RESEARCH ARTICLE

INTERNATIONAL MICROBIOLOGY (2009) 12:29-38
DOI: 10.2436/20.1501.01.79 ISSN: 1139-6709 **www.im.microbios.org**

INTERNATIONAL MICROBIOLOGY

# Identification of a cell-wall channel in the corynemycolic acid-free gram-positive bacterium *Corynebacterium amycolatum*

**Ursula Dörner,[1] Bettina Schiffler,[1] Marie-Antoinette Lanéelle,[2] Mamadou Daffé,[2] Roland Benz[1]***

[1]Department of Biotechnology, Biocenter, University of Würzburg, Würzburg, Germany. [2]Institute for Pharmacology and Structural Biology, Mixed Unit of Research, National Center for Scientific Research, Paul Sabatier University, Toulouse, France

Received 10 January 2009 · Accepted 15 March 2009

**Summary.** As part of a comparative study of the cell wall of corynebacteria, a channel-forming protein was characterized in *Corynebacterium amycolatum*, a species devoid of corynemycolic acids. *Corynebacterium amycolatum* cells were disrupted and the cell envelope subjected to two different separation procedures, differential centrifugation to separate the different fractions of the cell envelope, and sucrose-step-gradient density centrifugation. The fractions obtained by the two methods were analyzed for lipid composition, NADH oxidase activity, and the formation of ion-permeable channels in lipid bilayers. High channel-forming activity was always detected in fractions expected to contain only cell-wall components. The highest NADH-oxidase activity was found in other fractions, indicating that the cell-wall fraction was distinct from the membrane fraction. The cell wall was found to contain an ion-permeable channel with a single-channel conductance of about 3.8 nS in 1 M KCl. The channel-forming protein, with an apparent molecular mass of 45 kDa, was purified to homogeneity using FPLC and preparative SDS-PAGE. Single-channel experiments suggested that the cell-wall channel is wide and water-filled and has a narrow selectivity for cations. Analysis of the fatty-acid composition of extractable lipids and delipidated cells suggested that the cell wall of *C. amycolatum* contains enough lipids to form an additional permeability barrier on the surface of the bacteria, thus accounting for the presence of the cell-wall channel. [**Int Microbiol** 2009; 12(1):29-38]

**Key words:** *Corynebacterium amycolatum* · cell-wall channel · corynemycolic acids · reconstitution experiments · fatty-acid composition

## Introduction

There is increasing evidence that the cell wall of mycolic-acid-containing actinomycetes, the mycolata, contains one or several cell-wall channels [11,26,39,44]. These channels represent the major route for the uptake of hydrophilic solutes through the thick mycolic-acid layer, which, in some mycolata, forms an efficient permeability barrier [8,18]. Thus, the cell wall of mycolata functionally resembles the outer membrane of gram-negative bacteria, an efficient cell-surface permeability barrier composed of lipids and lipopolysaccharides. Hydrophilic compounds can diffuse through the outer membrane via the porin pathway [3] or by receptor-mediated uptake mechanisms [33]. The cell wall of gram-positive bacteria other than the mycolata is believed not to form a permeability barrier on the surface, because it is rather porous, allowing the diffusion even of large molecules. Nevertheless, there is some evidence that *Streptomyces griseus* and *Micromonospora purpurea*, both of which belong to the non-mycolic-acid-containing Actinomycetales, contain cell-wall channels with properties similar to those of the mycolata [18].

***Corresponding author:** R. Benz
Department of Biotechnology, Biocenter
University of Würzburg, Am Hubland
D-97074 Würzburg, Germany
Tel. +49-9318884501. Fax +49-9318884509
E-mail: roland.benz@mail.uni-wuerzburg.de

Corynebacteria are widely distributed in nature and are important, for instance, in the industrial production of amino acids, such as glutamic acid and lysine, by *Corynebacterium glutamicum* or *Corynebacterium efficiens* [14,22,25,41]. They belong to a distinctive suprageneric actinomycete taxon, as do mycobacteria, nocardia, rhodococci, and closely related genera. All these bacteria have an unusual cell-envelope composition and architecture, different from those of other gram-positive microorganisms. The envelope of corynebacteria consists of three layers: a typical plasma membrane of phospholipid and protein, a characteristic thick wall of peptidoglycan, and a complex outer layer composed of trehalose corynemycolates and other lipids [1,12,29,42]. The corynemycolic fatty acids comprise homologous mixtures of $C_{22}$–$C_{36}$ saturated and unsaturated acids [30]. The cell wall is made up of a thick *meso*-diaminopimelic acid-containing peptidoglycan covalently linked to arabinogalactan, which is in turn esterified by mycolic acids (long-chain α–alkyl, β-hydroxy fatty acids), with the notable exception of *Corynebacterium amycolatum* [2,10], (for reviews, see [12,31,42]).

*Corynebacterium amycolatum* is only distantly related to other corynebacteria, according to alignment analysis of 16S rRNA of different *Corynebacterium* species with NCBI-BLAST, and is located at the periphery of the genus *Corynebacterium* [35]. It has been isolated from human skin and from dairy cows with mastitis [16]. Nosocomial endocarditis and septic arthritis are caused by *C. amycolatum* [9,21]. *Corynebacterium minutissimum* and *Corynebacterium xerosis* are closely related to *C. amycolatum* and represent the most frequently isolated corynebacteria in clinical samples. However, most strains reported in the literature, such as *C. xerosis* and *C. minutissimum*, are probably misidentified and are instead *C. amycolatum* [15,45]. *Corynebacterium amycolatum* does not have corynemycolic acids and also contains only small amounts of extractable lipids [2,10,37]. In freeze-etch electron microscopy, all strains of *C. amycolatum* consistently exhibit a fracture plane only within the plasma membrane and not within the cell wall, whereas the reverse is true for most mycolate-containing corynebacteria examined [37]. Similarly, proteins from different cell fractions of *C. amycolatum* do not interact with polyclonal antibodies directed against PorA of *C. glutamicum* [37], although *C. amycolatum* may contain proteins homologous to corynemycoloyl transferase PS1 [36]. Taken together, these observations suggests that the cell wall of *C. amycolatum* does not act as a permeability barrier—because of the lack of corynemycolates and a typical corynebacterial porin. However, the present study demonstrates that the cell-wall fraction obtained by sucrose density centrifugation of *C. amycolatum* showed channel-forming activity in a lipid-bilayer assay. The protein responsible for the channel-forming activity was purified to homogeneity. The pure protein formed channels in lipid bilayers that were indistinguishable from those observed in cell-wall extracts obtained by two different methods. Analysis of the fatty acid composition of delipidated cells showed that the cell wall of *C. amycolatum* indeed contains enough lipids to account for the presence of a cell-wall channel. Therefore, the cell wall of *C. amycolatum* may represent a permeability barrier in which channels are needed to facilitate the uptake of nutrients.

## Materials and methods

**Bacterial strain and growth conditions.** *Corynebacterium amycolatum* ATCC 49368 [2], was routinely grown at 30°C with shaking (180 rpm) for 24 h in brain heart infusion (BHI) medium (3.7%, Difco).

**Sucrose-step-gradient density centrifugation.** *Corynebacterium amycolatum* cells were harvested by centrifugation (6000 rpm for 15 min in a Beckman J2-21M/E centrifuge, rotor JA17) and washed twice in 10 mM Tris-HCl (pH 8.0). The cells were passed three times through a French pressure cell at 900 psi. Unbroken cells were removed by centrifugation at 5000 ×*g* for 15 min. The cell envelopes (cytoplasmic membrane and cell wall) were obtained by centrifugation of the supernatant at 170,000 ×*g* for 60 min (Beckman Omega 90 XL ultracentrifuge, rotor 70.1 Ti). The pellet containing the cell envelope was resuspended in 2 ml 10 mM Tris-HCl (pH 7) and applied to a sucrose step-gradient of 20% (3 ml), 40% (4 ml), and 70% (3 ml) sucrose, similar to that used previously to separate cytoplasmic membranes and cell walls of *Mycobacterium chelonae* [44] and *Corynebacterium glutamicum* [27]. The gradient was centrifuged at 170,000 ×*g* for 16 h in a Beckman Optima 90 XL ultracentrifuge (rotor SW40Ti). Nine fractions of the gradient were collected and analyzed for protein content by SDS-PAGE, for the presence of cytoplasmic membrane by NADH-oxidase activity, and for pore-forming activity by reconstitution experiments in lipid bilayers. NADH-oxidase activity was measured by detecting the decrease of absorbance at 340 nm [32]. The reaction followed first-order kinetics. Specific activity was calculated from the rate constants by normalizing to the protein concentration.

**Differential centrifugation and purification of the channel-forming activity.** Wet *C. amycolatum* cells obtained by centrifugation (5 g) were suspended in 20 ml Tris-HCl buffer (10 mM, pH 8.0) and passed three times through a French pressure cell at 900 psi. Unbroken cells were removed by centrifugation at 3000 rpm for 15 min. The cell walls were recovered from the supernatant by centrifugation at 10,000 ×*g* for 60 min at 4°C (Beckman Omega 90 XL ultracentrifuge, rotor 70.1Ti). The cell-wall pellet was resuspended in 1 ml of 10 mM Tris-HCl (pH 8.0) supplemented with 1% Genapol and shaken for 4 h at room temperature. The supernatant of the centrifugation step was applied to a HiTrap Q column (Amersham Pharmacia Biotech, Freiburg, Germany) that had been washed with Tris-HCl (10 mM, pH 8) and 1% Genapol X-80 (Fluka, Buchs, Switzerland). The fractions were eluted with the same buffer, supplemented with increasing concentration of NaCl. Linear gradients between 0 and 1 M NaCl were applied to the column and 40 1-ml fractions were collected. The protein concentration in the fractions was detected at 280 nm and inspected for channel-forming activity in a lipid-bilayer assay. The channel-forming activity eluted at a NaCl concentration of about 250 mM. This fraction contained several protein bands, as judged by SDS-PAGE. Preparative SDS-PAGE was carried out to identify the channel-forming protein. Very low or no channel-forming

activity was found in the supernatant of the 10,000 ×*g* centrifugation, which is considered to contain the plasma membrane [37].

**SDS-PAGE.** SDS-PAGE was carried out using the Laemmli gel system [23]. The gels were stained with Coomassie brilliant blue or silver stain [7].

**Extraction and analysis of lipids.** Lipids were extracted from 20 g of wet cells, 500 mg of cell wall, and 500 mg of plasma membrane with 10 ml chloroform/methanol (1:2, v/v) for 16 h at room temperature with continuous stirring. The residues were re-extracted for 16 h with 10 ml chloroform/methanol (1:1, v/v) followed by an additional extraction of the residues for 16 h with 10 ml chloroform/methanol (2:1, v/v). The organic phases were pooled and concentrated to yield extractable lipids that were analyzed on silica plates (Macherey Nagel, Durasil 25, silica gel 60, 0.25-mm thickness). The plates were developed with $CHCl_3$/MeOH/$H_2O$ (65:25:4), $CHCl_3$/MeOH (9:1) to analyze phospholipids and trehalose mycolates, respectively. Lipids analyzed by thin-layer chromatography (TLC) were detected by spraying the plates with either rhodamine B or molybdophosphoric acid (10% in ethanol, w/v) followed by heating. The Dittmer-Lester reagent [13] was used for visualizing phosphorous-containing lipids. Glycolipids were revealed by spraying the plates with 0.2 % anthrone (w/v) in concentrated $H_2SO_4$, followed by heating at 110°C.

Whole cells, delipidated cells, or lipid extracts were saponified with 1 ml of 5% KOH (w/v) in methanol/benzene (8:2 v/v) overnight at 80°C for analysis of fatty-acid content. After acidification with 2 ml of 20% $H_2SO_4$, the resulting fatty acids were extracted with diethyl ether and converted to methyl esters with diazomethane. Fatty esters were first analyzed by TLC with dichloromethane as an eluent as described above and then characterized by gas chromatography (GC) and GC/mass spectrometry (GC/MS). Acid methanolysis was used as an alternative to analysis by GC, for both fatty-acid and sugar constituents. Dry material placed in a screw-capped tube was treated overnight with HCl/MeOH (1.5 M) at 80°C. The reaction mixture was evaporated under nitrogen. This evaporation was repeated three times with the addition of a few drops of methanol. At this step, fatty-acid methyl esters were directly obtained, but silylation was necessary to convert monosaccharides into volatile compounds for GC analysis. For this purpose, the dry material from acid methanolysis was treated with trimethylsilyl reagents [43]. After drying under nitrogen, the product was dissolved in petroleum ether and then analyzed by GC/MS and MS. GC was performed using a Hewlett Packard HP4890A equipped with a fused silica capillary column (25-m length by 0.22-mm internal diameter) containing WCOT OV-1 (0.3-mm film thickness, Spiral). A temperature gradient of 5°C/min was used from 100 to 310°C. GC/MS analysis was conducted on a Hewlett Packard 5889A mass spectrometer (electron energy, 70 eV) working in electron impact (EI) coupled with a Hewlett-Packard 5890 gas chromatograph fitted with a similar OV1 column (0.30 mm × 12 m).

**Isolation of acylated arabinose-containing fragments.** Delipidated bacterial cells (300 mg) were treated twice overnight at 50°C with 1 M HCl in alcohol/diethylether (1:1). After filtration, the pooled extracts were concentrated under vacuum to dryness, chromatographed on a Florisil column, and then eluted with a gradient of $CH_3OH$ in $CHCl_3$. Fractions were analyzed by TLC (solvent $CHCl_3$/$CH_3OH$ 95:5, v/v) to detect anthrone-positive lipid spots.

**Lipid bilayer experiments.** The methods used for the lipid bilayer experiments were described previously in detail [5]. Black-lipid bilayer membranes were obtained from a 1% (w/v) solution of diphytanoyl phosphatidylcholine (PC) (Avanti Polar Lipids, Alabaster, AL, USA) in n-decane. The temperature was maintained at 20°C during all experiments. Zero-current membrane potentials were measured by establishing a salt gradient across membranes containing 100–1000 channels, as described earlier [6].

## Results

**Identification of channel-forming activity in fraction F5 following sucrose-step-gradient density centrifugation.** Nine fractions were obtained following sucrose-step-gradient density centrifugation of the cell envelope of *C. amycolatum* (Fig. 1A). The fractions were inspected for protein content, NADH-oxidase activity, and channel-forming ability. Fractions F7, F8, and F9 accounted for 54, 13, and 20%, respectively, of the total NADH-oxidase activity and thus contained most of the plasma membrane, as

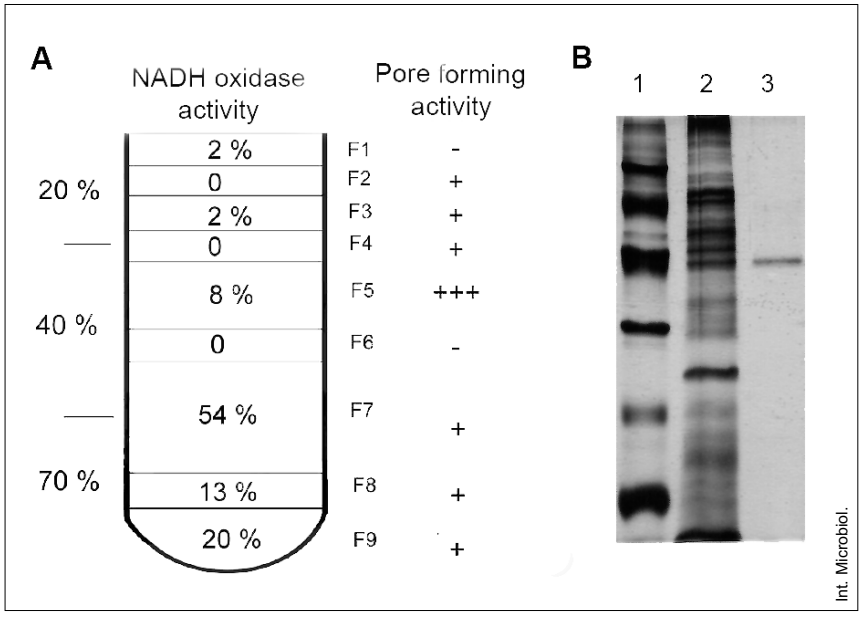


**Fig. 1.** (**A**) Fractions formed in a sucrose-step-gradient density centrifugation of the cell envelope of *Corynebacterium amycolatum*. The sucrose steps were 20% (w/v; 3 ml), 40% (w/v; 4 ml), and 70% (w/v; 3 ml). The sucrose concentration before centrifugation is indicated on the left side of the panel. The different zones formed after 16 h of centrifugation and could be clearly distinguished by the density of the material. The sizes of the zones correspond to their real sizes in the centrifugation tube. Nine fractions were collected, as indicated in the second column from the left in the figure. Fraction F7 contained the highest NADH-oxidase activity, and fraction F5 the highest pore forming activity as measured by the lipid-bilayer assay. (**B**) 12% SDS-PAGE of the total protein extract of the cell wall of *C. amycolatum* and the pure 45-kDa protein. The gel was stained with Coomassie blue G 250. Lane 1: Molecular mass markers 66, 45, 36, 29, 24, 20 and 14.2 kDa: lane 2: 5 µl of the Genapol extract of the cell-wall fraction solubilized at 100°C for 5 min in 5 µl sample buffer; lane 3: 5 µg of the pure 45-kDa protein solubilized at 100°C for 5 min in 5 µl sample buffer.

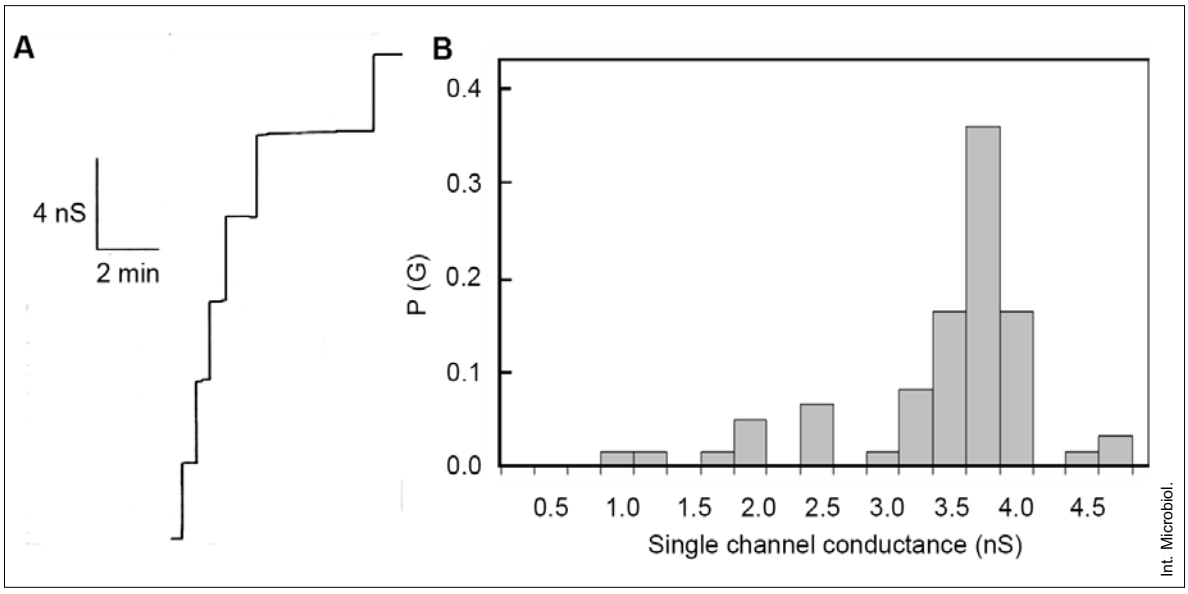


**Fig. 2.** (**A**) Single-channel recording of a diphytanoyl phosphatidylcholine/n-decane membrane in the presence of 20 ng of the 45-kDa cell wall protein of *Corynebacterium amycolatum*/ml. The aqueous phase contained 1 M KCl. The applied membrane potential was 20 mV, the temperature 20°C. (**B**) Histogram of the probability of the occurrence of certain conductivity units observed with membranes made up of diphytanoyl phosphatidylcholine/n-decane in the presence of 20 ng of the 45-kDa cell wall channel protein of *C. amycolatum*/ml. P(G) is the probability that a given conductance increment G is observed in the single-channel experiments. It was calculated by dividing the number of fluctuations with a given conductance increment by the total number of conductance fluctuations. The aqueous phase contained 1 M KCl. The applied membrane potential was 20 mV, the temperature 20°C. The average single-channel conductance was 3.8 nS for 121 single-channel events.

they accounted for roughly 90% of the total NADH-oxidase activity. The different fractions were also inspected for channel-forming activity in the lipid-bilayer assay, and highest activity was observed for fraction F5. This fraction contained only 8% of the total NADH-oxidase activity, which means that it was depleted of cytoplasmic membrane. Minor channel-forming activity was also found in other fractions of the sucrose-step-gradient density centrifugation but considerably less than in fraction F5 (Fig. 1A). This result indicated that the NADH-oxidase and the channel-forming protein were localized in different fractions of the cell envelope of *C. amycolatum*.

**Channel-forming activity in cell-wall extracts of *Corynebacterium amycolatum*.** Purification of the channel-forming protein in fraction F5 of the sucrose-step-gradient density centrifugation was difficult because of the low protein content of this fraction and the considerable loss of material during this procedure. To avoid this problem, the protein-isolation protocol was started from the cell-wall fraction of *C. amycolatum*, obtained according to the method of Puech et al. [37], by treatment with the detergent Genapol. The supernatant from the extraction showed high channel-forming activity in the lipid-bilayer assay, similar to that observed previously with porins or cell-wall channels from gram-negative and gram-positive bacteria [3,44]. The channels had a single-channel conductance of 3–4 nS in 1 M KCl.

**Purification of the channel-forming protein.** The aim of the next step was to identify and purify the channel-forming component using FPLC across a HiTrap Q column. Figure 1B (lane 2) shows the protein composition of the Genapol-treated cell-wall extract applied to the column. The column was first washed with buffer and then eluted with buffer supplemented with increasing concentrations of NaCl. The wash was essentially free of channel-forming activity. Elution with increasing concentrations of NaCl revealed several protein peaks that were subsequently investigated for channel-forming activity. Activity was observed only in the protein peak that eluted at about 250 mM NaCl. This peak showed several bands on SDS-PAGE. To identify the protein responsible for channel-forming activity, the different bands were eluted from preparative SDS-PAGE and again inspected for channel formation in reconstitution experiments. Only the band with a molecular mass of about 45 kDa (Fig. 1B, lane 3) was able to form the 3.8 nS channel in 1 M KCl solution. The other bands had no activity in the lipid-bilayer assay.

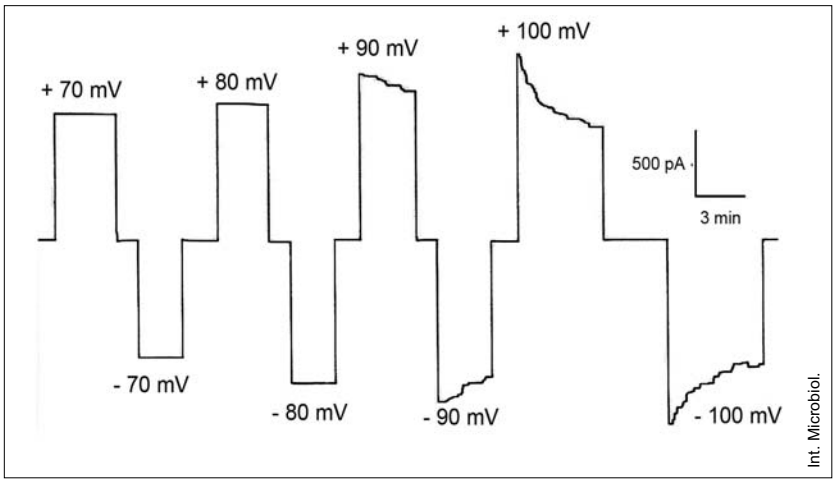


**Fig. 3.** Study of the voltage-dependence of *Corynebacterium amycolatum* cell-wall porin. Cell wall channel protein (200 ng/ml) was added to the cis-side of a diphytanoyl phosphatidylcholine/n-decane membrane and reconstitution of the channels was followed for about 30 min. Increasing positive (70 to 100 mV; upper traces) and negative voltages (–40 to –70 mV; lower traces) were applied to the cis-side of the membrane and the membrane current measured as a function of the time. The aqueous phase contained 1 M KCl, pH 6, the temperature was 20°C.

**Analysis of the channels formed by the 45-kDa channel-forming protein.** Figure 2A shows a single-channel recording of a phosphatidylcholine (PC) membrane in the presence of the pure 45-kDa protein of *C. amycolatum*, which was added to a black-membrane preparation at a concentration of about 20 ng/ml. The recording demonstrated that the protein formed defined channels. The single-channel conductance of most channels formed by the 45-kDa protein was about 3.8 nS in 1 M KCl (almost 40%), with only a few channels formed at other conductances (Fig. 2B). It is noteworthy that the channels formed by the 45-kDa protein of *C. amycolatum* had a long lifetime, similar to those previously described for porins of gram-negative [4] and gram-positive bacteria [44]. All these channel-forming proteins from the cell walls of bacteria form channels with a long lifetime at small transmembrane potentials in lipid-bilayer membranes (mean lifetime at least 5 min). Furthermore, only a minor voltage-dependence closure was observed in KCl solution, ranging from ± 70 to ± 80 mV (Fig. 3).

Single-channel experiments were also done with salts other than KCl to obtain information on the size and selectivity of the channels formed by the 45-kDa protein of *C. amycolatum*. The results are summarized in Table 1. The conductance sequence of the different salts within the channel was KCl > K acetate > LiCl, implying that the single-channel conductance followed approximately the bulk aqueous conductivity of the different salts. The influence of the anions and cations of different mobility on conductance was moderate, as seen in Table 1, suggesting only a small selectivity of the channel. Table 1 also shows the average single-channel conductance, G, as a function of KCl concentration in the aqueous phase. As in the case of many general-diffusion porin channels of gram-negative bacteria [4], the relationship between conductance and KCl-concentration was approximately linear; this is a characteristic of wide and water-filled channels without point net charges and/or ion-binding sites.

Further information about the structure of the channel formed by the 45-kDa protein of *C. amycolatum* was obtained from zero-current membrane-potential measurements in the presence of KCl gradients. A five-fold KCl gradient (100 vs. 500 mM) across a lipid-bilayer membrane in which about 100–1000 channels were reconstituted resulted in an asymmetry potential of, on average, about 15 mV at the more dilute side (mean of 3 measurements). This result indicated preferential movement of potassium ions over chloride through the channel at neutral pH. The zero-current membrane potentials were analyzed using the Goldman-Hodgkin-Katz equation [6] The ratio obtained by dividing the potassium permeability, $P_K$, by the chloride permeability, $P_{Cl}$, was about 2.6, which indeed indicated a narrow cation selectivity of the channel formed by the 45-kDa protein of *C. amycolatum*.

**Table 1.** Average single-channel conductance, G, of the cell wall channel of *Corynebacterium amycolatum* in different salt solutions[a]

| Salt | Salt concentration (M) | Single-channel conductance G (nS) |
|---|---|---|
| LiCl | 1.0 | 1.8 |
| KCl | 0.03 | 0.18 |
| | 0.10 | 0.45 |
| | 0.30 | 1.0 |
| | 1.0 | 3.8 |
| | 3.0 | 8.0 |
| $KCH_3COO$ | 1.0 | 2.0 |

[a]Membranes were formed from diphytanoyl phosphatidylcholine dissolved in n-decane. Aqueous solutions were buffered with 10 mM Tris-HCl and had a pH of 8 unless otherwise indicated. The applied voltage was 20 mV, and the temperature was 20°C. The average single-channel conductance, G, was calculated from at least 80 single events.

**Voltage-dependence.** In single-channel recordings, the 45-kDa protein from *C. amycolatum* exhibited some flickering at higher voltages, i.e., transitions between the open and closed configurations. This may have been caused by voltage-dependent closure of the channel, which was studied by increasing the membrane voltage in multi-channel recordings. Figure 3 shows an experiment of this type. The 45-kDa protein was added in a concentration of 200 ng/ml to one side of a black PC/n-decane membrane. After 30 min, about 10 channels were reconstituted into the membrane. At that time, different potentials were applied to the cis-side of the membrane: beginning with 70 mV (upper trace of Fig. 3) and then –70 mV (lower trace of Fig. 3). These experiments were repeated with 80, 90, and 100 mV. For negative and positive potentials at the cis-side, the membrane current decreased exponentially starting at about 70–80 mV. The data of Fig. 3 are consistent with a symmetric voltage-dependence of the 45-kDa protein when it was added to the cis-side. This result indicated either symmetric insertion of the protein into the membranes or a symmetric response to the applied voltage.

**Fatty acids in the cell wall of *Corynebacterium amycolatum*.** The presence of a channel-forming protein in the cell wall fraction of *C. amycolatum* suggested that the cell wall represents a permeability barrier similar to that of the mycolic acid layer of the mycolata [18]. However, *C. amycolatum* does not contain corynemycolic acids, shows no fracture plane in the cell wall, and has a low content of free lipid (around 2%) [2,10,37]. To better understand the environment of the 45-kDa protein, lipid distributions within the envelope, whole cells, plasma membrane, and cell wall were compared. These fractions were characterized prior to extraction by GC analysis of an aliquot of whole cells, plasma membrane, and cell wall treated by acid methanolysis. As expected, the cell wall was found to contain arabinose and galactose, the main characteristic sugar constituents of the cell wall, accompanied by a small amount of glucose [37], while in the plasma membrane only mannose was present due to the presence of phosphatidylinositolmannoside (PIM). The different subcellular fractions were then extracted with chloroform/methanol and the lipids analyzed by TLC. The lipid composition of the whole cells, cell wall, and plasma membrane was approximately the same (Fig. 4 and data not shown) and was similar to the phospholipid composition previously described for *C. amycolatum* [46]: acylphosphatidylglycerol (APG), diphosphatidylglycerol (DPG) or cardiolipin, phosphatidylglycerol (PG), phosphatidylinositol (PI), and acylated PIM ($Ac_3PIM_2$). In contrast to other corynebacteria [37], no glycolipids other than PIM were detected. Substantial amounts of free lipid were extracted from the cell wall (1% relative to cell dry mass) compared to the amounts extracted from the plasma membrane (2% relative to cell dry mass), meaning that the cell wall does not contain enough extractable lipid for the formation of a complete outer permeability barrier [37]. Nevertheless, it was important to determine whether the lipids extracted from the cell wall were not simply contaminants of the plasma membrane. For this purpose, the two fractions were saponified and the fatty acids extracted, methylated, analyzed by GC, and characterized by their fragmentation patterns in GC/MS [40]. The relative percentages of fatty-acid esters in lipids from the cell wall and plasma membrane are shown in Table 2. Overall, there was little difference between the two fractions. However, lipids of the plasma membrane contained a high concentration of branched pentadecanoic acid (28.4 vs. 3.4% in the cell wall), whereas free lipids from the cell wall contained almost all C15:0 fatty acid (23.2%), not detectable in lipids from the plasma membrane. Nevertheless it could not completely be excluded that a portion of the extractable lipids in the cell-wall fraction was due to contamination by the plasma membrane [37].

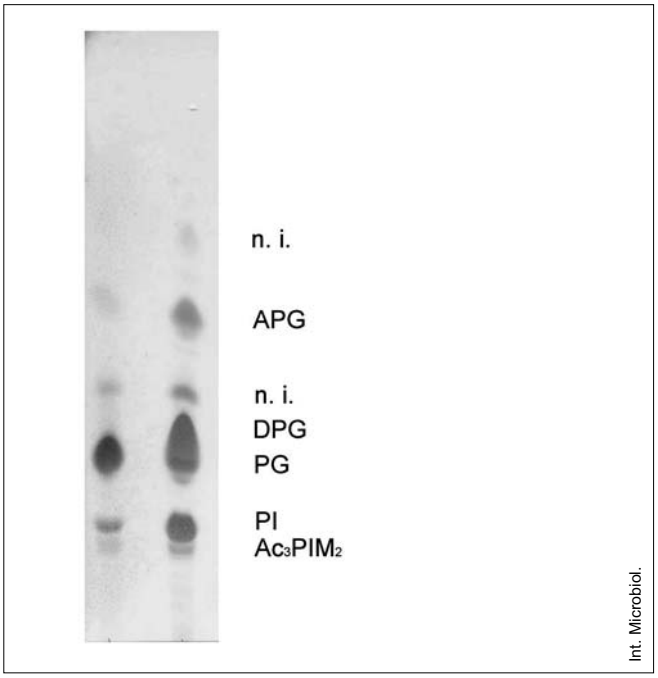


**Fig. 4.** TLC analysis of the extracted lipids of the cell wall and plasma membrane of the *Corynebacterium amycolatum* cell envelope. The fractions were extracted with chloroform/methanol and applied to a silica gel TLC plate. The plate was run in chloroform/methanol/water in the proportion 60/25/4 (v/v/v). Lane 1: lipids of the cell wall; lane 2: lipids of the whole cell. The TLC plate was stained with Dittmer reagent. Detected phospholipids: acylphosphatidylglycerol (APG), diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylinositol (PI) and triacylphosphatidylinositol dimannosides (Ac3PIM2). Two spots were not identified (n.i.).

**Table 2.** Fatty-acid composition (relative percentage) of the cell envelope of *Corynebacterium amycolatum*[a]

| | Fatty acids from extractable lipids | | |
|---|---|---|---|
| Fatty acid | Cell wall | Plasma membrane | Fatty acids from delipidated cells |
| 14:1 | 0.8 | 1.0 | ND |
| 14:0 | 0.9 | 1.1 | ND |
| 15 branched | 3.4 | 28.4 | ND |
| 15:0 | 23.2 | ND | 11.6 |
| 16 branched | 1.0 | 1.7 | ND |
| 16:1 | 0.7 | ND | ND |
| 16:0 | 17.5 | 15.3 | 10.5 |
| 17 branched | 10.0 | 10.0 | 1.5 |
| 17:1 | ND | ND | 3.9 |
| 17:0 | 1.1 | 1.2 | ND |
| 18 branched | ND | 0.5 | ND |
| 18:1 | 22.4 | 21.3 | 12.5 |
| 18:0 | 12.3 | 10.9 | 43.7 |
| 19 branched | 2.9 | 4 | ND |
| 19:1 | 1.2 | ND | ND |
| 19:0 | 0.8 | 1.1 | ND |
| Tuberculostearic acid (TBS) | ND | ND | 1.5 |

[a]The cell wall and plasma membrane were obtained by differential centrifugation of the supernatant of disrupted cells, as previously described [37]. Extractable lipids and delipidated cells were treated with 5% KOH in MeOH/benzene (8:2) to obtain methylated fatty acids. Methyl esters were characterized by their retention times and by interpreting their fragmentation patterns in GC/MS [40]. The results are expressed as the relative percentage of fatty-acid methyl esters calculated from the GC profile. ND, not detectable, i.e., fatty-acid concentration < 0.1% of the total fatty acids of the corresponding fraction.

It is well-established that mycolic acids covalently attached to cell-wall arabinogalactan provide the major contribution to the permeability barrier of the mycolata [10,14,21,37]. Although no corynemycolic acids are present in *C. amycolatum* [2,10,37], it was possible that the cell-wall polysaccharide of this species is acylated by fatty acids. To address this question, 1 g of dry delipidated cells of *C. amycolatum* was subjected to alkaline treatment in order to release fatty acids presumably attached to arabinogalactan. On average, about 20 mg of fatty acids (2% w/w, average of three experiments) were released. Interestingly, the fatty-acid distribution as derived from this treatment showed several remarkable differences with that of the free lipids extracted from the plasma membrane and cell wall: the most prominent fatty acid was $C_{18:0}$, which comprised almost 44% of the total fatty acid content of the delipidated cells, whereas the extractable lipids contained only about 11–12% of this fatty acid. To firmly establish that these fatty-acid residues were attached to cell-wall polysaccharide, delipidated cells were partially hydrolyzed with acid to release putative arabinooligosaccharides linked to fatty acids which would then be extractable with organic solvents [24]. Surprisingly, no glycolipid was detected from the treatment, suggesting that either the fatty acids were not covalently linked to arabinogalactan, but rather esterified to yet unknown compounds, or the amounts of acylated arabinose-containing compounds were too low to be detected. Taken together, these results indicated that the cell wall of *C. amycolatum* contains free lipids as well as covalently bound lipids, as indicated by the release of fatty acids from the cell wall.

## Discussion

**Presence of an ion-permeable channel in *Corynebacterium amycolatum*.** The present work provides evidence for the presence of a channel in the cell envelope of *C. amycolatum*. This species belongs to the corynebacteria but does not contain corynemycolates [2,10]. The channel was identified by lipid-bilayer assay in detergent extracts of the cell wall fraction and in fraction F5 of the sucrose-step density gradient centrifugation. Both the detergent extracts and fraction F5 showed very high channel-forming activity, which makes it extremely unlikely that the

channel was caused by an artifact. Similarly, high channel-forming activity was also found in the cell-wall fraction obtained by differential centrifugation of the cell wall [37]. Sucrose-gradient density centrifugation also showed that the cell wall had a lower density than the cytoplasmic membrane (Fig. 1A). In sucrose-gradient density centrifugations of the cell envelopes of members of the mycolata, the cell wall was shown to have a higher density than the cytoplasmic membrane [26,27,44]. However, the cell walls of *S. griseus* and *M. purpurea* have a lower density than the cytoplasmic membrane [21,22]. Thus, a lower density is not completely unusual, in particular, because cell walls of gram-negative bacteria that are devoid of lipopolysaccharides, such as *Borrelia* species, also have a lower density than the respective bacteria's cytoplasmic membrane [38].

The channel-forming protein was purified by FPLC using a HiTrap-Q column and a linear NaCl-gradient. High channel-forming activity of the same channels detected in fraction F5 was also found in fractions eluted from the column at 250 mM NaCl. These fractions were enriched in a 45-kDa protein. Final purification of the channel-forming protein was achieved by preparative SDS-PAGE of the FPLC fractions. The channel-forming protein was determined to have a molecular mass of about 45 kDa, which interestingly, is much higher than the channel formers of *C. glutamicum*, all of which have molecular masses below 10 kDa and thus probably form oligomers in the cell wall of this species [11,17,26,28].

**Properties of the cell-wall channel of *C. amycolatum*.** In experiments with lipid bilayers, the 45-kDa cell-wall channel of *C. amycolatum* had a high single-channel conductance of about 3.8 nS in 1 M KCl, higher than the channels of most gram-negative bacteria and PorB of *C. glutamicum* but similar to the channels formed by PorA and PorH of the same organism [11,17,26,28]. The channel is wide and water-filled since the movement of ions inside the channel was similar to their movements in the aqueous phase (Table 1). Furthermore, the single-channel conductance was an almost linear function of the bulk aqueous salt concentration, which provides evidence in favor of an aqueous channel. Selectivity measurements suggested that cations and anions enter the channel: the cell-wall channel showed only a slight preference for cations, when single-channel conductance in KCl, LiCl, and K-acetate and selectivity measurements were considered. The channel showed slight selectivity for potassium over chloride without any indication for the presence of the point net charges typical of the cell-wall channels of *C. glutamicum* [11,17,26]. This result suggested that the interior aspect of the channel contains negatively and positively charged groups that are almost perfectly balanced. The size of the cell-wall channel could not be deduced from single-channel measurements but it is definitely large and presumably also shows high permeability for neutral solutes. It is quite conceivable that mycolata and actinomycetes that do not contain mycolic acids possess different cell-wall channels depending on the growth conditions of the cells, similar to the situation in certain gram-negative bacteria, in which the chromosome includes several genes coding for different porins [3,4].

**Fatty acids in the cell wall of *C. amycolatum*.** The 45-kDa protein present in the cell envelope of *C. amycolatum* was determined to form wide and water-filled channels and was found in fractions characteristic of the cell wall. The presence of the protein in the plasma membrane is not considered likely because the cells would not survive the resulting leakiness. If the 45-kDa protein forms a channel for hydrophilic compounds in the cell wall of *C. amycolatum*, the presence of lipid or other components of a permeability barrier would also be required. Otherwise there is little need for the channel. Our search for lipids in the different fractions of the cell envelope provided evidence that the cell-wall fraction of the differential centrifugation contained extractable lipids. The amount of this lipid (1% relative to cell dry mass) was relatively small compared with that of the plasma membrane (2% relative to cell dry mass), which agrees with the literature [37]. The composition of these lipids was very similar to that of lipids from the cytoplasmic membrane (Fig. 4), raising the question whether the cell-wall lipids were cytoplasmic membrane contaminants. However, this was rather unlikely because of the remarkable differences between the fatty-acid compositions of lipids from the two fractions (Table 2): C15:0 branched fatty acids were almost exclusively found in high proportion (>25% of total fatty acids) in lipids from the cytoplasmic membrane, whereas C15:0 fatty acids were found in high proportion (23% of total fatty acids) exclusively in lipids from the cell wall. This result indicated that the cell wall indeed contains extractable lipids that could contribute to a permeability barrier.

*C. amycolatum* does not contain corynemycolic acids but it is possible that other lipids are covalently linked to the peptidoglycan layer. Alkaline treatment of delipidated cells resulted in the recovery of fatty acids. The fatty acid composition of the lipids bound to the cell wall suggested that the lipids are not a cytoplasmic membrane contaminant (Table 2). Their fatty-acid composition differed from that of the extractable lipids because they were enriched in stearic acid ($C_{18:0}$), which comprised more than 40% of the total fatty acids. Interestingly, the cell wall contained tuberculostearic acid

(TBS), which was not found in the extractable lipids. Thus, the fatty acids appear to be localized by the acylation of a polymer in the cell wall. However, the amount of this lipid was relatively small; compared with the amount in the plasma membrane and with the amount theoretically needed to form a lipid monolayer around the cell [34,37]. Consequently, the low content of lipid could not explain the formation of a permeability barrier at the surface of *C. amycolatum* cells, as discussed for mycobacteria [34]. This observation is reinforced by the failure to detect a second fracture plane in corynebacteria with a low amount of lipids [37]. On the other hand, it is possible that free lipid together with the bound fatty acids present in the cell wall of *C. amycolatum* form a permeability barrier with some other not-yet-identified components, such as protein. A structure combined of lipid and protein would fail to form a fracture plane. It is noteworthy that water-filled channels were already demonstrated in two non-mycolata gram-positive bacteria, *S. griseus* [19] and *M. purpurea* [20]. Interestingly, the protein that forms the channel in *S. griseus* contains a binding site for streptomycin [19]. In this context, the role of *C. amycolatum* porin remains to be elucidated in future experiments. It may have a role as a component of a putative permeability barrier or function as a binding protein for specific compounds.

**Acknowledgements.** This study was supported by grants of the Deutsche Forschungsgemeinschaft (project Be 865/13-1) and the Fonds der Chemischen Industrie.

## References

1. Barksdale L (1970) *Corynebacterium diphtheriae* and its relatives. Bacteriol Rev 34:378-422
2. Barreau C, Bimet F, Kiredjian M, Rouillon N, Bizet C (1993) Comparative chemotaxonomic studies of mycolic acid-free coryneform bacteria of human origin. J Clin Microbiol 31:2085-2090
3. Benz R (1994) Solute uptake through bacterial outer membrane. In: Ghuysen JM, Hakenbeck R (eds) Bacterial cell wall. Elsevier, Amsterdam, pp 397-423
4. Benz R (2001) Porins–structure and function. In: Winkelmann G (ed) Microbial transport systems. Wiley, Weinheim, pp 227-246
5. Benz R, Janko K, Boos W, Läuger P (1978) Formation of large, ion-permeable membrane channels by the matrix protein (porin) of *Escherichia coli*. Biochim Biophys Acta 511:305-319
6. Benz R, Schmid A, Hancock REW (1985) Ion selectivity of gram-negative bacterial porins. J Bacteriol 162:722-727
7. Blum H, Beier H, Gross HJ (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Elektrophoresis 8:93-99
8. Brennan PJ, Nikaido H (1995) The envelope of mycobacteria. Annu Rev Biochem 64:29-63
9. Clarke R, Qamruddin A, Taylor M, Panigrahi H (1999) Septic arthritis caused by *Corynebacterium amycolatum* following vascular graft sepsis. J Infect 38:26-127
10. Collins MD, Burton RA, Jones D (1988) *Corynebacterium amycolatum* sp. nov, a new mycolic acid-less *Corynebacterium* species from human skin. FEMS Microbiol Lett 49:349-352
11. Costa-Riu N, Maier E, Burkovski A, Krämer R, Lottspeich F, Benz R (2003) Identification of an anion-specific channel in the cell wall of the gram-positive bacterium *Corynebacterium glutamicum*. Mol Microbiol 50:1295-1308
12. Daffé M, Draper P (1998) The envelope layers of mycobacteria with reference to their pathogenicity. Adv Microb Physiol 39:131-203
13. Dittmer JCF, Lester RL (1964) A simple, specific spray for the detection of phospholipids on thin layer chromatograms. J Lipid Res 5:126-127
14. Eggeling L, Sahm H (1999) Glutamate and L-lysine: traditional products with impetuous developments. Appl Microbiol Biotechnol 52:146-153
15. Funke G, Lawson PA, Bernard KA, Collins MD (1996) Most *Corynebacterium xerosis* strains identified in the routine clinical laboratory correspond to *Corynebacterium amycolatum*. J Clin Microbiol 34:1124-1128
16. Hommez J, Devriese LA, Vaneechoutte M, Riegel P, Butaye P, Haesebrouck F (1999) Identification of nonlipophilic corynebacteria isolated from dairy cows with mastitis. J Clin Microbiol 37:954-957
17. Hünten P, Costa-Riu N, Palm D, Lottspeich F, Benz R (2005) Identification and characterization of PorH, a new cell wall channel of *Corynebacterium glutamicum*. Biochim Biophys Acta 1715:25-36
18. Jarlier V, Nikaido H (1994) Mycobacterial cell wall: structure and role in natural resistance to antibiotics. FEMS Microbiol Letters 123:11-18
19. Kim B-H, Andersen C, Benz R (2001) Identification of a cell wall channel of *Streptomyces griseus*: the channel contains a binding site for streptomycin. Mol Microbiol 41:665-673
20. Kim B-H, Wengerter K, Benz R (2002) The cell wall of *Micromonospora purpurea* contains a high conductance channel. Arch Microbiol 177:184-191
21. Knox KL, Holmes AH (2002) Nosocomial endocarditis caused by *Corynebacterium amycolatum* and other nondiphtheriae Corynebacteria. Emerg Infect Dis 8:97-99
22. Krämer R (1994) Secretion of amino acids by bacteria: physiology and mechanism. FEMS Microbiol Rev 13:75-94
23. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685
24. Lanéelle MA, Asselineau J (1977) Glycolipids of *Brevibacterium vitarumen*. Biochim Biophys Acta 486:205-208
25. Leuchtenberger W (1996) Amino acids–Technical production and use. In: Roehr M (ed) Products of primary metabolism, vol. 6. VCH Weinheim, Germany, pp 455-502
26. Lichtinger T, Burkovski A, Niederweis M, Krämer R, Benz R (1998) Biochemical and biophysical characterization of the cell wall porin of *Corynebacterium glutamicum*: the channel is formed by a low molecular mass polypeptide. Biochemistry 37:15024-15032
27. Lichtinger T, Reiss G, Benz R (2000) Biochemical identification and biophysical characterization of a channel-forming protein from *Rhodococcus erythropolis*. J Bacteriol 182:764-770
28. Lichtinger T, Riess FG, Burkovski A, et al. (2001) The low-molecular-mass subunit of the cell wall channel of the Gram-positive *Corynebacterium glutamicum*. Immunological localization, cloning and sequencing of its gene porA. Eur J Biochem 268:462-469
29. Marienfeld S, Uhlemann EM, Krämer R, Burkovski A (1997) Ultrastructure of the *Corynebacterium glutamicum* cell wall. Antonie Van Leeuwenhoek 72:291-297
30. Minnikin DE, Goodfellow M, Collins MD (1978) Lipid composition in the classification and identification of coryneform and related taxa. In: Bousfield IJ, Callely AG (eds) Coryneform bacteria. Academic Press, London, pp 85-160
31. Minnikin DE, Goodfellow M (1980) Lipid composition in the classification and identification of acid-fast bacteria. Soc Appl Bacteriol Symp Ser 8:189-256
32. Mizuno T, Kageyama M (1978) Separation and characterization of the outer membrane of *Pseudomonas aeruginosa*. J Biochem (Tokyo) 84:179-191

33. Nikaido H (1992) Porins and specific channels of bacterial outer membranes. Mol Microbiol 267:512-514
34. Nikaido H, Kim SH, Rosenberg EY (1993) Physical organisation of lipids in the cell wall of *Mycobacterium chelonae*. Mol Microbiol 8:1025-1030
35. Pascual C, Lawson PA, Gimenez MN, Collins MD (1995) Phylogenetic analysis of the genus *Corynebacterium* based on 16S rRNA gene sequences. Int J Syst Bacteriol 45:724-728
36. Puech V, Bayan N, Salim K, Leblon G, Daffé M (2000) Characterization of the *in vivo* acceptors of the mycoloyl residues transferred by the corynebacterial PS1 and the related mycobacterial antigens 85. Mol Microbiol 35:1026-1041
37. Puech V, Chami M, Rouja C, et al. (2001) Structure of the cell envelope of corynebacteria: importance of the non-covalently bound lipids in the formation of the cell wall permeability barrier and fracture plane. Microbiology 147:1365-1382
38. Radolf JD, Goldberg MS, Bourell K, Baker SI, Jones JD, Norgard MV (1995) Characterization of outer membranes isolated from *Borrelia burgdorferi*, the Lyme disease spirochete. Infect Immun 63:2154-2163
39. Riess FG, Lichtinger T, Cseh R, Yassin AF, Schaal KP, Benz R (1998) The cell wall porin of *Nocardia farcinica*: biochemical identification of the channel-forming protein and biophysical characterization of the channel properties. Mol Microbiol 29:139-150
40. Ryhage R, Stenhagen E (1960) Mass spectrometry in lipid research. J Lipid Res 1:361-390
41. Sahm H, Eggeling L, Eikmanns B, Krämer R (1996) Construction of L-lysine, L-threonine-, and L-isoleucine-overproducing strains of *Corynebacterium glutamicum*. Ann NY Acad Sci 782:25-39
42. Sutcliffe IC (1997) Macroamphiphilic cell envelope components of *Rhodococcus equi* and closely related bacteria. Vet Microbiol 56:287-299
43. Sweeley CC, Bentley R, Makita M, Wells WW (1963) Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances. J Am Chem Soc 85:2497-2507
44. Trias J, Jarlier V, Benz R (1992) Porins in the cell wall of mycobacteria. Science 258:1479-1481
45. Zinkernagel AS, von Graevenitz A, Funke G (1996) Heterogeneity within *Corynebacterium minutissimum* strains is explained by misidentified *Corynebacterium amycolatum* strains. Am J Clin Pathol 106:378-383
46. Yagüe G, Segovia M, Valero-Guillén PL (2003) Phospholipid composition of several clinically relevant *Corynebacterium* species as determined by mass spectrometry: an unusual fatty acyl moiety is present in inositol-containing phospholipids of *Corynebacterium urealyticum*. Microbiology 149:1675-1685