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Acyl-CoA Carboxylases (*accD2* and *accD3*), Together with a Unique Polyketide Synthase (*Cg-pks*), Are Key to Mycolic Acid Biosynthesis in *Corynebacterianeae* Such as *Corynebacterium glutamicum* and *Mycobacterium tuberculosis**

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The *Corynebacterianeae* such as *Corynebacterium glutamicum* and *Mycobacterium tuberculosis* possess several unique and structurally diverse lipids, including the genus-specific mycolic acids. Although the function of a number of genes involved in fatty acid and mycolic acid biosynthesis is known, information relevant to the initial steps within these biosynthetic pathways is relatively sparse. Interestingly, the genomes of *Corynebacterianeae* possess a high number of *accD* genes, whose gene products resemble the β-subunit of the acetyl-CoA carboxylase of *Escherichia coli*, providing the activated intermediate for fatty acid synthesis. We present here our studies on four putative *accD* genes found in *C. glutamicum*. Although growth of the *accD4* mutant remained unchanged, growth of the *accD1* mutant was strongly impaired and partially recovered by the addition of exogenous oleic acid. Overexpression of *accD1* and *accBC*, encoding the carboxylase α-subunit, resulted in an 8-fold increase in malonyl-CoA formation from acetyl-CoA in cell lysates, providing evidence that *accD1* encodes a carboxyltransferase involved in the biosynthesis of malonyl-CoA. Interestingly, fatty acid profiles remained unchanged in both our *accD2* and *accD3* mutants, but a complete loss of mycolic acids, either as organic extractable trehalose and glucose mycolates or as cell wall-bound mycolates, was observed. These two carboxyltransferases are also retained in all *Corynebacterianeae*, including *Mycobacterium leprae*, constituting two distinct groups of orthologs. Furthermore, carboxyl fixation assays, as well as a study of a *Cg-pks* deletion mutant, led us to conclude that *accD2* and *accD3* are key to mycolic acid biosynthesis, thus providing a carboxylated intermediate during condensation of the mero-chain and α-branch directed by the *pks*-encoded polyketide synthase. This study illustrates that the high number of *accD* paralogs have evolved to represent specific variations on the well known basic theme of providing carboxylated intermediates in lipid biosynthesis.

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, 2). In addition, nonpathogenic bacteria belong to this taxon, such as *Corynebacterium glutamicum*, which is used in the industrial production of amino acids (3). A common feature to all these bacteria is that they possess unusual lipids, such as mycolic acids (1).

Mycolic acids are long chain α-alkyl-β-hydroxylated fatty acids (R-CH(OH)-CH(R′)-COOH), where R represents the meromycolate chain consisting in *M. tuberculosis* of up to 56 carbons possessing additional structural modifications, and R′ represents a shorter aliphatic branch possessing 22–26 carbons). These two chains are then condensed together via a specialized Claisen condensation enzyme, followed by reduction to yield mature mycolic acids (4). In contrast, mycolic acids from *Corynebacterium* species, including *C. glutamicum*, represent the simplest form of these lipids, whereby two C_{16} fatty acids condense together, followed by reduction to afford a C_{32} mycolic acid. These α-branched, β-hydroxy fatty acids are found primarily as esters of the nonreducing arabinan terminus of arabinogalactan (5). In addition, mycolic acids can also be found as extractable "free" lipids within the cell wall, mainly linked to glucose and trehalose (6). These mycolic acids and their derivatives are believed to play a crucial role in the architecture of the cell envelope (1).

Interestingly, *M. tuberculosis* is characterized by an exceptionally high number of additional lipids and glycolipids, which are thought to aid in the persistence of the bacterium, thereby fueling the promise of the identification of new drug targets in the context of tuberculosis (7). The rich diversity of lipids present in *M. tuberculosis* is reflected at the genomic level by a panoply of genes involved in lipid biosynthesis (8), some of them constituting sets of paralogous genes. For instance, *M. tuberculosis* has 35 *fadD* genes (annotated as fatty acid-CoA ligases), 16 *pks* genes (annotated as polyketide synthases), and six *accD* genes (annotated as acyl-CoA carboxylases), and their detailed function in lipid biosynthesis and relevance for persistence are only now emerging (8, 9). Importantly, *Corynebacterium* species are considered the archetype within *Corynebacterianeae*, including *M. tuberculosis*, due to a low frequency of gene duplications and structural alterations giving rise to a strong conservation at the genomic level within this subgroup of the Corynebacterianeae (10). Together with their simpler chemical composition (as outlined above for mycolic acids), it can therefore be assumed that species of *Corynebacterium* have

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at their disposal just the core set of genes and reactions characteristic for the Corynebacterianeae. Indeed, *C. glutamicum* possesses just three *fadD*, one *pks*, and four *accD* genes (11). As a result, comparative studies using *C. glutamicum* have been employed in understanding the role of several *M. tuberculosis* proteins, *e.g.* Ppm1/D2 in lipoarabinomannan biosynthesis (12) and the "antigen 85" mycolyltransferases in cell wall mycolylation (13–16).

The group of four *accD* genes in *C. glutamicum* is interesting, and little is known about the function of these genes in *Corynebacterianeae*. These genes encode polypeptides with similarities to the β-subunits of acetyl/propionyl-CoA carboxylases, and basically only one of them would be sufficient for the carboxylation of acetyl-CoA to provide malonyl-CoA for fatty acid biosynthesis. This suggests additional and specific carboxylations, probably involved in the synthesis of unusual lipids.

In this context, the still controversial issue of mycolic acid synthesis is very attractive to consider carboxylation reactions. Cell-free extracts of *Corynebacterium* have been shown to utilize [^{14}C]palmitic acid (17, 18), with the newly synthesized mycolic acid exclusively labeled at C-1 and C-3 (19). The Claisen condensation reaction was hypothesized to involve a carboxylation step since it is inhibited by avidin, an inhibitor of biotin-dependent enzymes in extracts of *Corynebacterium diphtheriae* that produces the putative precursor of mycolic acids, 2-tetradecyl-3-oxo-octadecanoic acid (19, 20). In contrast, avidin has no effect on the Claisen condensation reaction in a cell-free extract of *Corynebacterium matruchotii*, which synthesizes mature mycolic acids (17, 18). What seems to support this last observation is the incorporation of [2,2-^{2}H]palmitic acid in whole cells of *C. matruchotii* (21). During the preparation of this manuscript, a polyketide synthase from *C. glutamicum* (*Cg-pks*), the equivalent of *M. tuberculosis pks13*, that apparently plays a key role in mycolic acid biosynthesis was identified (22).

The study is centered on the four *accD* genes, with the latter located at the 3′-end of *Cg-pks*. As a result, we present our systematic study on lipid and mycolic acid biosynthesis based on defined *C. glutamicum* mutants, focusing on the relevance of the *accD* genes along with a phylogenomic analysis of these genes in *Corynebacterium* and *Mycobacterium* species whose genomes are established (8, 11, 23–25).

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—*Escherichia coli* DH5αmcr and *C. glutamicum* ATCC 13032 (the wild-type strain; referred to throughout as *C. glutamicum*) were grown in LB broth (Difco) at 37 and 30 °C, respectively. The mutants generated in this study were grown on LBHIS complex medium (5 g/liter tryptone, 5 g/liter NaCl, 2.5 g/liter yeast extract, 18.5 g/liter brain-heart infusion (Difco), and 90.1 g/liter sorbitol). Kanamycin and ampicillin were used at a concentration of 50 μg/ml. CGIII medium and CGXII medium (26) were used for *C. glutamicum* with 30 mg/liter protocatechuic acid being added to CGXII as a chelating agent. Samples for lipid analyses were prepared by harvesting cells at A = 10–15, followed by a saline wash and freeze-drying. Growth comparisons of the four *accD* mutants were performed on unsupplemented CGXII and LBHIS plates and on LBHIS plates supplemented with 0.03% (w/v) sodium oleate, 0.03% (w/v) Tween 40, 0.04% (w/v) Brij 35, and 0.03% (w/v) butter hydrolysate.

Construction of Plasmids—To enable chromosomal inactivation of the four *accD* genes of *C. glutamicum*, internal fragments were amplified by PCR and blunt end-ligated using the Sure cloning kit (Amersham Biosciences) into the SmaI site of the non-replicative vector pK18mob (27). The primers used were accD1MuF10 (5′-GCA TGT GCA GGT GGC AAC GC-3′), accD1MuR11 (5′-GGT AAT CTT TGG AAC GGT TGC-3′), accD2MuF30 (5′-GTC ACG TGT ACT CCC CT-3′), accD2MuR31 (5′-CAA GCG AAT ACG AGG TC-3′), accD3MuF40 (5′-GTT GTA GGC GTC GCA GAT AC-3′), accD3MuR41 (5′-GCG TCC TCT GAA GAA GAG-3′), paccD4intfor (5′-TGG GGT TCA TCT GGG CAT CTC AC-3′), and paccD4intrev (5′-TGC CCC CAA CGT TTC CAT AAT CTC-3′). The inactivation vectors derived were termed pK18mobaccD1-int, pK18mobaccD2-int, pK18mobaccD3-int, and pK18mobaccD4-int.

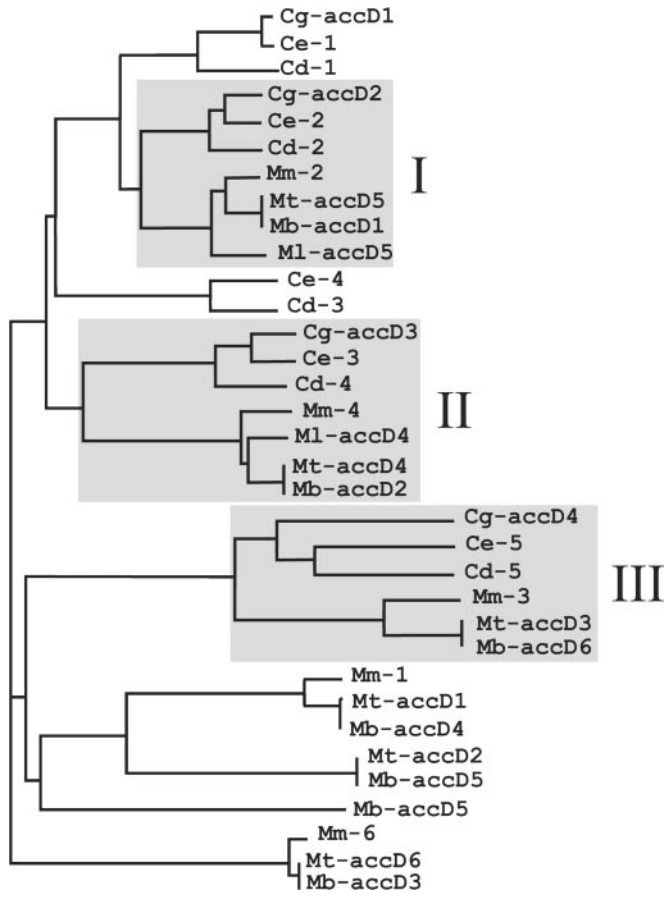

FIG. 1. **Phylogenomic analysis of mycobacterial and corynebacterial *acc* genes.** Groups of ortholog carboxyltransferases are highlighted in *gray* and labeled *I–III*. *Cg*, *C. glutamicum*; *Ce*, *C. efficiens*; *Cd*, *C. diphtheriae*; *Mm*, *M. marinum*; *Mt*, *M. tuberculosis*; *Mb*, *M. bovis*; *Ml*, *M. leprae*.

The in-frame deletion of *Cg-pks* in *C. glutamicum* was achieved with pK19mobsacBΔpks. Cross-over PCR was used to enable one-step integration of fragments containing upstream and downstream sequences of *Cg-pks* into pK19mobsacB. In the first PCR round, two separate amplification products were generated using primer pair pCipks (5′-TGT TTA AGT TTA GTG GAT GGG AGT CGC CGC ATT GAT GAG ATT TC-3′) and pCopks (5′-GGA ATT CGA CAG CGG AAG CTG ACG ACG-3′) and primer pair pNopks (5′-GGA ATT CCG TTG GCA CTG CAC ACG GTG-3′) and pNipks-2 (5′-CCC ATC CAC TAA ACT TAA ACA CTT CTG ATC CGA CGA TTG GCT CTG-3′). Both PCR products were purified and used as a template for amplification (using primers pCopks and pNopks) of a fragment devoid of *Cg-pks* that was treated with EcoRI and ligated with EcoRI-cleaved pK19mobsacB, yielding pK19mobsacBΔpks. The inserts in constructs used in this study were verified by sequencing.

Genomic Mutations—The non-replicative integration and deletion vectors were usually introduced via electroporation, but conjugation was used when electroporation failed. Conjugation was carried out using *E. coli* S17-1 as the donor, and its sensitivity to nalidixic acid (50 μg/ml) was used after plating for counterselection. Selection of recombinant *C. glutamicum* strains for integration of the four pK18mob-int vectors was performed using 15 μg/ml kanamycin. The correct integration of sequences into the chromosome and the absence of sequences, respectively, in the resulting recombinants were verified by PCR using two different primer pairs and by Southern analysis.

To achieve deletion of *Cg-pks*, plasmid pK19mobsacBΔpks was introduced into *C. glutamicum* by electroporation. Selection for resistance to kanamycin in at least 20 independent assays yielded regularly a number of clones indicating integration of the vector in the chromosome by homologous recombination. At least 100 clones derived from independent electroporation experiments were subjected to the subsequent selection for the second homologous recombination event. In this round, the presence of *sacB* (together with the addition of 10% sucrose to the

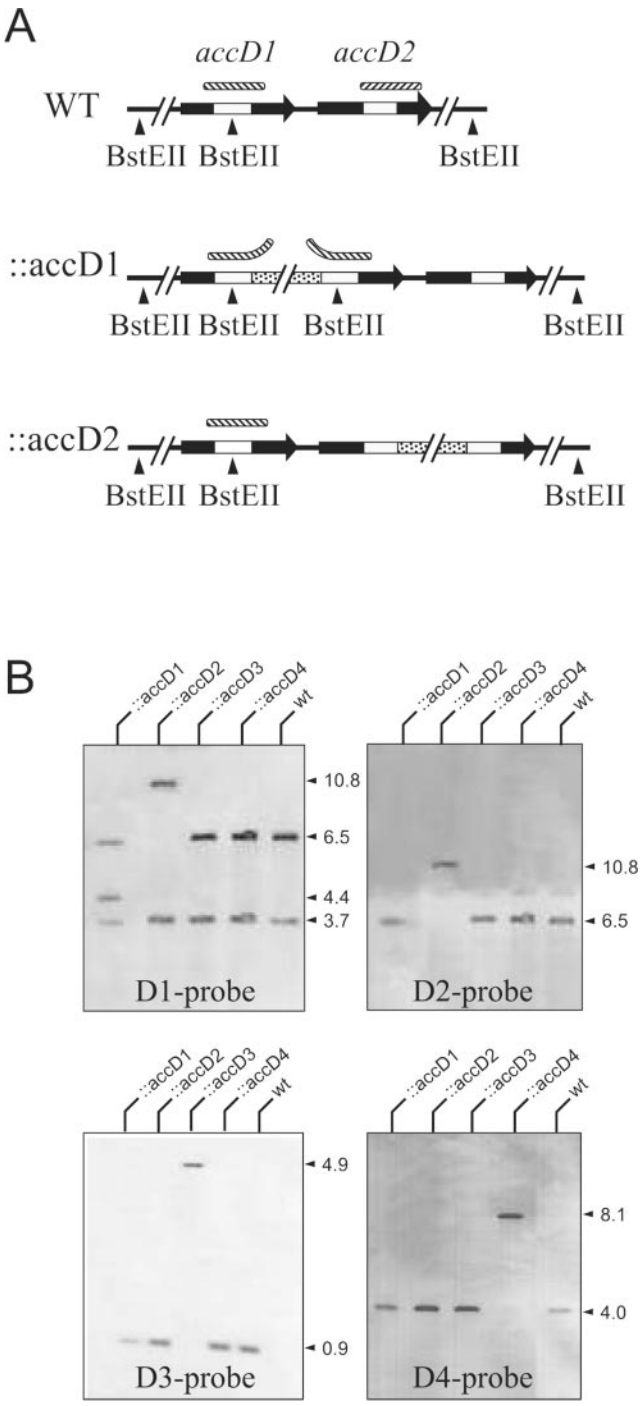

FIG. 2. *A*, schematic diagram of the construction of *accD* mutants from *C. glutamicum*. The scheme illustrates the situation for the two adjacent genes, *accD1* and *accD2*. The wild-type (*WT*) situation is given, where the two internal fragments used for gene disruption are indicated as *open boxes* within the genes. Also shown are the three relevant BstEII restriction sites as well as the location of the *accD1* and the *accD2* probes, which are shown as *hatched boxes* above their complementary chromosomal DNA regions. Homologous recombination and selection for kanamycin resistance resulted in strain *accD1* with disrupted *accD1* (*::accD1*). Since the BstEII cut was present on the internal fragment, it was duplicated after correct integration of the vector, thus resulting in three fragments hybridizing with the *accD1* probe. Note that there are two complementary regions of the internal fragment. One of the three fragments (3.7 kb) corresponds to the left BstEII fragment, one to the 4.4-kb middle BstEII fragment containing the vector (*stippled boxes*), and one to the 6.5-kb right BstEII fragment containing also *accD2*. With chromosomal DNA of strain *accD2*, the *accD1* probe resulted in two fragments (*::accD2*) due to the BstEII cut within *accD1*. Therefore, also with the *accD1* probe, an enlarged fragment with vector sequences was detected in the *accD2* mutant. The sizes of the BstEII fragments are not drawn to scale. *B*, final confirmation of the constructed strains via Southern blot analyses using chromosomal DNA from the *accD1–4* inactivation mutants and the wild-type (*wt*) strain. The lanes are marked accordingly, and the fragment sizes are given in kilobases on the right. For specific detection, the *accD1* probe (*upper left panel*), the *accD2* probe (*upper right panel*), the *accD3* probe (*lower left panel*), and the *accD4* probe (*lower right panel*) were used.

medium) resulted in a positive selection of clones in which vector sequences were lost. Small colonies picked after 10 days were analyzed for deletion of *Cg-pks*. Of 60 colonies analyzed, 19 exhibited the loss of *Cg-pks*. One of these was further analyzed by Southern blot analysis, and the resulting strain, *Cg-*Δ*pks*, was used in all subsequent studies.

Southern Blot Analysis—Genomic DNA was extracted from *accD1–4* mutants and the *Cg-pks* mutant and cleaved with BstEII. 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 fragments of the *accD* genes as probes that were labeled with digoxigenin (DIG labeling and detection kit, Roche Applied Science). For *accD1–4*, the SacI-BglII, PvuII-SgrAI, ClaI-StuI, and SfuI-PvuI fragments were used, respectively. For *Cg-*Δ*pks* analysis, the 645-bp fragment used was generated by PCR with primers pNopks and ppksACPintrev (5′-CAA CAT CGC GAG AGG AAA GG-3′).

Extraction and Analysis of ^{14}C-Labeled Lipids—LBHIS medium (5 ml) was inoculated with a single colony of the *accD1–4* and *Cg-pks* mutants, respectively, and shaken at 120 rpm overnight at 30 °C. An aliquot (1 ml) of this culture was used to inoculate 5 ml of CGIII medium and grown again overnight. This second pre-culture was used to inoculate 5 ml of CGXII medium to give a starting absorbance of ~0.2. Cells were incubated at 30 °C until A = 0.4, at which point, 5 μCi/ml [^{14}C]acetate (62 mCi/mmol; Amersham Biosciences) was added, followed by overnight incubation with shaking at room temperature. Cells were harvested, washed, and freeze-dried.

Free lipids were extracted by two consecutive extractions with 2 ml of $CHCl_3/CH_3OH/H_2O$ (10:10:3, v/v/v) for 3 h at 50 °C. These lipid extracts were combined with 1.75 ml of $CHCl_3$ and 0.75 ml of water, mixed, and centrifuged. The lower organic phase was recovered and washed twice with 2 ml of $CHCl_3/CH_3OH/H_2O$ (3:47:48, v/v/v), and the resulting organic phase was dried and resuspended in 200 μl of $CHCl_3/CH_3OH/H_2O$ (10:10:3, v/v/v). An aliquot (20,000 cpm) from each strain was subjected to TLC using Silica Gel 60 F_{254} plates (Merck) developed in $CHCl_3/CH_3OH/H_2O$ (60:16:2, v/v/v). Autoradiograms were produced by 2–3-day exposure to Kodak X-Omat AR film to reveal ^{14}C-labeled lipids and compared with known standards (28).

The bound lipids from the delipidated extracts were released by the addition of 2 ml of a 5% aqueous solution of tetrabutylammonium hydroxide, followed by overnight incubation at 95 °C. After cooling, water (2 ml), CH_2Cl_2 (4 ml), and CH_3I (500 μl) were added and mixed thoroughly for 30 min. The lower organic phase was recovered following centrifugation, 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 CH_2Cl_2 (200 μl). An aliquot (10,000 cpm) from each strain was subjected to TLC using Silica Gel 60 F_{254} plates developed in petroleum ether/acetone (95:5, v/v). Autoradiograms were produced by overnight exposure to Kodak X-Omat AR film to reveal ^{14}C-labeled fatty acid and mycolic acid methyl esters and compared with known standards (21).

Acyl Carboxylation Assay—To assay acyl carboxylase activity, we adapted the method of Rainwater and Kolattukudy (29). Briefly, a clarified lysate of the appropriate strain was prepared by resuspending the bacterial cultures to a density of 0.5 g (wet weight)/ml in 0.1 M potassium phosphate buffer (pH 8.0) and 5 mM 2-mercaptoethanol. The cell paste was lysed by two passages through a French pressure cell (19,000 p.s.i., cell pre-chilled to 4 °C), and the lysate was clarified by centrifugation at 27,000 × g for 30 min at 4 °C. The supernatant was removed and stored on ice until assayed. Assay mixtures (100 μl) contained 100 mM potassium phosphate (pH 8.0), 5 mM ATP, 5 mM $MgCl_2$, 2% Me_2SO, 0.9 mM acyl substrate (acetyl-CoA, palmitoyl-CoA, or palmitic acid), 65 mg of bovine serum albumin, 0.5 μCi of $NaH^{14}CO_3$ (0.1 Ci/mol; CFA421, Amersham Biosciences), and 250 μg of total protein from the lysate. All of the components of the assay excluding the enzyme extract were premixed and then mixed with the enzyme extract to initiate the reaction, which was held at 30 °C for 30 min. The reaction was quenched by the addition of 50 μl of concentrated HCl. This mixture was then held at 95 °C under a stream of air to remove non-fixed $^{14}CO_2$. After evaporation to dryness, the residue was dissolved in 1 ml

of water and added to 10 ml of Ecoscint A (National Diagnostics, Inc.) prior to liquid scintillation counting.

Acetyl-CoA Carboxylation Assay—To assay for acetyl-CoA-dependent malonyl-CoA formation, cells grown on CGIII medium were washed twice with 0.9% NaCl, resuspended in 60 mM Tris-HCl (pH 7.2), and disrupted as described above. Assay mixtures (250 μl) contained 60 mM Tris-HCl (pH 7.2), 65 mM $KHCO_3$, 1 mM ATP, 1.5 mM $MgCl_2$, and 135 μl of cell extract. The mixture was preincubated at 30 °C for 1 min, and the reaction was initiated by the addition of acetyl-CoA (lithium salt, Roche Applied Science) to give a final concentration of 2 mM and further incubated at 30 °C. Samples were taken at the given time intervals and mixed immediately with 5 μl of 30% perchloric acid to quench the reaction. The assay tubes were centrifuged subsequently (14,000 × *g*, 4 °C, 5 min), and 50 μl of the supernatant was withdrawn, neutralized with 12.5 μl of Na_2CO_3, and analyzed via HPLC.[1] Acyl-CoA synthesis was monitored by reversed-phase chromatography using a LiChrospher 1000 RP 18-EC-5μ column (125 × 4 mm; Merck) on an Agilent 1100 series HPLC apparatus. Samples (12 μl) were automatically injected and separated by an increasing gradient consisting of 50 mM sodium phosphate buffer (pH 5.0) containing 2% acetonitrile, increasing up to 26% acetonitrile in the same buffer at a flow rate of 0.3 ml/min, and monitored at 254 nm. Reactions were compared with known standards of acetyl-CoA, malonyl-CoA, and CoA (0.1–1 mM) dissolved in 200 mM sodium phosphate buffer (pH 3.0).

RESULTS

accD Genes of Corynebacterianeae—From the recent Himar1-based transposon mutagenesis in *M. tuberculosis* (30), *accD4* and *accD6* were found to be essential genes, whereas *accD1* and *accD3* were found to be nonessential. In terms of *accD1* (previously *dtsR1*) of *C. glutamicum*, an involvement in L-glutamate formation that is related to fatty acid synthesis was demonstrated (31). To address the problem regarding functional similarities of the carboxyltransferase proteins, we performed a phylogenomic analysis of the *accD* genes present in the genomes of the sequenced *Corynebacterianeae* (8, 11, 23–25, 32).

In fatty acid biosynthesis, the malonyl-CoA required for each elongation step is generated by carboxylation of acetyl-CoA. The process is catalyzed by an enzyme consisting of a biotinylated α-subunit and a β-subunit, the latter being the actual carboxyltransferase. In *E. coli* and *Bacillus subtilis*, each of these subunits consists of two polypeptides, whereas in all Corynebacterianeae analyzed, we have found that the α- and β-subunits consist of a single polypeptide. *C. glutamicum* has four putative carboxyltransferase β-subunits (*accD* genes); its close relatives, *Corynebacterium efficiens* and *C. diphtheriae*, possess five; and *M. tuberculosis, Mycobacterium bovis*, and *Mycobacterium marinum* possess as many as six carboxyltransferases. The sequence identities of the polypeptides within one organism are also in part exceptional. For instance, AccD1 and AccD2 of *M. tuberculosis* share an identity of 49%, and even the least homologous pair, AccD1 and AccD3, still share 22% identity.

The primary structures of 35 polypeptides were analyzed using ClustalW (33), and the results are presented in Fig. 1. There are several remarkable features. First, there are two distinct groups (I and II) of closely related proteins in which each organism analyzed is represented only once with one carboxyltransferase. Group III is clearly distant from groups I and II, whereas the latter two are more closely related. The distinct clustering of these groups suggests that the members of each group are orthologs with an identical function. Second, there are three additional clusters in which *M. bovis* and *M. tuberculosis* are represented once. Also, *M. marinum* is present in two of these clusters. These results indicate that these carboxyltransferases serve more specialized functions within the mycobacterial branch of *Corynebacterianeae*, whereas those of the first and second clusters represent fundamental core functions specific for lipid biosynthesis in all bacteria. In addition, *M. leprae* possesses three pseudogenes sharing in part identities with the other carboxyltransferases (25). However, the fact that in *M. leprae* the two single carboxyltransferases fully retained are members of groups I and II

[1] The abbreviations used are: HPLC, high performance liquid chromatography; ACP, acyl carrier protein.

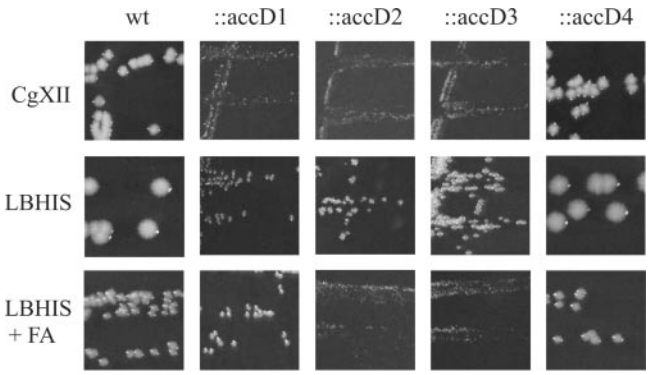

FIG. 3. **Growth of *accD* mutants of *C. glutamicum*.** The *accD* mutants of *C. glutamicum* and the wild-type (*wt*) strain were grown for 3 days at 30 °C. The plates used were CGXII salt medium with glucose, LBHIS complex medium, and LBHIS complex medium supplemented with sodium oleate (300 mg/liter) and butter hydrolysate (300 mg/liter) together with the detergents Tween 40 (300 mg/liter) and Brij 35 (400 mg/liter). *FA*, fatty acids.

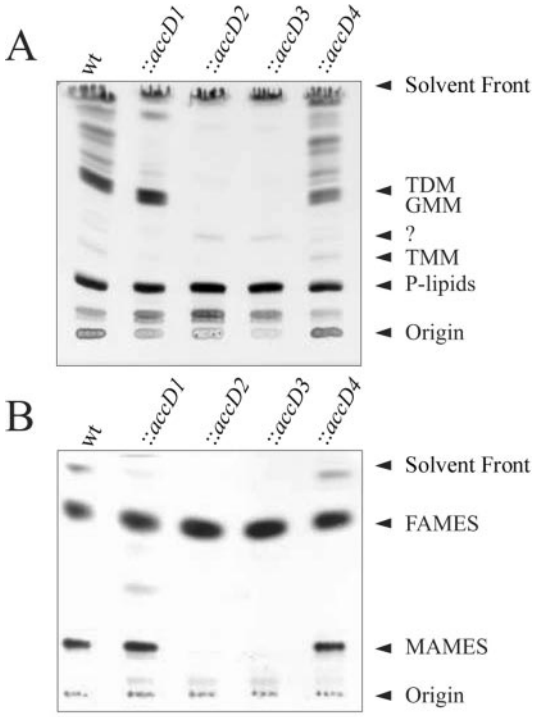

FIG. 4. **Lipid analysis of *accD* mutants of *C. glutamicum*.** *A*, free lipids were extracted, and an aliquot (20,000 cpm) from each strain was subjected to TLC analysis using Silica Gel 60 F_{254} plates developed in $CHCl_3/CH_3OH/H_2O$ (60:16:2, v/v/v). Autoradiograms were produced by 2–3-day exposure to Kodak X-Omat AR film to reveal ^{14}C-labeled lipids and compared with known standards (trehalose monomycolates (*TMM*), trehalose dimycolates (*TDM*), and glucose monomycolates (*GMM*)) (28). *B*, the bound lipids from the delipidated extracts were released by the addition of tetrabutylammonium hydroxide, followed by overnight incubation at 95 °C. Following preparation of methyl esters, an aliquot (10,000 cpm) from each strain was subjected to TLC analysis using Silica Gel 60 F_{254} plates developed in petroleum ether/acetone (95:5, v/v). Autoradiograms were produced by overnight exposure to Kodak X-Omat AR film to reveal ^{14}C-labeled fatty acid methyl esters (*FAMES*) and mycolic acid methyl esters (*MAMES*) and compared with known standards (21). *wt*, wild-type.

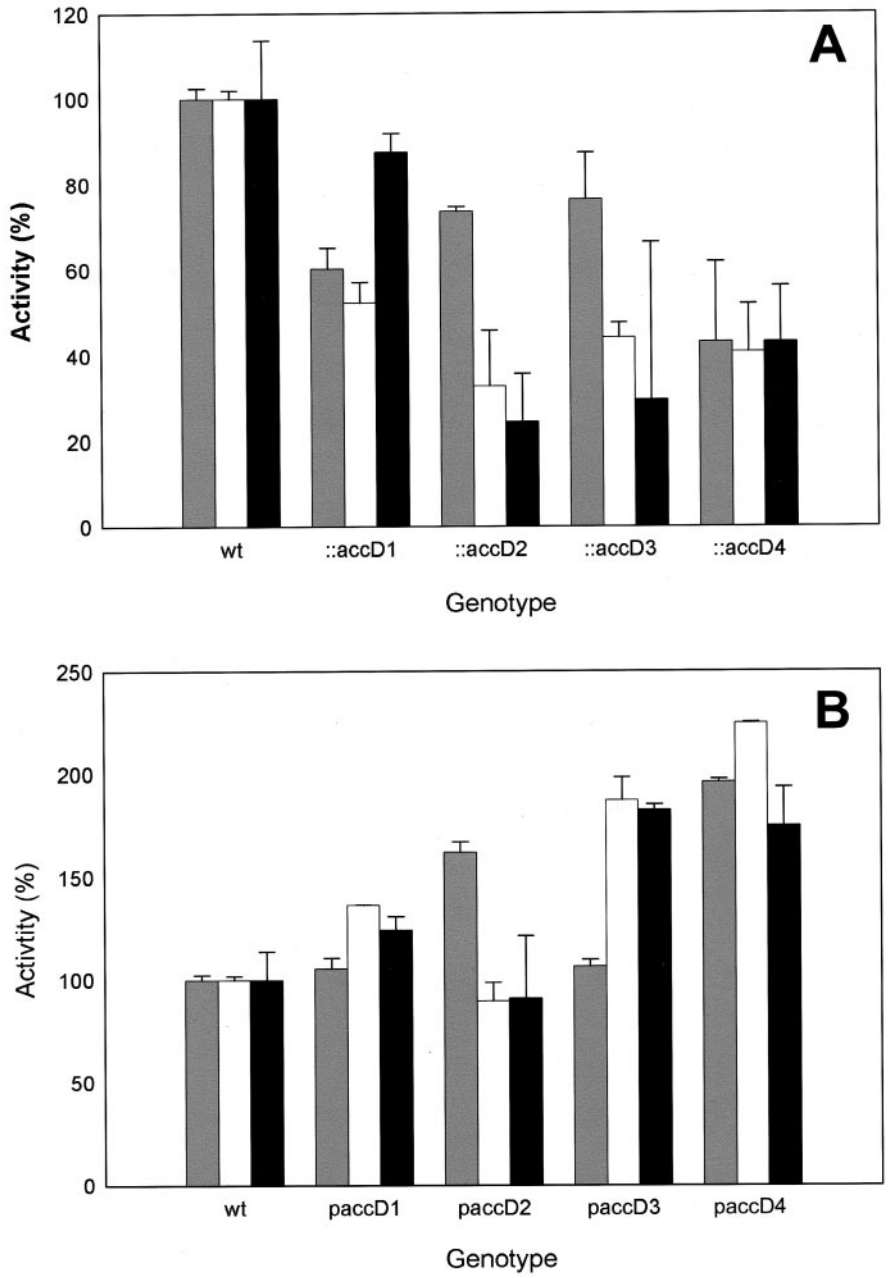

FIG. 5. **Relative acyl carboxylase activity associated with the *C. glutamicum* AccD family.** The relative acyl carboxylase activity (percent of wild-type (*wt*) activity for each substrate) in extracts of *C. glutamicum* with either disruption (*A*) or overexpression (*B*) of the various *accD* family members was determined using acetyl-CoA (*gray bars*), palmitoyl-CoA (*white bars*), or palmitic acid (*black bars*). Carboxylation activity was followed by fixation of $H^{14}CO_3^-$. Fixed radioactive carbon was measured by liquid scintillation counting after acidification of the reaction mixtures and evaporation to dryness.

strengthens the view that these members encode essential functions in lipid biosynthesis, as is the case with mycobacterial mycolic acid biosynthesis (4). Since carboxyltransferase activity requires an activated carboxyl group derived from a biotinylated α-subunit, we searched the genomes for the corresponding proteins. The three *Corynebacterium* species and *M. leprae* have only one such subunit, whereas *M. tuberculosis, M. bovis*, and *M. marinum* possess three.

Inactivation of the Corynebacterial β-Subunit Genes—To systematically investigate the function of the *accD* genes, each was inactivated in *C. glutamicum*. For this purpose, we constructed non-replicative vectors to enable their specific disruption by use of internal fragments of *accD1–4*, respectively (see "Experimental Procedures;" Fig. 2*A*). After transformation, kanamycin-resistant clones were assayed for vector integration by PCR with two different primer sets. Their correct chromosomal integration was further confirmed by a scrupulous Southern blot analysis, with the final result shown for each mutant in Fig. 2*B*. The three chromosomal fragments derived from the *accD1* inactivation mutant hybridized with the *accD1* probe (Fig. 2*B*, *upper left panel*), which is in agreement with the correct integration and inactivation of *accD1*. The fact that a shift occurred with the same *accD1 probe* with DNA prepared from the *accD2* mutant was due to the immediate proximity of both *accD1* and *accD2*, separated by 344 bp (Fig. 2*A*). One of these genes probably originated by a recent gene duplication event since the paralogs share >74% identical nucleotides over 75% of their length. With the *accD2–4* probes, the chromosomal fragment of each respective mutant carrying the inactivation vector was shifted to the expected fragment increased by the vector length (3.99–4.35 kb) (Fig. 2*B*). These results confirm the specific inactivation of each *accD* gene and the integrity of the gene locus in the mutants generated.

As a control, the four inactivation mutants were made competent and transformed with pEC7accD1–4, respectively. The resulting recombinants were confirmed by plasmid preparations and PCR analysis to have the chromosomal locus disrupted with an intact copy of the respective gene plasmid encoded. Growth of these complemented mutants was undistinguishable from that of wild-type *C. glutamicum* either on

LBHIS or CGXII minimal medium (data not shown). This makes it unlikely that the *accD* disruptions result in any polar effects.

Phenotypic Characterization of Mutants—As shown in Fig. 3, the *accD4* mutant (compare *left* and *right panels*) did not exhibit a phenotype, whereas growth of the *accD1–3* mutants was strongly reduced on CGXII salt medium (*upper row*). The worst growth was in fact seen with the *accD1* inactivation mutant, and the colonies visible in Fig. 3 are due to revertants. Also on LBHIS complex medium, growth of the three mutants was still impaired, illustrating that these mutants are not rescued by specific components supplied by yeast extract and tryptone, which are present in LBHIS medium. However, when this complex medium was supplemented with sodium oleate together with butter hydrolysate (and detergents for emulsification), growth of the *accD1* mutant was markedly improved, whereas that of the *accD2* and *accD3* mutants was still impaired, indicating a direct relation of *accD1* with fatty acid biosynthesis and confirming previous observations with a *dtsR1* deletion mutant (31). The colony surface of both the *accD2* and *accD3* mutants was rougher than that of wild-type colonies, and these two mutants tended to clump in CGXII liquid culture (data not shown). Overall, the *accD4* mutation has no apparent phenotype, whereas *accD1* appears to be involved in lipid biosynthesis, as it was rescued by sodium oleate, suggesting that it may be involved in the synthesis of oleate from malonyl-CoA. The *accD2* and *accD3* mutants are likely to be very specific and essential carboxyltransferase β-subunits, as also illustrated by the phylogenomic analysis.

Lipid Analysis of the accD Mutants—To relate the phenotypic changes in the *accD* mutants to their cellular composition, the mutant and wild-type bacteria were grown on CGXII salt medium for 4 h and then labeled with [^{14}C]acetate. After additional incubation, the cells were harvested and dried, and their lipid composition was analyzed. The free lipids were extracted with chloroform/methanol/water and analyzed by TLC as shown in Fig. 4*A*. The *accD1* and *accD4* mutations did not considerably change the relative amounts of phospholipids, trehalose monomycolates and dimycolates, and glucose monomycolates. However, the consequences of the *accD2* and *accD3* mutations were dramatic. Surprisingly, both mutations resulted in the complete loss of free and extractable mycolates (trehalose monomycolates and dimycolates and glucose monomycolates), without affecting phospholipid synthesis and the possible accumulation of an unknown intermediate. The extracted lipids were also hydrolyzed, and their methyl esters were separated by TLC, confirming that the synthesis of fatty acids was not affected in the *accD2* and *accD3* mutants (data not shown).

The remaining cell wall-bound lipids were analyzed by hydrolysis and the preparation of methyl esters. The profile of the extracted fatty acid and mycolic acid methyl esters is shown in Fig. 4*B*. In the *accD2* and *accD3* mutants, cell wall-bound mycolic acids were absent, whereas the relative amounts of fatty acids and mycolic acids in the *accD1* and *accD4* mutants were comparable. These results illustrate the specific involvement of both *accD2* and *accD3* in mycolic acid synthesis and that neither of these two genes themselves can complement each other or *accD1* and *accD4*.

Carboxylation Reactions in the accD Mutants—To assess the validity of the assignment of the AccD family members as acyl carboxylase components, we assayed the fixation of ^{14}C from radiolabeled bicarbonate in extracts of the *C. glutamicum* mutants. In these assays, we used acetyl-CoA and palmitoyl-CoA, as these were the obvious substrates for *de novo* fatty acid biosynthesis and corynomycolate condensation, respectively.

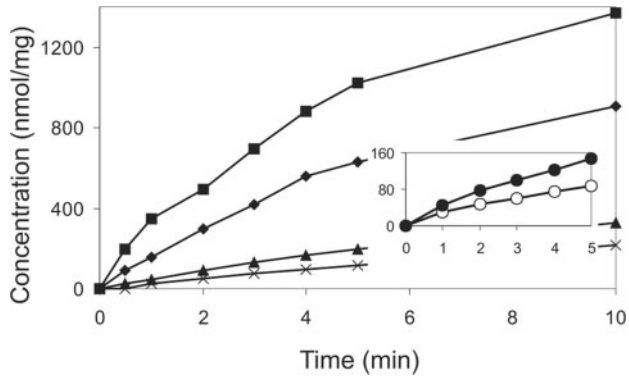

FIG. 6. **Malonyl-CoA formation with extracts of recombinant *C. glutamicum*.** Malonyl-CoA synthesis (nanomoles/mg of protein) is shown in recombinant *C. glutamicum* Δpyc with the genotypes pJC1accBCΔpyc pEC7accD1 (■), pJC1accBCΔpyc pEC7 (♦), pJC1Δpyc pEC7accD1 (▲), and the control (×) after incubation at 30 °C at the given time intervals. The *inset* shows comparable analyses using the separate *accD1*-overexpressing strain pVWEx2accD1 (●) compared with the empty vector pVWEx2 control (○).

The recent demonstration that the product of *M. tuberculosis fadD32*, which occupies the locus adjacent to *pks13*, activates fatty acids by adenylation (34) led us to incorporate palmitic acid into our experiments to determine whether, in the absence of commercially available palmitoyl-AMP, it may generate a suitable substrate for carboxylation. When assaying acyl carboxylase activity in clarified lysates of the parent strain, we detected that carbon fixation was 40-fold higher with acetyl-CoA than with palmitoyl-CoA and palmitic acid as acyl substrates, yielding specific activities of 2.45, 0.06, and 0.06 nmol/min/mg, respectively. The following reaction rates were normalized to these values. Basically, with each *accD* mutant and overexpressing strain, a significant influence on carbon fixation was apparent (Fig. 5). The most distinct effect was present for the *accD4* mutant, where inactivation resulted in a uniform and significant decrease in ^{14}C fixation, and overexpression of *accD4* led to a uniform increase in activity, in both cases independent of the substrate used. The *accD1* mutant also possessed lower levels of carboxyltransferase activity with respect to acetyl-CoA and palmitoyl-CoA effects; however, extracts prepared from the *accD1*-overexpressing strain possessed similar levels of activity in comparison with the wild-type extract. When analyzing extracts from *accD2* and *accD3*, we observed substrate-specific variations in acyl carboxylase activity. With the *accD2* inactivation mutant, acyl carboxylase activity with acetyl-CoA present was slightly reduced, whereas it was most pronounced with the C_{16} substrates. A similar activity profile was observed with the extract of the *accD3* inactivation mutant, with both observations being wholly consistent with roles for AccD2 and AccD3 specific to corynomycolate biosynthesis. When we assayed extracts from bacteria overexpressing *accD2* or *accD3*, we observed clear differences in their substrate preference. In the extract enriched in AccD3, ^{14}C fixation detected using acetyl-CoA was similar to that in the wild-type extract, whereas activity with C_{16} substrates was increased. In contrast, overexpression of *accD2* coincided with increased ^{14}C fixation when using acetyl-CoA rather than the longer acyl chain length substrates.

Acetyl-CoA Carboxylation by AccD1—We inferred from the growth response of the *accD1* inactivation mutant (Fig. 3) that *accD1* might encode the β-subunit of the carboxylase, transferring the carboxyl group from the β-subunit onto acetyl-CoA to yield malonyl-CoA. Therefore, an HPLC assay was used to follow malonyl-CoA formation. As shown in Fig. 6, use of extracts derived from *C. glutamicum* Δpyc resulted in a constant

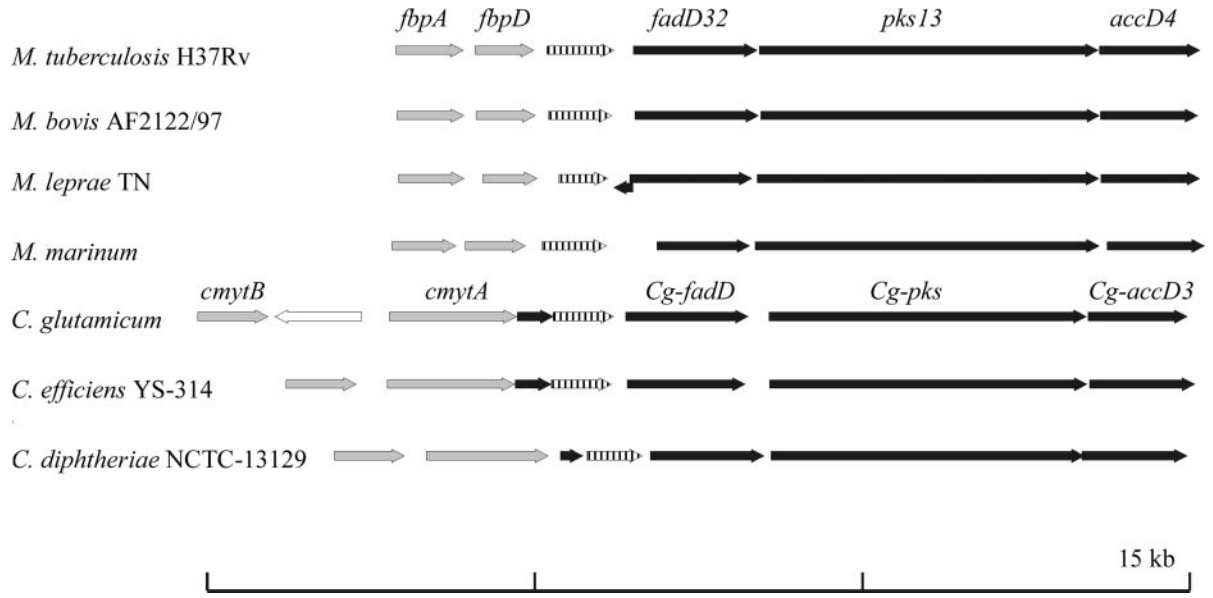

FIG. 7. **The *pks* locus of mycolic acid-containing bacteria responsible for mycolic acid biosynthesis.**

increase in malonyl-CoA formation over time that was dependent upon acetyl-CoA and ATP (data not shown). The derived specific activity was 23.1 nmol/min/mg, which is in the range of that determined for *M. tuberculosis* and *M. bovis* (29). When *accD1* was overexpressed (pJC1accD1), the specific activity increased by 2-fold to 40.7 nmol/min/mg. This significant increase was confirmed in a separate experiment using a separate construct (pVWEx2accD1) (Fig. 6, *inset*), where the specific activity was increased from 15.1 to 27.2 nmol min/mg. Unexpectedly, the overexpression of the biotin-containing α-subunit of *C. glutamicum* (pJC1accBC) resulted in a sharp rise in acetyl-CoA carboxylation activity, yielding a specific activity of 132.8 nmol/min/mg. These results indicate a possible limiting availability of this subunit for sufficient carboxylation to occur. This was reconfirmed by the mutual overexpression of *accBC* and *accD1* (pJC1accBC paccD1), where the specific activity was additionally increased to 180.3 nmol/min/mg. These results were confirmed in several independent experiments. A low activity of malonyl-CoA formation was observed in the *accD1* inactivation mutant (specific activity of 8 nmol/min/mg); poor growth in liquid culture and malonyl-CoA detection at the threshold level prevented a more detailed quantification in this mutant. In summary, the acyl carboxylase assays described above for AccD1–4 demonstrate that these enzymes possess carboxylase activity, with AccD3 (and possibly AccD2) preferring long chain substrates, consistent with their involvement in mycolic acid biosynthesis, and that AccD1 uses acetyl-CoA as a substrate.

The pks locus of Corynebacterianeae—Inspection of the chromosomal organization of the *accD* genes in *C. glutamicum* revealed the localization of *accD3* close to the single *Cg-pks* gene in this bacterium (Fig. 7). *Cg-pks* has high identity to *pks13* of *M. tuberculosis* and is located in a locus strictly conserved in all *Mycobacterium* and *Corynebacterium* species (Fig. 7), as it is also in *Rhodococcus* (22). It consists of at least six genes, all of them transcribed in the same direction and extending over >12 kb. In *M. tuberculosis*, the first two genes encode the mycolyltransferase FbpA and its non-catalytic paralog FbpD, respectively (13). These are followed by an essential gene (30), whose product is predicted to be anchored in the membrane by one transmembrane-spanning helix. The remaining segment (amino acids 35–336) is probably directed toward the periplasm and is predicted to possess an esterase

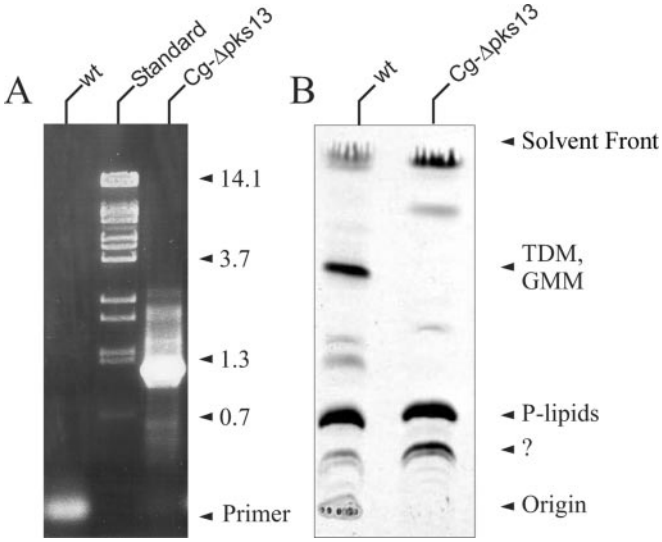

FIG. 8. **Analysis of the *Cg-pks* deletion mutant of *C. glutamicum*.** *A*, PCR analysis of the *Cg-pks* locus of the wild-type (*wt*) strain and the in-frame deletion mutant (*Cg-Δpks*). A standard was applied in the *second lane*, with the molecular masses given on the right in kilodaltons. Whereas with the primers annealing upstream and downstream of *Cg-pks*, the expected amplification product of 1.11 kb was obtained with chromosomal DNA from *Cg-Δpks*, no product could be formed under the conditions used with the wild-type DNA due to the length of *Cg-pks*. *B*, TLC analysis of extractable lipids from *C. glutamicum* (*wt*) and the deletion mutant (*Cg-Δpks*), illustrating the absence of the dominant lipids, trehalose dimycolates (*TDM*) and glucose monomycolates (*GMM*), upon *Cg-pks* deletion.

activity. Farther located downstream is *fadD32*, recently shown to activate long chain fatty acids as acyl adenylates (34), followed by the polyketide synthase *pks13*. The gene locus is completed with the β-chain of the acyl carboxylase *accD4* (*accD3* in *C. glutamicum*). The overall organization of the entire locus in all species analyzed is almost identical, although a slight difference is apparent in the mycolyltransferase region (Fig. 7, *gray arrows*). In *C. glutamicum*, a transposase is located between *cmytA* and *cmytB*. The strong conservation of this entire locus, the known mycolyltransferase activities in *M. tuberculosis* (13) and *C. glutamicum* (14, 15), the absence of mycolic acids in *accD3* and *Cg-pks* mutants (see below and Ref.

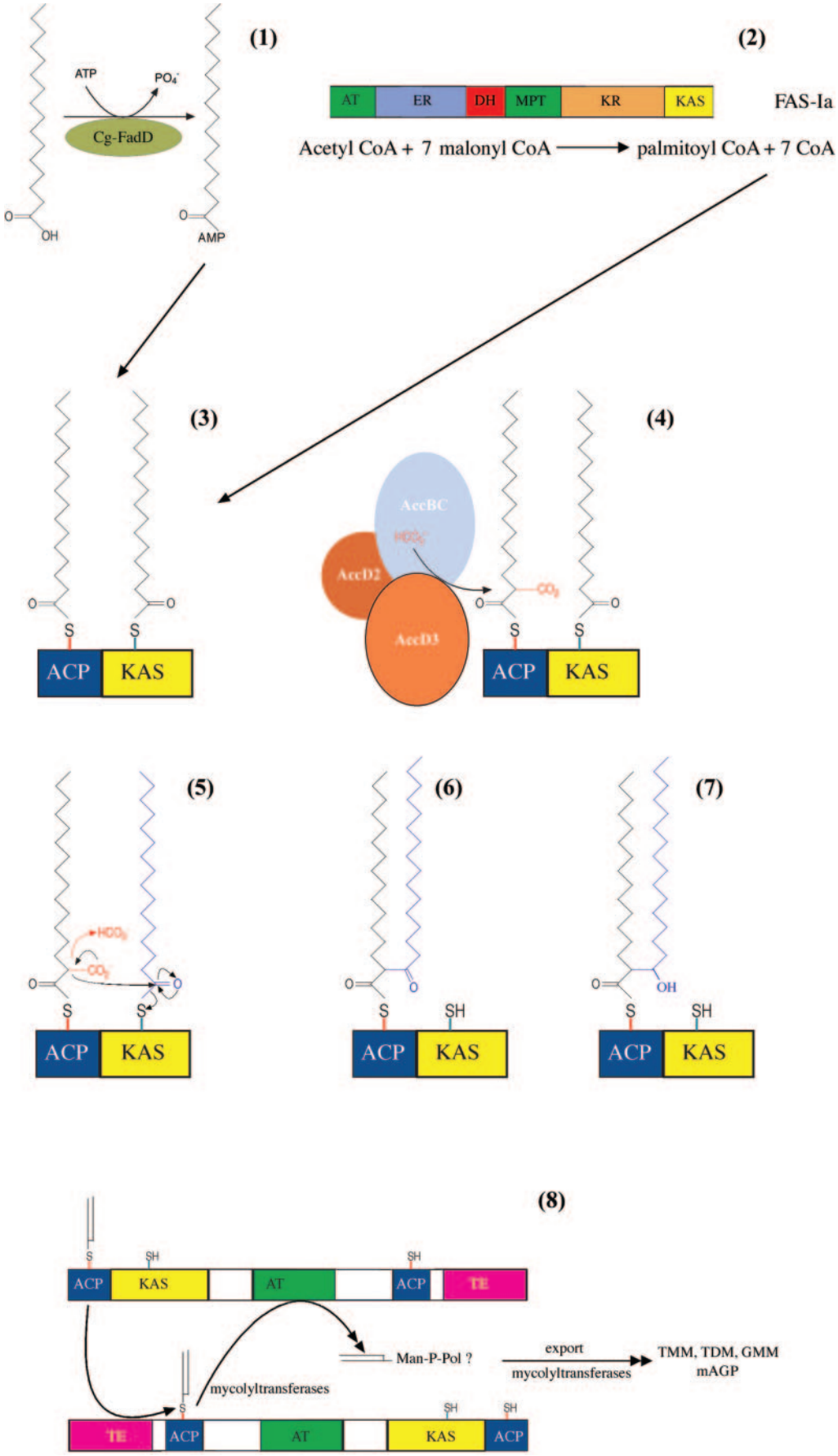

FIG. 9. **Proposed mechanism for mycolic acid biosynthesis in *Corynebacterianeae*.** Protein domains are shown as colored segments. *ACP* represents the N-terminal phosphopantetheinylated (*Ppant*; *red bar*) acyl carrier protein-like domain, and *KAS* represents the predicted β-ketoacyl synthase domain. *Step 1* depicts the charging of fatty acids (C_{16}) by the fatty acid-ADP ligase FadD. Step 2 depicts *de novo* fatty acid synthesis via

22), and the acyl-AMP ligase identified (34) suggest a fundamental and essential role of these genes in mycolic acid biosynthesis and translocation of mature mycolic acids to the cell wall and the outer lipid layer.

Deletion of pks13—To examine the phenotype of a *Cg-pks* mutant, we attempted to delete the *pks* gene while preserving the entire gene locus. For this purpose, the upstream and downstream sequences adjacent to *Cg-pks* were fused, with the in-frame deletion in the construct verified by sequencing. The construct was used in two rounds of positive selection (see "Experimental Procedures"). The original clones obtained after 3 days were all shown to have the wild-type locus restored. This indicates a strong disadvantage of cells deleted of *pks*. However, when we analyzed the small colonies that appeared after 10 days, these had lost *Cg-pks*. One of these clones was chosen, verified by PCR (Fig. 8*A*) and Southern blot analysis (data not shown), and confirmed to have *Cg-pks* deleted.

This strain, *Cg-*Δ*pks*, exhibited poor growth and a rough colony surface. Lipid analysis revealed that *Cg-*Δ*pks* was devoid of extractable mycolic acids (Fig. 8*B*) and cell wall-bound mycolic acids (data not shown), although fatty acids were still present in phospholipids. Since the *Cg-pks* deletion has virtually the same consequences on mycolic acid synthesis as *accD2* and *accD3*, this illustrates that these genes are equally important and possibly act in concert in the final assembly of mature mycolic acids.

DISCUSSION

Our combined mutational and functional analyses on the *accD* genes, which we propose are key to lipid biosynthesis, revealed that growth of the *accD1* mutant of *C. glutamicum* on minimal medium was severely restricted compared with the other mutants. This mutant was extremely unstable, and the phospholipids derived from the mutant culture are, in all likelihood, due to revertants. The recovery of growth by sodium oleate addition is strong evidence that AccD1 is a constituent of the acetyl carboxyltransferase enzyme, generating oleate from malonyl-CoA. This is further substantiated by the increased malonyl-CoA formation upon overexpression of *accD1*, yielding a higher specific activity of 40.7 nmol/min/mg compared with wild-type extracts (23.1 nmol/min/mg). In addition, when overexpression of *accD1* and *accBC* was combined, an 8-fold increase in specific activity was observed, indicating that both polypeptides are components of acetyl-CoA carboxyltransferase, which is in agreement with an acyl-CoA carboxylase isolated from *M. bovis* (29), and a propionyl-CoA carboxylase from *Mycobacterium smegmatis* (35), both of which are composed of two individual polypeptides, with one being biotinylated.

Extracts of the *accD4* mutant possessed lower levels of acyl carboxylase activity, and extracts from the *accD4*-overexpressing strain possessed increased levels of acyl carboxylase activity, suggesting that AccD4 is a carboxylase enzyme. However, the absence of a phenotype for the *accD4* mutant, as well as its unaltered lipid profiles, led us to conclude that AccD4 is not directly involved in lipid biosynthesis. In sharp contrast are the *accD2* and *accD3* mutants; lipid analyses revealed that both proteins are essential for corynomycolate biosynthesis. We cannot be sure whether the general carboxylation assays reported here represent an indication of relative acyl carboxylase activity or if they simply reflect the half-life of the carboxylated products, which, we presume, are readily decarboxylated in a Claisen-type condensation reaction. However, the assays revealed a profound decrease in carboxylation activity for the *accD2* and *accD3* inactivation mutants with respect to the C_{16} substrates. The data would appear wholly consistent with the proposed function of AccD2 and AccD3 in the later stages of corynomycolate biosynthesis. As expected, overexpression of *accD3* led to a similar increase in acyl carboxylase activity with both C_{16} substrates, whereas with acetyl-CoA, carboxylation activity was comparable with extracts from the parent strain. Intriguingly, overexpression of *accD2* led to an increase in the acyl carboxylase activity with acetyl-CoA only. Maybe the peculiar substrate specificities implied by the above *in vitro* data indicate the formation of hetero-oligomeric assemblies whose composition defines substrate specificity. However, as both AccD2 and AccD3 are required for corynomycolate biosynthesis, we can rule out any complementation by AccD1 and may speculate that AccD2 and AccD3 operate as a heterodimer. Furthermore, our *in vitro* analyses of *accD2* and *accD3* overexpression strongly suggest that AccD3 might define the strict substrate specificity of such a complex. Sequence analysis of the AccD paralogs supports this hypothesis, as AccD2 is more closely related to AccD1 than AccD3.

The *M. tuberculosis* antigen 85 complexes, which possess mycolyltransferase activity, are located outside of the cytoplasm in the cell envelope and are often found in culture medium (13), as observed for *C. glutamicum* (14, 15). The localization of these enzymes suggests that they use mature mycolic acids once they are synthesized to transfer them to their final destination (5, 13–15). The mycolyltransferase locus is slightly different in the related *Corynebacterium* species (Fig. 7). CmytA in *C. glutamicum* is fused to a larger polypeptide with a domain of unknown function. In addition, a small open reading frame is present downstream (166 amino acids). A distinct possibility exists that *cmytA* and *cmytB* have different specificities, as they are conserved in *Corynebacterianeae*. Interestingly, downstream of the mycolyltransferase locus is a gene essential for *M. tuberculosis* (30). The encoded Pro-rich protein is predicted to be membrane-anchored, with the remaining segment directed toward the periplasm and exhibiting identity to an acetylxylan esterase from *Penicillium purpurogenum* possessing the characteristic serine esterase motif. A tempting hypothesis is that this particular gene may be responsible for

fatty-acid synthase Ia (*FAS-Ia*). The domains shown are acyltransferase (*AT*), enoyl reductase (*ER*), dehydratase (*DH*), malonyl/palmitoyltransferase (*MPT*), ACP, β-ketoacyl reductase (*KR*), and β-ketoacyl synthase (*KAS*). In *step 3*, transacylation reactions charge the phosphopantetheine residue of ACP and the active-site cysteine residue (Cys^{288}; *green bar*) of the β-ketoacyl synthase domain with the C_{16} fatty acid chains. The latter is likely to occur spontaneously, as in the case of *M. tuberculosis* KasA/B (40) and *M. tuberculosis* FabH (41), but the acylation of ACP may require an acyltransferase activity. In *step 4*, AccD2 and AccD3 and their biotinylated partner AccBC activate the ACP-bound C_{16} acyl group for condensation by α-carboxylation. The removal of the carboxyl group (*step 5*), presumably now translocated into the active site of the β-ketoacyl synthase domain, forms a carbanion that attacks the thioester bound acyl chain occupying Cys^{288}, thus forming the oxomycolyl intermediate (*step 6*). The latter stages of synthesis of mycolic acid, *e.g.* reduction (*step 7*) and its transfer to extracytoplasmic trehalose and cell wall arabinan (summarized in *step 8*), are not certain. If the quaternary structure of polyketide synthase resembles that of fatty-acid synthase I, a head-to-tail homodimer, the following reaction series can be envisaged. The predicted thioesterase (*TE*) domain may transfer the mycolic acid moiety to the phosphopantetheine residue of the C-terminal ACP-like domain of the other monomer molecule, thus facilitating the transfer of the mycolate residue by the predicted acyltransferase domain to a suitable acceptor for export. The gene adjacent to those encoding the mycolyltransferases (FbpA proteins CmytA and CmytB, respectively), a putative membrane-anchored esterase, may act to transfer the mycolate residue to the mannosylated polyprenyl phosphate (*Man-P-Pol*) carrier described in mycobacteria (36) that presumably facilitates its export. However, other mechanisms are also possible. *TMM*, trehalose monomycolates; *TDM*, trehalose dimycolates; *GMM*, glucose monomycolates; mAGP, mycolyl-arabinogalactan-peptidoglycan complex.

the first steps of mature mycolic acid transfer from a putative carrier molecule, such as acyl carrier protein (ACP)/CoA, or from an intermediate polyprenol carrier (36) to possibly either trehalose or the cell wall arabinogalactan. This seems meaningful, as it is located in close conjunction with the mycolyltransferases and together with the *fadD-pks-accD3* locus and warrants further investigation as a possible specific mycolyltransferase. Another structural characteristic is the gene arrangement of *pks* and its downstream *accD3* gene. In the three *Corynebacterium* species analyzed, the genes are separated by <20 nucleotides; however, both genes overlap by 4 nucleotides in *M. tuberculosis*, *M. bovis*, and *M. leprae*. Such intimate structural organization has also been observed for other important genes in the *Corynebacterianeae*, such as *ppm1*, in which even protein fusion has occurred (12, 37, 38).

The β-ketoacyl synthases catalyze the formation of new carbon–carbon bonds by condensation of a variety of acyl chain precursors with an elongation substrate, usually malonyl or methylmalonyl residues that are covalently attached in a thioester linkage to an ACP. The data obtained in this work, along with the structural characteristics of the *fadD-pks-accD3* locus and recent findings on FadD32 (34) and *pks13* (22), yield three distinct features that are very likely to represent the basis of discrete steps in the mechanism of mycolic acid biosynthesis. (i) FadD, recently shown to constitute a new class of fatty acyl-AMP ligases, activates long chain fatty acids as acyl adenylates, which are loaded onto multifunctional polyketide synthase for further chain extension (34); (ii) polyketide synthase possesses a β-ketoacyl synthase domain with two phosphopantetheinyl-binding sites and a thioesterase domain; and (iii) the Acc proteins have carboxyltransferase activity, with that of AccD2 and AccD3 of *C. glutamicum* necessary for mycolic acid biosynthesis. An explanation for the two phosphopantetheinyl arms is that one site is occupied by the acyl chain forming the mero-chain and the other by the acyl chain resulting in the incoming α-branch. The role of AccD3 as shown in these studies would be to generate an activated carboxylated acyl derivative, which would then react with the bound acyl chain to form a 3-oxo intermediate, which, after reduction, would form a mature mycolic acid (Fig. 9). The experimental data and the phylogenomic analysis indicate that in addition to AccD3 in *C. glutamicum*, a second Acc protein (AccD2) is also apparently required for mycolic acid biosynthesis. One possibility is that after the fixation of carbon by AccBC, this is not directly transferred via AccD3 (AccD4 in *M. tuberculosis*), but transmitted via AccD2 (AccD5 in *M. tuberculosis*). The carboxylating transferase might fall into a group of multienzyme complexes such as transcarboxylase from *Propionibacterium shermanii* (39), which is a 1.2-MDa multienzyme complex that couples two carboxylation reactions, transferring CO_2^- from methylmalonyl-CoA to pyruvate, yielding propionyl-CoA and oxalacetate. Cg-AccD3 also exhibits a high degree of identity to the 12 S subunit of this enzyme. Shuttling via intermediates between different catalytic subunits provides a complex and intriguing mechanism for regulation and modulation of this specificity. Clearly, further experiments and, in particular, an *in vitro* system for the Claisen condensation reaction involved in mycolic acid biosynthesis are required (studies in progress). The mechanism proposed (Fig. 9) for the Claisen condensation could well be a general mechanism of relevance for other lipids or antibiotics produced via polyketide synthases.

REFERENCES

1. Brennan, P. J., and Nikaido, H. (1995) *Annu. Rev. Biochem.* **64,** 29–63
2. Stackebrandt, E., Rainey, F. A., and Ward-Rainey, N. L. (1997) *Int. J. Syst. Bacteriol.* **47,** 479–491
3. Eggeling, L., Pfefferle, W., and Sahm, H. (2001) in *Basic Biotechnology* (Ratledge, C., and Kristiansen, B., eds) pp. 281–302, Cambridge University Press, Cambridge, United Kingdom
4. Kremer, L., Baulard, A. R., and Besra, G. S. (2000) in *Molecular Genetics of Mycobacteria* (Hatfull, G. F., and Jacobs, W. R., Jr., eds) pp. 173–190, ASM Press, Washington D. C.
5. McNeil, M. R., Daffe, M., and Brennan, P. J. (1991) *J. Biol. Chem.* **266,** 13217–13223
6. Minnikin, D. E. (1982) in *The Biology of Mycobacteria* (Ratledge, C., and Stanford, J., eds) pp. 95–184, Academic Press Ltd., London
7. Kremer, L. S., and Besra, G. S. (2002) *Expert Opin. Investig. Drugs* **11,** 1033–1049
8. 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, R., Squares, S., Sulston, J. E., Taylor, K., Whitehead, S., and Barrell, B. G. (1998) *Nature* **393,** 537–544
9. Glickman, M. S., and Jacobs, W. R. (2001) *Cell* **104,** 477–485
10. Nakamura, Y., Nishio, Y., Ikeo, K., and Gojobori, T. (2003) *Gene* (*Amst.*) **317,** 149–155
11. Kalinowski, J., Bathe, B., Bartels, D., Bischoff, N., Bott, M., Burkovski, A., Dusch, N., Eggeling, L., Eikmanns, B. J., Gaigalat, L., Goesmann, A., Hartmann, M., Huthmacher, K., Kraemer, R., Linke, B., McHardy, A. C., Meyer, F., Mockel, B., Pfefferle, W., Puhler, A., Rey, D. A., Ruckert, C., Rupp, O., Sahm, H., Wendisch, V. F., Wiegrabe, I., and Tauch, A. (2003) *J. Biotechnol.* **104,** 5–25
12. Gibson, K. J., Eggeling, L., Maughan, W. N., Krumbach, K., Gurcha, S. S., Nigou, J., Puzo, G., Sahm, H., and Besra, G. S. (2003) *J. Biol. Chem.* **278,** 40842–40850
13. Belisle, J. T., Vissa, V. D., Sievert, T., Takayama, K., Brennan, P. J., and Besra, G. S. (1997) *Science* **276,** 1420–1422
14. De Sousa-D'Auria, C., Kacem, R., Puech, V., Tropis, M., Leblon, G., Houssin, C., and Daffe, M. (2003) *FEMS Microbiol. Lett.* **224,** 35–44
15. Brand, S., Niehaus, K., Puehler, A., and Kalinowski, J. (2003) *Arch. Microbiol.* **180,** 33–44
16. Kacem, R., De Sousa-D'Auria, C., Tropis, M., Chami, M., Gounon, P., Leblon, G., Houssin, C., and Daffe, M. (2004) *Microbiology* (*Read.*) **150,** 73–84
17. Shimakata, T., Iwaki, M., and Kusaka, T. (1984) *Arch. Biochem. Biophys.* **229,** 329–339
18. Shimakata, T., Tsubokura, A. K., and Kusaka, T. (1985) *Arch. Biochem. Biophys.* **238,** 497–508
19. Walker, R. W., Prome, J. C., and Lacave, C. S. (1973) *Biochim. Biophys. Acta* **326,** 52–62
20. Ahibo-Coffy, A., Aurelle, H., Lacave, C., Prome, J. C., Puzo, G., and Savagnac, A. (1978) *Chem. Phys. Lipids* **22,** 185–195
21. Lee, R. E., Armour, J. W., Takayama, K., Brennan, P. J., and Besra, G. S. (1997) *Biochim. Biophys. Acta* **1346,** 275–284
22. 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
23. Garnier, T., Eiglmeier, K., Camus, J. C., Medina, N., Mansoor, H., Pryor, M., Duthoy, S., Grondin, S., Lacroix, C., Monsempe, C., Simon, S., Harris, B., Atkin, R., Doggett, J., Mayes, R., Keating, L., Wheeler, P. R., Parkhill, J., Barrell, B. G., Cole, S. T., Gordon, S. V., and Hewinson, R. G. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 7877–7882
24. Cerdeno-Tarraga, A. M., Efstratiou, A., Dover, L. G., Holden, M. T., Pallen, M., Bentley, S. D., Besra, G. S., Churcher, C., James, K. D., De Zoysa, A., Chillingworth, T., Cronin, A., Dowd, L., Feltwell, T., Hamlin, N., Holroyd, S., Jagels, K., Moule, S., Quail, M. A., Rabbinowitsch, E., Rutherford, K. M., Thomson, N. R., Unwin, L., Whitehead, S., Barrell, B. G., and Parkhill, J. (2003) *Nucleic Acids Res.* **31,** 6516–6523
25. Cole, S. T., Eiglmeier, K., Parkhill, J., James, K. D., Thomson, N. R., Wheeler, P. R., Honore, N., Garnier, T., Churcher, C., Harris, D., Mungall, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R. M., Devlin, K., Duthoy, S., Feltwell, T., Fraser, A., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Lacroix, C., Maclean, J., Moule, S., Murphy, L., Oliver, K., Quail, M. A., Rajandream, M. A., Rutherford, K. M., Rutter, S., Seeger, K., Simon, S., Simmonds, M., Skelton, J., Squares, R., Squares, S., Stevens, K., Taylor, K., Whitehead, S., Woodward, J. R., and Barrell, B. G. (2001) *Nature* **409,** 1007–1011
26. Keilhauer, C., Eggeling, L., and Sahm, H. (1993) *J. Bacteriol.* **175,** 5595–5603
27. Schafer, A., Tauch, A., Jaeger, W., Kalinowski, J., Thierbach, G., and Puehler, A. (1994) *Gene* (*Amst.*) **145,** 69–73
28. Puech, V., Bayan, N., Salim, K., Leblon, G., and Daffe, M. (2000) *Mol. Microbiol.* **35,** 1026–1041
29. Rainwater, D. L., and Kolattukudy, P. E. (1982) *J. Bacteriol.* **151,** 905–911
30. Sassetti, C. M., Boyd, D. H., and Rubin, E. J. (2003) *Mol. Microbiol.* **48,** 77–84
31. Kimura, E., Abe, C., Kawahara, Y., Nakamatsu, T., and Tokuda, H. (1997) *Biochem. Biophys. Res. Commun.* **234,** 157–161
32. Nishio, Y., Nakamura, Y., Kawarabayasi Y., Usuda, Y., Kimura, E., Sugimoto, S., Matsui, K., Yamagishi, A., Kikuchi, H., Ikeo, K., and Gojobori, T. (2003) *Genome Res.* **13,** 1572–1579
33. Pearson, W. R. (1990) *Methods Enzymol.* **183,** 63–98
34. Trivedi, O., Arora, P., Sridharan, V., Tickoo, R., Mohanty, D., and Gokhale, R. S. (2004) *Nature* **428,** 441–445
35. Haase, F. C., Henrikson, K. P., Treble, D. H., and Allen, S. H. (1982) *J. Biol. Chem.* **257,** 11994–11999
36. Besra, G. S., Sievert, T., Lee, R. E., Slayden, R. A., Brennan, P. J., and Takayama, K. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 12735–12739

37. Gurcha, S. S., Baulard, A. R., Kremer, L., Locht, C., Moody, D. B., Muhlecker, W., Costello, C. E., Crick, D. C., Brennan, P. J., and Besra, G. S. (2002) *Biochem. J.* **365,** 441–450
38. Baulard, A. R., Gurcha, S. S., Engohang-Ndong, J., Gouffi, K., Locht, C., and Besra, G. S. (2003) *J. Biol. Chem.* **278,** 2242–2248
39. Hall, P. R., Wang, Y. F., Rivera-Hainaj, R. E., Zheng, X., Pustai-Carey, M., Carey, P. R., and Yee, V. C. (2003) *EMBO J.* **22,** 2334–2337
40. Kremer, L., Dover, L. G., Carrère, S., Nampoothiri, K. M., Lesjean, S., Brown, A. K., Brennan, P. J., Minnikin, D. E., Locht, C., and Besra, G. S. (2002) *Biochem. J.* **364,** 423–430
41. Choi, K. H., Kremer, L., Besra, G. S., and Rock, C.O. (2000) *J. Biol. Chem.* **275,** 28201–28207