THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 23, pp. 15653–15661, June 9, 2006

Identification of a Novel Arabinofuranosyltransferase (AftA) Involved in Cell Wall Arabinan Biosynthesis in *Mycobacterium tuberculosis*[*]

Received for publication, January 3, 2006, and in revised form, March 31, 2006 Published, JBC Papers in Press, April 4, 2006, DOI 10.1074/jbc.M600045200

Luke J. Alderwick[‡1,2], **Mathias Seidel**[§1], **Hermann Sahm**[‡3], **Gurdyal S. Besra**[‡4], **and Lothar Eggeling**[§3]

From the [‡]*School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom and* [§]*Institute for Biotechnology 1, Research Centre Juelich, D-52425 Juelich, Germany*

The cell wall mycolyl-arabinogalactan-peptidoglycan complex is essential in mycobacterial species, such as *Mycobacterium tuberculosis*, and is the target of several anti-tubercular drugs. For instance, ethambutol targets arabinogalactan biosynthesis through inhibition of the arabinofuranosyltransferases Mt-EmbA and Mt-EmbB. Following a detailed bioinformatics analysis of genes surrounding the conserved *emb* locus, we present the identification and characterization of a novel arabinofuranosyltransferase AftA (Rv3792). The enzyme catalyzes the addition of the first key arabinofuranosyl residue from the sugar donor β-D-arabinofuranosyl-1-monophosphoryldecaprenol to the galactan domain of the cell wall, thus "priming" the galactan for further elaboration by the arabinofuranosyltransferases. Because *aftA* is an essential gene in *M. tuberculosis*, we deleted its orthologue in *Corynebacterium glutamicum* to produce a slow growing but viable mutant. Analysis of its cell wall revealed the complete absence of arabinose resulting in a truncated cell wall structure possessing only a galactan core with a concomitant loss of cell wall-bound mycolates. Complementation of the mutant was fully restored to the wild type phenotype by Cg-*aftA*. In addition, by developing an *in vitro* assay using recombinant *Escherichia coli* expressing Mt-*aftA* and use of cell wall galactan as an acceptor, we demonstrated the transfer of arabinose from β-D-arabinofuranosyl-1-monophosphoryldecaprenol to galactan, and unlike the Mt-Emb proteins, Mt-AftA was not inhibited by ethambutol. This newly discovered glycosyltransferase represents an attractive drug target for further exploitation by chemotherapeutic intervention.

The Corynebacterianeae represent a distinct group within Gram-positive bacteria, with prominent members being the human pathogens *Mycobacterium tuberculosis*, *Mycobacterium leprae*, and *Corynebacterium diphtheriae* (1). In addition, nonpathogenic bacteria belonging to this taxon, such as *Corynebacterium glutamicum* and *Corynebacterium efficiens*, are used in the industrial production of amino acids (2). A common feature of the Corynebacterianeae is that they possess an unusual cell wall architecture (3–5). The cell wall is dominated by an essential heteropolysaccharide, arabinogalactan (AG),[5] linked to both peptidoglycan and mycolic acids, forming the mycolyl-arabinogalactan-peptidoglycan (mAGP) complex (3–6). The biosynthesis of the arabinan domain of AG, which is made up of α1→5-, α1→3-, and β1→2-glycosyl linkages, results from the sequential addition of arabinofuranose (Ara*f*) residues from the sugar donor β-D-arabinofuranosyl-1-monophosphoryldecaprenol (DPA) (7–9), by a set of unique arabinofuranosyltransferases termed the Emb proteins, of which three paralogues exist in *Mycobacterium avium* (10) and *M. tuberculosis* (11).

The anti-tuberculosis drug ethambutol (EMB) specifically inhibits AG biosynthesis (12), and the molecular target of EMB occupies the *embCAB* locus in *M. tuberculosis* (11). Upon individual disruption of *embC*, *embA*, and *embB* in *Mycobacterium smegmatis*, the resultant mutants are viable (13, 14). However, the crucial terminal Ara_6 motif, which is the structural motif for mycolylation in AG (5), is altered in both the Ms-*embA* and Ms-*embB* mutants (13). These results suggest that EmbA and EmbB are involved in the formation of the terminal Ara_6 motif in AG and also presumably compensated for each other in the respective Ms-*embA* and Ms-*embB* mutants, whereas Ms-*embC* is probably involved in the formation of the arabinan domains of lipoarabinomannan (LAM) (14). This is in agreement with the initial studies of the Ms-*embC* mutant (14) and recent findings that indicate when point mutations were re-introduced into the Ms-*embC* mutant on a multicopy plasmid expressing the mutant allele, a truncated LAM was synthesized, which retained the basic glycosyl linkage profile of LAM (15). However, attempts to obtain deletion mutants of *embA* and *embB* in *M. tuberculosis* or *embAB* in *M. smegmatis* have proved unsuccessful,[6] presumably because of the essentiality of the cell wall mAGP in these bacteria (16–19). In contrast, *C. glutamicum* has proven useful in the study of orthologous *M. tuberculosis* genes essential for cell viability. For instance, Cg-*pks* has been shown to be the key Claisen condensation enzyme involved in mycolic acid biosynthesis through the construction of a deletion mutant of *C. glutamicum* and its complementation with the Mt-*pks13* orthologue (19, 20).

In a recent study (6), deletion of the single Cg-*emb* orthologue in *C. glutamicum* resulted in a slow growing yet viable mutant that synthesized a novel truncated AG structure possessing a galactan core and only *t*-Ara*f* residues. Moreover, partial acid hydrolysis and matrix-assisted laser desorption ionization time-of-flight analysis identified the precise location of the three singular *t*-Ara*f* residues attached to the galactan core on the 8th, 10th, and 12th galactofuranose (Gal*f*) residue, thus representing the anchor points of the intact arabinan domains of AG (6). Treatment of *C. glutamicum* with EMB resulted in an identical phenotype comparable with the *C. glutamicum* Δ*emb* mutant (6). Thus,

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Both authors contributed equally to this work.

[2] Biotechnology and Biological Sciences Research Council Quota Student.
[3] Supported by the Fonds der Chemischen Industrie.
[4] Supported as a Lister Institute-Jenner Research Fellow and the Medical Research Council (UK). To whom correspondence should be addressed. Tel.: 121-415-8125; Fax: 121-414-5925; E-mail: g.besra@bham.ac.uk.

[5] The abbreviations used are: AG, arabinogalactan; Ara, arabinose; CMAME, corynomycolic acid methyl ester; DPA, decaprenol phosphoarabinose; DPPR, decaprenylphosphoryl-5-phosphoribose; EMB, ethambutol; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; mAGP, mycolyl-arabinogalactan-peptidoglycan; Rha, rhamnose; Araf, arabinofuranose; MOPS, 4-morpholinepropanesulfonic acid.

[6] G. S. Besra, unpublished results.

in contrast to the disruption of Ms-*embA* and Ms-*embB*, deletion of Cg-*emb* leads to an almost entire absence of arabinose in the cell wall, apart from specific *t*-Ara*f* residues that are directly linked to the galactan backbone. This suggested the presence of a novel enzyme responsible for "priming" the galactan domain for further elaboration by the Emb proteins, resulting in the final maturation of the native AG polysaccharide. It is the aim of this study to identify this novel arabinofuranosyltransferase, which catalyzes the addition of the first key Ara*f* residues to the galactan domain of AG.

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 in the remainder of the text it is referred to as *C. glutamicum*) and *Escherichia coli* DH5αMCR were grown in Luria-Bertani broth (LB; Difco) at 30 and 37 °C, respectively. The mutants generated in this study were grown on complex medium BHIS (21). Kanamycin and ampicillin were used at a concentration of 50 μg/ml. The minimal medium CGXII was used for *C. glutamicum* (21). Samples for lipid analyses were prepared by harvesting cells at an absorbance of 10–15, followed by a saline wash and freeze drying. Cultivation of *C. glutamicum* Δ*aftA* for lipid and cell wall analysis required two pre-cultures. First, a 5-ml BHIS culture was grown for 8 h, which was then used to inoculate a 50-ml BHIS culture for 15 h. This was then used to inoculate a 100-ml BHIS culture to an absorbance of 1, which was harvested after reaching absorbance of 3.

Construction of Plasmids and Strains—The vectors made were pET23b-Mt-*aftA* (Rv3792), pEKEx2Cg-*aftA* (NCgl0185), pEKEx2Mt-*aftA*, and pK19mobsacBΔ*aftA*, with the gene number of the *M. tuberculosis* and *C. glutamicum aftA* orthologue added in parentheses. To construct the *E. coli* expression vector pET23b-Mt-*aftA*, the primer pair 5′-GATCGATCCATATGCCGAGCAGACGCAAAAGCCCCCAATTC-3′ and 5′-GATCGATCAAGCTTCGCGCTCTCCTGCGGCTTGCGGATGGC-3′ was used with the restriction sites NdeI and HindIII underlined, with *M. tuberculosis* H37Rv chromosomal DNA as a template. The purified PCR fragment was ligated with accordingly digested pET23b (Novagen). To overexpress *C. glutamicum aftA*, the primer pair 5′-TCCCCCGGGAAGGAGATATAGATATGATTAACACCTCTGAAGATGAAG-3′ and 5′-TCCCCCGGGTTACTCATTGTGCGTTACCACCAC-3′ was used to amplify *C. glutamicum aftA*, which was ligated with SmaI-cleaved pEKEx2 to generate pEKEx2Cg-*aftA*. Similarly, primer pairs 5′-CAGGATCCAAGGAGATATAGATATGCCGAGCAGACGCAAAAG-3′ and 5′-CAGGATCCCCATCCGCGCTCTCCTGCGGCTTGC-3′were used to clone *M. tuberculosis aftA* into the BamHI site of pEKEx2. To construct the deletion vector pK19mobsacBΔ*aftA*, crossover PCR was applied with primer pairs AB (A, 5′-CGTGGATCCGGTGCC-3′; B, 5′-CCCATCCACTAAACTTAAACATTCAGAGGTGTTAATCAT-3′) and CD (C, 5′-TGTTTAAGTTTAGTGGATGGGGTGGGACCTTTCGTGGTGGTAACG-3′; D, 5′-GGCGTCCGTACTGTCCAG-3′) and *C. glutamicum* genomic DNA as template. Both PCR products were used in a second PCR with primer pairs AD to generate a fragment consisting of sequences adjacent to Cg-*aftA*, which was blunt end-ligated with SmaI-cleaved pK19mobsacB. All plasmids were finally confirmed by sequencing. The chromosomal deletion of Cg-*aftA* was performed as described using two rounds of positive selection (22), and its successful deletion was verified by use of different primer pairs. Plasmid pET23b-Mt-*aftA* was used to transform chemically competent cells of *E. coli* C43 (DE3) to ampicillin resistance (100 μg/ml), and

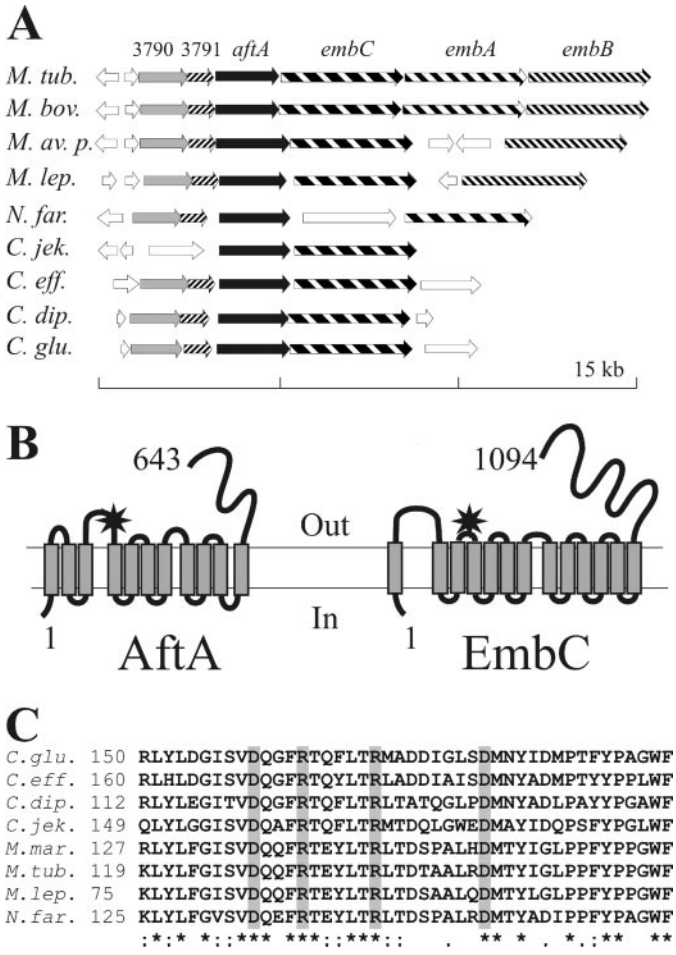

FIGURE 1. **Comparison of the *aftA* locus within the Corynebacterianeae.** *A*, the locus in *M. tuberculosis* (*M. tub.*) consists of *aftA* with its two upstream genes *Rv3790* and *Rv3791* leading to the formation of DPA (24, 25). Downstream of *aftA* the genes *embC*, *embA*, and *embB* are located encoding known arabinofuranosyltransferases (11). The organization of *Rv3790, Rv3791, aftA,* and *embC* is retained in a number of Corynebacterianeae indicative of a basic functional unit. In *N. farcinica* (*N. far.*), a glycosyltransferase of unknown function is located between *aftA* and *embC*. In *C. jeikeium* (*C. jek.*), *Rv3790* and *Rv3791* are clustered but are located at another locus. The abbreviations used are as follows: *M. bov.*, *M. bovis*; *M. av. p.*, *M. avium paratuberculosis*; *M. lep.*, *M. leprae*; *C. eff.*, *C. efficiens*; *C. dip.*, *C. diphtheriae*; and *C. glu.*, *C. glutamicum*. *B*, topology of AftA and EmbC of *M. tuberculosis*. The topology is predicted using dense alignment surface (30). AftA spans the membrane 11 times and EmbC 13 times, and both have a C-terminal extension located in the periplasm covering about one-third of the protein. The *star* indicates a highly conserved region that resides in the periplasmic loop and is probably concerned with glycosyltransferase activity. *C*, partial sequence comparison of region I of AftA proteins (*star* in *B*), indicating their high degree of identity. The conserved negative residues possibly involved in glycosyltransferase activity are *shaded* in *gray*. The abbreviations are as above including *Mycobacterium marinum* (*M. mar.*).

pEKEx2Cg-*aftA* was introduced into *C. glutamicum* Δ*aftA* by electroporation with selection to kanamycin resistance (25 μg/ml).

Extraction and Analysis of Cell Wall-bound Mycolic Acids—Cells were grown as described above, harvested, washed, and freeze-dried. Cells (100 mg) were extracted by two consecutive extractions with 2 ml of $CHCl_3/CH_3OH/H_2O$ (10:10:3, v/v) for 3 h at 50 °C. The bound lipids from the delipidated extracts or purified cell walls (see below) were released by the addition of 5% aqueous solution of tetrabutylammonium hydroxide, followed by overnight incubation at 100 °C, methylated as described previously (6), and analyzed by TLC using known standards (6).

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* (4, 6, 23). The pelleted material was extracted three times with 2% SDS in phosphate-

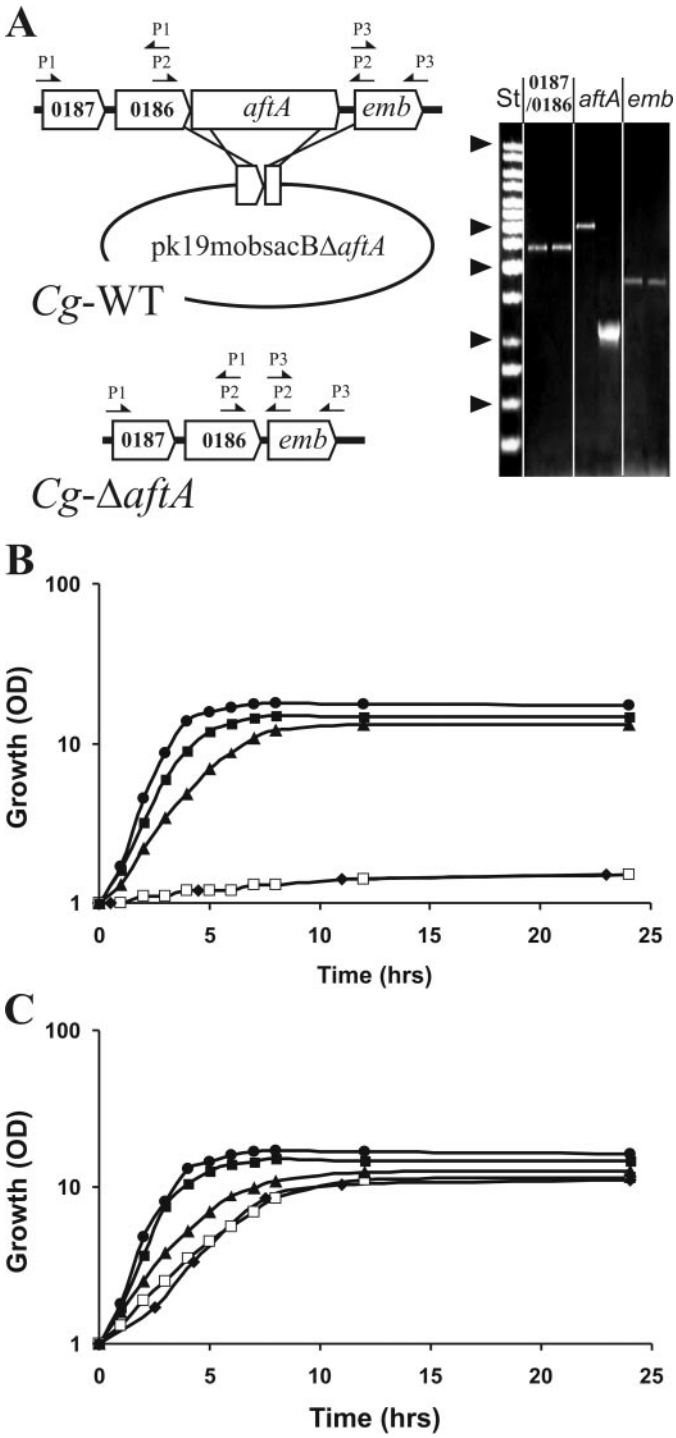

FIGURE 2. **Construction and characteristics of *C. glutamicum* Δ*aftA*.** *A,* illustrated is Cg-*aftA* with its adjacent genes NCgl0186 and Cg-*emb* and the strategy to delete Cg-*aftA* by using the deletion vector pK19mobsacBΔ*aftA*. This vector carries 18 nucleotides of the 5′-end of Cg-*aftA* and 36 nucleotides of its 3′-end, thereby enabling the in-frame deletion of almost the entire Cg-*aftA* gene. The *arrows* marked *P2* locate the primers used for the PCR analysis to confirm the absence of Cg-*aftA*. Primers P1 were used to detect NCgl0187 and NCg10186, the orthologue of Rv3790 and Rv3791, and P3 to detect Cg-*emb*. Distances are not drawn to scale. The results of the PCR analysis are shown on the *right*, where NCg10187 and NCg10186 mark the result obtained with primers P1, Cg-*aftA* with P2, and Cg-*emb* with P3. Samples were applied pairwise with the PCR product obtained from the wild type applied in the *left lane* and that of the deletion mutant in the *right lane*. *St* marks the standard, where the *arrowheads* are located at 10, 3, 2, 1, and 0.5 kb, respectively. *B*, the consequences of Cg-*aftA* deletion on growth in rich medium BHI. Growth of *C. glutamicum* (●), *C. glutamicum* Δ*aftA* (□), as well as the same strain expressing plasmid encoded Cg-*aftA* (■), Mt-*aftA* (▲), and Cg-*emb* (♦) is shown. *C,* consequences of Cg-*aftA* deletion on the same medium as in *B* but supplemented with 0.5 M sorbitol for osmotic stabilization. Symbols are same as in *B*.

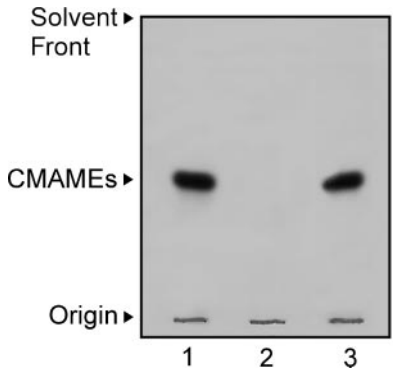

FIGURE 3. **Analysis of cell wall-bound CMAMEs from delipidated cells of *C. glutamicum*, *C. glutamicun* Δ*aftA*, and *C. glutamicum* Δ*aftA* pEKEx2*aftA*.** *Lane 1, C. glutamicum*; *lane 2, C. glutamicum* Δ*aftA*; *lane 3, C. glutamicum* Δ*aftA* pEKEx2*aftA*. The bound corynomycolic acids from the delipidated extracts or purified cell walls were released by the addition of tetrabutylammonium hydroxide at 100 °C overnight and methylated as described under "Experimental Procedures." An aliquot from each strain was subjected to TLC using silica gel plates (5735 Silica Gel $60F_{254}$, Merck), and developed in petroleum ether/acetone (95:5, v/v) and charred using 5% molybdophosphoric acid in ethanol at 100 °C to reveal CMAMEs and compared with known standards (6, 19).

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 (4, 6, 23).

Glycosyl Composition and Linkage Analysis of Cell Walls by Alditol Acetates—Cell wall preparations were hydrolyzed using 2 M trifluoroacetic acid, reduced with NaB^2H_4, and the resultant alditols per-*O*-acetylated and examined by gas chromatography (GC) as described previously (4, 6, 23). Cell wall preparations were per-*O*-methylated using dimethyl sulfinyl carbanion as described previously (4, 6, 23). The per-*O*-methylated cell walls were hydrolyzed using 2 M trifluoroacetic acid, reduced with NaB^2H_4, per-*O*-acetylated, and examined by gas chromatography/mass spectrometry (GC/MS) as described previously (4, 6, 23). 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 (6). GC/MS was carried out on a Finnigan Polaris/GCQ Plus™. The column used was a BPX5 (Supelco).

DPA and Cg-Emb Biosynthetic Activity within Membrane Preparations of C. glutamicum and C. glutamicum Δ*aftA*—Membranes from *C. glutamicum* and *C. glutamicum* Δ*aftA* were prepared as described previously to determine DPA biosynthetic activity (24, 25). Membrane protein (1 mg) was added to 5-phospho[^{14}C]ribofuranose pyrophosphate (2×10^6 cpm), 50 μg of decaprenol monophosphate, 60 μM ATP, 0.5 mM NADP in 50 mM MOPS (pH 7.9), 5 mM β-mercaptoethanol, and 10 mM $MgCl_2$ (buffer A) to a final volume of 160 μl. The reaction mixture was incubated for 1 h at 37 °C and stopped by the addition of 3 ml of $CHCl_3/CH_3OH$ (2:1, v/v). Radiolabeled lipid linked sugars were extracted, as described previously, prior to scintillation counting and subjected to TLC using silica gel plates (5735 Silica Gel $60F_{254}$, Merck) in $CHCl_3/CH_3OH/H_2O/NH_4OH$ (65:25:3.6:0.5, v/v) with reaction products visualized by autoradiography (24, 25). Analysis of Cg-Emb activity was determined using the synthetic α-D-Ara*f*-(1→5)-α-D-Ara*f*-*O*-$C_{10:1}$ acceptor in a cell-free assay as described previously (8).

Expression and Analysis of Mt-aftA Gene Product—For expression studies, *E. coli* (C43) was transformed with pET23b-Mt-*aftA* and cultured in Terrific Broth at 37 °C until an absorbance of 0.5, followed by the addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside and further incubation for 12 h at 16 °C. Cells were harvested by centrifugation

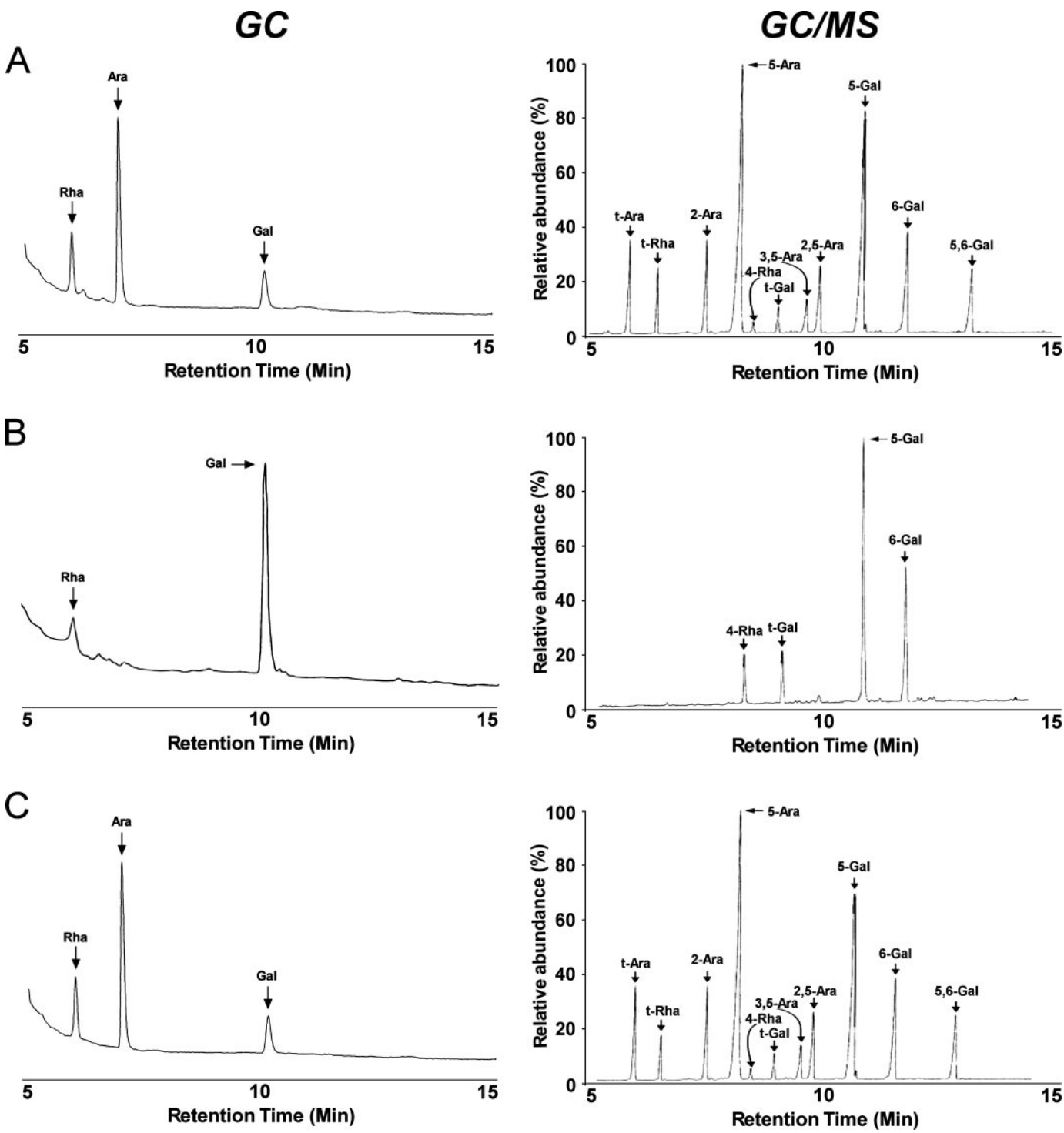

FIGURE 4. **Glycosyl compositional and glycosyl linkage analysis of cell walls of *C. glutamicum* (*A*), *C. glutamicum* Δ*aftA* (*B*), and *C. glutamicum* Δ*aftA* pEKEx2*aftA* (*C*).** Samples of purified cell walls were hydrolyzed using 2 M trifluoroacetic acid, reduced, per-*O*-acetylated, and subjected to GC as described under "Experimental Procedures." Alditol acetate standards (Supelco) of Rha, Ara, and Gal were analyzed with retention times of 6, 7 and 10.1 min, respectively. Cell walls were per-*O*-methylated, hydrolyzed using 2 M trifluoroacetic acid, reduced, and per-*O*-acetylated. The resulting partially per-*O*-methylated and per-*O*-acetylated glycosyl derivatives were analyzed by GC/MS as described previously (4, 6, 23).

at 5000 rpm, and the resulting pellet was resuspended in buffer A. Resuspended cells were sonicated and centrifuged at 23,000 × *g* for 20 min at 4 °C, and the resulting supernatant was recentrifuged at 100,000 × *g* for 90 min at 4 °C to isolate cell membranes that were collected and concentrated to a protein concentration of 15–20 mg/ml.

Decaprenol phospho[^{14}C]arabinose (100,000 cpm (45 μM) prepared as described previously (9, 26) and stored in $CHCl_3/CH_3OH$, 2:1 (v/v)) was dried under a stream of argon in a microcentrifuge tube (1.5 ml) and placed in a vacuum desiccator for 15 min to remove any residual solvent. The dried decaprenol phospho[^{14}C]arabinose was then resuspended in 30 μl of buffer A supplemented with 10% IgePal CA-630 (Sigma). An aliquot of this decaprenol phospho[^{14}C]arabinose solution (10,000 cpm, 4.5 μM, 3 μl) was added to the remaining constituents of the assay, which included 1 mg of membranes containing Mt-AftA and increasing

amounts of purified cell wall galactan polymer (0.1–1.0 mg, which represents ~0.015–0.15 mM galactan acceptor (6, 27, 28)) from *C. glutamicum* Δ*aftA* in buffer A to a final volume of 300 μl. The reaction mixture was incubated for 1 h at 37 °C, and the pellet was recovered following centrifugation at 18,000 × *g*. The pellet was washed twice with 1 ml of buffer A containing 1% IgePal CA-630 and further washed with 1 ml of $CHCl_3/CH_3OH$ (2:1, v/v) until the radioactivity was recorded as background in the final supernatant washes (less than 30 cpm). The pellet was finally resuspended in buffer A and transferred to a scintillation vial, and 10 ml of EcoScint was added, and the amount of [^{14}C]arabinose incorporation into cell wall galactan was measured by scintillation counting.

Analysis of Mt-AftA Assay Product—The basic assay was repeated five times as described above, but using nonradiolabeled DPA (200 μg, 0.75 mM) (9, 26), and 1 mg of cell wall galactan polymer prepared from *C. glutamicum* Δ*aftA*. Following an initial incubation at 37 °C for 1 h, the assay was replenished with fresh membranes containing Mt-AftA (1 mg) and re-incubated for 1 h at 37 °C with the entire process repeated three times with the addition of fresh membranes. The reaction mixture was processed as described above using two 1% IgePal CA-630 detergent washes in buffer A (1 ml) and five $CHCl_3/CH_3OH$ (1 ml, 2:1, v/v) extractions followed by centrifugation at 18,000 × *g*. The recovered pellets were pooled, per-*O*-methylated, hydrolyzed using 2 M trifluoroacetic acid, reduced with NaB^2H_4, per-*O*-acetylated, and analyzed by GC/MS as described previously (6).

RESULTS

Genome Comparison of the Emb Locus—Based on our previous observation that *C. glutamicum* Δ*emb* possessed no known arabinofuranosyltransferase activity yet contained single *t*-Ara*f* units attached to the galactan core (6), we analyzed a 14-kb chromosomal region of *M. tuberculosis* encompassing *embC*, *embA*, and *embB* in detail and compared it with that of other Corynebacterianeae (Fig. 1*A*). This region includes the recently discovered decaprenylphosphoryl-5-phosphoribose (DPPR) epimerizing enzymes encoded by Rv3790 and Rv3791, which eventually provide Ara*f* units from the sugar donor DPA (8, 24, 25, 29). These genes are followed by *Rv3792* which is adjacent to *embC*. This particular region consisting of four genes is syntenic with regions in *Mycobacterium bovis, M. avium* subsp. *paratuberculosis,* and *M. leprae* as well as with those of *C. efficiens, C. diphtheriae,* and *C. glutamicum*. In addition, *Nocardia farcinica* also shows ample synteny to the *M. tuberculosis* gene locus, as well as *Corynebacterium jeikeium*, indicating a fundamental function of Rv3792. Based on the results described below, the *Rv3792* gene and its orthologues was designated *aftA* (acronym for arabinofuranosyltransferase A).

M. tuberculosis aftA is predicted to encode a membrane protein of 643 amino acid residues. The predicted topology of the N-terminal region (residues 1–459) contains several hydrophobic segments based on dense alignment surface analysis (30), which probably form 11 transmembrane-spanning helixes, whereas the C-terminal region (residues 460–643) is predicted to be directed toward the periplasm. This domain organization and localization somewhat resemble that of EmbC (Fig. 1*B*). Nevertheless, the AftA proteins show no significant sequence similarity to the Emb proteins, and unlike the Emb proteins, they are not included in the CAZy data base of glycosyltransferases (31). However, the similarity of the AftA proteins among each other is very high over their entire sequence. Even for the most distant pairs, *M. tuberculosis* and *C. diphtheriae*, there is still 35.1% identity spanning 555 amino acid residues. The three regions of maximal similarity extend from 111 to 191 (I), 474 to 498 (II), and 516 to 551 (III) (amino acid residues of *M. tuberculosis* AftA). These regions contain several strictly conserved acidic and polar side chains as exemplified for part of region I of AftA (Fig. 1*C*), which are also known to play roles of general base and nucleophilic residues in glycosyl hydrolysis (31). Interestingly, region I (marked by a *star* in Fig. 1*B*) contains a conserved stretch of amino acids similar to that of the GT-C motif in EmbC, which is located in a periplasmic loop between membrane-spanning domains 3 and 4 (15). Taken together, the structural features of AftA as well as the localization of *aftA* within the gene cluster involved in arabinan biosynthesis suggest that AftA represents a putative glycosyltransferase involved in arabinan polymerization.

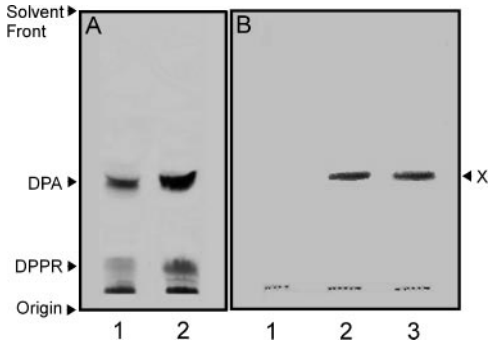

FIGURE 5. **Production of DPA (*A*) and Cg-Emb activity (*B*) within membrane preparations of *C. glutamicum* and *C. glutamicum* Δ*aftA*.** Radiolabeled lipid-linked sugars (*A*) were extracted following incubation with membranes and 5-phospho[^{14}C]ribofuranose pyrophosphate, counted, and subjected to TLC using silica gel plates (5735 Silica Gel 60F_{254}, Merck) in $CHCl_3/CH_3OH/H_2O/NH_4OH$ (65:25:3.6:0.5, v/v) with reaction products (DPA and DPPR) visualized by autoradiography. *Lane 1*, *C. glutamicum*; *lane 2*, *C. glutamicum* Δ*aftA*. Cg-Emb activity (*B*) was determined using the synthetic α-D-Araf-(1→5)-α-D-Araf-*O*-$C_{10:1}$ acceptor in a cell-free assay as described previously (8). The product X (α-D-[^{14}C]Araf-(1→2/5)-α-D-Araf-(1→5)-α-D-Araf-*O*-$C_{10:1}$) was resuspended prior to scintillation counting and subjected to TLC using silica gel plates (5735 silica gel 60F_{254}, Merck) in $CHCl_3$:CH_3OH:H_2O (65:25:4, v/v) with the reaction products visualized by autoradiography. *Lane 1*, control, no membranes; *lane 2*, *C. glutamicum*; *lane 3*, *C. glutamicum* Δ*aftA*.

Construction and Growth of C. glutamicum Δ*aftA*—Despite the fact that Rv3792 is essential for *M. tuberculosis* (32), we attempted to delete its orthologue in *C. glutamicum*. The nonreplicative plasmid pK19mobsacBΔ*aftA* was constructed carrying sequences adjacent to Cg-*aftA*. The vector was introduced into *C. glutamicum*, and in several electroporation assays kanamycin-resistant clones were obtained, indicating integration of pK19mobsacBΔ*aftA* into the genome by homologous recombination (Fig. 2*A*). The *sacB* gene enables positive selection of a second homologous recombination event that can result either in the original wild type genomic organization or in clones deleted of *aftA* (20). More than 200 clones were obtained after 2–4 days and analyzed by PCR, but all were wild type, illustrating a strong disadvantage of *aftA* deletion. We continued with this analysis to eventually obtain in three independent approaches three rough textured colonies appearing after about 15 days. These were shown by PCR to have *aftA* deleted, whereas inspection of the adjacent *C. glutamicum* open reading frames resulted in identical PCR products to that of controls derived from the wild type (see Fig. 2*A*).

Growth of *C. glutamicum* Δ*aftA* in liquid brain-heart-infusion medium is shown in Fig. 2*B*. The mutant was almost unable to grow on this medium, whereas the presence of plasmid-encoded Cg-*aftA* (pEKEx2Cg-*aftA*) almost fully restored growth. Also, upon complementation of the mutant with Mt-*aftA* (pEKEx2Mt-*aftA*), growth restoration was obtained, albeit somewhat reduced, which might be due to the biased codon usage of *M. tuberculosis*. Transformation of *C. glutamicum* Δ*aftA* with pEKEx2*emb* (6) did not restore the growth defect, showing that overexpression of Cg-*emb* is unable to substitute Cg-*aftA*, thus confirming the unique specificity of both AftA and Emb. The iden-

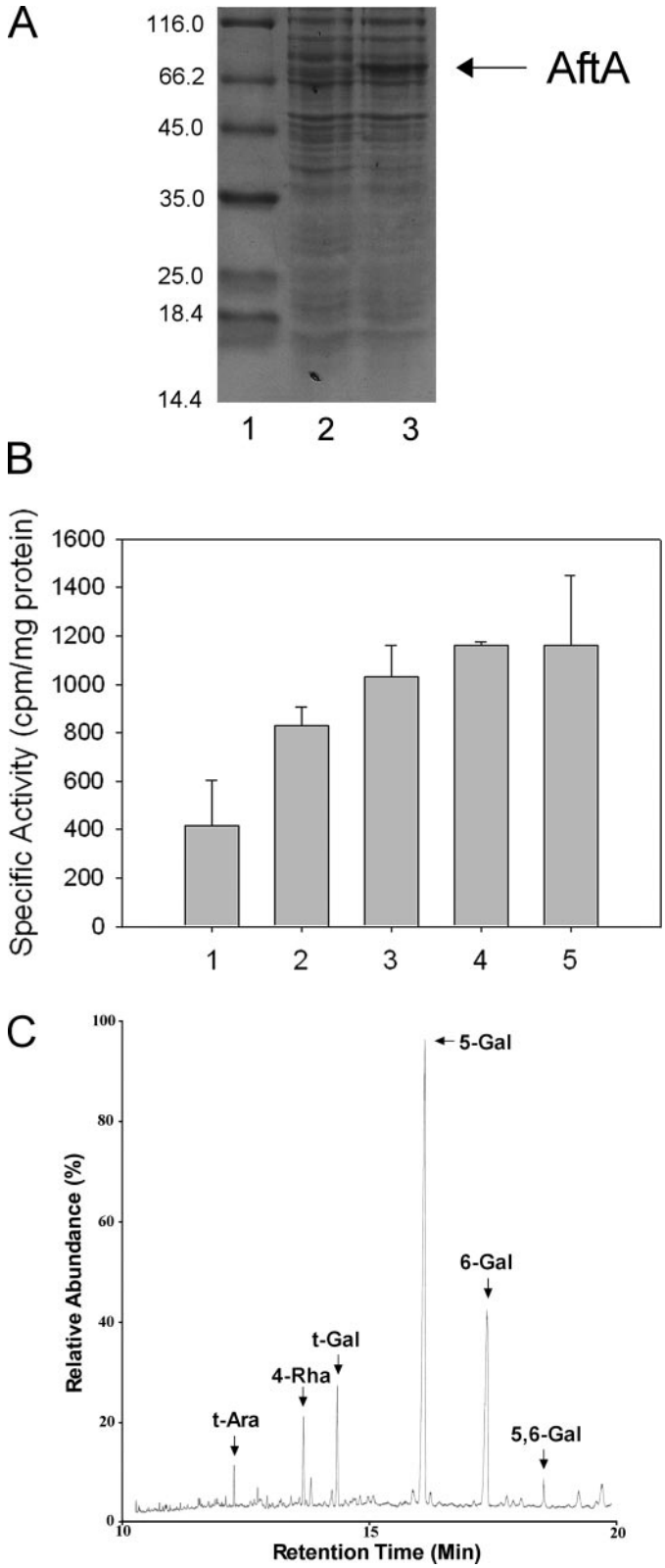

FIGURE 6. **Expression and functional characterization of recombinant Mt-*aftA*.** *A*, expression of Mt-*aftA* within *E. coli* (C43) membranes was confirmed by SDS-PAGE analysis. *Lane 1*, molecular mass standards (kDa); *lane 2*, *E. coli* (C43) uninduced membranes; *lane 3*, *E. coli* (C43) membranes expressing Mt-AftA. *B*, the data illustrate an increase in [^{14}C]arabinose incorporation into cell wall galactan from decaprenol phospho[^{14}C]arabinose with a fixed amount of *E. coli* (C43) membranes containing recombinant Mt-AftA (1 mg/ml). Background counts (less than 30 cpm) have been subtracted to give a final value for [^{14}C]arabinose incorporation with 0.1 mg (*lane 1*), 0.25 mg (*lane 2*), 0.5 mg (*lane 3*), and 1.0 mg (*lane 4*) of cell wall galactan. *Lane 5* represents the same reaction as *lane 4* supplemented with 100 μg/ml ethambutol. In addition, control assays were performed with membranes prepared from uninduced *E. coli* or *E. coli* harboring empty pET23b also resulted in background counts (less than 30 cpm) for the [^{14}C]Ara*f* incorporation from decaprenol phospho[^{14}C]arabinose in the presence of increasing cell wall galactan acceptor. *C*, the reaction identical to that described above (*B*, *lane 4*) was performed but using nonradiolabeled DPA. After incubation the reaction product was extracted, per-*O*-methylated, hydrolyzed, reduced with NaB^2H_4, per-*O*-acetylated, and analyzed by GC/MS as described under "Experimental Procedures."

tical strains were also grown on the same medium as before (brain-heart-infusion) but osmotically stabilized with 0.5 M sorbitol (Fig. 2*C*). Surprisingly, under these conditions, substantial growth was possible for *C. glutamicum* Δ*aftA*, which is presumably indicative of sorbitol stabilizing the cell wall mutant. On this medium the growth rate obtained for the wild type was 0.66 h^{-1} with a final absorbance of 17 and that for the deletion mutant was 0.31 h^{-1} with a final absorbance of 15.

Cell Wall-bound Corynomycolic Acid and Glycosyl Compositional and Linkage Analysis of Cell Walls—To relate the above growth phenotypic changes of *C. glutamicum* Δ*aftA* to its cellular composition, *C. glutamicum* Δ*aftA* and its complemented strain along with *C. glutamicum* were analyzed for arabinogalactan-esterified corynomycolic acids. *C. glutamicum* exhibited the known profile of corynomycolic acid methyl esters (CMAMEs) (Fig. 3, *lane 1*), whereas cell wall-bound corynomycolic acids were absent in *C. glutamicum* Δ*aftA* (Fig. 3, *lane 2*). The complementation of *C. glutamicum* Δ*aftA* with Cg-*aftA* (Fig. 3, *lane 3*) led to the restoration of cell wall-bound corynomycolic acids. These results suggest that Cg-*aftA* was involved in a key aspect of arabinan biosynthesis, whereby deletion perturbs tethering of corynomycolic acids to AG. Analysis of alditol acetate derivatives prepared from purified cell walls of *C. glutamicum* by GC revealed the sugar composition rhamnose (Rha), Ara, and Gal (Fig. 4*A*) (6). GC analysis of alditol acetates prepared from *C. glutamicum* Δ*aftA* (Fig. 4*B*) revealed a total loss of cell wall arabinose, which was restored upon complementation with plasmid pEKEx2Cg-*aftA* (Fig. 4*C*). GC/MS of per-*O*-methylated alditol acetate derivatives of *C. glutamicum*, *C. glutamicum* Δ*aftA*, and *C. glutamicum* Δ*aftA* pEKEx2Cg-*aftA* is shown in Fig. 4, *A*–*C*. Apart from the presence of 2,5-Ara*f* and *t*-Rha*p* associated with the arabinan domain of AG in *C. glutamicum*, other glycosidic linkages are comparable between *M. tuberculosis* (4, 33) and *C. glutamicum* (6, 27, 33) and include *t*-Ara*f*, 2-Ara*f*, 5-Ara*f*, 3,5-Ara*f*, 4-Rha*p*, *t*-Gal*f*, 5-Gal*f*, 6-Gal*f*, and 5,6-Gal*f* (Fig. 4*A*). As expected, *C. glutamicum* Δ*aftA* was devoid of *t*-Ara*f*, *t*-Rha*p*, 2-Ara*f*, 5-Ara*f*, 3,5-Ara*f*, and 2,5-Ara*f*, whereas the galactan domain (apart from the 5,6-Gal*f* branching residues resulting from arabinan side chains) was completely unaffected by the deletion of *Cg-aftA* and contained 4-Rha*p*, *t*-Gal*f*, 5-Gal*f*, and 6-Gal*f* (Fig. 4*B*). Complementation of *C. glutamicum* Δ*aftA* with plasmid pEKEx2Cg-*aftA* restored the glycosyl linkage profile to that of *C. glutamicum* (Fig. 4, *A* and *C*). The previous deletion of *emb* in *C. glutamicum* and chemical analysis of the cell wall revealed a drastically truncated AG structure possessing only *t*-Ara*f* residues and an unaltered galactan domain (6). Thus, the results indicate that *aftA* represents a putative arabinofuranosyltransferase responsible for priming the galactan domain with Ara*f* residues for subsequent elaboration by Emb proteins.

Analysis of DPA Synthesis and Cg-Emb Activity—In order to ensure that in *C. glutamicum* Δ*aftA* the biosynthesis of DPA is not reduced thereby disabling Ara*f* delivery to the cell wall, we examined DPA and DPPR biosynthesis. Based on previous studies (24, 25) using 5-phospho[^{14}C]ribofuranose pyrophosphate and decaprenol phosphate to monitor DPA formation, membranes were prepared from *C. glutamicum* and *C. glutamicum* Δ*aftA*. Both preparations afforded DPA synthesis (Fig. 5*A*), demonstrating that there was no reduced ability of *C. glutamicum* Δ*aftA* to synthesize DPA. In fact, an accumulation of DPA and DPPR was

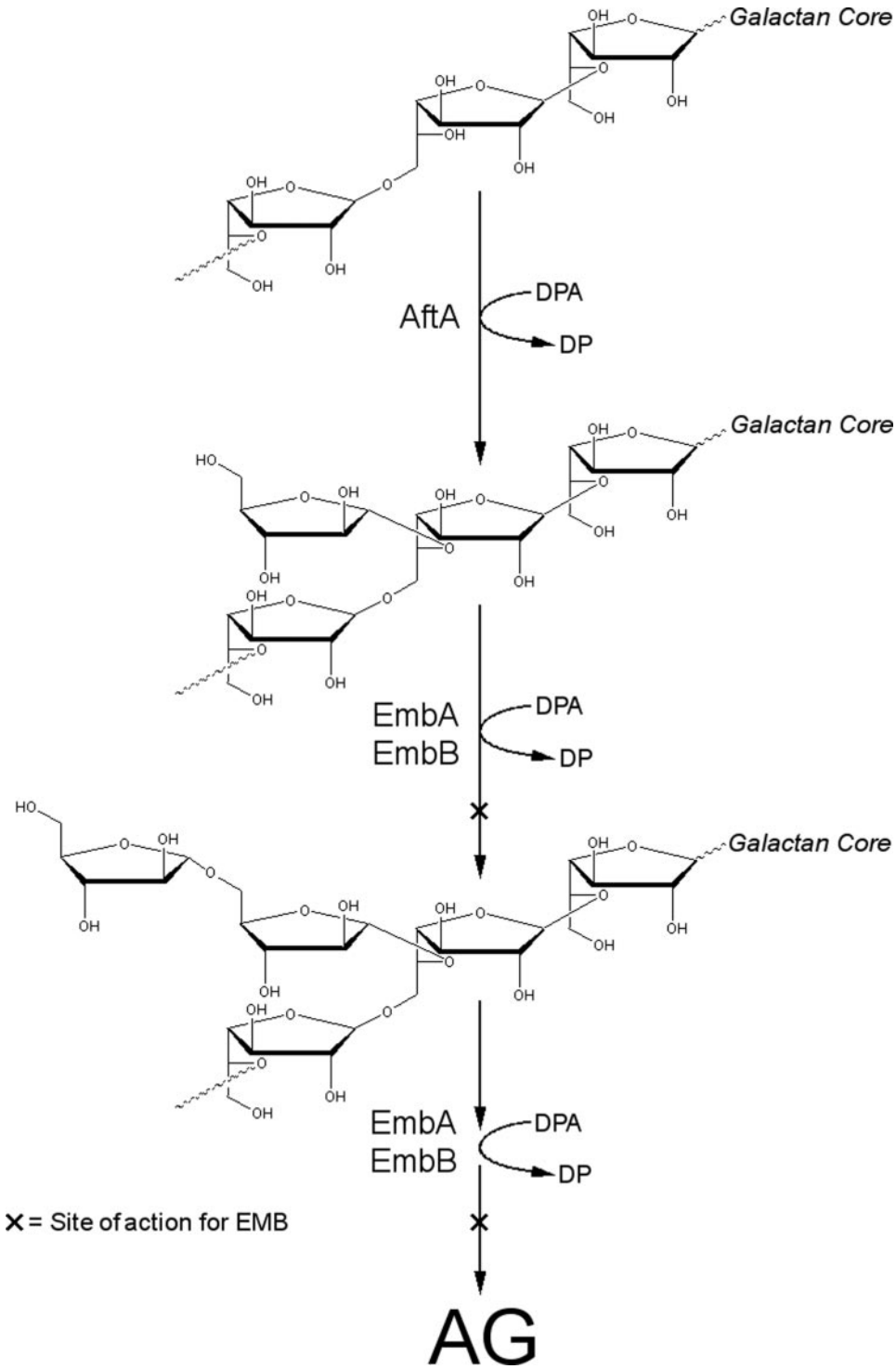

FIGURE 7. **Proposed biosynthetic pathway leading to arabinan formation in *M. tuberculosis* AG.**

evident in *C. glutamicum* Δ*aftA* (~75 and 80%, respectively) as compared with *C. glutamicum*. Using endogenous acceptor and membrane preparations of either *C. glutamicum* or *C. glutamicum* Δ*aftA*, we observed no apparent Emb transferase activity with *C. glutamicum* Δ*aftA* compared with a sustained level of activity with *C. glutamicum* (data not shown), thus suggesting that the endogenous acceptor from *C. glutamicum* Δ*aftA* requires priming with Ara*f* residues. In contrast, use of the synthetic acceptor α-D-Ara*f*-(1→5)-α-D-Araf-*O*-$C_{10:1}$ (8, 9) resulted in a significant transfer of [^{14}C]arabinose from decaprenol phospho[^{14}C]arabinose (8) affording an organic soluble trisaccharide product with membrane preparations from both strains (Fig. 5*B*). Thus, Emb is fully functional in *C. glutamicum* Δ*aftA*, as demonstrated above and by the earlier genetic experiments (Fig. 2, *B* and *C*). Furthermore, these results follow our hypothesis that the galactan domain of AG requires priming by the addition of a single Ara*f* unit to the C-5 OH of a β(1→6)-linked Gal*f* sugar for recognition and further extension by the Emb proteins.

Cloning and Functional Characterization of Recombinant Mt-aftA—By using *M. tuberculosis* H37Rv chromosomal DNA as a template, Mt-*aftA* was amplified by PCR, and the purified fragment was ligated with pET23b enabling expression of His_6-tagged Mt-*aftA*. Mem-

branes were prepared from *E. coli* (C43) pET23b-Mt-*aftA* and analyzed by SDS-PAGE revealing a weakly staining protein band with an apparent molecular mass of 69.5 kDa, which was absent in uninduced cultures, and a size predicted for Mt-His$_6$AftA (Fig. 6*A*). According to the current data, the reaction catalyzed by Mt-AftA is the transfer of Ara*f* from DPA to galactan. Therefore, an arabinose-free cell wall galactan acceptor was prepared from *C. glutamicum* Δ*aftA* and incubated with the *E. coli* membrane preparation expressing His$_6$-tagged Mt-*aftA*. Following incubation, the residual decaprenol phospho[^{14}C]arabinose substrate was removed from the cell wall galactan by detergent and several repeated extractions using $CHCl_3/CH_3OH$ (2:1, v/v). The remaining insoluble cell wall core was then subjected to scintillation counting revealing an increased amount of [^{14}C]Ara*f* incorporation in the presence of increasing amounts of cell wall galactan acceptor (Fig. 6*B*). Surprisingly, a 10-fold increase in cell wall galactan acceptor resulted in only a 3-fold increase in transferase activity. This poor turnover is presumably because of the inefficiency of the assay, which utilizes insoluble cell wall galactan as an acceptor. In addition, the activity of Mt-AftA remained unaffected in the presence of 100 μg/ml EMB (Fig. 6*B*), a known inhibitor of Cg-Emb (6), Mt-EmbA, and Mt-EmbB (11).

The conversion of the galactan acceptor to a sugar polymer containing *t*-Ara*f* units was further confirmed by glycosyl linkage analysis of the enzymatic reaction product (Fig. 6*C*). The newly synthesized product of several scaled up nonradiolabeled reactions was recovered, per-*O*-methylated, and derivatized to alditol acetates, which were analyzed by GC/MS. The linkages identified included those associated with the original cell wall galactan acceptor (Fig. 4*B*) plus the appearance of *t*-Ara*f*, and as a result the branched 5,6-Gal*f* residues (Fig. 6*C*).

Thus, our results describe the first report of a novel EMB-resistant arabinofuranosyltransferase, AftA, as the key initial glycosyltransferase involved in cell wall arabinan biosynthesis in Corynebacterianeae (Fig. 7) like *M. tuberculosis* and *C. glutamicum*.

DISCUSSION

The mAGP represents one of the most important cell wall components of the Corynebacterianeae and is essential for the viability of *M. tuberculosis* (16–19). It is therefore not surprising that one of the most effective anti-mycobacterial drugs, EMB, targets its biosynthesis. However, the emergence of multidrug-resistant tuberculosis has accelerated the need to discover new drug targets (34). We previously hypothesized the presence of a new priming enzyme that would link the initial Ara*f* unit with the C-5 OH of a β(1→6)-linked Gal*f* of a presynthesized galactan core (6). This was derived from a thorough analysis of a *C. glutamicum* mutant deleted of its single arabinofuranosyltransferase Emb (6), which still synthesizes a linear galactan extending from the reducing Rha with single *t*-Ara*f* residues attached to the 8th, 10th, and 12th Gal*f* residue. Apparently, these specific Ara*f* residues serve for recognition and extension by the known Emb proteins resulting in the formation of the mature arabinan chains (6, 10, 11).

The *in vivo* analysis of *C. glutamicum* Δ*aftA*, as well as the *in vitro* study with the AftA protein of *M. tuberculosis*, identifies a *bona fide* arabinofuranosyltransferase. In principle, the absence of Ara*f* residues in *C. glutamicum* Δ*aftA* could be due to the unavailability of precursors, as we demonstrated previously with a *C. glutamicum* mutant devoid of the polyprenyl transferase (UbiA) activity involved in the synthesis of DPA (6). However, we established that DPA biosynthesis was maintained in *C. glutamicum* Δ*aftA*, as well as Emb-catalyzed arabinan biosynthesis *in vitro*. This, together with our previous study on *C. glutamicum* Δ*emb*, shows that AftA functions to link the first Ara*f* residue to the cell wall galactan core. More importantly, the recombinant Mt-AftA transferred Ara*f* units from DPA to a cell wall galactan core acceptor *in vitro*, thus further confirming the unique activity of the enzyme. Although both Emb and AftA are arabinofuranosyltransferases, the proteins cannot functionally replace each other. Thus, despite some functional relationship, both glycosyltransferases have inherent specific features as is also evident from the insensitivity of Mt-AftA and Cg-AftA toward EMB, whereas the single Cg-Emb (6, 35) and Mt-Emb proteins are sensitive toward EMB (11).

The discovery of AftA sheds new light on the key arabinofuranosyltransferases that build the arabinan domain, which is typical for Corynebacterianeae. An elementary structure of this sugar polymer is apparent in *C. glutamicum*, and this bacterium has proven useful for a number of studies on mAGP biosynthesis (6, 19, 20). It represents the archetype of the Corynebacterianeae and has a low frequency of structural alterations as manifested, for instance in a low number of gene duplications (36). *Corynebacterium* species have only one *emb* gene, whereas *Mycobacterium* species have up to three. Nevertheless, the glycosidic linkage analysis of *C. glutamicum* AG shows that 2-Ara*f*, 5-Ara*f*, and 3,5-Ara*f* linkages are present, which are analogous to those found in the AG of *M. tuberculosis* (4). Furthermore, in *C. diphtheriae* and *C. glutamicum* 2,5-Ara*f* linkages are also evident (6, 37). This suggests the possibility that one single Emb glycosyltransferase enables the formation of different linkage types, a feature reminiscent of the bi-functional galactofuranosyltransferase (GlfT) of *M. tuberculosis* that produces alternating 5-Gal*f* and 6-Gal*f* linkages within the galactan core of AG (38, 39). The high syntenies of the *M. tuberculosis* Rv3790, Rv3791, *aftA*, and *embC* to the maps of all other *Mycobacterium* and *Corynebacterium* species are in agreement with this view (Fig. 1*A*). Also in *N. farcinica* this general organization is retained with an additional membrane protein-encoding gene. This largely retained organization, as well as the separation of the paralogous *embAB* genes in *M. leprae* and *M. avium* subsp. *paratuberculosis* is indicative of an ancient core function of Rv3790, Rv3791, *aftA*, and *embC* within the Corynebacterianeae involved in the synthesis of Ara*f* donors and their use to assemble a basic periplasmic arabinan domain that serves as a scaffold to tie mycolic acids. The identification of new cell wall biosynthetic drug targets is of great importance, especially with the emergence of multidrug-resistant tuberculosis. This newly discovered DPA-dependent arabinofuranosyltransferase represents a promising candidate for further exploitation as a potential drug target to disrupt the essential mycolyl-arabinogalactan-peptidoglycan complex in mycobacterial species, such as *M. tuberculosis*.

Acknowledgments—M. tuberculosis H37Rv DNA was obtained from the Tuberculosis Research Materials Contract (National Institutes of Health) at Colorado State University. We thank Graham Burns for technical assistance.

REFERENCES

1. Bloom, B. R., and Murray, C. J. (1992) *Science* **257,** 1055–1064
2. Sahm, H., Eggeling, L., and de Graaf, A. A. (2000) *Biol. Chem.* **381,** 899–910
3. McNeil, M., Daffe, M., and Brennan, P. J. (1990) *J. Biol. Chem.* **265,** 18200–18206
4. Besra, G. S., Khoo, K. H., McNeil, M. R., Dell, A., Morris, H. R., and Brennan, P. J. (1995) *Biochemistry* **34,** 4257–4266
5. McNeil, M., Daffe, M., and Brennan, P. J. (1991) *J. Biol. Chem.* **266,** 13217–13223
6. Alderwick, L. J., Radmacher, E., Seidel, M., Gande, R., Hitchen, P. G., Morris, H. R., Dell, A., Sahm, H., Eggeling, L., and Besra, G. S. (2005) *J. Biol. Chem.* **280,** 32362–32371
7. Wolucka, B. A., McNeil, M. R., de Hoffmann, E., Chojnacki, T., and Brennan, P. J. (1994) *J. Biol. Chem.* **269,** 23328–23335
8. Lee, R. E., Brennan, P. J., and Besra, G. S. (1997) *Glycobiology* **7,** 1121–1128
9. Lee, R. E., Mikusova, K., Brennan, P. J., and Besra, G. S. (1995) *J. Am. Chem. Soc.* **117,** 11829–11832
10. Belanger, A. E., Besra, G. S., Ford, M. E., Mikusova, K., Belisle, J. T., Brennan, P. J., and Inamine, J. M. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 11919–11924

11. Telenti, A., Philipp, W. J., Sreevatsan, S., Bernasconi, C., Stockbauer, K. E., Wieles, B., Musser, J. M., and Jacobs, W. R., Jr. (1997) *Nat. Med.* **3,** 567–570
12. Takayama, K., and Kilburn, J. O. (1989) *Antimicrob. Agents Chemother.* **33,** 1493–1499
13. Escuyer, V. E., Lety, M. A., Torrelles, J. B., Khoo, K. H., Tang, J. B., Rithner, C. D., Frehel, C., McNeil, M. R., Brennan, P. J., and Chatterjee, D. (2001) *J. Biol. Chem.* **276,** 48854–48862
14. Zhang, N., Torrelles, J. B., McNeil, M. R., Escuyer, V. E., Khoo, K. H., Brennan, P. J., and Chatterjee, D. (2003) *Mol. Microbiol.* **50,** 69–76
15. Berg, S., Starbuck, J., Torrelles, J. B., Vissa, V. D., Crick, D. C., Chatterjee, D., and Brennan, P. J. (2005) *J. Biol. Chem.* **280,** 5651–5663
16. Pan, F., Jackson, M., Ma, Y., and McNeil, M. (2001) *J. Bacteriol.* **183,** 3991–3998
17. Mills, J. A., Motichka, K., Jucker, M., Wu, H. P., Uhlik, B. C., Stern, R. J., Scherman, M. S., Vissa, V. D., Pan, F., Kundu, M., Ma, Y. F., and McNeil, M. (2004) *J. Biol. Chem.* **279,** 43540–43546
18. Vilcheze, C., Morbidoni, H. R., Weisbrod, T. R., Iwamoto, H., Kuo, M., Sacchettini, J. C., and Jacobs, W. R., Jr. (2000) *J. Bacteriol.* **182,** 4059–4067
19. Gande, R., Gibson, K. J., Brown, A. K., Krumbach, K., Dover, L. G., Sahm, H., Shioyama, S., Oikawa, T., Besra, G. S., and Eggeling, L. (2004) *J. Biol. Chem.* **279,** 44847–44857
20. Portevin, D., De Sousa-D'Auria, C., Houssin, C., Grimaldi, C., Chami, M., Daffe, M., and Guilhot, C. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 314–319
21. Eggeling, L., and Reyes, O. (2005) in *Handbook of Corynebacterium glutamicum*, (Eggeling, L. and Botts, M., eds) pp. 535–566, CRC Press, Inc., Taylor Francis Group, Boca Raton, FL
22. Schafer, A., Tauch, A., Jager, W., Kalinowski, J., Thierbach, G., and Puhler, A. (1994) *Gene* (*Amst.*) **145,** 69–73
23. Daffe, M., Brennan, P. J., and McNeil, M. (1990) *J. Biol. Chem.* **265,** 6734–6743
24. Mikusova, K., Huang, H., Yagi, T., Holsters, M., Vereecke, D., D'Haeze, W., Scherman, M. S., Brennan, P. J., McNeil, M. R., and Crick, D. C. (2005) *J. Bacteriol.* **187,** 8020–8025
25. Huang, H., Scherman, M. S., D'Haeze, W., Vereecke, D., Holsters, M., Crick, D. C., and McNeil, M. R. (2005) *J. Biol. Chem.* **280,** 24539–24543
26. Lee, R. E., Brennan, P. J., and Besra, G. S. (1998) *Bioorg. Med. Chem. Lett.* **8,** 951–954
27. Puech, V., Chami, M., Lemassu, A., Laneelle, M. A., Schiffler, B., Gounon, P., Bayan, N., Benz, R., and Daffe, M. (2001) *Microbiology* **147,** 1365–1382
28. Acharya, P. V., and Goldman, D. S. (1970) *J. Bacteriol.* **102,** 733–739
29. Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., Barry, C. E., III, Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M. A., Rajandream, M.-A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, S., Squares, R., Sulston, J. E., Taylor, K., Whitehead, S., and Barrell, B. G. (1998) *Nature* **393,** 537–544
30. Cserzo, M., Wallin, E., Simon, I., von Heijne, G., and Elofsson, A. (1997) *Protein Eng.* **10,** 673–676
31. Liu, J., and Mushegian, A. (2003) *Protein Sci.* **12,** 1418–1431
32. Sassetti, C. M., Boyd, D. H., and Rubin, E. J. (2003) *Mol. Microbiol.* **48,** 77–84
33. Daffe, M., McNeil, M., and Brennan, P. J. (1993) *Carbohydr. Res.* **249,** 383–398
34. Brennan, P. J., and Nikaido, H. (1995) *Annu. Rev. Biochem.* **64,** 29–63
35. Radmacher, E., Stansen, K. C., Besra, G. S., Alderwick, L. J., Maughan, W. N., Hollweg, G., Sahm, H., Wendisch, V. F., and Eggeling, L. (2005) *Microbiology* **151,** 1359–1368
36. Nakamura, Y., Nishio, Y., Ikeo, K., and Gojobori, T. (2003) *Gene* (*Amst.*) **317,** 149–155
37. Dover, L. G., Cerdeno-Tarraga, A. M., Pallen, M. J., Parkhill, J., and Besra, G. S. (2004) *FEMS Microbiol. Rev.* **28,** 225–250
38. Kremer, L., Dover, L. G., Morehouse, C., Hitchin, P., Everett, M., Morris, H. R., Dell, A., Brennan, P. J., McNeil, M. R., Flaherty, C., Duncan, K., and Besra, G. S. (2001) *J. Biol. Chem.* **276,** 26430–26440
39. Mikusova, K., Yagi, T., Stern, R., McNeil, M. R., Besra, G. S., Crick, D. C., and Brennan, P. J. (2000) *J. Biol. Chem.* **275,** 33890–33897