-aftB restored the mutant to a wild type phenotype. For the purpose of significance, C. glutamicum ΔaftB comple-
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Non-Polar lipids

TDCM

TMC

Phospholipids

Origin

Solvent Front

CMAMEs

FIGURE 3. Quantitative analysis of extractable [14C]lipids from C. glutamicum, C. glutamicumΔaftB, and C. glutamicumΔaftB pMSX-Mt-aftB. Lipids were extracted from cells by a series of organic washes as described under “Experimental Procedures.” An aliquot (25,000 cpm) from each strain was subjected to TLC using silica gel plates (5735 silica gel 60F254, Merck) developed in CHCl3/CH3OH/H2O (60:16:2, v/v/v) and either charred using 5% molybdophosphoric acid in ethanol at 100 °C to reveal the extracted lipids and compared with known standards (9, 16) or quantified using phosphorimaging after exposure to Kodak X-Omat film for 24 h. The TLC autoradiogram is representative of three independent experiments. Lane 1, C. glutamicum; lane 2, C. glutamicumΔaftB; lane 3, C. glutamicumΔaftB pMSX-Mt-aftB.

omented with Mt-aftB was used throughout this investigation to study the corresponding mutant phenotype; however, similar results were also obtained with C. glutamicumΔaftB complemented with Cg-aftB (data not shown).

Cell Wall-associated and Bound Corynomycolic Acid Analysis—Our initial qualitative investigations involved the analysis of cell wall-associated lipids and bound CMAMEs TLC analysis. Analysis of free lipids from other previously identified cell wall mutants, such as C. glutamicumΔemb (9) and C. glutamicumΔaftA (19), highlighted an apparent increase in trehalose monocorynomycolate (TDCM), indicating a defect in cell wall biosynthesis. This phenotype was also consistently observed for the aftB deletion mutant in several independent experiments (data not shown). In addition, we also compared quantitatively through [14C]acetate labeling of cultures and equal loading of radioactivity the extractable free lipids from C. glutamicum, C. glutamicumΔaftB, and the complemented C. glutamicumΔaftB pMSX-Mt-aftB strains. Typically, C. glutamicum exhibited the known free lipid profile for wild type C. glutamicum, including phospholipids (3945 cpm), TDCM (3217 cpm), trehalose dicorynomycolate (TDCM) (3217 cpm), and non-polar lipids migrating at the solvent front (8753 cpm) (Fig. 3, lane 1). In contrast, after equivalent loading of radioactive and quantitative analysis by phosphorimaging analyzes, C. glutamicumΔaftB possessed an approximate significant 3-fold increase in TMC (10185 cpm) and a decrease in TDCM (6539 cpm), phospholipids (1275 cpm), and nonpolar lipids (5439 cpm) (Fig. 3, lane 2). Complementation of C. glutamicumΔaftB with pMSX-Mt-aftB reverted the deletion mutant back to a phenotype similar to the wild type, TMC (3331 cpm), TDCM (9123 cpm), phospholipids (4011 cpm), and non-polar lipids (8901 cpm) (Fig. 3, lane 3). To relate the above growth phenotypic changes of C. glutamicumΔaftB to its cellular composition, C. glutamicumΔaftB and C. glutamicumΔaftB pMSX-Mt-aftB along with wild type C. glutamicum were analyzed for arabinogalactan-esterified corynomycolic acids released from the above 14C-delipidated cells. As expected, the wild type exhibited a typical profile of CMAMEs (Fig. 4, lane 1, 28,562 cpm), whereas these products were significantly reduced in C. glutamicumΔaftB (Fig. 4, lane 2, 8,947 cpm). In addition, complementation of C. glutamicumΔaftB with pMSX-Mt-aftB (Fig. 4, lane 3, 27,523 cpm) led to the restoration of normal “levels” of cell wall-bound corynomycolic acids. These results suggested that Mt-aftB was involved in a key aspect of arabinan biosynthesis whereby deletion perturbs tethering of corynomycolic acids to AG but not as severely as in C. glutamicumΔemb and C. glutamicumΔaftA mutants (9, 19).

Cell Wall Glycosyl Compositional and Linkage Analysis of Cell Walls—Alditol acetate derivatives of highly purified mAGP from C. glutamicum, C. glutamicumΔaftB, and C. glutamicumΔaftB pMSX-Mt-aftB were prepared for glycosyl compositional analysis. All strains exhibited a similar Ara:Gal ratio of 3.7:1. However, glycosyl linkage analysis of per-O-methylated alditol acetate derivatives of mAGP extracted from these strains highlighted an obvious difference in linkage profiles (Fig. 5). All glycosyl linkages could be accounted for in wild type C. glutamicum (Fig. 5A) as described previously (9, 19); however, mAGP from C. glutamicumΔaftB was devoid of β(1→2) Ara linkages (Fig. 5B). Complementation of C. glutamicumΔaftB with pMSX-Mt-aftB restored the β(1→2) Ara linkage, thus reverting the deletion mutant to a wild
Further to this, we analyzed the cell wall glycosyl composition of *C. glutamicum* /H9004 aftB complemented with either pMSX-Mt-aftB-D29A or pMSX-Mt-aftB-D30A. Each of these complemented stains exhibited a phenotype identical to that of *C. glutamicum* /H9004 aftB, with a complete loss of 2-Araf linkages (data not shown). As confirmed in Fig. 6 the Mt-AftB muteins are synthesized in vivo, and the failure to establish the /H9252(1→2) Araf linkage is, therefore, most likely due to a catalytically inactive AftB, thus highlighting the importance of these particular aspartic acid residues in enzyme function.

In Vitro Arabinofuranosyltransferase Activity of *C. glutamicum*, *C. glutamicum* AftB, and *C. glutamicum* AftB pMSX-Mt-aftB—Initial attempts to develop an in vitro assay using either purified recombinant expressed Mt-AftB or E. coli membranes expressing Mt-aftB have thus far proved unsuccessful. As an alternative approach, we assessed the capacity of membrane preparations from *C. glutamicum*, *C. glutamicum* /H9004 aftB, and *C. glutamicum* /H9004 aftB complemented with pMSX-Mt-aftB to catalyze arabinofuranosyltransferase activity in the presence of an exogenous synthetic α-D-Araf and per-O-acetylated. The resulting partially per-O-methylated, per-O-acetylated glycosyl derivatives were analyzed by GC/MS as described previously (5, 8, 9). Rha, rhamnose.
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A

B

FIGURE 7. Arabinofuranosyltransferase activity in membranes prepared from C. glutamicum, C. glutamicumΔaftB, and C. glutamicumΔaftB pMSX-Mt-aftB. A, biosynthetic reaction scheme of products formed in arabinofuranosyltransferase assays using α-α-Araf-(1→5)-α-α-Araf-O-C8. B, arabinofuranosyltransferase activity was determined using the synthetic α-α-Araf-(1→5)-α-α-Araf-O-C8 acceptor in a cell-free assay as described previously (24). The products of the assay were resuspended before scintillation counting and subjected to TLC using silica gel plates (5735 silica gel 60F254, Merck) in CHCl3:CH3OH:H2O:NH4OH (65/25/3.6/0.5, v/v/v/v/v) with the reaction products visualized by autoradiography. DP, decaprenol phosphate.

(1 → 5)-α-α-Araf-O-C8, neoglycolipid acceptor (24), and DP[14C]A (35). TLC analysis of the products, when assayed with wild type C. glutamicum membranes, resulted in the formation of two products (A and B) (Fig. 7A) when analyzed by TLC (Fig. 7B). The enzymatic synthesis of products A and B are consistent with our previous studies (24) using mycobacterial assays utilizing membranes from C. glutamicum and EMB, identified the new glycosyl linkage as a β(1 → 2)-linked Araf residue (Figs. 8C and 7A). By analogy, this new glycosidic linkage corresponds to a terminal β(1 → 2)-linked Araf residue (24). These analyses were further confirmed by 1H NMR studies (data not shown) by the assignment of α(1 → 5) and β(1 → 2) membrane preparations resulting in trisaccharide products as a result of the addition of α(1 → 5)- and β(1 → 2)-linked Araf residues to the disaccharide acceptor (Fig. 7A) (24). The addition of EMB in several experiments, even at high concentrations of up to 1 mg/ml to the reaction mixture, resulted in the complete loss of only product A. However, when assays were performed using membranes prepared from C. glutamicumΔaftB, only a single band migrating to a position akin to that of product A could be observed, and no product formation could be identified upon the addition of 100 μg/ml of EMB (Fig. 7B). Membranes prepared from C. glutamicumΔaftB complemented with pMSX-Mt-aftB restored product A and B formation back to that of the wild type (Fig. 7B), and only product B was synthesized when EMB (up to 1 mg/ml) was added to the reaction mixtures.

ES-MS and GC/MS Analysis of Product A and B—Newly synthesized products A and B prepared using C. glutamicum treated with EMB and C. glutamicumΔaftB membranes, as described above, were further characterized. ES-MS analysis of the reaction products A (data not shown) and B extracted through preparative TLC (Fig. 8A) revealed a strong molecular ion m/z 549.3 (M + Na+) which corresponds to a trisaccharide product Araf-(1 → ?)-Araf-(1 → 5)-α-α-Araf-O-C8. GC/MS analysis of the partially per-O-methylated, per-O-acetylated alditol acetate derivative of product A, synthesized in assays with C. glutamicumΔaftB membranes, revealed the addition of only an α(1 → 5)-linked Araf residue (Figs. 8B and 7A) (24). However, GC/MS analysis of the partially per-O-methylated, per-O-acetylated alditol acetate derivative of product B, synthesized in enzyme
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Araf anomic protons in comparison to the acceptor Araf-(1→5)-α-D-Araf-O-C₆ and are consistent with our previous studies (24). Finally, the results clearly establish both from in vivo and in vitro experiments that Mt-AftB catalyzes the addition of a β(1→2) Araf unit and that this enzyme is resistant to EMB (Fig. 7B).

DISCUSSION

The biosynthesis of AG in M. tuberculosis has been the subject of intense research over the past decade (5, 9, 12, 19, 21, 37, 38). Because cell wall biosynthesis is the target for several antitubercular agents, such as EMB, the requirement for a complete understanding of the enzymes involved is imperative. We recently identified a unique DPA-dependent α-D-arabinofuranosyltransferase (AftA) that is responsible for the deposition of the first Araf residue onto the galactan moiety of AG, thus “priming” the polysaccharide for further extension by the Emb proteins (19). However, our current understanding of further downstream arabinan biosynthesis of AG is limited to that of the Emb proteins and is poorly defined (9, 39). M. tuberculosis possesses three Emb proteins encoded within the embCAB operon, of which EmbA and EmbB have been implicated in cell wall arabinan biosynthesis (39), whereas EmbC is involved in lipoarabinomannan biosynthesis (31, 40, 41). The catalytic mechanism of how these enzymes are able to synthesize the array of arabinan glycosidic linkages α(1→5), α(1→3), and β(1→2), present in both M. tuberculosis and C. glutamicum, remains to be elucidated. This catalytic conundrum is further questioned by the fact that members belonging to the Corynebacteria, such as C. glutamicum and C. diphtheriae, contain only a single emb gene (18). Therefore, one might assume that other arabinofuranosyltransferases could be involved in concert with the Emb proteins to build the arabinan domain of AG.

In this study we have identified Rv3805c, which we have termed AftB, as a novel retaining arabinofuranosyltransferase which is likely to form a new family that is distinct from the inverting arabinofuranosyltransferase enzymes (EmbA, -B, -C, and AftA) in GT-83/85 families (42). More precisely, AftB adds the nonreducing end of the arabinan domain of AG β(1→2) Araf residues as shown through both in vivo and in vitro experiments. For instance, incubation of membranes prepared from C. glutamicum with DP[14C]A and the disaccharide neoglycolipid acceptor resulted in the appearance of two trisaccharide products (A and B), which equate to the transfer of both α(1→5) and β(1→2)Araf residues, respectively. Through further chemical characterization of the products by TLC, ES-MS, and glycosyl linkage analyses, an α(1→5)-linked trisaccharide product could only be identified in assays conducted with membranes prepared from C. glutamicum AftB. This clear loss of β(1→2)Araf activity corroborates the cell wall analysis of the C. glutamicum AftB mutant, where the loss of β(1→2)-linked Araf residues could also be observed. We also attempted to inhibit AftB activity by incubation of the assay components in the presence of high concentrations of EMB (up to 1 mg/ml), a known inhibitor of the Emb proteins in M. tuberculosis and C. glutamicum. In doing so, analysis of the corresponding products synthesized from C. glutamicum membranes after EMB treatment clearly show evidence of an EMB-resistant β(1→2)
arabinofuranosyltransferase activity and an EMB-sensitive α(1 → 5) arabinofuranosyltransferase activity. In addition, since we have previously established that the EMB-resistant AftA introduces the priming Ara residue at the 8th, 10th, and 12th Gal residue of the galactan backbone, it can be concluded that the bulk α(1 → 5)Araf stems of AG represent the primary target of EMB. It is interesting to note that EMB resistance is simply not due to AftB being a retaining arabinofuranosyltransferase, in contrast to the inverting arabinofuranosyltransferase Mt-EmbA and Mt-EmbB, since AftA, which is also an inverting arabinofuranosyltransferase, is also EMB resistant (19).

A modified scheme for terminal cell wall arabinan biosynthesis in Corynebacteriaceae is presented in Fig. 9. It is possible that the AftB protein is responsible for the successive addition of two β(1 → 2) Araf residues at a 3,5-Araf-branched residue. Although this may be a reasonable inference from the in vivo structural work with the aftB deletion strain, it has not been completely verified by our in vitro assay. Therefore, it is formally possible that the AftB-dependent addition of one β(1 → 2) Araf residue is required before a second GT-C-related arabinofuranosyltransferase adds the second terminal β(1 → 2) Araf residue as shown in Fig. 9.

The arabinofuranosyltransferases of the Emb family (EmbC, EmbA, and EmbB) (12, 13, 31, 39) and AftA (19) and AftB possess some sequence similarity. This relates to a modified glycosyltransferase motif, which is defined in the GT-C glycosyltransferase superfamily as either DXD, EXD, DDX, or DEX (29). The most distant is probably AftA with only one negatively charged D residue, however, possessing an adjacent polar Gln residue (19). In AftB there are two adjacent Asp residues (Fig. 1B), which due to our mutational study are likely to be directly involved in glycosyl hydrolysis and transfer. Also, the high number of charged aminoacyl residues of the strongly conserved loop region after the first TM helix might contribute to the proper orientation of substrates at the catalytic center. The glycosyltransferase motif of arabinofuranosyltransferases so far identified is always located in a periplasmic loop region, which connects TM III-IV in EmbC, TM III-IV in AftA, and TM I-II in AftB (Fig. 1B). A

FIGURE 9. Proposed biosynthetic pathway leading to arabinan formation in M. tuberculosis AG. For reasons of simplicity, it is shown that one of the β(1 → 2)-linked Araf residues is added by AftB, whereas the second β(1 → 2)-linked Araf residue may be catalyzed by AftB or via an unknown GT-C arabinofuranosyltransferase, presumably closely related to AftB. Mycolylation is shown to occur after the final step of introducing both terminal β(1 → 2)-linked Araf residues of AG. However, mycolylation at the α(1 → 5)-linked Araf residue may occur before completion or simultaneously during establishment of the unique Araf motif of AG in M. tuberculosis. In addition, mycolylation of the penultimate Araf residue may also occur before the β(1 → 2)-linked Araf residue is attached. DP, decaprenol phosphate.
further feature common of the Emb, AftA, and AftB proteins is that they consist of an N-terminal region, which has a number of hydrophobic segments spanning the TM, and a large C-terminal domain, which in Emb has been demonstrated to be located toward the periplasmic side (30). The number of TMs is different among these proteins, but the involvement of these TMs could be considered as important for the translocation of DPA, the lipid-linked substrate of these glycosyltransferases. The weak structural identities of the membrane-embedded part of the arabinofuranosyltransferases indicate that transport and presentation of DPA to the catalytic site might be different for these enzymes. A Pro-motif, as identified in the Emb proteins (31), is not present in AftB and AftA. This motif is typical for polysaccharide co-polymerases and is assumed to control the chain length in polysaccharide biosynthesis. Its absence in AftA and AftB seems plausible, since these enzymes add only singular Ara residues, but the Emb proteins presumably add a number of α(1 → 5)-linked Ara residues to form the inner chain of the AG domain.

It is noteworthy that deletion of aftB in C. glutamicum results in only a weak phenotype (Fig. 2B). In M. tuberculosis mycolic acids are attached to the terminal β(1 → 2)Araf and penultimate α(1 → 5)Araf residue of the Ara₉ motif of AG (6). This appears to be similar in C. glutamicum, since in the absence of terminal β(1 → 2) Araf residues mycolic acids are still bound to AG, thus emphasizing in this respect the cell wall similarity of these bacteria. However, in C. glutamicum a maximal 5% of the mycolic acids are covalently attached to AG (43), whereas this value is about 10% in M. tuberculosis (43). The fact that the aftB deletion mutant of C. glutamicum possesses less AG-bound mycolic acids also results in an increased abundance of TMCM. This situation can be entirely different in M. tuberculosis due to the essentiality of aftB in M. tuberculosis (44) and requires further investigation.

We conclude that AftB represents a novel arabinofuranosyltransferase in Corynebacteriaceae, such as M. tuberculosis, which is responsible for the addition of the terminal β(1 → 2)-linked Araf residues. In doing so, we now propose a contemporary revision of cell wall arabinan biosynthesis (Fig. 9), which may aid in a more detailed understanding of the pathogenicity and persistence of M. tuberculosis.

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