Arabinan-deficient mutants of Corynebacterium glutamicum and the consequent flux in decaprenylmonophosphoryl-D-arabinose metabolism

Luke J. Alderwick², Lynn G. Dover², Mathias Seidel³, Roland Gande³, Hermann Sahm³, Lothar Eggeling³, and Gurdyal S. Besra¹,²

¹School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK; and ²Institute for Biotechnologie ¹, Research Centre Juelich, D-52425 Juelich, Germany

© The Author 2006. Published by Oxford University Press. All rights reserved. For permissions, please e-mail: journals.permissions@oxfordjournals.org

Introduction

Corynebacterianeae, which include the human pathogens Corynebacterium diphtheriae and Mycobacterium tuberculosis, represent an extremely important group of Actinomycetales (Coyle and Lipsky, 1990; Bloom and Murray, 1992; Funke et al., 1997). They belong to the same suborder and share a similar genome, cell wall ultrastructure, and corresponding cell wall biosynthetic machinery (Dover et al., 2004). The cell envelope of these bacteria consist of a basal peptidoglycan, which is coupled to a lipid-rich mycolic acid layer by a heterogeneous polysaccharide, arabinogalactan (AG) (Daffe et al., 1990; McNeil et al., 1990, 1991; Besra et al., 1995; Dover et al., 2004). AG consists of alternating β(1→5) and β(1→6) linked galactofuranose residues and is polymerized by the bifunctional galactofuranosyltransferase, GlfT (Mikusova et al., 2000; Kremer et al., 2001), which uses UDP-Galf as a high-energy sugar donor (Weston et al., 1997; Sanders et al., 2001), to produce a linear galactan domain. Three branched arabinan motifs are attached to the C5 position of a β(1→6) linked galactose (Gal) residue at the 8th, 10th, and 12th Gal residues of the galactan domain (Alderwick et al., 2005). The arabinan motifs consist of a linear α(1→5) arabinan core with branching introduced at specific C3 positions along the arabinan polysaccharide (Daffe et al., 1990, 1991; Besra et al., 1995; Alderwick et al., 2005). The AG terminates with β(1→2) linked Araf units in a unique hexa-arabinofuranosyl motif, which is the site of mycolation (McNeil et al., 1991). The final stages of AG biosynthesis are unknown, but at some point, the entire mAG is ligated to the peptidoglycan (Hancock et al., 2002; Yagi et al., 2003). AG has been shown to be an essential macromolecule in Corynebacterianeae, such as M. tuberculosis (Pan et al., 2001; Mills et al. 2004). However, the generation of viable arabinan-deficient mutants of Corynebacterium glutamicum has proven to be inherently useful in the study of "essential" genes involved in mycobacterial cell wall biosynthesis (Alderwick et al., 2005, 2006).

Mycobacterium tuberculosis utilizes the genes encoded by aftA, embA, and embB to perform arabinan polymerization in AG (Belanger et al., 1996; Escuyer et al., 2001; Alderwick et al., 2005, 2006) and by embC in lipoarabinomannan (Zhang et al., 2003). In contrast, C. glutamicum possesses only one ethambutol (Emb) paralog (Alderwick et al., 2005), which is the sole enzyme used in AG biosynthesis. However, both M. tuberculosis and C. glutamicum utilize the lipid-linked sugar donor decaprenylmonophosphoryl-D-arabinose (DPA) as its high-energy sugar donor (Wolucka et al., 1994; Lee et al., 1995, 1998). DPA is initially synthesized via the pentose shunt pathway (Scherman et al., 1996), where 5-phospho-ribofuranose-pyrophosphate (pRpp) is transferred to decaprenylmonophosphate forming decaprenylphosphoryl-5-phosphoribose (DPPR) by the decaprenyl transferase UbiA (Huang et al., 2005) (Rv3806c and NCgI2789 in M. tuberculosis and C. glutamicum, respectively).
were aligned using ClustalW and rendered with ESPRINT, as shown in Figure 2. A solid line indicates regions that correspond to transmembrane (TM) spanning domains, whereas regions predicted to be placed on the cytoplasmic side and extracellular side of the membrane are designated by dashed and dotted lines, respectively. A total of nine TM spanning domains were predicted using the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/). An interesting aspect of this alignment is the high homology surrounding intracellular loop 1 between TM-2 and TM-3. This region is 95% identical and contains a speculative NDXRD motif (Figure 2), which is presumably involved in Mg^{2+}, phosphate, and hydroxyl coordination of pRpp. This notion is further substantiated by the fact that Mt-UbiA exhibits high similarity with respect to sequence and topology to the ribose oligoprenyltransferase NeoC of Azorhizobium caulinodans (Mergaert et al., 1996), which also has three aspartic acid residues retained in the corresponding loop region (data not shown).

**Results**

**Disruption of Cg-ubiA**

The *ubiA* gene product was shown in prior work to synthesize DPPR, which is converted to DPA, thus supplying the substrate for the “priming” arabinosyltransferase AftA (Alderwick et al., 2006). We inactivated the mycobacterial ortholog of *C. glutamicum*, NCgl2781, by transforming the wild type to kanamycin resistance conferred by the vector borne *aph* gene product of pCg::ubiA. The vector was integrated into the chromosomal *ubiA* gene, thus disrupting *ubiA*, as confirmed by two independent polymerase chain reaction (PCR) analyses with two different primer pairs (data not shown). As expected, the resulting strain *C. glutamicum*:::ubiA exhibited a strong reduced growth, as shown in Figure 1. It thus resembles the *aftA* inactivation mutant of *C. glutamicum* exhibiting an almost identical growth behavior (Alderwick et al., 2006).

**UbiA sequence analysis**

*UbiA* sequences from *M. tuberculosis*, *Mycobacterium bovis*, *Mycobacterium avium paratuberculosis*, and *C. glutamicum* were aligned using ClustalW and rendered with ESPRINT, as shown in Figure 2. A solid line indicates regions that correspond to transmembrane (TM) spanning domains, whereas regions predicted to be placed on the cytoplasmic side and extracellular side of the membrane are designated by dashed and dotted lines, respectively. A total of nine TM spanning domains were predicted using the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/). An interesting aspect of this alignment is the high homology surrounding intracellular loop 1 between TM-2 and TM-3. This region is 95% identical and contains a speculative NDXRD motif (Figure 2), which is presumably involved in Mg^{2+}, phosphate, and hydroxyl coordination of pRpp. This notion is further substantiated by the fact that Mt-UbiA exhibits high similarity with respect to sequence and topology to the ribose oligoprenyltransferase NeoC of Azorhizobium caulinodans (Mergaert et al., 1996), which also has three aspartic acid residues retained in the corresponding loop region (data not shown).

**Mycol-arabinogalactan composition of C. glutamicum::ubiA**

We have previously reported on the generation of *C. glutamicum* mutants that are truncated in their arabinan domains of AG or are completely arabinan deficient (Alderwick et al., 2005, 2006). This study highlights the generation of *C. glutamicum* mutants as an aid to probe underlying cell wall biosynthetic enzymes within Corynebacteriaceae, which are predicted to be essential (Alderwick et al., 2005). Glycosyl linkage analysis of cell walls prepared from a *C. glutamicum*:::ubiA mutant indicates that this mutant is completely devoid of an arabinan domain, whereas the galactan core remains unaffected compared with *C. glutamicum* (Figure 3), thus further corroborating our previous findings (Alderwick et al., 2005). The loss of myclic acid esterification sites was also confirmed by analysis of cell-wall-bound corynomycolic acids, which proved to be absent in *C. glutamicum*:::ubiA (Figure 4).

**Identification of cell-wall-associated and exported lipids**

Because the disruption of arabinan in *C. glutamicum* essentially results in a complete loss of the “upper” cell wall mycolyl-arabinogalactan peptidoglycan (mAGP) complex, we investigated the production and profile of cell-wall-associated and exported lipids in *C. glutamicum* and *C. glutamicum* arabinan-deficient mutants. Analysis of cell-wall-associated lipids, such as trehalose dicorynomycolates (TDCM), trehalose monosaccharomycolates (TMCM), and phospholipids from *C. glutamicum*, in comparison with *C. glutamicum*:::ubiA, *C. glutamicum*Δemb, and *C. glutamicum*ΔaftA suggested that *C. glutamicum* possessed a higher degree of cell-wall-associated lipids (Figure 5A). It was interesting to note the increased level of free TMCM in comparison with TDCM and phospholipids in *C. glutamicum*:::ubiA, *C. glutamicum*Δemb, and *C. glutamicum*ΔaftA compared with *C. glutamicum*. This suggests that perhaps those lipids, which would otherwise be present in larger quantities, were emancipated due to the loss of cell-wall-bound corynomycolic acids and arabinan, thus removing their intercalation with the mAGP complex. Metabolic labeling

---

![Graph](image.png)

**Fig. 1.** Growth characteristics of *Corynebacterium glutamicum*:::ubiA. Consequence of Cg::ubiA (■) disruption on growth in rich medium (BHI) in comparison with wild-type *C. glutamicum* (■).
Arabinan-deficient mutants of *Corynebacterium glutamicum*

using $[^{14}C]$acetate of growing cultures of *C. glutamicum*, *C. glutamicum::ubiA*, *C. glutamicumΔemb*, and *C. glutamicumΔaftA* and analysis of spent culture filtrates revealed a dramatic increase in “exported” TDCM, TMCM, and phospholipids from the *C. glutamicum* mutants compared with *C. glutamicum* (Figure 5B).

**Endogenous arabinofuranosyltransferase activity of C. glutamicum, C. glutamicum::ubiA, and C. glutamicumΔemb**

We assessed the capacity of membrane preparations from *C. glutamicum*, *C. glutamicum::ubiA*, and *C. glutamicumΔemb* to elicit arabinofuranosyltransferase activity in the presence of an endogenous cell wall acceptor. Analysis of membranes along with cell wall material extracted from *C. glutamicum*, and exogenous radiolabeled p$[^{14}C]$Rpp, indicated severely reduced activity from *C. glutamicumΔemb* to turn over p$[^{14}C]$Rpp via DP$[^{14}C]$A and incorporation into cell wall polymer compared with *C. glutamicum* (Figure 6A). This was not surprising, because Emb is responsible for the majority of cell wall arabinan biosynthesis. However, the level of arabinan incorporation was not completely diminished due to the activity of Cg-AftA (Alderwick et al., 2006), which is able to transfer single arabinofuranosyl residues to the galactan core from DPA. Conversely, we could not observe any glycosyltransferase activity using membranes prepared from *C. glutamicum::ubiA* (Figure 6A). However, restoration of Emb activity could be observed in membranes prepared from *C. glutamicum::ubiA* when, rather than providing p$[^{14}C]$Rpp as a substrate, exogenous DP$[^{14}C]$A was included in assays (Figure 6B). This suggests that both Emb and UbiA work in a mutually exclusive manner and also indicates that Emb is unaffected by Cg-ubiA disruption. We could observe a slight increase in arabinofuranosyltransferase activity in membranes produced from *C. glutamicumΔemb* (Figure 6B), this however could be attributed to a higher level of DP$[^{14}C]$A in the assay components, compared with the assay containing p$[^{14}C]$Rpp which would require further endogenous metabolism into DP$[^{14}C]$A.

**UbiA activity in membranes prepared from C. glutamicum, C. glutamicum::ubiA, C. glutamicumΔemb, and C. glutamicumΔaftA**

It has been previously reported (Huang et al., 2005) that UbiA is a 5-phospho-α-D-ribose-1-diphosphate: decaprenyl-phosphate 5-phosphoribosyltransferase. We assayed membranes prepared from *C. glutamicum* and from various mutants described in this report for DP$[^{14}C]$A biosynthetic activity. Membranes prepared from *C. glutamicum* were able to produce radiolabeled products that migrate on thin layer chromatography (TLC) corresponding to DPA (Lee et al., 1995, 1998; Scherman et al., 1996) and DPPR (Huang et al., 2005) standards (Figure 7A). This result was also observed with membranes prepared from *C. glutamicumΔemb* and *C. glutamicumΔaftA*, but with a highly significant increase in band density corresponding to DPP$[^{14}C]R$ and DP$[^{14}C]$A/DP$[^{14}C]R$ in *C. glutamicumΔemb* and

---

**Fig. 2.** Alignment of UbiA amino acid sequences from *Mycobacterium tuberculosis*, *M. bovis*, *M. avium paratuberculosis* and *Corynebacterium glutamicum*. Sequences were aligned using ClustalW and rendered with ESPRIPiPT. Boldface-type line indicates transmembrane spanning regions, whereas dashed and dotted lines indicate intracellular and extracellular loops, respectively. *Proposed catalytic residues.*
As expected, we could not observe formation of DPP[14C]R or DP[14C]A/DP[14C]R in membrane preparations from \textit{C. glutamicum}::ubiA (Figure 7A). To confirm the sugar content of each individual product in these reactions, we extracted the bands by preparative TLC and analyzed the product hydrolysates in terms of sugar content (Figure 7B). Extraction and sugar analysis of the band corresponding to DPPR from \textit{C. glutamicum} (Figure 7B, lane 1) indicated that this product contained exclusively ribose. Analysis of the band equivalent to DPA/decapenylmonophosphoryl-D-ribose (DPR) from \textit{C. glutamicum} (Figure 7B, lane 2) indicated that there was a heterogeneous mix of ribose and arabinose (Ara), corresponding to a mixture of DPR and DPA, respectively. Interestingly, sugar analysis of products migrating to the positions of DPPR and DPA/DPR from \textit{C. glutamicum}\Delta aftA and \textit{C. glutamicum}\Delta emb gave different band density profiles for ribose and Ara (Figure 7B, lanes 4–6). It has not escaped our notice that the utilization of membranes prepared from \textit{C. glutamicum}\Delta aftA would be a useful tool for the enzymatic synthesis of DPA (Figure 7A and B, lane 6). As a control, we excised bands in the regions which related to DPPR and DPA/DPR from \textit{C. glutamicum}::ubiA, and, as expected, no sugars were observed (Figure 7B, lanes 7 and 8).
Discussion

Understanding the biosynthetic pathways involved in mycobacterial arabinan biosynthesis is paramount to identifying potential new drug targets for the treatment of tuberculosis. We have shown recently that gene deletion in *C. glutamicum* provides a useful tool in the understanding of mycobacterial cell wall biosynthesis, because deletion of orthologous genes in mycobacterial species often causes lethality (Gande et al., 2004; Alderwick et al., 2005, 2006). More specifically, our earlier studies (Alderwick et al., 2005) examined the potential disruption of *ubiA* in *C. glutamicum* and its consequence at a whole cell level due to the observation that the singular Ara residues in the *emb*-deleted strain could possibly arise from another sugar donor, that is, we attempted to disrupt arabinan biosynthesis completely and examine the final incorporation of Ara into the cell wall at a whole cell level. Analysis of alditol acetates prepared from purified cell walls from the *Cg::ubiA* mutant established DPA as the sole donor of Ara residues in *C. glutamicum*. In this present report, we have now more carefully analyzed the *in vitro* biochemical phenotype of the *Cg::ubiA* mutant in terms of cell-wall-associated TDCM and TMCM glycolipids, “exported” glycolipids and phospholipids, *in vitro* arabinofuranosyltransferase activity of *C. glutamicum*, *C. glutamicum::ubiA*, and *C. glutamicum::emb* mutant in terms cell wall arabinan polymerization, and UbiA activity in membranes prepared from *C. glutamicum*, *C. glutamicum::ubiA*, *C. glutamicum::emb*, and *C. glutamicum::aftA* in terms of the biosynthesis of DPPR and DPA. These *in vitro* biochemical experiments were not described in our earlier studies (Alderwick et al., 2005) and provide a more comprehensive analysis of arabinan biosynthesis in our panel of mutants as discussed below. Huang and others (2005) clearly established that UbiA is a *bona fide*
5-phospho-α-D-ribose-1-diphosphate: decaprenyl-phosphate-5-phosphoribosyltransferase; however, the scope of our current studies was to examine the consequences of arabinan biosynthesis and relationship between cell wall arabinan biosynthesis and DPPR/DPA precursors in a panel of mutants rather than characterization of an enzyme that was overexpressed within an *Escherichia coli* membrane fraction and assayed for UbiA activity.

AftA, a potential drug target, is responsible for the addition of the first key Ara residue to the galactan core, thus priming the polysaccharide for further decoration by the Emb proteins (Alderwick *et al*., 2005, 2006). Furthermore, UbiA represents another ideal druggable enzyme within the arabinan biosynthetic pathway, as it is responsible for the production of the only Ara sugar donor involved in mycobacterial arabinan biosynthesis and is essential for growth and survival of the organism, as discussed in Dover *et al.* (2004). The biosynthesis of DPA via DPPR was recently reported (Huang *et al*., 2005; Mikusova *et al*., 2005). However, it was unclear what catalytic mechanism is utilized by these enzymes; nevertheless, inspection of the UbiA sequence and further topological analysis highlights a hypothetical mechanism. Because UbiA is a membrane-bound enzyme consisting of approximately nine TM spanning domains, it would be prudent to suggest that the decaprenyl phosphate substrate is probably coordinated by these TM spanning regions anchoring the lipid acceptor in place for addition of the phosphoribose moiety from pRpp. Indeed, the most conserved region of the enzyme resides on an intracellular cytoplasmic loop, which contains the proposed catalytic motif, NDXRD, which presumably coordinates pRpp through a Mg$^{2+}$ ion allowing either $S_N1$ or $S_N2$ nucleophilic substitution.

By interruption of the gene encoding UbiA in *C. glutamicum* (NcgI2781), we now demonstrate that the cell wall produced from this strain is devoid of bound corynomycolic acids, deficient of arabinan, and is incapable of synthesizing DPA via DPPR. Nevertheless, arabinofuranosyltransferase activity remains unaffected by the disruption of Cg-ubiA indicating that there is no other Ara-containing donor involved in arabinan biosynthesis. Interestingly, we observed a moderate level of arabinofuranosyltransferase activity from *C. glutamicum* Δemb, which is attributed to a fully functional Cg-AftA (Alderwick *et al*., 2006), illustrating that Cg-Emb and Cg-AftA work independently from each other and thus mutually exclusively from Cg-UbiA.

Analysis of *C. glutamicum*Δemb and *C. glutamicum*ΔaftA DPA biosynthetic activity resulted in two very different glycolipid profiles when the reaction products were analyzed by TLC and compared with wild-type *C. glutamicum*. A buildup of DPPR and DPR was observed in the *C. glutamicum*Δemb strain, with the levels of DPA remaining consistent with wild-type *C. glutamicum*. This phenomenon is most likely due to a low-to-moderate turnover of DPA, which is being recruited by the first arabinofuranosyltransferase Cg-AftA, causing a buildup of the precursors DPPR and DPR. However, *C. glutamicum*ΔaftA produces a large reservoir of DPA, which builds up because of a complete lack of arabinofuranosyltransferase activity, both initial and downstream arabinan biosyntheses. This effect can be accredited to the fact that any endogenous cell wall

---

**Fig. 7.** Analysis of DPA/DPR and DPPR glycolipid precursors from *Corynebacterium glutamicum*, *C. glutamicum*Δemb, *C. glutamicum*ΔaftA, and *C. glutamicum*:ubiA. (A) Membranes prepared from *C. glutamicum* and the various mutants were assayed for DPA biosynthetic activity and analyzed via TLC and autoradiography as described in Materials and methods. (B) Individual bands corresponding to either DPPR or DPA/DPR (as numbered in panel A) were excised, hydrolyzed using 2 M trifluoroacetic acid (TFA), analyzed by TLC, and visualized by autoradiography.
polysaccharide present in the membranes prepared from *C. glutamicumΔaftA* is devoid of arabinan and is therefore “unprimed” for further elongation by a fully functional Emb. Nevertheless, one would expect to observe an accumulation of DPRR and DPR in tandem with DPA buildup. Here, we propose that a critical level of DPA accumulation causes an inhibitory effect on Cg-UbiA, thus acting in a negative feedback response mechanism. Any remaining DPRR and DPR would be shuttled through the pathway via the phosphatase, Rv3790 and Rv 3791 (DPR epimerase complex), respectively, which are unaffected by the level of DPA (Mikusova et al., 2005). The thermodynamics of this mechanism makes energetic sense because of the fact that a continued incorporation of pRpp with decaprenyl phosphate would be inefficient to the metabolism of the cell. Further work is required to establish the enzymatic mechanisms utilized by UbiA before potential compounds can be designed to target this essential protein in mycobacterial species.

### Materials and methods

#### Strains and culture conditions

*Corynebacterium glutamicum* ATCC 13032 (the wild-type strain, and referred for the remainder of the text as *C. glutamicum*) and *E. coli DH5α* were grown in Luria–Bertani (LB) broth (Difco, Detroit, MI) at 30 and 37°C, respectively. The *C. glutamicumΔaftA* mutant generated in this study was grown on complex medium brain heart infusion (Difco). Kanamycin and ampicillin were used at a concentration of 50 μg/mL. Samples for lipid analyses were prepared by harvesting cells at an optical density (OD) of A600~10–15, followed by a saline wash and freeze drying. Cultivation of *C. glutamicumΔemb* and *C. glutamicumΔaftA* were carried out as described in Alderwick et al. (2005, 2006).

#### Construction of plasmids

The vector used for inactivation of *C. glutamicumΔaftA* is as follows: pCG::ubiA (NCgl2781, Rv3806c). For inactivation of *ubiA*, an internal fragment of 321 bp was amplified (pubA-for: ATC TTC AAC CAG CGC ACG ATC; pubA-rev: AAT ATC GAT CAC TGG CAT GTG C), which was made blunt and ligated into the Smal site of the nonreplicative vector pK18mob to yield pCG::ubiA. 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 Cg-ubiA was confirmed with different primer pairs annealing in the vector and the bacterial chromosome.

#### Extraction and analysis of cell-wall-bound mycolic acids from *C. glutamicum* strains

Cells were grown as described in Strains and culture conditions, 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/v) for 3 h at 50°C. The bound lipids from the delipidated extracts or purified cell walls (see Isolation of the mAGP complex) were released by the addition of 2 mL of 5% aqueous solution of tetra-butyl ammonium hydroxide (TBAH), followed by overnight incubation at 100°C. After cooling, water (2 mL), CH2Cl2 (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 CH2Cl2 (100 μL). An aliquot (10 μL) from each strain was subjected to TLC using silica gel plates (5735 silica gel 60F254, Merck, Darmstadt, Germany) and developed in petroleum ether/acetone (95:5, v/v) and charred using 5% molybdoephosphoric acid in ethanol at 100°C to reveal corynomycolic acid methyl esters (CMAMES) and compared with known standards (Gande et al., 2004).

#### Isolation of the mAGP complex

The thawed bacterial cells were resuspended in phosphate buffered saline (PBS) containing 2% Triton X-100 (pH 7.2), disrupted by sonication and centrifuged at 27,000 × g (Daffe et al., 1990; Besra et al., 1995). The pelletted material was extracted three times with 2% sodium dodecyl sulphate (SDS) in PBS 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 highly purified cell wall preparations (Daffe et al., 1990; Besra et al., 1995).

### Glycosyl linkage analysis of cell walls

Chemical derivitization of highly purified cell walls was carried out as described in Besra et al. (1995) and Alderwick et al. (2005, 2006). Briefly, cell wall preparations (10 mg) were suspended in 0.5 mL of dimethyl sulfoxide (anhydrous) and 100 μL of 4.8 M dimethyl sulfinyl carbaniion (Daffe et al., 1990; Besra et al., 1995). The reaction mixture was stirred for 1 h, and then, CH3I (100 μL) was slowly added and the suspension stirred for a further 1 h, and this process was repeated for a total of three times. After dialysis and Sep-Pak purification, the resulting per-O-methylated cell wall samples were hydrolyzed, reduced, per-O-acetylated, and examined by gas chromatography/mass spectrometry (GC/MS) carried out on a BPX5 column (Supelco, Bellefonte, Pennsylvania) and a Finnigan Polaris/GCQ PlusTM, as described in Daffe et al. (1990) and Besra et al. (1995).

### Analysis of C. glutamicum-free lipids

*Corynebacterium glutamicum*, *C. glutamicumΔemb*, and *C. glutamicumΔaftA* were cultured at 30°C in 5-mL BHIS media and supplemented with antibiotic, where appropriate. Cell-wall-associated free lipids were extracted twice from 100 mg of dried cells using 2 mL of CHCl3/CH3OH/H2O (10:10:3, v/v/v) for 3 h at 50°C. Organic extracts were combined with 1.75 mL of CHCl3 and 0.75 mL of H2O, mixed, and centrifuged. The lower organic phase was recovered, back washed twice with 2 mL of CHCl3/CH3OH/H2O (3:47:48, v/v/v), dried, and resuspended with 200 μL of CHCl3/CH3OH/H2O (10:10:3, v/v/v). A 10 μL of aliquot was subjected to TLC analysis using silica gel plates (5735 silica gel 60F254, Merck) developed in CHCl3/CH3OH/H2O (60:16:2, v/v/v) and charred using 5% molybdophosphoric acid in ethanol at 100°C to reveal cell-wall-associated free lipids. Exported lipids were analyzed in a similar manner. *C. glutamicum*, *C. glutamicumΔaftA*, *C. glutamicumΔemb*, and *C. glutamicumΔaftA* were confirmed with different primer pairs annealing in the vector and the bacterial chromosome.

### Arabinin-deficient mutants of *Corynebacterium glutamicum*

[1079]
C. glutamicumΔemb, and C. glutamicumΔaftA were cultured as described in Strains and culture conditions. Once the A600 reached ~0.5, cultures were labeled with 1 μCi [14C]-acetic acid and further incubated for 8 h. Cells were harvested by centrifugation at 27,000 × g for 30 min, and the supernatant was carefully removed and dried using a Savant SpeedVac. The supernatant was dried and extracted using the method described in Extraction and analysis of cell wall bound mycolic acids from C. glutamicum strains. An aliquot of each extraction was subjected to scintillation counting and analyzed by TLC using silica gel plates (5735 silica gel 60F254, Merck) developed in CHCl₃/CH₃OH/H₂O (60:16:2, v/v/v). TLCs were exposed to X-ray film (Kodak X-Omat) for 2 days to visualize radiolabeled lipids by autoradiography.

Preparation of corynebacterial membranes and cell wall material

Cells (10 g) from C. glutamicum, C. glutamicum::ubiA, C. glutamicumΔemb, and C. glutamicumΔaftA were resuspended in 35 mL of 50 mM MOPS (pH 7.9), 10 mM MgSO₄, 5 mM β-mercaptoethanol (buffer A) and subjected to probe sonication for 60 s on and 90 s off (repeated for a total of 10 cycles). The cell slurry was centrifuged at 27,000 × g for 20 min at 4°C; the pellet was recovered and the resulting supernatant further centrifuged at 100,000 × g for 90 min at 4°C. Purified C. glutamicum membranes were recovered and resuspended in buffer A to a final concentration of 15–20 mg/mL. The pellet was centrifuged at 27,000 × g for 90 min at 4°C, the pellet was recovered, re suspended in 4 mL of CHCl₃/CH₃OH (2:1, v/v), mixed for 15 min and centrifuged at 3000 × g for 15 min. The supernatant was removed, combined with 340 μL of buffer A, mixed for 15 min, and centrifuged at 3000 × g for 15 min. The lower organic phase was removed and washed twice with 1 mL of CHCl₃/CH₃OH/H₂O (3:47:48, v/v/v), centrifuged at 3000 × g for 15 min, recovered, and dried under nitrogen. The resulting residue was resuspended in 20 μL of CHCl₃/CH₃OH (2:1, v/v) and an aliquot subjected to TLC analysis using silica gel plates (5735 silica gel 60F254, Merck) developed in CHCl₃/CH₃OH/H₂O/ NH₄OH (65:25:3.6:0.5, v/v/v/v) and visualized by autoradiography by exposure of TLCs to X-ray film (Kodak X-Omat). Major bands corresponding to lipid-linked sugars were excised directly from their migrating position and extracted by incubation overnight in 5 mL of CHCl₃/CH₃OH (2:1, v/v). Samples were then centrifuged at 5000 × g to remove silica gel, and the supernatant was recovered and dried under nitrogen. The products were then hydrolyzed by using 100 μL of 2 M trifluoroacetic acid (TFA) for 1 h at 120°C, dried, resuspended in 2 mL of CHCl₃/H₂O (1:1, v/v), and the upper aqueous phase recovered and dried. The residue was then resuspended in 20 μL of H₂O and subjected to TLC using cellulose-coated aluminum plates (HPTLC-Aluminum Cellulose, Merck) and developed three times in formic acid/H₂O/methyl ethyl ketone (3:3:8.6, v/v/v/v). Sugars were visualized by TLC exposure to X-ray film (Kodak X-Omat) and compared with known sugar standards.

Arabinofuranosyltransferase activity

Membranes prepared from C. glutamicum, C. glutamicum::ubiA, and C. glutamicumΔemb were assayed for their ability to incorporate [14C]-Ara into endogenous cell wall polymer using either p[14C]Rpp or DP[14C]A as substrate. p[14C]Rpp and DP[14C]A were prepared, as described in Lee et al. (1995, 1998) and Scherman et al. (1996). Assays consisted of 2-mg cell membranes, 2-mg cell wall P60, 1 mM ATP, and 1 mM NADP in a final volume of 200 μL buffer A. Assays were initiated by the addition of 45,000 cpm p[14C]Rpp and incubated at 37°C for 2 h. The reaction was quenched by the addition of 3 mL of CHCl₃/CH₃OH (2:1, v/v), for 15 min. The supernatant was removed, combined with 340 μL of buffer A, mixed for 15 min, and centrifuged at 3000 × g for 15 min. The lower organic phase was removed and washed twice with 1 mL of CHCl₃/CH₃OH/H₂O (3:47:48, v/v/v), centrifuged at 3000 × g for 15 min, recovered, and dried under nitrogen. The resulting residue was resuspended in 20 μL of CHCl₃/CH₃OH (2:1, v/v) and an aliquot subjected to TLC analysis using silica gel plates (5735 silica gel 60F254, Merck) developed in CHCl₃/CH₃OH/H₂O/ NH₄OH (65:25:3.6:0.5, v/v/v/v) and visualized by autoradiography by exposure of TLCs to X-ray film (Kodak X-Omat). Major bands corresponding to lipid-linked sugars were excised directly from their migrating position and extracted by incubation overnight in 5 mL of CHCl₃/CH₃OH (2:1, v/v). Samples were then centrifuged at 5000 × g to remove silica gel, and the supernatant was recovered and dried under nitrogen. The products were then hydrolyzed by using 100 μL of 2 M trifluoroacetic acid (TFA) for 1 h at 120°C, dried, resuspended in 2 mL of CHCl₃/H₂O (1:1, v/v), and the upper aqueous phase recovered and dried. The residue was then resuspended in 20 μL of H₂O and subjected to TLC using cellulose-coated aluminum plates (HPTLC-Aluminum Cellulose, Merck) and developed three times in formic acid/H₂O/methyl ethyl ketone (3:3:8.6, v/v/v/v). Sugars were visualized by TLC exposure to X-ray film (Kodak X-Omat) and compared with known sugar standards.

DPA biosynthetic activity

Cell membranes from C. glutamicum, C. glutamicum::ubiA, C. glutamicumΔemb, and C. glutamicumΔaftA were assayed for DPA biosynthesis activity. Decaprenol phosphate (50 μg, 5 mg/mL stored in ethanol, 10 μL) was dried under nitrogen and was resuspended by the addition of 50 μL of a 1% IgePal CA-630 (Sigma Aldrich) solution in buffer A and sonicated. The basic assay mix consisted of 2 μg of membranes, 1 mM ATP, and 1 mM NADP in a final volume of 160 μL of buffer A and initiated by the addition of 45,000 cpm p[14C]Rpp. Reactions were incubated at 37°C for 2 h and quenched by the addition of 3 mL of CHCl₃/CH₃OH (2:1, v/v), mixed for 15 min, and centrifuged at 3000 × g for 15 min. The supernatant was removed, combined with 340 μL of buffer A, mixed for 15 min, and centrifuged at 3000 × g for 15 min. The lower organic phase was removed and washed twice with 1 mL of CHCl₃/CH₃OH/H₂O (3:47:48, v/v/v), centrifuged at 3000 × g for 15 min, recovered, and dried under nitrogen. The resulting residue was resuspended in 20 μL of CHCl₃/CH₃OH (2:1, v/v) and an aliquot subjected to TLC analysis using silica gel plates (5735 silica gel 60F254, Merck) developed in CHCl₃/CH₃OH/H₂O/ NH₄OH (65:25:3.6:0.5, v/v/v/v) and visualized by autoradiography by exposure of TLCs to X-ray film (Kodak X-Omat). Major bands corresponding to lipid-linked sugars were excised directly from their migrating position and extracted by incubation overnight in 5 mL of CHCl₃/CH₃OH (2:1, v/v). Samples were then centrifuged at 5000 × g to remove silica gel, and the supernatant was recovered and dried under nitrogen. The products were then hydrolyzed by using 100 μL of 2 M trifluoroacetic acid (TFA) for 1 h at 120°C, dried, resuspended in 2 mL of CHCl₃/H₂O (1:1, v/v), and the upper aqueous phase recovered and dried. The residual radiolabeled sugars were resuspended in 20 μL of H₂O and subjected to TLC using cellulose-coated aluminum plates (HPTLC-Aluminum Cellulose, Merck) and developed three times in formic acid/H₂O/methyl ethyl ketone (3:3:8.6, v/v/v/v). Sugars were visualized by TLC exposure to X-ray film (Kodak X-Omat) and compared with known sugar standards.

Acknowledgments

L.J.A. is a Biotechnology and Biological Sciences Research Council Quota Student. G.S.B. acknowledges support in the form of a Personal Research Chair from Mr James Bardrick and as a former Lister Institute-Jenner Research Fellow, and the Medical Research Council (UK). H.S. and L.E. are supported by the Fonds der Chemischen Industrie. We also thank Graham Burns for technical assistance.
Conflict of interest statement

None declared.

Abbreviations

AG, arabinogalactan; Ara, arabinose; CMAME, corynomycolic acid methyl ester; DPA, decaprenylmonophosphoryld-arabinose; DPPR, decaprenylphosphoryl-5-phosphoribose; DPR, decapenylmonophosphoryld-ribose; EMB, ethambutol; Gal, galactose; mAGP, mycolyl-arabinogalactan peptidoglycan; pRpp, 5-phospho-ribofuranose-pyrophosphate; TDCM, trehalose dicorynomycolates; TLC, thin layer chromatography; TM, transmembrane; TMCM, trehalose monomycolates.

References


