J. Microbiol. Biotechnol. (2001), **11**(2), 294–301

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Characterization of the Cell-Surface Barriers to Plasmid Transformation in *Corynebacterium glutamicum*

JANG, KI-HYO[1,4]*, PAUL J. CHAMBERS[2], UCK-HAN CHUN[3], AND MARGARET L. BRITZ[1,5]

[1]*Centre for Bioprocessing and Food Technology,* [2]*Department of Biological and Food Science, Victoria University of Technology, PO Box 14428, MCMC, Melbourne 8001, Australia*
[3]*Department of Food Science and Technology, Institute of Life Science, Kyung Hee University, Suwon 449-701, Korea*
[4]*Graduate School of East-West Medical Science, Kyung Hee University, Hoekidong 1, Dongdaemoon-ku, Seoul 130-701, Korea*
[5]*Department of Food Science and Agribusiness, Gilbert Chandler Campus, The University of Melbourne, Sneydes Rd., Werribee 3030, Australia*

Received: November 27, 2000
Accepted: March 8, 2001

Abstract The effects of including glycine and isonicotinic acid hydrazide (INH) in the growth medium (Luria broth, LBG) on the subsequent lysozyme-induced protoplast formation and transformation efficiency of *Corynebacterium glutamicum* were studied. The transformation efficiency of *C. glutamicum* AS019 increased up to 100-fold as the concentration of glycine in the media increased from 0% to 5% (w/v), relative to cells grown in the absence of glycine. The presence of 5 mg/ml INH in the growth medium led to a further 10-fold increase in transformation efficiency. In addition, this transformation protocol was successfully applied to other strains of *C. glutamicum*. Both chemicals affected the mycolic acid attachment to the cell surface of *C. glutamicum*, when INH was present at 8 mg/ml in growth media. In the presence of glycine and INH, the relative percentage of fatty acids of AS019 to the total lipids (mycolic acid plus fatty acids) decreased from 76.9% (in LBG) to 72.9% (in LBG-2% glycine) and 66.4% (in LBG-8 mg INH/ml), thereby suggesting that these chemicals also inhibit fatty acid synthesis.

Key words: *Corynebacterium glutamicum*, mycolic acid, glycine, isonicotinic acid hydrazide

Nonpathogenic corynebacteria, including *Brevibacterium flavum*, *B. lactofermentum*, *B. ammoniagenes*, and *Corynebacterium glutamicum*, have been used traditionally for industrial production of amino acids and nucleotides [22, 24, 38]. Over the last two decades, strain improvement has progressed from using mutation-selection approaches to developing recombinant DNA methods for improving both endogenous biochemical pathways and the expression of heterologous genes [3, 34]. Accordingly, the development of an efficient method for introducing DNA into *C. glutamicum* is needed to utilize these advances. However, two major factors have been noted as contributing to the poor transformation efficiencies of *C. glutamicum* and related species: the presence of a restriction-modification system(s) [12, 17, 18] and a complex cell wall structure [2, 15]. The latter is evident from the very low transformation frequencies seen when cells are not cultured in the presence of media additives, such as penicillin G, Tween 80, glycine, or isonicotinic acid hydrazide (INH), that target cell surface structures [6, 22, 34, 39]. The saprophytic corynebacteria and *Brevibacterium* species are taxonomically related to *Mycobacteria*, *Rhodococcus*, and *Nocardia*, which are distinguished by the unique presence of mycolic acids in their cell walls, that otherwise contain peptidoglycan and arabinogalactan [1, 7, 8, 23, 36]. In mycobacteria, the covalently-connected structure of the cell wall is made of peptidoglycan, to which arabinogalactan is linked *via* a phosphodiester bridge [5]. About 10% of the arabinose residues in arabinogalactan are, in turn, bound to mycolic acid [25]. Indeed, mycolic acids are a major component of the cell wall structure of mycolic acid-containing bacteria where, for instance, up to 60% by weight of the mycobacterial cell wall is composed of mycolic acids [21]. Mycolic acids have a structural role which gives rise to the resistance of cells to harsh environments and causes the acid-fastness of mycobacteria; they probably play an important role in the restricted permeability of these microbes to water-soluble molecules [20]. Glycine and INH in the growth medium are known to modify cell wall structures in Gram-positive species and mycobacteria. In the present study, we investigated the impact of these chemicals on protoplast formation and the

*Corresponding author
Phone: 82-2-961-0506; Fax: 82-2-961-9215;
E-mail: kihyojang@hotmail.com

subsequent efficiency of plasmid transformation. Using these chemicals, we also analyzed the effect of glycine and INH on the fatty acid and mycolic acid compositions of *C. glutamicum* cells. Based on these observations and our previously published results, we proposed a putative model for the structure of the corynebacteria cell wall.

MATERIALS AND METHODS

Bacterial Strains, Media, and Growth Conditions

The strains of *C. glutamicum* used were: AS019, a spontaneous rifampicin-resistant mutant of ATCC 13059 [39]; CG2, a wild-type strain obtained from the Queensland University Culture Collection (No. 2740), Australia. The plasmid used was pCSL17, a 7.2 kb *Escherichia coli*-corynebacteria shuttle vector [16]. All strains were routinely grown in Luria broth [26] supplemented with 0.5% glucose (LBG), at 30°C with agitation (200 rpm) in a shaker incubator. The glycine (0–10%, w/v) and INH (0–10 mg/ml) were added from sterile stock solutions (20%, w/v, glycine and 100 mg INH per ml). The specific growth rates (μ) was determined based on changes in A_{600} in the *C. glutamicum* strains in shake-flask cultures of LBG supplemented with up to 8 mg/ml INH or 10% (w/v) glycine, and expressed as a percentage relative to the growth in the LBG.

Plasmid DNA Isolation

The plasmid DNA was isolated from *C. glutamicum* using the alkaline lysis method, as described by Britz and Best [6], and further purified using CsCl-EtBr density gradient centrifugation [32].

Protoplast Formation

The protoplasts of *C. glutamicum* were prepared as described previously [2]. The starter cultures were grown overnight in LBG and then subcultured into a fresh LBG medium or LBG supplemented with glycine and/or INH. The protoplasts were routinely prepared from cells harvested by centrifugation during the mid-exponential phase. To obtain the total viable count before and after transformation, the cells were diluted in an SMMC buffer (0.7 M sucrose; 50 mM maleic acid; 10 mM $MgCl_2 \cdot 6H_2O$; 10 mM $CaCl_2$, pH 6.5) or in water and spread onto NAG (Nutrient Broth no. 2 with 1.2% (w/v) agar plus 0.5% glucose) or ET agar [2]. The protoplasting efficiencies were determined by comparing the differences in the counts on NAG and ET plates following dilution in SMMC buffer or distilled water. Dilution in SMMC and plating onto ET media produced counts for the total number of cells (spheroplasts, protoplasts, and normal cells), whereas dilution in SMMC followed by plating onto NAG determined the spheroplasts and normal cells, as the protoplasts failed to recover on NAG, since it provided insufficient osmotic protection. When the cells were diluted in water and spread onto either ET or NAG, only normal cells grew, as the osmotically sensitive cells (spheroplasts and protoplasts) were lyzed during dilution.

Transformation and Regeneration of Protoplasts

C. glutamicum strains are short rods, yet following lysozyme treatment and the formation of spheroplasts and protoplasts, they form spherical shapes of different sizes when viewed under a phase contrast microscope, where the protoplasts are almost transparent and the spheroplasts are dark in various phases. This difference in morphology was used to follow protoplasting to assess the progress of the lysozyme treatment. Osmotically-sensitive cells were transformed using the polyethylene glycol-mediated transformation method, as described by Britz and Best [6]. Viable counts were also performed on the NAG and ET plates before and after transformation, using SMMC as the diluent, to determine the initial number of cells in the transformation (surviving the protoplasting treatment) and the survivors of this treatment. The transformants were regenerated after 2 to 10 days at 30°C. The transformation efficiency was calculated as the number of transformants/10^9 cells used/ μg of plasmid DNA added. Results reported for each type of experiment were consistent between replicates within any one experiment but some quantitative variations in the transformation efficiency occurred between separate experiments, which has also been reported by other workers [35].

Isolation and Analysis of Fatty and Mycolic Acids from Whole Cells and Culture Fluids

The preparation and gas chromatography (GC) analysis of the fatty and mycolic acids were carried out as described by Jang *et al.* [19], with minor modifications. Cells (50 mg, dry weight, depending on the culture) were harvested during stationary phase by centrifugation (2,000 ×g, 4°C, 10 min); the supernatant was then collected and used as the source for the fatty and mycolic acids in the culture fluids. Both samples were stored at -20°C until analysis. Stored cells were lyophilized and subjected to acid methanolysis (methanol/toluene/H_2SO_4, 30:15:1, v/v/v, 3 ml) in sealed tubes for 16 h at 80°C, as previously described by Minnikin *et al.* [27]. The liberated mycolic acid methyl esters (MAMEs) and fatty acid methyl esters (FAMEs) were extracted using petroleum ether (b.p. 60–80°C, 3×2 ml). The extracts were combined, then concentrated by drying under a N_2 flow, and the residues were treated with 100 μl of trimethylsilylimidazole (TRI-SIL Z, Pierce) for 20 min at 60°C to form trimethylsilyl (TMS) ethers. The samples were analyzed by GC or GC-MS.

To quantify the fatty and mycolic acids in the culture supernatant samples from the flask studies, the fatty and mycolic acids in the culture fluids were extracted as described by Bligh and Dyer [4] (for data reported in

Tables 2 and 3). The filtered culture fluids, 30 ml or 100 ml, were mixed at 30°C for 1 h, 100 rpm, with 2.5 volumes of chloroform/methanol (1:2, v/v), then held stationary for 4 h. Subsequently, one volume of chloroform and one volume of deionized water were added, incubated for 1 h at 100 rpm, then the mixture was transferred to a separating funnel and allowed to separate for 16 h. After collecting the bottom layer, the extract was dried, derivatized by acid methanolysis; the FAMEs and MAMEs were extracted into petroleum ether, then concentrated under nitrogen before trimethylsilylation, as above.

Gas Chromatography and Mass Spectrometry (GC-MS)

When determining the effect of growth in the presence of glycine and INH on the mycolic acid composition, 100 µl of lignoceric acid methyl ester ($C_{25}H_{50}O_2$, Sigma St. Louis, U.S.A.) was added as an internal standard to each sample prior to extraction and all analyses standardized using the area obtained following extraction and derivatization of this C_{25} internal standard. The TMS ethers were analyzed by GC using a Varian Star 3400 *CX* gas chromatograph fitted with flame ionization detector (FID). One ml of the solution was injected onto a 25 m nonpolar BPX5 (0.22 mm i.d.; 0.33 mm o.d., fused silica, SGE, Scientific Pty, Ltd, Australia) with the split ratio set at 85:1. The oven temperature program was 150°C isothermally for 1 min, then increased by 5°C/min to 165°C, then increased to 185°C by 0.3°C/min. The column temperature was increased to 260°C by 6°C/min, increased by 2°C/min to 320°C, and held at 320°C for 6.5 min. The injection temperature and detector temperature were 300°C. Nitrogen was used as the carrier gas (0.9 ml/min).

The identity of the fractionated mycolic acid TMS ethers obtained using a GC was determined by GC-MS (Carlo Erba/Kratos High Resolution GC/MS or a Varian Saturn II GC/MS) equipped with a direct insertion probe. For the GC-MS analysis, the column (25 m nonpolar BPX5) was directly coupled to the ion source which was held at 300°C, and helium (1.0 ml/min) was used as the carrier gas. The oven temperature was programmed at 260°C for 1 min, then increased by 10°C/min to 340°C. Electron impact ionization was used and the ionization potential was 70 eV.

The reproducibility of the analyses was verified as follows. Five injections were carried out using various concentrations of mycolic acids. As a result, a linear relationship was observed between the peak areas detected corresponding to each mycolic acid and the amount of mycolic acids applied, with less than a 2% variation in the detected amounts between injections. For the analyses of both the fatty acid and mycolic acid compositions, replicates were performed within each experiment and several independent experiments were performed for each condition. The trends seen for the relative proportions of mycolic acids present were identical in every analysis for each set of experimental conditions.

Results and Discussion

Effect of Glycine and INH on Growth of *C. glutamicum*

The *C. glutamicum* strains AS019 and CG2 were grown in LBG in the presence of a range of either glycine or INH concentrations. For both strains, the growth rate decreased with an increase in the glycine or INH concentration. For *C. glutamicum* CG2, the growth rate of the cultures was inhibited by 50% when grown in the presence of either 2.0–3.0% (w/v) glycine or about 3.0 mg/ml INH. The growth rate of *C. glutamicum* AS019 was reduced by 50% when grown in the presence of either 4% (w/v) glycine or 8.0 mg/ml INH (Fig. 1).

Effect of Growth in INH and Glycine on Protoplast Formation and Transformation Efficiency of Corynebacteria

The efficiency of transformation in bacteria is typically dependent on the growth phase during which the host cells are harvested. To test whether this general phenomenon applies to *C. glutamicum*, AS019 was harvested at different stages during its growth in LBG with 2.5% glycine, and the protoplasting and transformation efficiencies were tested following lysozyme treatment. The cells were harvested at optical densities ranging from 0.11 to 1.53 (A_{600}), covering growth from the lag to the late exponential-early stationary phase. The harvested cells were then adjusted to the same cell density, equivalent to an A_{600} of 0.2, by concentration using centrifugation or dilution. The results indicated that the cells harvested earlier in the growth cycle were protoplasted and transformed more efficiently (data not shown). This observation is consistent with the data obtained by Haynes and Britz [15] for the transformation of corynebacteria

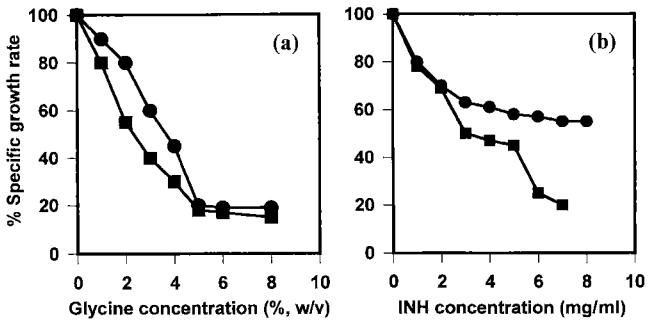

Fig. 1. Effect of glycine (a) and INH (b) on specific growth rates of *C. glutamicum* strains AS019 (●) and CG2 (■).
Each point on the graphs is based on the mean of two independent experiments. The specific growth rates are expressed as a percentage relative to the rates seen in LBG without supplementation.

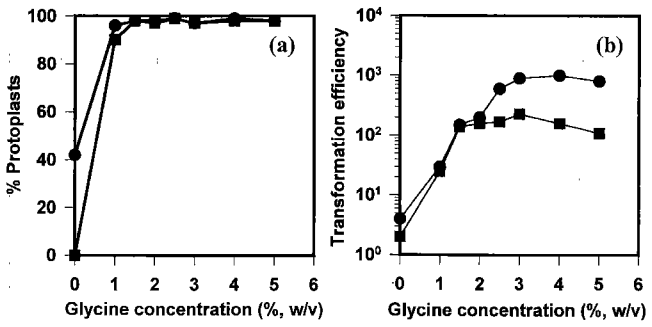

Fig. 2. Effect of glycine concentration on protoplast formation (a) and protoplast transformation efficiency for *C. glutamicum* AS019 (b) for cells cultured in LBG (■) or LBG plus 5 mg/ml INH (●).

The transformations were carried out on cells harvested from cultures at A_{600} between 0.08–0.24 and treated with 2.5 mg/ml lysozyme for 2 h. The transformation mixture (80 µl) contained 1.26 µg of homologous pCSL17 DNA. The enumeration of the transformants was done in triplicate on ET plates containing kanamycin (50 µg/ml). The % protoplast is the number of viable osmotically sensitive cells divided by the total number of viable cells after lysozyme treatment.

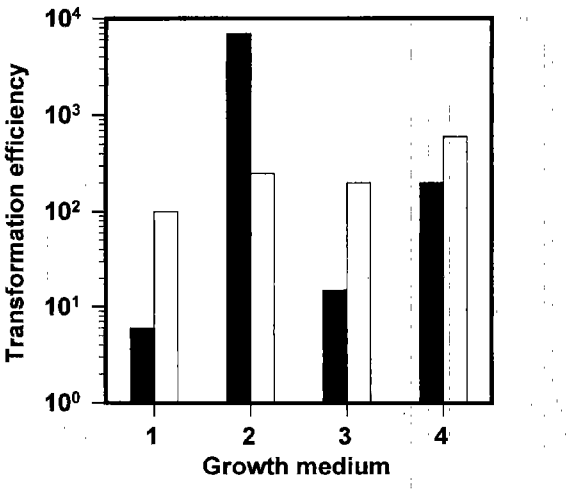

Fig. 3. Transformation efficiencies of two strains of *C. glutamicum* [AS019 (□) and CG2 (■)] with pCSL17 DNA from cultures grown in LBG supplemented with 2% glycine (1), 2% glycine plus 5 mg INH/ml (2), 2.5% glycine (3), and 2.5% glycine plus 5 mg INH/ml (4).

The cells grown on the four different media were harvested at A_{600} between 0.16–0.23 and protoplasted as described in Materials and Methods. The transformation mixture (80 µl) contained 1.26 µg of homologous pCSL17 DNA. The enumeration of the transformants was performed in triplicate on ET plates containing kanamycin (50 µg/ml).

using electroporation. From Fig. 2, it is evident that *C. glutamicum* AS019 cells grown in LBG without INH or glycine were recalcitrant to lysozyme-induced protoplast formation; no protoplasting was detected in the cells from such cultures. In contrast, *C. glutamicum* AS019 grown in the presence of 5.0 mg/ml INH did form protoplasts following lysozyme treatment. The AS019 grown in the presence of 1% (w/v) glycine was even more sensitive to lysozyme treatment, with about 90% of its cells forming protoplasts, plus the addition of INH in the medium improved this marginally. The transformation efficiencies for the protoplasted *C. glutamicum* AS019 grown to early-exponential phase in either LBG or LBG containing 5.0 mg/ml INH were found to be very low (Fig. 2). However, the transformation efficiency was markedly improved when the cells were grown in the presence of glycine. When glycine was present at concentrations >2%, the transformation efficiency was 100-fold higher relative to cells grown in LBG without glycine. The transformation efficiency was further increased by the presence of 5 mg/ml INH. This enhancement by INH was particularly evident at glycine concentrations of 4.0% and 5.0%, which exhibited values 10-fold above those seen for cells grown in glycine alone. The optimum growth conditions for protoplast transformation of strain AS019 were seen for cells cultured in LBG containing 2.5–3% glycine plus 4–5 mg/ml INH, which resulted in good recovery rates of protoplasts (data not shown) and maximum transformation efficiencies. A possible explanation for the effectiveness of the glycine-supplemented medium in predisposing the cells to lysozyme treatment for protoplast transformation is that lysozyme and glycine both disrupt the peptidoglycan structure of the cell wall, and therefore perhaps act synergistically. INH is known to affect mycolic acid synthesis in mycobacteria and is likely to play a similar role in corynebacteria species, although the concentrations of INH that inhibit growth of *C. glutamicum* are much higher than those needed to inhibit mycobacteria. This may also contribute to improving access of lysozyme to the peptidoglycan, depending on the arrangement of lipids on the cell surface.

To confirm the above finding, *C. glutamicum* CG2 was tested for transformation efficiency following growth in the presence of glycine and glycine plus INH (Fig. 3). The results indicated that, like *C. glutamicum* AS019, CG2 grown in the presence of glycine or glycine plus INH showed a higher protoplast transformation efficiency; the cells grown in the absence of glycine, or glycine plus INH, transformed poorly, however, this was improved when the cells were cultured in glycine concentrations of 2–5% glycine (data not shown). The presence of 5.0 mg/ml INH with glycine in the growth medium further improved the transformation efficiency by at least an order of magnitude. Strain CG2 was more sensitive to growth inhibition by glycine, so that the combination of 2% glycine and 5 mg/ml INH used produced a very significant change in efficiency of transformation. At higher concentrations of glycine plus INH, the cells may well have protoplasted too efficiently so that fewer cells survived transformation, corresponding to the lower transformation efficiency seen. These results indicate that there is strain-to-strain variation in the impact of glycine and INH on both growth and protoplast transformation, so that it is important to optimize the

concentrations used for each strain to obtain optimum transformation efficiency.

Effect of Growth in Presence of Glycine and INH on Fatty and Mycolic Acids Compositions

The changes observed in cell growth rate, protoplasting ability, and efficiency of transformation of AS019 following growth in the presence of glycine and INH may be explained by the modulation of the cell-surface components. To evaluate any changes in the synthesis of fatty and mycolic acids in AS019, this strain was grown in a shake-flask culture in LBG and LBG containing either 2% (w/v) glycine or 5 mg/ml INH, or a combination of both, and the cells harvested in the late exponential-phase. This growth phase was used because it allowed sufficient quantities of lipids to be collected for analysis, particularly from extracellular fluids. Our previous results indicated that the type and proportion of lipids did not change significantly over the growth cycle, except for a small increase in extracellular mycolic acids [19]. Using the protocol described in Materials and Methods, the fatty acids and mycolic acids were both fractionated in the same chromatogram. Figure 4 shows a typical GC profile for *C. glutamicum* AS019 and the fragment pattern of the TMS ethers, which

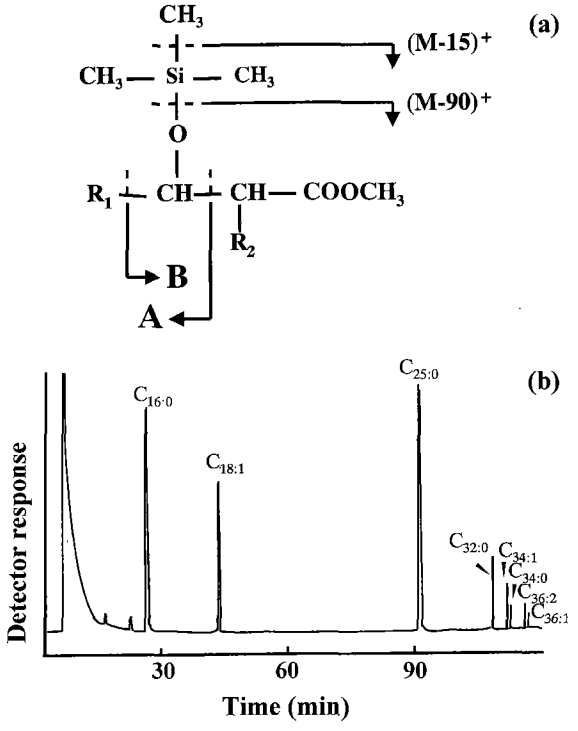

Fig. 4. Fragmentation scheme for TMS ethers of MAMEs (a) and gas chromatogram of TMS derivatives of FAMEs and MAMEs from *C. glutamicum* AS019 grown in LBG (b).
The conditions for the gas chromatography are described in Materials and Methods. For each peak, the first number indicates the number of carbon atoms and the second indicates the number of double bonds.

Table 1. Mycolic acid structures of *C. glutamicum*.

Formula of acids	Side chain		No. of double bonds
	R1	R2	
$C_{32}H_{64}O_3$	$C_{15}H_{31}$	$C_{14}H_{29}$	0
$C_{34}H_{66}O_3$	$C_{17}H_{33}$ or $C_{15}H_{31}$	$C_{14}H_{29}$ $C_{16}H_{31}$	1
$C_{34}H_{68}O_3$	$C_{17}H_{35}$ or $C_{15}H_{31}$	$C_{14}H_{29}$ $C_{16}H_{33}$	0
$C_{36}H_{68}O_3$	$C_{17}H_{33}$	$C_{16}H_{31}$	2
$C_{36}H_{70}O_3$	$C_{17}H_{35}$	$C_{16}H_{31}$	1

See Fig. 4, for overall structure of mycolic acids.

were analyzed using a GC-MS to determine the overall structure of the mycolic acids. Palmitic ($C_{16:0}$) and oleic ($C_{18:1}$) acids constituted 96–98% of the total fatty acids detected. Myristic acid ($C_{14:0}$), pentadecanoic acid ($C_{15:0}$), palmitoleic acid ($C_{16:1}$), heptadecanoic acid ($C_{17:0}$), and stearic acid ($C_{18:0}$) were also detected as minor components. Five types of mycolic acid were fractionated and identified ($C_{32:0}$, $C_{34:0}$, $C_{34:1}$, $C_{36:1}$, and $C_{36:2}$) (Table 1). The approach used for the present investigation involved derivatizing the fatty and mycolic acids into FAMEs and MAMEs, then converting the FAMEs and MAMEs into TMS ethers. The conversion of MAMEs into TMS ethers protects the molecules from pyrolysis and enables their analysis by conventional GC at a high temperature. Table 2 shows that a relatively small proportion of the total mycolic acids was found in the filtered culture fluids and the mycolic acid profiles for the whole cells and culture fluids were similar for the four media tested (LBG alone and LBG containing 2% [w/v] glycine, 5 mg/ml INH, or both glycine and INH).

Table 2. Comparison of mycolic acids found in whole cells and culture fluids of *C. glutamicum* AS019 grown in four different media.

Mycolic acids	Growth medium			
	LBG[a]	LBG-G	LBG-I	LBG-GI
Mycolic acid from whole cells				
$C_{32:0}$	53.0	52.7	49.1	55.9
$C_{34:1}$	30.0	27.4	32.9	27.9
$C_{34:0}$	8.8	10.8	9.0	10.6
$C_{36:2}$	5.3	6.3	6.2	4.1
$C_{36:1}$	2.9	2.9	2.9	2.4
Mycolic acid from culture fluids				
$C_{32:0}$	54.2	54.1	53.6	57.4
$C_{34:1}$	30.4	28.2	31.9	27.9
$C_{34:0}$	7.6	10.3	7.5	9.3
$C_{36:2}$	6.1	4.4	5.0	3.7
$C_{36:1}$	2.1	2.9	2.1	1.6
Relative % of total[b]	4.5	15.9	4.2	15.9

[a]Abbreviations: LBG-G, LBG-I or LBG-GI, LBG supplemented with 2% glycine and/or 5 mg INH/ml.
[b]Proportion (%) of mycolic acids found in culture fluids relative to total mycolic acids detected (whole cells plus culture fluids).

However, the presence of glycine (2%, w/v) caused an increase in the proportion of mycolic acids found in the extracellular fluids and the presence of INH (5 mg/ml) did not further increase this: the proportions of extracellular mycolic acids were 4.5% (LBG alone and LBG supplemented with INH) and 15.9% (LBG supplemented with glycine or glycine plus INH) (Table 2). The proportion of extracellular mycolic acids for cells grown in LBG-I only increased when INH concentrations were 8 mg/ml or higher (data not shown).

The putative target sites of glycine and INH are different and glycine affects the biosynthesis of peptidoglycan [14]. Nam *et al.* [28] reported that in the jar fermentor culture with the glycine at an initial concentration of 2% (w/v), L-ornithine production increased by 28%, compared to that of the culture with no glycine added. However, the present observation suggests that glycine impacts either directly or indirectly on the attachment of mycolic acids to the cell surface, where the latter may involve dislocation of peptidoglycan plus the attached mycolic acids during growth in glycine. Although the cell surface of *C. glutamicum* has not been studied in detail, based on previous work on related species and mycobacteria, it is likely that mycolic acids link covalently to the arabinose units of the peptidoglycan *via* a phosphodiester bridge [5, 30]. In contrast to this, INH is known to affect mycolic acid synthesis in mycobacteria species. INH (trivial name, isoniazide) is a potent antitubercular agent which has been used for 40 years for the chemotherapy of tuberculosis, caused by *Mycobacterium* species [9, 36]. Although the mode of INH is not fully understood, INH decreases the amount of mycolic acids and possible intermediates, very long-chain nonpolar fatty acids, in *Nocardia* species [36], and INH inhibited the enzymatic biosynthesis of very long-chain fatty acids in *M. tuberculosis* $H_{37}Ra$ [31]. Recently, one of the target sites of INH in several mycobacterial species was identified as the InhA protein, which is a 2-trans-enoyl-acyl carrier protein involved in fatty acid synthesis [9]. The presence of an *inhA* gene homolog has also been detected in *C. glutamicum* (Hafeez *et al.* [13]), thereby supporting the hypothesis that the biosynthetic pathway for mycolic acids is also related to fatty acid metabolism in this species [11, 33, 37]. Shimakata *et al.* [33] demonstrated biosynthetic activity for mycolic acids ($C_{34:0}$, $C_{34:1}$, $C_{36:0}$, and $C_{36:1}$) in the *Bacterionema* (now *Corynebacterium*) *matruchotti*. They found that when cells were grown in [1-^{14}C]-stearic acid, the label is incorporated into two major radioactive peaks: one corresponding to the peak of ($C_{34:0}$+$C_{34:1}$), the other to ($C_{36:0}$+$C_{36:1}$) mycolic acid. Similar condensations were established for other groups of corynebacteria [11, 37]. Table 3 shows the relative proportion of fatty acids of *C. glutamicum* strain AS019 after growth in three different media. AS019 showed approximately the same amount of $C_{16:0}$ and $C_{18:1}$ when the cells were grown in LBG. The relative proportions of fatty acids to total esters (fatty acids plus mycolic acids) were 76.9% (in cells) and 88.2% (in culture fluids). A small proportion of a $C_{18:3}$ fatty acid was also found in extracellular culture fluids (data not shown). In the presence of 2% glycine or 8 mg/ml INH in LBG, the fatty acid composition was hardly changed: the relative proportions of $C_{16:0}$ ranged between 48.9% (in INH medium) to 51.4% (in LBG medium). The most significant observation was changes in the percentage of fatty acids to total acids (fatty acids plus mycolic acids). In the presence of glycine or INH, the relative percentage of cellular fatty acids decreased from 76.9% in LBG to 72.9% in LBG-2% glycine, and 66.4% in LBG-8 mg/ml INH. Similar decreases in the relative proportion of extracellular fatty acids were also observed (Table 3). This indicates that growth in glycine and INH influenced not only the mycolic acid biosynthesis but also the biosynthesis of fatty acids. The results presented here also showed the presence of extracelluar fatty acids in the culture fluids (5.0 to 8.5% of the total detected). An analysis of the extracellular fatty acids suggested that some fatty acids of *C. glutamicum* are probably located near the cell surface and leak out during growth. In mycobacteria, fatty acids are found in both the cell wall and the cytoplasmic membrane [30]. In the present study of fatty acids in *C. glutamicum*, since the starting materials were obtained from whole cells rather than the cell wall, it is difficult to know with any certainty whether the changes caused by the presence of INH or glycine in the relative proportion of fatty acids occurred at the cell surfaces, the membranes, or both. However, the main effect of the addition of glycine (2%) or INH (8 mg/ml) was to decrease the proportion of fatty acids relative to mycolic acids found in both the cell-associated and

Table 3. Quantitative analysis of fatty acids of *C. glutamicum* AS019 grown in three different media.

Medium[a]	Fatty acids[b]		% FAMEs[c]	% extracellular[d] FAMEs
	$C_{16:0}$	$C_{18:1}$		
Fatty acids from whole cells				
LBG	51.4	48.7	76.9	
LBG-G	50.0	50.0	72.9	
LBG-I	48.9	51.2	66.4	
Fatty acids from culture fluids				
LBG	52.1	47.8	88.2	8.2
LBG-G	47.0	53.0	64.7	5.0
LBG-I	49.0	51.0	66.4	8.5

[a]Abbreviations: LBG-G, LBG supplemented with 2% (w/v) glycine; LBG-I, LBG supplemented with 8 mg INH/ml.
[b]Proportion of each TMS derivative of FAMEs was calculated as % of total in terms of peak area detected.
[c]Proportion (%) of FAMEs relative to total ethers (FAMEs plus MAMEs).
[d]Proportion of FAMEs found in culture fluids relative to total fatty acids detected (whole cells plus culture fluids).

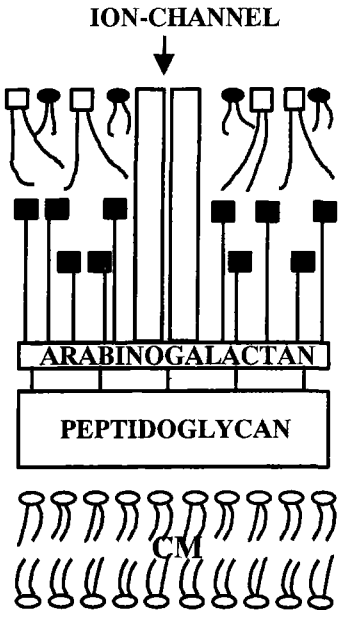

Fig. 5. Proposed model for structure of corynebacterial cell wall. CM, cytoplasmic membrane.
Legends: (■) mycolic acid; (□) extractable mycolic acid; (●) extractable fatty acid.

extracellular fractions. Accordingly, if these two major fatty acids are used as substrates for the biosynthesis of mycolic acids, it can be postulated that the presence of glycine or INH in the medium will inhibit both the mycolic acid synthesis and fatty acid synthesis. In the latter case, the inhibitory mode seems to be more significant. This observation indicates indirectly that fatty acids are present in the cell wall, and INH may inhibit the early stage of fatty acid synthesis. Based on the present information and that reported by other sources, we propose a model for the structure of the *C. glutamicum* cell wall (Fig. 5), which is based on the model of the mycobacterial cell wall [30]. Unlike the mycobacterial model, since the presence of solvent-extractable mycolic acids (approximately 50% of total cellular mycolic acids) has been previously reported [19] and the present work indicated the presence of fatty acids in the cell wall, both extractable mycolic and fatty acids are included in this model. The model also shows the presence of an ion-channel, which was recently established by Niederweis *et al.* [29]. Furthermore, Dufréne *et al.* [10] reported that the surface of *Corynebacterium* (*Corynebacterium* species strains DSM 44016 and DSM 6688) is rich in hydrocarbon-like compounds (about 40%), including mycolic acids. Both strains of corynebacteria were found to include 23-27% (dry weight percentage of cell wall constituents) of peptidoglycan and 14% protein. Therefore, the cell wall structure of *C. glutamicum* is quite a complicated structure which contains peptidoglycan, arabinogalactan, free mycolic acids, covalently-bound mycolic acids, free or covalently-bound fatty acids (not yet experimentally studied), proteins and peptides, and an ion-channel. We are currently pursuing further information on the biochemistry of the mycolic acid synthesis in *C. glutamicum* and the properties of the *inh*A gene in this species to provide some insight into the mycolic- and fatty acids synthesis in this species.

REFERENCES

1. Barksdale, L. and K. S. Kim. 1977. *Mycobacterium. Bacteriol. Rev.* **41:** 217-372.
2. Best, G. R. and M. L. Britz. 1986. Facilitated protoplasting in certain auxotrophic mutants of *Corynebacterium glutamicum*. *Appl. Microbiol. Biotechnol.* **23:** 288-293.
3. Billman-Jacobe, H., L. Wang, A. Kortt, D. Stewart, and A. Radford. 1995. Expression and secretion of heterologous protease by *Corynebacterium glutamicum*. *Appl. Environ. Microbiol.* **61:** 1610-1613.
4. Bligh, E. G. and W. J. Dyer. 1959. A rapid method for total lipid extraction and purification. *Can. J. Biochem. Phys.* **37:** 911-917.
5. Brennan, P. J. and H. Nikaido. 1995. The envelope of mycobacteria. *Annu. Rev. Biochem.* **64:** 29-63.
6. Britz, M. L. and G. R. Best. 1986. Expression of chloramphenicol resistance specified by plasmid pHY416 hosted in *Corynebacterium glutamicum*. *Current Microbiol.* **14:** 13-17.
7. Chevalier, J., M.-T. Pommier, A. Cremieux, and G. Michel. 1988. Influence of tween 80 on the mycolic acid composition of three cutaneous corynebacteria. *J. Gen. Microbiol.* **134:** 2457-2461.
8. Collins, M. D., M. Goodfellow, and D. E. Minnikin. 1982. A survey of the structures of mycolic acids in coryneform bacterium and related taxa. *J. Gen. Microbiol.* **128:** 129-149.
9. Dessen, A., A. Quemard, J. S. Blanchard, W. R. Jacobs Jr., and J. C. Sacchettini. 1995. Crystal structure and function of the isoniazid target of *Mycobacterium tuberculosis*. *Science* **267:** 1638-1641.
10. Dufréne, Y. F., A. van der Wal, W. Norde, and P. G. Rouxhet. 1997. X-ray photoelectron spectroscopy analysis of whole cells and isolated cell walls of Gram-positive bacteria: Comparison with biochemical analysis. *J. Bacteriol.* **179:** 1023-1028.
11. Gastambide-Odier, M. and E. Lederer. 1959. Biosynthesis of corynemycolic acid from two molecules of palmitic acid. *Nature* **184:** 1563-1564.
12. Ha, G. S., J. Kim, and Y. J. Choi. 1999. Transformation of *Bacillus strearothermophilus* No. 236 by changing incubation temperature after electroporation. *J. Microbiol. Biotechnol.* **9:** 687-690.
13. Hafeez, S. A., M. D. E. Serificia, and M. L. Britz. 2000. *Corynebacterium glutamicum* Inh A (*inhA*) gene. Genebank accession number AF139472.
14. Hammes, W., K. H. Schleifer, and O. Kandler. 1973. Mode of action of glycine on the biosynthesis of peptidoglycan. *J. Bacteriol.* **116:** 1029-1053.

15. Haynes, J. A. and M. L. Britz. 1990. The effect of growth conditions of *Corynebacterium glutamicum* on the transformation frequency obtained by electroporation. *J. Gen. Microbiol.* **136:** 255-263.
16. Hodgson, A. L. M., J. A. Hayes, M. L. Britz, and I. T. Nisbet. 1989. Construction and utilisation of a *Corynebacterium-E. coli* shuttle vector. *In: Proceeding of the VIIIth Biotechnology Conference*, Sydney, Australia. Australian Biotechnology Association (Melbourne, Australia). pp. 181-184.
17. Jang, K. H. and M. L. Britz. 2000. Improved electrotransformation frequencies of *Corynebacterium glutamicum* using cell-surface mutants. *Biotechnol. Lett.* **22:** 539-545.
18. Jang, K. H., P. J. Chambers, and M. L. Britz. 1996. Analysis of nucleotide methylation in DNA from *Corynebacterium glutamicum* and related species. *FEMS Microbiol. Lett.* **136:** 309-315.
19. Jang, K. H., D. Pierotti, G. W. Kemp, G. R. Best, and M. L. Britz. 1997. Mycolic acid composition of *Corynebacterium glutamicum* and its cell surface mutants: Effects of growth in glycine and isonicotinic acid hydrazide. *Microbiol.* **143:** 3209-3221.
20. Jarlier, V. and H. Nikaido. 1990. Permeability barrier to hydrophilic solutes in *Mycobacterium cheloni. J. Bacteriol.* **172:** 1418-1423.
21. Jarlier, V. and H. Nikaido. 1994. Mycobacterial cell wall; Structure and role in natural resistance to antibiotics. *FEMS Microbiol. Lett.* **123:** 11-18.
22. Jetten, M. S. and A. J. Sinskey. 1995. Recent advances in the physiology and genetics of amino acid-producing bacteria. *Critical Rev. Biotechnol.* **15:** 73-103.
23. Kim, M. J., J. H. Lee, and J. C. You. 2000. A new and rapid testing method for drug susceptibility of *Mycobacterium leprae* using RT-PCR. *J. Microbiol. Biotechnol.* **10:** 685-689.
24. Kim, T. H., N. G. Suck, J. H. Kwak, S. Y. Lee, and H. S. Lee. 2000. Effect of *tktA*, *aroF*^{FBR}, and *aroL* expression in the tryptophan-producing *Escherichia coli. J. Microbiol. Biotechnol.* **10:** 789-796.
25. McNeil, M. and P. J. Brennan. 1991. Structure, function and biogenesis of the cell envelope of mycobacteria in relation to bacterial physiology, pathogenesis and drug resistance; some thoughts and possibilities arising from recent structure information. *Res. Microbiol.* **142:** 451-463.
26. Miller, J. H. 1972. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
27. Minnikin, D. E., I. G. Hutchinson, A. B. Caldicott, and M. Goodfellow. 1980. Thin layer chromatography of methanolysates of mycolic acid containing bacteria. *J. Chromatogr.* **188:** 221-233.
28. Nam, S. W., D. K. Choi, W. S. Ryu, H. W. Jang, B. H. Chung, and Y. H. Park. 1994. Effect of glycine on L-ornithine production by a citrulline auxotroph of *Brevibacterium ketoglutamicum* and stoichiometric analysis. *J. Microbiol. Biotechnol.* **4:** 95-101.
29. Niederweis, M., E. Maier, T. Lichtinger, R. Benz, and R. Krämer. 1995. Identification of channel-forming activity in the cell wall of *Corynebacterium glutamicum. J. Bacteriol.* **177:** 5716-5718.
30. Nikaido, H., S. H. Kim, and E. Y. Rosenberg. 1993. Physical organization of lipids in the cell wall of *Mycobacterium chelonae. Mol. Microbiol.* **8:** 1025-1030.
31. Quémard, A., J. C. Sacchettine, A. Dessen, C. Vilcheze, R. Bittman, W. R. Jacobe-Jr., and J. S. Blanchard. 1995. Enzymatic characterization of the target for isoniazid in *Mycobacterium tuberculosis. Biochemistry* **34:** 8235-8241.
32. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual.* 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
33. Shimkata, T., M. Iwaki, and T. Kusaka. 1984. *In vitro* synthesis of mycolic acids by the fluffy layer fraction of *Bacterionema matuchotii. Archiv. Biochem. Biophys.* **229:** 329-339.
34. Smith, M. D., J. L. Flickinger, D. W. Lineberger, and B. Schmidt. 1986. Protoplast transformation in coryneform bacteria and introduction of α-amylase gene from *Bacillus amyloliquifacients* into *Brevibacterium lactofermentum. Appl. Environ. Microbiol.* **51:** 634-639.
35. Thierbach, G., A. Schwarzer, and A. Pühler. 1988. Transformation of spheroplasts and protoplasts of *Corynebacterium glutamicum. Appl. Microbiol. Biotechnol.* **29:** 356-362.
36. Tomiyasu, I. and I. Yano. 1984. Isonicotinic acid hydrazide induced changes and inhibition in mycolic acid synthesis in *Nocardia* and related taxa. *Arch. Microbiol.* **137:** 316-323.
37. Walker, R. W., J.-C. Prome, and C. Lacave. 1973. Biosynthesis of mycolic acids: Formation of C_{32} β-keto ester from palmitic acid in a cell-free system of *Corynebacterium diphtheriae. Biochim. Biophys. Acta* **326:** 52-62.
38. Yoon, K. H., H. Yim, and K. H. Jung. 1998. Cloning, expression and nucleotide sequencing of the gene encoding glucose permease of phosphotransferase system from *Brevibacterium ammoniagenes. J. Microbiol. Biotechnol.* **8:** 214-221.
39. Yoshihama, M., K. Higarshiro, E. A. Rao, M. Akedo, W. G. Shanabruch, M. T. Follettie, G. C. Walker, and A. J. Sinskey. 1985. Cloning vector system for *Corynebacterium glutamicum. J. Bacteriol.* **162:** 591-597.