Deletion of Cg-emb in Corynebacterianeae Leads to a Novel Truncated Cell Wall Arabinogalactan, whereas Inactivation of Cg-ubiA Results in an Arabinan-deficient Mutant with a Cell Wall Galactan Core*

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The cell wall of Mycobacterium tuberculosis has a complex ultrastructure that consists of mycolic acids connected to peptidoglycan via arabinogalactan (AG) and abbreviated as the mAGP complex. The mAGP complex is crucial for the survival and pathogenicity of M. tuberculosis and is the target of several anti-tubercular agents. Apart from sharing a similar mAGP and the availability of the complete genome sequence, Corynebacterium glutamicum has proven useful in the study of orthologous M. tuberculosis genes essential for viability. Here we examined the effects of particular genes involved in AG polymerization by gene deletion in C. glutamicum. The anti-tuberculosis drug ethambutol is thought to target a set of arabinofuranosyltransferases (Emb) that are involved in arabinan polymerization. Deletion of emb in C. glutamicum results in a slow growing mutant with profound morphological changes. Chemical analysis revealed a dramatic reduction of arabinose resulting in a novel truncated AG structure possessing only terminal arabinofuranoside (t-Araf) residues with a corresponding loss of cell wall bound mycolic acids. Treatment of wild-type C. glutamicum with ethambutol and subsequent cell wall analyses resulted in an identical phenotype comparable to the C. glutamicum emb deletion mutant. Additionally, disruption of ubiA in C. glutamicum, the first enzyme involved in the biosynthesis of the sugar donor decaprenol phosphoarabinose (DPA), resulted in a complete loss of cell wall arabinan. Herein, we establish for the first time, (i) that in contrast to M. tuberculosis embA and embB mutants, deletion of C. glutamicum emb leads to a highly truncated AG possessing t-Araf residues, (ii) the exact site of attachment of arabinan chains in AG, and (iii) DPA is the only Araf sugar donor in AG biosynthesis suggesting the presence of a novel enzyme responsible for “priming” the galactan domain for further elaboration by Emb, resulting in the final maturation of the native AG polysaccharide.

The Corynebacterianeae represent a distinct and unusual group within Gram-positive bacteria, with the most prominent members being the human pathogens Mycobacterium tuberculosis and Mycobacterium leprae (1). In addition, the human pathogen Corynebacterium diphtheriae is the causal agent of diphtheria, and serious economic losses occur from the infection of animals by corynebacterial strains, such as Corynebacterium pseudotuberculosis and Corynebacterium matruchotii (2, 3). Furthermore, non-pathogenic bacteria belong to this taxon, such as Corynebacterium glutamicum, which is used in the industrial production of amino acids (4). A common feature to all these bacteria is that they possess an unusual cell wall matrix composed of mycolic acids, arabinogalactan, and peptidoglycan and is often referred to as the mycolyl-arabinogalactan-peptidoglycan (mAGP) complex (5–9).

Arabinogalactan (AG) plays a crucial role in covalently anchoring the outer lipid layer to peptidoglycan. Synthesis of AG begins with the formation of the linker unit through the transfer of GlcNAc-1-P and Rha from their respective sugar nucleotides (UDP-GlcNAc and dTDP-Rha) to form polyproprenyl-P-P-GlcNAc and polyproprenyl-P-P-Glcnac-Rha lipid intermediates (10, 11). The intermediates polyproprenyl-P-P-GlcNAc and polyproprenyl-P-P-GlcNAc-Rha then serve as acceptors for the sequential addition of galactofuranose (Galp) residues from UDP-Galp (generated from UDP-Galp via Gf (12, 13)) to form polyproprenyl-P-P-GlcNAc-Rha-Galp through a novel enzyme designated GfT (Rv3808c). This latter enzyme expresses two glycosyltransferase activities, a UDP-Galp:β-D-(1→5)-Galp and a UDP-Galp:β-D-(1→6)-Galp, both activities being required for alternating β(1→5) and β(1→6) linkages during galactan polymerization (11, 14). Chemical analysis of the mature lipid-linked galactan, synthesized in vitro (11), suggests that this intermediate then serves as the acceptor for the subsequent addition of arabinofuranose (Araf) residues from the arabinose sugar donor β-D-arabinofuranosyl-1-monophosphoryldecaprenol (DPA) in the formation of the Araf portion (α1→5), α1→3, and β1→2 linkages of AG (15–18). The AG-lipid intermediate at some point is mycolylated and transglycosylated to peptidoglycan (19, 20).

Early studies demonstrated that administration of ethambutol (EMB) led to a rapid cessation of mycolic acid transfer to the cell wall and an

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The abbreviations used are: mAGP, mycolyl arabinogalactan peptidoglycan; AG, arabinogalactan; Ara, arabinose; CMAME, Corynomyccum acid methyl ester; DPA, decaprenol phosphoarabinose; EMB, ethambutol; Gal, galactose; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; GlcNAc, N-acetyl-galactosamine; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; Rha, rhamnose; OD, optimal density.
accumulation of trehalose monomycolate and trehalose dimycolate (21). Subsequently, EMB was shown to inhibit specifically AG biosynthesis (22). The precise molecular target of EMB occupies the emb locus in Mycobacterium avium and M. tuberculosis. The locus consists of embRAB in M. avium (23) and embCAB in M. tuberculosis (24). To further define the role of EmbCAB proteins in arabinoxylan biosynthesis, embA, embB, and embC genes were inactivated individually in Mycobacterium smegmatis (25, 26). Although all three mutants were viable, only the crucial terminal Ara6 motif, which is the template for mycolylation in AG, was altered in both embA and embB mutants with the remaining AG structure intact (25). This suggested that both EmbA and EmbB are involved in the formation of the terminal Ara6 motif in AG, remaining AG structure intact (25). This suggested that both EmbA and EmbB are involved in the formation of the terminal Ara6 motif in AG, remaining AG structure intact (25). This suggested that both EmbA and EmbB are involved in the formation of the terminal Ara6 motif in AG, remaining AG structure intact (25). This suggested that both EmbA and EmbB are involved in the formation of the terminal Ara6 motif in AG, remaining AG structure intact (25). This suggested that both EmbA and EmbB are involved in the formation of the terminal Ara6 motif in AG, remaining AG structure intact (25). This suggested that both EmbA and EmbB are involved in the formation of the terminal Ara6 motif in AG, remaining AG structure intact (25). This suggested that both EmbA and EmbB are involved in the formation of the terminal Ara6 motif in AG, remaining AG structure intact (25). This suggested that both EmbA and EmbB are involved in the formation of the terminal Ara6 motif in AG, remaining AG structure intact (25). This suggested that both EmbA and EmbB are involved in the formation of the terminal Ara6 motif in AG, remaining AG structure intact (25). This suggested that both EmbA and EmbB are involved in the formation of the terminal Ara6 motif in AG, remaining AG structure intact (25). This suggested that both EmbA and EmbB are involved in the formation of the terminal Ara6 motif in AG, remaining AG structure intact (25).}

In the present study we have established through comparative genomic analyses the first biochemical and molecular description of a gene that is required for the formation of cell wall arabinans in a non-mycobacterial strain, and we highlight the inherent usefulness of examining related spp. to probe complex biosynthetic pathways.

MATERIALS AND METHODS

Strains and Culture Conditions—C. glutamicum ATCC 13032 (the wild-type strain, and referred for the remainder of the text as C. glutamicum) and Escherichia coli DH5αmer were grown in Luria-Bertani (LB) broth (Difco) at 30 °C and 37 °C, respectively. The mutants generated in this study were grown on BHIS (5 g of Tryptone, 5 g of NaCl, 2.5 g of yeast extract, 18.5 g of brain heart infusion (Difco), and 90.1 g of sorbitol per liter). Kanamycin and ampicillin were used at a concentration of 50 μg/ml. The minimal medium CGXII was used for C. glutamicum (30). Samples for lipid analyses were prepared by harvesting cells at an optical density (OD) of 10–15, followed by a saline wash and freeze drying. Cultivation of C. glutamicum Δemb for lipid and cell wall analysis required two pre-cultures: Firstly, 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 OD 1, which was harvested after reaching an OD 3.

Construction of Plasmids—The vectors used for deletion and inactivation were as follows: pK19mobsacBΔemb (pNCg0184, embC), pCg::ubiA (NCg2781, Rv3808c), with the gene numbers of the C. glutamicum and M. tuberculosis orthologs added in parentheses. The plasmid used for overexpression was pPEX2emb. To enable deletion of gene cross-over PCR was applied to generate the fragments carrying fused sequences adjacent to the gene in question. The resulting fragments were ligated with pK19mobsacB, and the final plasmids were confirmed by sequencing. For emb deletion, the primers used were emb_start_in 5’-CTT TAC TCA GAG AAT CTT TAA CAC TTA ACT TAC ATC TGA CAC GAT TAT C-3’, emb_start_out 5’-GCT TGG TGA GGT CCG AAA CAG GA-3’, emb_end_in 5’-GTT TTA GTT AGG GTA GGT GAT GGG CTC TGG ATT GAT GCA GGA GAT CAT ATG AAG-3’, and emb_end_out 5’-TCA CAC GAT GAG CCG ACA GAC-3’. For the second PCR the primer pair emb_start_out and emb_end_out was used again. The resulting fragment was ligated with Smal-cleaved pK19mobsacB to generate pK19mobsacBΔemb. For inactivation of ubiA an internal fragment of 321 bp was amplified (pubiA-for: ATC TGC AAT CAG CGG ACG ATC; publiA-rev: GAT ATC GAC TGG CAT GTG C), which was made blunt and ligated into the Smal site of the non-replicative vector pK18mob to yield pCg::ubiA.

Genomic Mutations—To enable chromosomal inactivation of ubiA, pCg::ubiA was introduced into C. glutamicum by electroporation. Selection for resistance to kanamycin yielded clones whose correct disruption of ubiA was confirmed with different primer pairs annealing in the vector and the bacterial chromosome.

Southern Blot Analysis—Genomic DNA was extracted from C. glutamicum Δemb and the wild-type strain and cleaved with EcoRV. The resulting fragments were separated on a 1% agarose gel and blotted onto a Nytran NY13N nitrocellulose membrane, with subsequent washings according to standard protocols. Detection was carried out with a fragment generated by PCR with primers pEmbΔ1 (5’-GTG TTA GTG GGG CTC TGG TGG G3’-3’) and pEmbΔ2 (5’-GGC AGC GTG CCG ATC ATC GCG C3’-3’) as probe that was labeled with digoxigenin (DIG labeling and detection kit, Roche Applied Science).

Extraction and Analysis of Cell Wall Bound Mycolic Acids from C. glutamicum Strains—Cells were grown as described above, harvested, washed, and freeze-dried. Cells (100 mg) were extracted by two consecutive extractions with 2 ml of CHCl3/CH3OH/H2O (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 2 ml of 5% aqueous solution of tetrabutylammonium hydroxide, followed by overnight incubation at 100 °C. After cooling, water (2 ml), CH3Cl2 (4 ml), and CH3I (500 μl) were added and mixed thoroughly for 30 min. The lower organic phase was recovered following centrifugation and washed three times with water (4 ml), dried, and resuspended in diethyl ether (4 ml). After centrifugation the clear supernatant was again dried and resuspended in CH3Cl2 (100 μl). An aliquot (10 μl) from each strain was subjected to TLC using silica gel plates (5735 silica gel 60F254, Merck), and developed in petroleum ether/acetone (95:5, v/v) and charred using 5% molybdenum phosphoric acid in ethanol at 100 °C to reveal corynomycolic acid methyl esters (CMAMES) and compared with known standards (31).

Isolation of the mAGP Complex—The thawed bacterial cells were resuspended in phosphate-buffered saline containing 2% Triton X-100 (pH 7.2), disrupted by sonication and centrifuged at 27,000 – g (6, 7). The pelletted material was extracted three 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) aceton in water, and acetone, and finally lyophilized to yield a highly purified cell wall preparation (6, 7).

Glycosyl Composition of Cell Walls by Alditol Acetates—Cell wall preparations were hydrolyzed in 250 μl of 2 M trifluoroacetic acid at 120 °C for 2 h as described (6, 7). Sugar residues were reduced with 50 μl of NaBH4 (10 mg/ml in ethanol:1 M NH3 (1:1)), and the resultant alditols were per-O-acetylated and examined by gas chromatography (GC) as described previously (6, 7).

Glycosyl Linkage Analysis of Cell Walls—Cell wall preparations (10 mg) were suspended in 0.5 ml of Me2SO (anhydrous) and 100 μl of 4.8 M dimethyl sulfinyl carbanion (6, 7). The reaction mixture was stirred for 1 h, and then CH3I was slowly added, and the suspension was stirred for a further 1 h; this process was repeated for a total of three times. The reaction mixture was then diluted with an equal volume of water, and the entire contents were dialyzed against water overnight. The resulting per-O-methylated cell wall samples were applied to a C18 Sep-Pak cartridge and purified as described previously (6, 7). The per-O-methylated cell walls were hydrolyzed using 250 μl of 2 M trifluoroacetic acid at 120 °C for 2 h. The resulting hydrolysate was reduced with NaBH4, per-O-acetylated, and examined by gas chromatography/mass spectrometry (GC/MS) as described previously (6, 7).

Mass Spectrometry of Per-O-methylated Cell Walls—Per-O-methylated cell walls were prepared as described above. Methanolic-HCl was 7 W. N. Maughan, unpublished results.
prepared by bubbling HCl gas into ~2 ml of methanol until hot to the touch (~1 molar). The reagent (100 µl) was added to the per-O-methylated cell wall sample, and aliquots were analyzed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) to monitor hydrolysis. The reaction was terminated by drying under nitrogen. MALDI-MS was performed using a PerSeptive Biosystems Voyager DE™ STR mass spectrometer (Applied Biosystems, CA) in the reflectron mode with delayed extraction. Samples were dissolved in methanol, and 1-µl aliquots were loaded onto a metal plate with 1 µl of the matrix 2,5-dihydrobenzoic acid. Sequazyme peptide mass standards were used as external calibrants (Applied Biosystems, CA).

**RESULTS**

**Genome Comparison of the emb Locus—** *M. tuberculosis*, *M. bovis*, *M. leprae*, and *M. avium* subsp. *paratuberculosis* have three *emb* genes (Fig. 1A), and at least one of these, *embB*, is suggested to be the target of EMB in mycobacteria (24, 32–34). However, *C. diphtheriae* and *C. glutamicum* have only one *emb* gene (35, 36). This is in accordance with the notion that the genome of Corynebacteriaceae is considered to represent the archetype of Corynebacteriaceae and has a low frequency of structural alterations and gene duplications (37). Interestingly, the single *emb* of *C. glutamicum*, Cg-emb, exhibits a higher identity to *embC* than to *embA* and *embB* of *M. tuberculosis*, and increased expression of Cg-emb increases resistance of *C. glutamicum* toward EMB (28). In *M. leprae* and *M. avium* subsp. *paratuberculosis* the paralogous *embAB* genes are separated by divergently transcribed genes that might indicate a more specific function and a separate regulation in these mycobacteria.

The above genomic comparison and the availability of the complete genome sequence of *C. glutamicum* has proven useful in the study of orthologous *M. tuberculosis* genes that are essential for viability. Therefore, in this study we examined the effects, in terms of arabinan biosynthesis and utilization of the sugar donor DPA, of firstly, Cg-emb by gene deletion in *C. glutamicum*, and secondly, disruption of Cg-ubiA (Fig. 1B), an enzyme recently shown to be involved in the biosynthesis of the sugar donor DPA.

**Construction of C. glutamicum Δemb**—In our recent studies on *emb* of *C. glutamicum* we placed the chromosomally encoded gene under the control of a tetracycline repressor (38) and observed a number of physiological consequences, including reduced growth in presence of repressor (38). These studies encouraged us to test whether it would be possible to obtain a deletion of *emb* in *C. glutamicum*. The non-repli- 

**GC and GC/MS of Sugar Composition and Sugar Linkage Analysis—** 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. GC/MS was carried out on a Finnigan Polaris/GCQ Plus™. The column was used a BPX5 (Supelco).

**In Vitro Growth Analysis of C. glutamicum Δemb**—The deletion mutant was transformed with pEKEx2emb (38), and growth was studied on brain-heart-infusion media supplemented with sorbitol for osmotic stabilization (30). Whereas growth of *C. glutamicum* was completed after 8 h at an OD of 16, *C. glutamicum Δemb* hardly reached an OD of 2 (Fig. 2D). However, complementation of the deletion mutant with pEKEx2emb restored the wild-type growth phenotype. mRNA transcript quantifications using LightCycler technology confirmed a 5-fold overexpression of *emb* due to pEKEx2emb when comparing expression of *emb* with its chromosomal copy (data not shown). *M. tuberculosis*
Arabinan-deficient Mutants of C. glutamicum

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**FIGURE 2.** Construction of C. glutamicum Δemb mutant and growth analysis. A, illustrated is emb with its adjacent genes of unknown function and the strategy to delete emb using the deletion vector pK19mobcBΔemb. The deletion vector carries 12 nucleotides of the 5’-end of emb and 12 nucleotides of its 3’-end thereby enabling the in-frame deletion of almost the entire emb gene. The hatched small box locates the probe used for the Southern blot analysis to detect hybridizing sequences on the 7.82-kb EcoRV fragment of the wild-type containing emb. Distances are not drawn to scale. B, situation of the original emb locus after deletion of emb, showing the intact organization of the originally adjacent genes in C. glutamicum Δemb with the digoxigenin-labeled probe given as hatched boxes in panels A and B. C, final confirmation of the constructed strain via Southern blot analysis using chromosomal DNA from C. glutamicum Δemb (lane 1), and Cg-WT (C. glutamicum) (lane 2). The right lane contains standards with their sizes given in kilobases. The left lane gives the sizes of the EcoRV fragments obtained from the wild-type, and the emb deletion mutant. The calculated sizes were 7.82 kb for the wild-type, and 4.35 kb for the deletion mutant. D, consequences of emb deletion on growth of C. glutamicum Δemb (■), C. glutamicum deleted of emb (C. glutamicum Δemb, △), as well as the same strain expressing plasmid encoded emb (C. glutamicum Δemb pEKE2Δemb (●)).
FIGURE 4. Glycosyl compositional analysis of cell walls of *C. glutamicum*, *C. glutamicumΔemb*, and *C. glutamicumΔemb pEKEx2emb*. Samples of purified cell walls were hydrolyzed with 2 M trifluoroacetic acid, reduced, per-O-acetylated, and subjected to GC as described under “Materials and Methods.” Alditol acetate standards (Supelco) of Rha, Ara, and Gal were analyzed with retention times of 6, 7, and 10.1 min, respectively.
mid-encoded Cg-emb restored the glycosyl composition to that of C. glutamicum (Fig. 4).

Glycosyl Linkage Analysis of Cell Walls from C. glutamicum, C. glutamicumΔemb, and Cg-emb-complemented C. glutamicumΔemb—Per-O-methylated alditol acetate derivatives of C. glutamicum, C. glutamicumΔemb, and C. glutamicumΔemb complemented with plasmid-encoded emb were shown in Fig. 5. Glycosyl linkages present in M. tuberculosis (data not shown) and C. glutamicum include t-Araf, 2-Araf, 5-Araf, 4-Rhap, t-Galp, 3,5-Araf, 5-Galp, 6-Galp, and 5,6-Galp. The major difference between C. glutamicum and M. tuberculosis AG includes the presence of 2,5-Araf and t-Rhap residues in C. glutamicum. In C. glutamicumΔemb a loss of 5-Araf, 3,5-Araf, 2,5-Araf, and t-Rhap is observed with only t-Araf residues appearing alongside 5-Galp, 6-Galp, and 5,6-Galp residues. These results suggest that Cg-Emb actually plays a much larger role in the arabinosylation of AG in comparison to the results previously obtained with M. smegmatis EmbA and EmbB mutants (25), possibly suggesting some partial complementation of EmbA and EmbB in the singular M. smegmatis emb disruption mutants (25).

In addition, the AG of C. glutamicum is unusual in that it would appear that the arabinan domains are also capped by t-Rha, because these are absent in the C. glutamicumΔemb mutant. Complementation of the emb mutant with plasmid-encoded Cg-emb restored the glycosyl linkage profile to that of C. glutamicum. Glycosyl linkage analysis of C. glutamicum treated with 100 μg/ml EMB yielded a CG/MS trace comparable to that of C. glutamicumΔemb (data not shown).

MALDI MS Analysis of Per-O-methylated Cell Walls from C. glutamicumΔemb—Cell walls derived from C. glutamicumΔemb were per-O-methylated and analyzed by MALDI-TOF MS, and the data are shown in Fig. 6A. The cluster of signals around m/z 4000 can be attributed to an AG polymer with truncated arabinan branching. The signals at m/z 3375, 3783, 4191, and 4599 are consistent with an AG glycan containing increasing numbers of Gal residues, AraGal13Rha, AraGal15-Rha, AraGal17-Rha, and AraGal19-Rha, respectively. The additional signals observed can be assigned to AG glycans lacking an Ara or Rha residue, possibly resulting from the derivatization process. To define the Ara branching pattern on the galactan polymer, the per-O-methylated sample was subjected to time course methanolysis followed by re-methylation. The data generated (Fig. 6B) show numerous partial hydrolytic products affording informative ion series. A key region of the spectrum is shown expanded in Fig. 6C, and the assignment of significant products containing Rha are presented in TABLE ONE. Collectively, the data indicate that a linear galactan polymer extends from the reducing Rha and that the first Ara branch appears on the eighth Gal residue with further Ara branches appearing on the tenth and twelfth Gal residues. These results are supported by data generated from partial hydrolysis followed by per-O-deuteromethylation of the resulting hydrolytic products. The mass shifts observed resulting from per-O-deuteromethylation (data not shown) support the proposed Ara branching pattern.

MALDI MS analysis of per-O-methylated cell walls derived from C. glutamicum treated with 100 μg/ml EMB revealed a similar profile to that observed for cell walls derived from C. glutamicumΔemb. In addition, partial hydrolysis confirmed that C. glutamicum treated with EMB produced an AG, which had the same Ara branching pattern as described above in C. glutamicumΔemb (data not shown) illustrating the effects of EMB and emb deletion are super imposable.

Disruption of Cg-ubiA—We were intrigued by UbiA, a putative 4-hydroxybenzoate polyprenyltransferase and the possibility that UbiA was perhaps involved in DPA formation from 5-phosphoribofuranose pyrophosphate and decaprenol phosphate. The ubiA gene is present in

FIGURE 5. Glycosyl linkage analysis of per-O-methylated cell walls prepared from C. glutamicum, C. glutamicumΔemb, and C. glutamicumΔemb pEKEx2emb. Cell walls were prepared as described under “Materials and Methods” per-O-methylated, hydrolyzed, reduced, and per-O-acetylated. The resulting partially per-O-methylated, per-O-acetylated glycosyl derivatives were analyzed by GC/MS as described (6, 7).
Corynebacterianae in synteny within the locus of other cell wall-related genes (Fig. 1B). The orthologue of *M. tuberculosis* Rv3806c in *C. glutamicum* is NCgl2781, and during compilation of this report was shown biochemically to perform the first step of DPA biosynthesis producing decaprenylphosphoryl-5-phosphoribose from 5-phosphoribofuranose pyrophosphate and decaprenol phosphate (39). To inactivate *ubiA* of *C. glutamicum*, plasmid pCg::ubiA was constructed and *C. glutamicum* transformed to kanamycin resistance. The resulting strain was confirmed by PCR analysis to have *ubiA* disrupted. This strain, *C. glutamicum::ubiA* was similar to *C. glutamicum*Δemb, exhibited

![Figure 6: MALDI-TOF MS of per-O-methylated cell walls derived from *C. glutamicum*Δemb.](image)

**TABLE ONE**

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<th>Assignment of the significant partial hydrolytic products detected by MALDI-TOF MS resulting from per-O-methylated cell walls derived from <em>C. glutamicum</em>Δemb</th>
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<sup>a</sup> Assignment of Ara, Gal, and Rha based on their identification by linkage analysis.
Arabinan-deficient Mutants of C. glutamicum

C. glutamicum::ubiA

FIGURE 7. Analysis of C. glutamicum::ubiA cell walls. Samples of purified cell walls were hydrolyzed with 2 M trifluoroacetic acid, reduced, per-O-acetylated, and subjected to GC as described under “Materials and Methods” to provide glycosyl compositional analysis. The inset shows the MALDI-TOF MS of per-O-methylated cell walls derived from C. glutamicum::ubiA.

poor growth, and was devoid of bound cell wall corynomycolic acids (data not shown).

Glycosyl Compositional and MALDI MS Analysis of Cell Walls from C. glutamicum::ubiA—Interestingly, glycosyl compositional analysis of the resulting cell wall of C. glutamicum::ubiA, in contrast to C. glutamicum::emb, revealed a complete ablation of arabinan (Fig. 7). The results support a functional role of Cg-ubiA in cell wall arabinan biosynthesis (39) and demonstrate that DPA is the sole donor of Ara residues in cell wall biosynthesis in Corynebacterianeae. Analysis of the per-O-methylated galactan derived from C. glutamicum::ubiA by MALDI MS revealed a cluster of signals consistent with a galactan backbone lacking any t-Ara residues as observed for the emb deletion mutant (see inset, Fig. 7). The signals at m/z 2895, 3303, 3711, and 4199 can be assigned the compositions Galα1-Rha, Galα1-Rha, Galα1-Rha, and Galα1-Rha, respectively. The additional signals observed can be assigned to galactan glycans lacking Rha or Gal, or retaining the GlcNAc attached to Rha.

DISCUSSION

The mAGP represents one of the most important cell wall components of members of the Corynebacterianeae, and it is essential for the viability of M. tuberculosis (27–29). It acts as a fulcrum between peptidoglycan and the impermeable hydrophobic mycolic acid layer. Furthermore, its biosynthesis is the target of the anti-mycobacterial drug EMB. However, the complete biosynthetic pathway and the cellular machinery involved in AG biosynthesis are still poorly understood (9).

As evident from the genome analyses (Fig. 1A) in Mycobacterium and Corynebacterium species analyzed to date, Cg-emb and its upstream region is strictly conserved, indicative of a core function common to all Corynebacterianeae and shown in this study to be involved in the majority of arabinan deposition in AG. Previous attempts to obtain embA, embB, and embAB deletion mutants in M. tuberculosis have been unsuccessful and probably reflects the importance of AG in the cell wall ultrastructure of Mycobacterial species. However, individual disruptions of embA and embB in M. smegmatis have been obtained resulting in viable cells with observable phenotypic alterations to AG (25). The embA and embB mutants led to an alteration of the terminal Ara6 motif of AG but still produced a highly arabinosylated AG polymer. The possibility existed that in either the embA or embB mutant partial complementation could ensue through the presence of either a functional copy of embA and embB, respectively, as gene duplication and redundancy appear common in M. tuberculosis (9, 25, 40). As a consequence the isolation of an arabinan-deficient cell wall mutant in M. tuberculosis appears fraught with difficulty.

With this in mind and because C. glutamicum possesses only a single emb gene, we attempted to construct an emb deletion mutant of C. glutamicum. The resultant deletion mutant produced a viable yet slow growing phenotype with profound morphological changes. Initial analysis of the corynomycolic acid content of C. glutamicum::emb showed that there was a complete absence of cell wall bound corynomycolates, hinting that there was a loss of corynomycolic acid esterification sites in the mutant, consistent with the loss of the terminal Ara6 motif. Upon glycosyl compositional and linkage analysis, we observed in contrast to M. smegmatis embA and embB mutants (25) a 90% loss of cell wall arabinan, with all 5-Araf, 3,5-Araf, and 2,5-Araf residues (also the capping t-Rhap residues) being absent in the cell wall of the C. glutamicum::emb, with the relative amounts of Gal unchanged. The minor amounts of Araf residues were present as t-Araf units. Furthermore, the AG derived from C. glutamicum::emb when analyzed by MALDI-TOF MS and partial acid hydrolysis indicated for the first time the location of the Ara branches of AG. A linear galactan extends from the reducing Rha and the first Ara branch appears on the tenth and twelfth Gal residues (Fig. 8). The observation of t-Araf residues in
It is tempting to postulate that this hypothetical “priming enzyme” could fix the initial arabinan units onto the galactan chain for further elaboration by Emb forming the fully matured AG. Interestingly, treatment of C. glutamicum with EMB results in a phenotype that is identical to the C. glutamicum Emb, with loss of esterified corymycolic acids and a dramatically reduced arabinan content in AG. These results show that Emb is indeed the target for EMB and that the arabinofuranosyltransferase activity of the “priming” enzyme remains unaffected. Given the importance of AG in biosynthesis, with the goal of deleting orthologues found in C. glutamicum, the donor, may use an alternative nucleotide sugar donor (41, 42). Although the two gene loci depicted in Fig. 1 (A and B) are separated in M. tuberculosis by only two open reading frames (not shown), this part is not conserved and is at variance in M. leprae and other Mycobacteria. However, genes of the subsequent region extending from accD4 to glfT are arranged in synteny in all Corynebacterianeae analyzed and are involved in some aspects of cell wall biosynthesis. For instance, accD4 (accD3 in C. glutamicum), which encodes the β-chain of an acyl carboxylase, which together with a second accD orthologue (accD2 in C. gluta-

**REFERENCES**


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* C. glutamicumΔemb was somewhat surprising, because it has long been thought that the gene products of embA and embB in mycobacterial ssp. are solely responsible for arabinan biosynthesis. Interestingly, the occurrence of arabinan deposition on an unaffected galactan backbone suggests that an unidentified arabinofuranosyltransferase might be responsible for the addition of the initial units onto the galactan domain. It is tempting to postulate that this hypothetical “priming enzyme” could fix the initial arabinan units onto the galactan chain for further elaboration by Emb forming the fully matured AG. Interestingly, treatment of C. glutamicum with EMR results in a phenotype that is identical to the C. glutamicum Emb, with loss of esterified corymycolic acids and a dramatically reduced arabinan content in AG. These results show that Emb is indeed the target for EMR and that the arabinofuranosyltransferase activity of the “priming” enzyme remains unaffected. Given the importance of AG in M. tuberculosis viability and pathogenicity, it is tempting to suggest that this “priming” enzyme might be an ideal candidate to exploit as a drug target, because its disruption would result in a completely arabinan-deficient cell wall.

Due to the presence of t-Ara residues in the emb-deleted strain of C. glutamicum, we endeavored to identify genes responsible for DPA biosynthesis, with the goal of deleting orthologues found in C. glutamicum for further phenotypic analysis. This was to rule out the possibility that the priming enzyme, unlike Emb, which utilizes DPA as a sugar donor, may use an alternative nucleotide sugar donor (41, 42).

Although the two gene loci depicted in Fig. 1 (A and B) are separated in M. tuberculosis by only two open reading frames (not shown), this part is not conserved and is at variance in M. leprae and other Mycobacteria. However, genes of the subsequent region extending from accD4 to glfT are arranged in synteny in all Corynebacterianeae analyzed and are involved in some aspects of cell wall biosynthesis. For instance, accD4 (accD3 in C. glutamicum), which encodes the β-chain of an acyl carboxylase, which together with a second accD orthologue (accD2 in C. gluta-

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* L. J. Alderwick, unpublished results.