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

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With the recent success of the heterologous expression of mycobacterial antigens in corynebacteria, in addition to the importance of these bacteria in biotechnology and medicine, a better understanding of the structure of their cell envelopes was needed. A combination of molecular compositional analysis, ultrastructural appearance and freeze-etch electron microscopy study was used to arrive at a chemical model, unique to corynebacteria but consistent with their phylogenetic relatedness to mycobacteria and other members of the distinctive suprageneric actinomycete taxon. Transmission electron microscopy and chemical analyses showed that the cell envelopes of the representative strains of corynebacteria examined consisted of (i) an outer layer composed of polysaccharides (primarily a high-molecular-mass glucan and arabinomannans), proteins, which include the mycoloyltransferase PS1, and lipids; (ii) a cell wall glycan core of peptidoglycan-arabinogalactan which may contain other sugar residues and was usually esterified by corynomycolic acids; and (iii) a typical plasma membrane bilayer. Freeze-etch electron microscopy showed that most corynomycolate-containing strains exhibited a main fracture plane in their cell wall and contained low-molecular-mass porins, while the fracture occurred within the plasma membrane of strains devoid of both corynomycolate and pore-forming proteins. Importantly, in most strains, the amount of cell wall-linked corynomycolates was not sufficient to cover the bacterial surface; interestingly, the occurrence of a cell wall fracture plane correlated with the amount of non-covalently bound lipids of the strains. Furthermore, these lipids were shown to spontaneously form liposomes, indicating that they may participate in a bilayer structure. Altogether, the data suggested that the cell wall permeability barrier in corynebacteria involved both covalently linked corynomycolates and non-covalently bound lipids of their cell envelopes.

Keywords: cell wall, corynebacteria, mycolic acid, polysaccharide, porin

INTRODUCTION

Corynebacteria are widely distributed in nature and are important in several ways; apart from the human pathogens, e.g. *Corynebacterium diphtheriae*, serious economic losses are caused by corynebacterial diseases

of animals (Coyle & Lipsky, 1990; Funke *et al.*, 1997). In addition, some corynebacteria are of considerable economic benefit, for instance in the industrial production of amino acids (Krämer, 1994). They belong to a distinctive suprageneric actinomycete taxon, which also includes mycobacteria, nocardiae, rhodococci and closely related

genera; all these bacteria share with corynebacteria the property of having an unusual cell envelope composition and architecture, compared to those of other Gram-positive micro-organisms. Based on most recently published data, the envelope layers of mycobacteria consist of a typical plasma membrane of phospholipid and protein, a characteristic wall of unusual structure and a complex outer layer (Daffé & Draper, 1998). Although detailed studies have not been carried out on the cell envelopes of corynebacteria and closely related genera, it is evident from published data that these are very similar to that of mycobacteria, specially in terms of ultrastructure and cell wall chemical composition (Barksdale, 1970; Marienfeld *et al.*, 1997; Sutcliffe, 1997).

The chemical structure of the cell wall skeleton of corynebacteria, mycobacteria and related genera has been extensively studied (for reviews see Daffé & Draper, 1998; Minnikin & Goodfellow, 1980; Minnikin & O'Donnell, 1984; Minnikin *et al.*, 1978; Sutcliffe, 1997); this is formed by a thick *meso*-diaminopimelic acid-containing peptidoglycan covalently linked to arabinogalactan, which in turn is esterified by long-chain α -alkyl, β -hydroxy fatty acids. While these fatty acids in mycobacteria, called eumycolic acids, possess a very long chain (C_{60-90}) and may contain various oxygen functions in addition to the β -hydroxyl group (Daffé & Draper, 1998), mycolic acids found in other actinomycetes consist of homologous mixtures of saturated and unsaturated acids and contain shorter chains, e.g. C_{40-50} in nocardomycolic acids and C_{22-36} in corynomycolic acids (Collins *et al.*, 1982; Minnikin & Goodfellow, 1980; Minnikin & O'Donnell, 1984; Minnikin *et al.*, 1978). Although they are Gram-positive bacteria, corynebacteria, mycobacteria and closely related micro-organisms share with Gram-negative bacteria the property of forming in their envelope an outer barrier that is distinct from the plasma membrane. While this additional barrier in Gram-negative micro-organisms is a typical bilayer of phospholipid and lipopolysaccharide, in mycobacteria and corynebacteria the cell-wall-linked mycolates and corynomycolates certainly participate in this barrier since the disruption of genes that code for mycoloyltransferases, the antigen-85 complex and PS1, respectively, causes a decrease in the amount of cell wall-bound mycolates and affects the permeability of the envelope of the mutants (Jackson *et al.*, 1999; Puech *et al.*, 2000). Evidence has also been presented that the chemical structure of mycolic acids plays a role in determining the fluidity and permeability of the mycobacterial cell wall (George *et al.*, 1995; Liu *et al.*, 1996; Dubnau *et al.*, 2000). The existence in mycobacteria, corynebacteria and related genera of an outer membrane diffusion barrier is reinforced by the characterization of cell envelope proteins with pore-forming ability (Kartmann *et al.*, 1999; Lichtinger *et al.*, 1998, 1999; Rieß *et al.*, 1998; Mukhopadhyay *et al.*, 1997; Trias *et al.*, 1992; Trias & Benz, 1994; Senaratne *et al.*, 1998).

In all currently proposed models (Minnikin 1982; Rastogi, 1991; Liu *et al.*, 1995) the outer permeability barrier of mycobacteria consists of a monolayer of mycoloyl residues covalently linked to the cell wall arabinogalactan and includes other lipids which are probably arranged to form a bilayer with the mycoloyl residues. Although no sign of a second lipid bilayer has ever been reported in thin sections of mycobacterial cells (Draper, 1998), freeze-fractured samples of mycobacteria, corynebacteria and related genera (Barksdale & Kim, 1977; Benedetti *et al.*, 1984; Chami *et al.*, 1995; Takeo *et al.*, 1984) showed that these organisms had two such planes of weakness in their envelopes, in addition to the expected plasma membrane fracture. A second fracture plane, close to the cell surface of mycobacteria, was observed. The existence of distinct lipid domains in the mycobacterial cell envelope has been indicated by studies using progressive erosion of the envelope (Ortalo-Magné *et al.*, 1996) or selective lipophilic probes (Christensen *et al.*, 1999). Freeze-fracture electron microscopy also showed the presence of ordered arrays on the surface of *Corynebacterium glutamicum* and other *Corynebacterium* spp. (Chami *et al.*, 1995; Peyret *et al.*, 1993; Soual-Hoebeke *et al.*, 1999); these surface layer proteins (S-layer) have been shown to be composed of the major corynebacteria-secreted protein, PS2 (Chami *et al.*, 1995; Peyret *et al.*, 1993; Soual-Hoebeke *et al.*, 1999). Such crystalline structures are not visible in published freeze-fractured micrographs of mycobacteria despite a singular report of a crystalline protein layer on the outside of *Mycobacterium bovis* BCG (Lounatmaa & Brander, 1989).

From recent advances in molecular biology, it appears that corynebacteria represent a model suitable for functional studies of expression of mycobacterial genes, primarily because (i) they possess the simplest cell wall structure, but one that is functionally and structurally close to those of mycobacteria and related genera; (ii) they are rapid growers and do not form clumps; and (iii) heterologous expression of mycobacterial antigens in *C. glutamicum* has proved to be effective (Salim *et al.*, 1997; Puech *et al.*, 2000). However, it has to be remembered that, unlike the other related micro-organisms, corynebacteria are widely used in biotechnology for the production of amino acids (Krämer, 1994); this singular property of corynebacteria suggests that, despite the global similarity of their cell envelope composition with those of related genera, they differ from them in the arrangement of its constituents. To gain further insights into the cell envelope structure of corynebacteria, various representative corynebacterial strains were examined in the present study by combining biochemical analyses and electron microscopy studies. These included the *Corynebacterium* genus type species *C. diphtheriae*, the amino-acid-producing strain *C. glutamicum*, corynomycolate-less strains, i.e. *C. amycolatum* (Collins *et al.*, 1988; Barreau *et al.*, 1993) and defined corynebacterial cell wall mutants (Chami *et al.*, 1995; Peyret *et al.*, 1993; Joliff *et al.*, 1992; Puech *et al.*, 2000).

METHODS

Strains and growth conditions. The strains used were *Corynebacterium amycolatum* ATCC 49368^T and other corynebacterial strains reclassified in this species (Barreau *et al.*, 1993), i.e. CIP (Collection Institut Pasteur) 79.37, CIP 100836, CIP 103818, CIP 103819, CIP 103820 and CIP 103821; *C. diphtheriae* strain C8_r(-) Tox⁻ (Barksdale, 1959); *C. glutamicum* CGL2005, its isogenic *csp1*-inactivated mutant CGL2022 (Puech *et al.*, 2000), *cspB*-disrupted mutant CGL2025 (Peyret *et al.*, 1993), and CGL1009; *C. pseudodiphtheriticum* (formerly *C. hofmannii*) strain Breuillaud (Welby-Giuseppe *et al.*, 1970); and *C. xerosis* ATCC 373^T, ATCC 7094, ATCC 7711 and ATCC 9016. All these strains were routinely grown at 30 °C with shaking (200 r.p.m.) for 24 h in BHI (3.7% Difco brain-heart infusion). For electron microscopy analysis, strains of *C. xerosis* were also grown for 16, 24, or 48 h in BHI, and *C. glutamicum* CGL2005 and its isogenic *cspB*-disrupted mutant CGL2025 were grown on BHI-containing agar (15 g l⁻¹) plates. For the structural analysis of polysaccharides, strains were grown on a corynebacterial minimal medium (Hucheng *et al.*, 1984) to avoid possible contamination with polysaccharides originating from the BHI medium.

Isolation of extracellular materials. Bacteria were grown for 48 h on minimal medium and the culture filtrates were recovered by centrifugation, filtered through a 0.2 µm sterile filter (Nalgene) and concentrated under vacuum to 1/10 of the original volume; the corresponding cells were dried and weighed. Chloroform and methanol were added to a portion of the filtrates to isolate lipids as described below and cold ethanol (6 vols) was added to the remaining portion; after centrifugation of the ethanol precipitates, the pellets were extensively dialysed against distilled water and extracellular materials were obtained and analysed for carbohydrate and protein content as described by Lemassu & Daffé (1994).

Isolation of the crude surface-exposed materials. Cells grown in either BHI or minimal medium were harvested by centrifugation, extensively washed and gently shaken for 1 min with 10 g glass beads (4 mm diameter) per 2 g of wet cells (Ortalo-Magné *et al.*, 1995). The resulting material was then suspended in distilled water and immediately filtered through a 0.2 µm sterile filter (Nalgene) and concentrated under vacuum to 1/10 of the original volume. Cold ethanol (6 vols) was added to a portion of the crude surface-exposed material, and the ethanol-precipitates were extensively dialysed against distilled water, lyophilized, and analysed for their carbohydrate and protein compositions.

CHCl₃ and CH₃OH were added to the remaining filtrate to give a final one-phase mixture of H₂O/CHCl₃/CH₃OH (0.8:1:2, by vol.; Bligh & Dyer, 1959) and lipids were extracted for 1 h at room temperature; a two-phase partition mixture was obtained by adding 1 vol. CHCl₃/H₂O (1:1, v/v) to the one-phase solution and lipids were recovered in the lower organic phase, dried and analysed by thin-layer chromatography (TLC) as described below.

Isolation, fractionation and analysis of whole-cell lipids. Lipids were obtained and analysed as previously described (Puech *et al.*, 2000). Briefly, lipids were extracted from wet cells for 16 h with CHCl₃/CH₃OH (1:1, v/v) at room temperature with continuous stirring; the bacterial residues were re-extracted three times with CHCl₃/CH₃OH (2:1, v/v) and the organic phases were pooled and concentrated. The crude lipid extracts were partitioned between the aqueous and the organic phases arising from a mixture of CHCl₃/

CH₃OH/H₂O (8:4:2, by vol.); the lower organic phases were collected, evaporated to dryness to yield the crude lipid extracts from each strain and comparatively examined by TLC on silica gel-coated plates (G-60, 0.25 mm thickness, Merck) developed with CHCl₃/CH₃OH (9:1, v/v) or CHCl₃/CH₃OH/H₂O (30:8:1 or 65:25:4, by vol.). Detection of all classes of lipids was performed by spraying the TLC plates with either rhodamine B or 20% H₂SO₄ in water, the latter followed by heating at 110 °C; glycolipids were revealed by spraying plates with 0.2% anthrone (w/v) in concentrated H₂SO₄, followed by heating at 110 °C. The Dittmer-Lester reagent (Dittmer & Lester, 1964) was used for visualizing phosphorus-containing lipids.

In a parallel experiment, surface-exposed lipids were isolated from *C. glutamicum* cells by extracting bacterial cells with octylglucoside. Briefly, *C. glutamicum* CGL2005 cells were grown overnight in BHI-rich medium. After extensive washing (twice the culture volume), cells were incubated for 1 h in 25 mM Tris/HCl, pH 6.8 buffer containing 1% octylglucoside. The crude detergent extract was recovered by centrifugation at 8000 r.p.m. for 15 min, extensively dialysed for 24 h against 25 mM Tris/HCl, pH 6.8 buffer, and then centrifuged at 200 000 g for 30 min; the pellet was analysed by freeze-fracture electron microscopy.

Quantification of corynomycolic acids was performed as follows. Delipidated cells (1.5 g dry weight) and lipid extracts (100 mg) of the various strains were dried under vacuum prior to weighing and were saponified (Daffé *et al.*, 1983). The saponified products were acidified with 20% H₂SO₄ and the resulting fatty acids were extracted with diethyl ether, converted to methyl esters with diazomethane and dried under vacuum, dissolved in petroleum ether and applied to a Florisil (60–100 mesh, Merck) column equilibrated in petroleum ether. The column was irrigated stepwise with increasing concentrations of diethyl ether in petroleum ether. Fractionations were monitored by TLC on silica gel-coated plates using dichloromethane and fractions containing the same lipid compounds (non-hydroxylated fatty acid methyl esters or corynomycolates) were pooled and weighed. Three sequential determinations from separate preparations of delipidated cells were performed (Puech *et al.*, 2000).

Fatty acid methyl esters from bacteria, delipidated cells and extractable lipids (1–2 mg) were treated with trimethylsilyl reagents (Sweeley *et al.*, 1963) to derivatize hydroxylated components of the mixtures, i.e. corynomycolates, and analysed by GC. The detector response for the various classes of fatty acid methyl esters was determined using authentic samples of C_{16:0} and C_{32:0} corynomycolate methyl esters. Identification of non-hydroxylated fatty acid methyl esters and corynomycolate derivatives was achieved by gas chromatograph-mass spectrometry (GC-MS).

Production of cell wall, plasma membrane and cytosol fractions. The cell fractions of the various corynebacteria were produced as previously described for mycobacteria (Daffé *et al.*, 1990; Raynaud *et al.*, 1998). Wet cells (5 g) were suspended in 20 ml phosphate buffer (50 mM, pH 7.5) and the resulting bacterial suspension was passed through a cell disrupter and then centrifuged at 3000 r.p.m. for 15 min to eliminate unbroken cells; cell walls were recovered from the supernatant by recentrifugation at 10 000 g for 1 h. The resulting pellet was extracted with aqueous 2% SDS at 95 °C for 1 h to remove soluble proteins and finally pelleted at 10 000 g; the cell walls were washed three times with 80% acetone to remove SDS and lyophilized (Daffé *et al.*, 1990). The 10 000 g supernatant was recentrifuged at 100 000 g for

2 h to yield the membrane fraction in the pellet; the supernatant was considered as the cytosol (Raynaud *et al.*, 1998).

Analysis of the cell wall arabinogalactans. O-Methylation of cell wall arabinogalactan was performed by suspending lyophilized walls (5 mg) in 3 ml of dimethylsulphoxide; 300 µl of dimethylsulphinyll carbanion was added and the mixture was stirred for 5 h. CH₃I was slowly added and the suspension was stirred for 2 h. A mixture of CH₃OH/H₂O (1:1, v/v) was added and the reaction mixture was dialysed against water. The addition of dimethylsulphoxide, dimethylsulphinyll carbanion and CH₃I, followed by dialysis, was repeated four times. The retentate was lyophilized and the per-O-methylated arabinogalactan was hydrolysed with 2 M CF₃COOH at 110 °C for 2 h. The hydrolysed products were reduced by NaBH₄, acetylated and the resulting partially O-methylated, partially O-acetylated alditols were analysed by GC-MS (Daffé *et al.*, 1990).

Production and analysis of lipoarabinomannans and lipomannan. Delipidated cells (7 g) were extracted with 100 ml ethanol/water (1/1, v/v) for 2 h at 70 °C; the bacterial residues were recovered by filtration and re-extracted with the same solvent mixture. The two extracts were pooled and dried; then, 100 ml hot phenol/water (1/1, v/v) was added and the mixture was heated for 5 min under continuous stirring followed by partitioning of the two phases. The phenol phase was discarded and the upper phase was extensively washed and dried. The extract was solubilized in water and Triton-X114 (2% w/v) was added to the cooled suspension; the mixture was stirred for 5 min and then heated at 50 °C until two phases had formed. The detergent phase was recovered, diluted by adding 2 ml water and washed three times with CHCl₃. The resulting aqueous phase was concentrated to a final volume of 0.5 ml and the macromolecules were precipitated with 6 vols cold ethanol. The precipitate was recovered, resuspended in water (0.5 ml) and the macromolecules were reprecipitated with 6 vols cold ethanol. The resulting pellet was dissolved in 100 mM sodium acetate buffer (pH 4.7), containing 15% (w/w) propan-1-ol and fractionated by hydrophobic interaction chromatography (Leopold & Fisher, 1993) by loading the material onto a column of octyl-Sepharose CL-4B (Pharmacia LKB) equilibrated with the buffer; the column was first irrigated with this buffer and then with the buffer containing 20, 30, 40, 50 and 60% (v/v) *n*-propanol. Fractions were collected in bulk, dialysed and monitored for carbohydrate; a portion of each dialysate was analysed for its carbohydrate and lipid compositions. The remaining portion was analysed by SDS-PAGE; authentic samples of mycobacterial lipoarabinomannan and lipomannan from *Mycobacterium bovis* BCG, kindly provided by T. Brando (IPBS, Toulouse, France) were used as standard.

Preparation of proteins for SDS-PAGE and Western blot analysis. Cell wall proteins from the various strains were released by incubating the cell pellet with 2% (w/v) SDS in 50 mM Tris/HCl (pH 6.8) at 100 °C for 2 min (50 µl buffer per equivalent of 0.4 ml bacterial suspension at OD₆₅₀ 1.2); the suspension was centrifuged at 12000 *g* for 3 min and the supernatant, which contains only cell wall-associated proteins and not cytoplasmic proteins (Peyret *et al.*, 1993), was collected. Cell wall proteins from roughly 0.4 ml bacterial suspension at OD₆₅₀ 1, were analysed by SDS-PAGE. The pore-forming proteins were isolated from the culture filtrates and different cell fractions as previously described (Lichtinger *et al.*, 1998). Proteins were separated by SDS-PAGE (Laemmli, 1970) with a 4% stacking gel and a 7% or a 10% running gel.

Samples were denatured in the presence of 2% SDS in 50 mM Tris/HCl (pH 6.8). After electrophoresis, gels were either stained with Coomassie brilliant blue R-250 or blotted onto nitrocellulose membrane (Towbin *et al.*, 1979). In this latter case proteins were probed with rabbit polyclonal antibodies anti-PS1 (Joliff *et al.*, 1992), anti-PS2 (Peyret *et al.*, 1993), or anti-*C. glutamicum* porin (Lichtinger *et al.*, 1998). Bands were detected using alkaline-phosphatase-conjugated antibodies and nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indoxyl phosphate (BCIP) *p*-toluidine (Promega) as substrates.

Sugar compositional analysis. The sugar constituents of the various materials were determined after acid hydrolysis either with 2 M CF₃COOH at 110 °C for 2 h or with 1 M methanolic HCl at 80 °C for 16 h; the mixture of hydrolysed products was dried, treated with trimethylsilyl reagents (Sweeley *et al.*, 1963) to derivatize monosaccharides and analysed by GC for their sugar.

Gas chromatography and mass spectrometry. 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 100–290 °C at 5 °C min⁻¹, followed by a 10 min isotherm plateau at 290 °C, was used.

GC-MS analysis was conducted on a Hewlett Packard 5890 gas chromatograph connected to a Hewlett Packard 5989A mass spectrometer. Samples were injected in the splitless mode. The column was a 12 m HP-1. A temperature gradient of 100–290 °C (8 °C min⁻¹) was used.

Experiments with lipid bilayer membranes. Black lipid bilayer membranes were formed as described previously (Benz *et al.*, 1978). The instrumentation consisted of a Teflon chamber with two aqueous compartments connected by a small circular hole. The hole had a surface area of about 0.5 mm². Membranes were formed across the hole by painting on a 1% solution of a mixture (molar ratio 4:1) of diphytanoyl phosphatidylcholine (PC) and phosphatidylserine (PS) (Avanti Polar Lipids, Alabaster AL) in *n*-decane.

Electron microscopy

Transmission electron microscopy. Bacterial pellets were fixed for 1 h at 4 °C with 2.5% (w/v) glutaraldehyde and 0.05 M lysine in a 0.1 M cacodylate buffer (pH 7.4) containing, or not, 0.075% (w/v) ruthenium red. Cells were washed five times in the same buffer, i.e. with or without 0.075% (w/v) ruthenium red, and postfixed for 1 h at room temperature in 1% (w/v) osmium tetroxide containing, or not, 0.075% (w/v) ruthenium red in cacodylate buffer (pH 7.4), and then rinsed with distilled water. Bacteria were suspended in 1% (w/v) aqueous uranyl acetate for 1 h at room temperature and then washed five times in distilled water. Suspended cells were embedded in 2% molten agar type IX (Sigma) before dehydration through a graded ethanol series. Dehydrated cells were embedded in Spurr medium with intermediate 1,2-epoxypropane infiltration. Blocks were conventionally cut, stained and examined with a Philips CM12 microscope operating under standard conditions.

Freeze-etched preparations for electron microscopy. Bacterial suspensions were centrifuged at 5000 *g*. A drop of the pellet was placed between thin copper holders and quenched in liquid propane. The frozen samples were fractured at –125 °C in a vacuum of about 10⁻⁷ torr as described by Aggerbeck & Gulik-Krzywicki (1986). The fractured samples were etched at –100 °C for 3 min at 1.3 × 10⁻⁵ Pa and then replicated with

1–1.5 nm of deposits of platinum-carbon, and backed with about 20 nm of carbon. The replicas were cleaned overnight with chromic acid, washed with distilled water and observed with a Philips 410 electron microscope.

RESULTS

Ultrastructure of the cell envelopes of corynebacteria

Electron microscopic examination of ultrathin sections from conventionally fixed corynebacterial cells (Fig. 1a, d, e) revealed a cell envelope structure composed of (i) a plasma membrane (PM) of 6–7 nm, (ii) a thick electron-dense layer (EDL) of 15–20 nm, (iii) an electron-transparent layer (ETL) of 7–8 nm and (iv) a thin outer layer (OL) of 2–3 nm. This ultrastructural appearance of the cell envelope of corynebacteria is similar to that found in mycobacteria (Daffé & Draper, 1998; Draper, 1982; Paul & Beveridge, 1992; Rastogi *et al.*, 1986). However, in contrast to mycobacteria, in which a space is observed between the PM and EDL corresponding to a hypothetical periplasmic space (Daffé & Draper, 1998), the PM is tightly associated to the EDL in thin sections of corynebacteria, an observation consistent with a previous report on *C. glutamicum* (Marienfeld *et al.*, 1997). When ultrathin sections were stained with ruthenium red, a stain that has been previously shown to react strongly with the surface of mycobacteria (Rastogi *et al.*, 1984), a much thicker OL (35–40 nm) was seen around the various corynebacteria (Fig. 1b, c, f). Thus,

as previously suggested (Daffé & Draper, 1998), the thin margin observed for OL in conventionally fixed cells and freeze-substituted samples of both corynebacteria (Marienfeld *et al.*, 1997) and mycobacteria (Paul & Beveridge, 1992) may not reflect the original thickness of the layer. In addition, no difference was seen between ultrathin sections of the parent strain of *C. glutamicum* (Fig. 1b) and its isogenic mutant devoid of S-layer (Fig. 1c), in terms of either staining properties or thickness; this observation indicated that, in contrast with a previous suggestion (Marienfeld *et al.*, 1997), the OL is not synonymous with the S-layer.

Nature of the outermost bacterial materials

The molecular composition of the outer bacterial surface of these bacteria is poorly documented, except for the presence of ordered arrays (S-layer) on the surface of *C. glutamicum* and some other corynebacterial strains (Chami *et al.*, 1995; Peyret *et al.*, 1993; Soual-Hoebeke *et al.*, 1999). Therefore, the extracellular material from strains grown on minimal medium, as well as the substances extracted by gentle shaking with glass beads (Ortalo-Magné *et al.*, 1995, 1996) from extensively washed actively growing cells (surface-exposed materials), from various strains of corynebacteria were analysed for their macromolecule and lipid compositions, in conditions of minimal bacterial lysis (as revealed by the absence in the filtrates of any detectable activity of the

Fig. 1. Thin sections of corynebacteria prepared by conventional embedding. Bacterial pellets were fixed for 1 h at 4 °C with 2.5 % (w/v) glutaraldehyde and 0.05 M lysine in a 0.1 M cacodylate buffer containing (b, c, f) or not (a, d, e) 0.075 % (w/v) ruthenium red. (a) and (b) *C. glutamicum* CGL2005 (PS2⁻); (c) *C. glutamicum* CGL2025 (PS2⁻); (d) *C. xerosis* (ATCC 373^T); (e) and (f) *C. amycolatum* (ATCC 49368^T). OL, outer layer; ETL, electron-transparent layer; EDL, electron-dense layer; PM, plasma membrane. Bars, 200 nm.

Table 1. Relative percentages of carbohydrate, proteins and lipids in the surface-exposed materials and culture media from corynebacteria

Exponentially grown corynebacteria were harvested. Surface-exposed materials (beads) were obtained from wet cells by extraction with 4 mm glass beads. The corresponding materials and culture media were sterilized by filtration. The surface-exposed materials and aliquots of the culture filtrates were assayed for carbohydrate and protein; the remaining culture media were extracted with organic solvents. The resulting lipids were dried and weighed as described in Methods. The lipid contents of the culture media were expressed relative to both macromolecules (carbohydrate + protein) and the cell dry masses (*).

Strain	Beads		Media		
	Carbohydrate	Protein	Carbohydrate	Protein	Lipid
<i>C. xerosis</i> ATCC 373 ^T	98	2	99	0.7	0.3 (10*)
<i>C. amycolatum</i> ATCC 49368 ^T	98	2	98	0.5	1.1 (17)
<i>C. diphtheriae</i> C8r(-)Tox ⁻	93	7	96	2	2 (33)
<i>C. glutamicum</i> CGL2005	99	1	94	3	3 (10)

cytosolic isocitrate dehydrogenase enzyme activity). The extracellular and cell surface macromolecules of corynebacteria which arose from the ethanol precipitation consisted primarily of carbohydrate (90%); protein represented less than 10% of the macromolecules (Table 1). Lipids extracted both from the culture filtrates and from surface-exposed materials consisted mainly of trehalose dicorynomycolate (TDCM) and trehalose monocorynomycolate (TMCM), except for *C. amycolatum*, where these glycolipids were absent; small amounts of phospholipids, notably phosphatidyl inositol dimannosides and phosphatidyl glycerol, were also detected (data not shown). Interestingly, the same qualitative lipid composition was found for the whole bacteria, showing that all the classes of lipid were exposed on the cell surface of corynebacteria, in sharp contrast to what has been observed for mycobacteria where selective classes of lipid were found exposed on the cell surface (Ortalo-Magné *et al.*, 1996).

Corynebacteria also differed from mycobacteria by the presence of relatively large amounts of lipids in their culture filtrates (Table 1) while only traces of lipid, if any, were found in the extracellular fluids of mycobacteria (Lemassu & Daffé, 1994; Lemassu *et al.*, 1996). Besides, extracellular lipids were present in both the *cspB*-disrupted *C. glutamicum* mutant CGL2025, which is devoid of a surface ordered S-layer (Peyret *et al.*, 1993), and its parent strain CGL2005, suggesting that the presence of an S-layer does not prevent the secretion of lipid into the culture medium.

Chemical analysis of the corynebacterial outermost polysaccharides

Analysis of the acid hydrolysis products originated from the extracellular and surface-exposed macromolecules of the various strains by GC showed that the sugar composition of the outermost constituents of the vast majority of the corynebacterial species analysed was similar to that in mycobacteria (Lemassu & Daffé,

1994; Lemassu *et al.*, 1996; Ortalo-Magné *et al.*, 1995); these were 10–20% arabinose (Ara), 20–35% mannose (Man) and 50–70% glucose (Glc). The outermost polysaccharides from *C. amycolatum* were almost exclusively composed of Glc. To characterize the polysaccharides that are composed of these sugar residues, the crude ethanol precipitates from the extracellular and surface-exposed macromolecules were further investigated; the precipitates were first treated with proteases and then chromatographed on an ion-exchange column (Lemassu & Daffé, 1994). In both cases, most of the carbohydrate material (> 90%) was eluted from the column with the buffer containing no salts (data not shown), indicating that they were composed of neutral substances; gel-filtration chromatography over a Bio-Gel P-10 column led to the isolation of three fractions (Fig. 2a). The major fraction (peak A) was exclusively composed of Glc and was eluted in the void volume; gel-filtration chromatography over a Sephadex G-200 column of the glucan fraction led to the isolation of a single peak A₁ at a position corresponding to an apparent molecular mass of 110 kDa (Fig. 2b). The second peak of the Bio-Gel P-10 column (peak B, Fig. 2a) contained Ara and Man (in a molar ratio of 1:1) and exhibited an apparent molecular mass of 13 kDa. The last fraction eluted from the Bio-Gel P-10 column was a large, broad peak (peak C, Fig. 2a) and contained Ara, Man and Glc; gel-filtration chromatography over a Bio-Gel P-4 column of this material gave two peaks (Fig. 2c) at positions corresponding to apparent molecular masses of 1.7 kDa (peak C₁, glucan, Fig. 2c) and 1 kDa (peak C₂, arabinomannan, Fig. 2c). A similar pattern was observed when the macromolecules derived from the culture filtrate of *C. xerosis* were analysed. Thus, like mycobacteria (Lemassu & Daffé, 1994; Lemassu *et al.*, 1996; Ortalo-Magné *et al.*, 1995), *C. xerosis* elaborates extracellular and surface-exposed polysaccharides composed of a 110 kDa high-molecular-mass glucan and a 13 kDa arabinomannan. In contrast to what was found in mycobacteria, low-molecular-mass arabinomannan and

Fig. 2. Gel filtration profiles of the extracellular and surface-exposed polysaccharides of *C. xerosis* (ATCC 373^T) grown on synthetic medium. (a) Neutral polysaccharides were fractionated on a column of Bio-Gel P-10 (90 cm × 1.8 cm); (b) fraction A from the Bio-Gel P-10 column was rechromatographed on a column of Sephadex G-200 (90 cm × 1.8 cm). (c) fraction C from the Bio-Gel P-10 column was rechromatographed on a column of Bio-Gel P-4 (90 cm × 1.8 cm). The eluant of the gel filtration columns consisted of 1% (a, b) and 0.5% acetic acid (c). Fractions, 5 ml in (a), 9 ml in (b) and 6.5 ml in (c), were collected, and the carbohydrate content of each fraction was determined by colorimetric method. V_0 , void volume.

glucan were present in the mixture of polysaccharides from the strains examined.

Non-covalently associated cell envelope lipopolysaccharides

The occurrence of arabinomannans in the extracellular and surface-exposed materials from corynebacteria led us to examine the existence of their lipidated forms, i.e. lipoarabinomannan (LAM) in the bacteria. On the basis of preliminary experiments using SDS-PAGE, which showed the occurrence of broad carbohydrate bands, the putative lipopolysaccharides were extracted from corynebacterial cells with hot phenol/water (Leopold & Fisher, 1993); as expected, Ara and Man were identified by GC analysis of the acid hydrolysis products of all the hot phenol/water extracts. Analysis by SDS-PAGE revealed two broad bands (in the region of 20–40 kDa)

Fig. 3. SDS-PAGE analysis and hydrophobic interaction chromatography of the lipoglycans extracted from corynebacteria. (a) Gel electrophoresis of an authentic sample of mycobacterial lipoarabinomannan (mLAM) and lipomannan (mLM) (lane 1) and of hot phenol/water extracts from *C. glutamicum* CGL2005 (lane 2), *C. diphtheriae* (strain C8, (–) Tox[–]) (lane 3), *C. xerosis* (ATCC 373^T) and *C. amycolatum* (ATCC 49368^T). Positions of size markers are shown on the left. cLAM₁, corynebacterial lipoarabinomannan with a long polysaccharide chain; cLAM₂, corynebacterial lipoarabinomannan with a short polysaccharide chain; cLM, corynebacterial lipomannan. (b) Hydrophobic interaction chromatography profile of the crude aqueous phase from the phenol/water extract from *C. glutamicum* on a column of octyl-Sepharose (10 cm × 0.5 cm). The column was equilibrated in 0.1 M sodium acetate buffer containing 15% propan-1-ol and first eluted with the buffer (3 ml) and then with a discontinuous gradient of propan-1-ol (15–100%, 3 ml each). Fractions of 1 ml were collected, and the carbohydrate content of each fraction was determined by a colorimetric method.

in all the crude aqueous extracts (Fig. 3a). Based on their electrophoretic properties, similar to those of authentic samples of mycobacterial LAM and lipomannan (LM), used as control, these data suggested the presence of LAM- and/or LM-like substances in corynebacteria and are in agreement with a previous observation (Sutcliffe, 1995). Compared to mycobacterial lipoglycans, however, the major amphiphilic substances from corynebacteria exhibited significantly faster mobilities on gels. To characterize these substances further, the crude amphiphilic fractions from *C. glutamicum* and *C. xerosis* were chromatographed on octyl-Sepharose gels (Leopold & Fisher, 1993) irrigated with a discontinuous gradient of propan-1-ol (15–100%); two carbohydrate peaks were obtained in both cases (Fig. 3b). The first peak contained non-lipidated polysaccharides, and probably nucleic acids (Leopold & Fisher, 1993), eluted at

Table 2. Glycosyl-linkage compositions of arabinogalactans from corynebacteria

Purified cell walls were O-methylated, hydrolysed, reduced and acetylated as described in Methods. The resulting partially O-methylated, partially O-acetylated samples were analysed by GC-MS. –, Not detected; t, terminal; Araf, arabinofuranosyl residue; Galf, galactofuranosyl residue; Glcp, glucopyranosyl residue; Manp, mannopyranosyl residue.

Glycosyl residue	<i>C. glutamicum</i> CGL2005	<i>C. diphtheriae</i> C8r(–)Tox [–]	<i>C. xerosis</i> ATCC 373 ^T	<i>C. amycolatum</i> ATCC 49368 ^T
t-Araf	10.0	6.0	22.0	20.0
2-Araf	8.0	7.0	–	–
5-Araf	61.0	48.0	38.0	35.0
3,5-Araf	10.0	–	11.0	12.5
2,5-Araf	–	3.5	5.0	6.0
t-Galf	–	2.0	2.0	1.0
5-Galf	6.0	13.0	8.0	12.0
6-Galf	2.5	3.5	2.0	2.5
5,6-Galf	2.5	10.0	–	–
2,5-Galf	–	–	5.0	2.5
t-Glcp	–	–	2.0	2.5
6-Glcp	–	–	2.0	1.0
2,6-Glcp	–	–	5.0	4.0
t-Manp	–	3.0	–	–
2-Manp	–	3.0	–	–

the initial concentration of 15–30% propanol. The second major carbohydrate peak, which was eluted from the gel with 50–60% propanol, contained most of the LAM- and LM-like substances, as judged by SDS-PAGE, suggesting that these occur in the aqueous phenol extracts in their acylated forms. Fatty acid methyl esters resulting from the methanolysis of the second peaks originated from both strains consisted of C_{16:0} and C_{18:1}, the major fatty acyl substituents identified in the saponification products of whole bacteria. Analysis of the acid hydrolysis products from this latter fraction showed a Ara:Man molar ratio of 1:8 and 1:2 for samples originating from *C. glutamicum* and *C. xerosis*, respectively. Based on the SDS-PAGE and GC profiles it was concluded that lipopolysaccharides of *C. glutamicum* corresponded primarily with LM-like substances (cLM, Fig. 3a), with small amounts of LAM-like molecules (cLAM₁, Fig. 3a); the lipoglycans of *C. xerosis* consisted of LAM-like compounds. It is suggested that *C. xerosis* possesses two types of LAMs, i.e. cLAM₁ and another class of LAM (cLAM₂, Fig. 3a) possessing a carbohydrate moiety much shorter than those of mycobacterial LAM (mLAM) and cLAM₁; this hypothesis is based on the carbohydrate content of the fraction and its mobility on SDS-PAGE, and is consistent with the occurrence of two types of arabinomannans and the lack of detection of mannan in the extracellular and surface-exposed materials of this species (see above).

Analysis of cell wall arabinogalactans

The cell walls of corynebacteria and closely related genera are typified by the presence of a polysaccharide composed mainly of D-arabinofuranosyl and D-gal-

actosyl (Gal) residues, arabinogalactan (AG), which is covalently attached to peptidoglycan. The glycosyl compositional analysis of the purified cell wall AG from the various corynebacteria examined demonstrated that all the strains, including those belonging to *C. amycolatum* which are devoid of corynomycolates, elaborated AG consisting primarily of Ara and Gal (Table 2), but may also have contained other sugar constituents. Based on their glycosyl compositions, the six species examined could be divided into three groups: (i) *C. glutamicum*, in which AG is composed only of Ara and Gal, (ii) *C. diphtheriae*, which contained significant amounts of Man residues in its AG, and (iii) *C. amycolatum* and *C. xerosis*, whose AG contained Glc; these data are consistent with the reported occurrence of Man and Glc in the AG of some corynebacteria (Abou-Zeid *et al.*, 1982).

Detailed structural studies of AG from mycobacteria, rhodococci and nocardiae (Daffé *et al.*, 1990, 1993) showed that the major structural features of AG are genus-specific in mycobacteria and species- or strain-specific in the two other bacterial groups; similar conclusions could also be drawn from the glycosyl-linkage compositions. Consequently, cell wall AGs from representative strains of the three types of glycosyl composition were isolated and analysed for their glycosyl-linkage composition through the characterization of the different partially O-methylated partially O-acetylated alditols (Table 2). While the glycosyl-linkage composition of AG from *C. glutamicum* was very similar to that of mycobacteria (Daffé *et al.*, 1990, 1993), those of AG from the other two groups of corynebacteria were unique; the glycosyl-linkage composition of AG from *C. diphtheriae* differed from those published so far by the

absence of 3,5-linked Ara whereas those of *C. amycolatum* and *C. xerosis* were typified by the absence of 2-linked Ara and the occurrence of 6- and 2,6-linked glucosyl units, two types of glucosyl units not present in the mycobacterial glucan (Lemassu & Daffé, 1994; Lemassu *et al.*, 1996). The glycosyl-linkage compositions of *C. diphtheriae*, *C. amycolatum* and *C. xerosis* were, however, related to those reported for AG from corynebacteria, nocardiae and rhodococci where 2,5-linked Ara has been found (Daffé *et al.*, 1993; Abou-Zeid *et al.*, 1982). A comparative chromatography on a Bio-Gel P-10 column of AGs from corynebacteria and mycobacteria indicated that both polysaccharides exhibited an apparent molecular mass of 15 kDa (data not shown).

Distribution of the corynomycoloyltransferase PS1 and the S-layer constituent PS2 in corynebacteria

It was recently demonstrated that one of the two major secreted protein from *C. glutamicum*, PS1, possesses a mycoloyltransferase activity in this species (Puech *et al.*, 2000). However, like the other major secreted S-layer constituent, PS2, the occurrence of PS1 in different corynebacteria has not been investigated. To determine the distribution of PS1, proteins extracted from the outermost cell envelope compartment were analysed by SDS-PAGE. Compared to those of mycobacteria (Ortalo-Magné *et al.*, 1995), the protein profiles of corynebacteria were less complex (Fig. 4a), consisting only of a few major bands, notably three bands with electrophoretic migrations similar to those of PS1 and PS2 (in the 60–70 kDa region). To characterize these proteins further, Western blot analyses were performed on them using polyclonal antibodies directed against PS1 and PS2. The anti-PS1 antibodies reacted strongly with polypeptide bands exhibiting the corresponding molecular mass in *C. glutamicum* (positive control, Fig. 4b, lane 1), one strain of *C. xerosis* (ATCC 9016; Fig. 4b, lane 3) and *C. amycolatum* (Fig. 4b, lane 5), and gave a faint reaction with proteins from *C. diphtheriae* (Fig. 4b, lane 4) and the type strain of *C. xerosis* (Fig. 4b, lane 2). In these latter strains, stronger reactions were observed with other polypeptides which may share some epitopes with PS1. Although the corynomycoloyltransferase activity of PS1 (Puech *et al.*, 2000) was not investigated, these data suggest the presence of PS1 in all the strains examined, including in those devoid of corynomycolates, i.e. *C. amycolatum* (Collins *et al.*, 1988; Barreau *et al.*, 1993). In contrast, the anti-PS2 antibodies reacted only with proteins originated from the PS2⁺ parent strain of *C. glutamicum* (positive control); proteins from the PS2⁻ strain CGL2025 of *C. glutamicum* (*cspB*-disrupted mutant, negative control), as well as those from the other corynebacterial strains examined did not react with the anti-PS2 antibodies (data not shown). Although the production of PS2 was shown to greatly depend on the carbon source (Soual-Hoebeke *et al.*, 1999), the failure to detect this protein was not attributed to the growth medium used to analyse the other corynebacterial strains since all the strains examined

Fig. 4. SDS-PAGE and Western blot analyses of cell wall proteins of corynebacteria. Protein associated with the cell wall of bacteria were extracted with 2% (w/v) SDS in 50 mM Tris/HCl at 100 °C for 2 min and analysed on acrylamide gels; protein bands were either stained with Coomassie blue (a) or blotted onto nitrocellulose membrane, probed with rabbit anti-PS1 polyclonal antibodies and bands detected using alkaline-phosphatase-conjugated antibodies and nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indoxyl phosphate (BCIP) *p*-toluidine as substrates (b). Positions of size markers are shown on the left. (a) Lane 1, *C. glutamicum* CGL1009 (overexpressing PS2); lane 2, *C. amycolatum* (ATCC 49368^T); lane 3, *C. xerosis* (ATCC 373^T); lane 4, *C. diphtheriae* (strain C8, (–) Tox⁻); lane 5, *C. pseudodiphtheriticum* (strain Breuillaud). (b) Lane 1, *C. glutamicum* CGL2005; lane 2, *C. xerosis* (ATCC 373^T); lane 3, *C. xerosis* (ATCC 9016); lane 4, *C. diphtheriae* (strain C8, (–) Tox⁻); lane 5, *C. amycolatum* (ATCC 49368^T).

were grown on the same BHI medium, which had been shown to allow *C. glutamicum* strain CGL2005 to produce a highly ordered surface layer composed of PS2 (Chami *et al.*, 1995; Peyret *et al.*, 1993). It was thus concluded that the S-layer component PS2 was absent from most of the strains examined herein.

Although PS2 was not detected by Western blotting of the outer surface proteins of most corynebacterial strains examined herein, the lack of detection of the protein could be due to the production of only a tiny amount of PS2 by these strains. In addition, some corynebacterial species may produce a surface layer composed of proteins other than PS2. Since freeze-etching electron

Fig. 5. For legend see facing page.

microscopy has proved to be a sensitive technique for the detection of crystalline S-layer, this technique was used to investigate the occurrence of ordered arrays on the various corynebacterial strains studied. Apart from the strain of *C. glutamicum* carrying the *cspB* gene (Peyret *et al.*, 1993; Chami *et al.*, 1995; data not shown from this study), no appearance of S-layer was visible on the other strains examined (Fig. 5). It was thus concluded that the other strains do not produce S-layer. Some interesting species-specific features were also observed; for instance, a filamentous surface, similar to that described for mycobacteria, was seen in *C. pseudodiphtheriticum* (Fig. 5c).

Presence, distribution and localization of porins in corynebacteria

Pore-forming polypeptides (porins) have been recently characterized in *C. glutamicum* as low-molecular-mass peptides (Lichtinger *et al.*, 1998) but the location of these proteins and their distribution in other members of the genus *Corynebacterium* have not been investigated. Thus, SDS-PAGE analysis was performed on the different cell fractions (extracellular, surface-exposed, crude cell wall, membrane proteins) of various corynebacterial strains and proteins were blotted and analysed by Western blotting using polyclonal antibodies directed against corynebacterial porins (Fig. 6a). Positive reactions were observed with polypeptide bands exhibiting small molecular masses from extracellular, surface-exposed, crude cell wall materials, but not with plasma membrane fractions of *C. glutamicum*, *C. diphtheriae* (Fig. 6a), *C. xerosis* ATCC 7711, 7094 and 9016, and *C. pseudodiphtheriticum*; no reaction was found, however, with the corresponding materials from *C. amycolatum* and the type strain of *C. xerosis* (Fig. 6a). To confirm that these polypeptide bands corresponded to porins, the various materials were examined for their ability to form channels for the entry of small hydrophilic molecules in lipid bilayer membranes. Materials derived from glass-bead-treated cells, cytosol and plasma fractions from *C. diphtheriae* and *C. pseudodiphtheriticum* gave a small amount of channel-forming activity but much higher channel-forming activity (Lichtinger *et al.*, 1998) was always found in the cell wall fractions. No channel-forming activity was detected with cell fractions from *C. xerosis*. An example of the channel-forming activity of cell wall material of *C. diphtheriae* is shown in Fig. 6(b). Small amounts of the cell wall fraction were treated with the detergent Genapol. The addition of this material to the aqueous phase bathing a lipid bilayer membrane resulted in a step-wise change in membrane conductance (see

Fig. 6c) caused by the formation of ion-permeable channels in the membrane. The channels were fairly homogeneous as Fig. 6(b) indicates and had a mean single channel conductance of about 2.3 nS in 1 M KCl. Only occasionally were channels observed that had twice the conductance of the 2.3 nS channel. It is noteworthy that the cell wall channel of *C. glutamicum* has a much higher single-channel conductance of 5.5 nS under the same conditions (Lichtinger *et al.*, 1998), an observation which may be related to the physiological differences between the two corynebacterial species; similar observations have been made in mycobacteria, where porins exhibiting different biochemical properties have been described, not only in various species, but also in the same mycobacterial strain (Kartmann *et al.*, 1999; Lichtinger *et al.*, 1999; Mukhopadhyay *et al.*, 1997; Trias *et al.*, 1992; Trias & Benz, 1994; Senaratne *et al.*, 1998).

Covalently linked corynomycolates and the cell wall fracture plane

Corynebacteria, mycobacteria and closely related genera are known to produce, in addition to the expected fracture plane within the plasma membrane, a second and main plane within the cell wall when freeze-fractured preparations of cells are observed by electron microscopy (Benedetti *et al.*, 1984; Chami *et al.*, 1995; Takeo *et al.*, 1984); this plane is postulated to propagate between the mycolate monolayer, formed by the mycoloyl residues which esterify the arabinan termini, and the lipid layer of the outer barrier of these organisms (Daffé & Draper, 1998; Minnikin, 1982). Thanks to the existence of a corynebacterial species devoid of mycolic acid, i.e. *C. amycolatum*, and of a pair of isogenic strains of *C. glutamicum* differing in their cell-wall-associated corynomycolate content (Puech *et al.*, 2000), the question of the contribution of mycolates in the main fracture plane was addressed. Cell wall corynomycolates were isolated from extensively delipidated cells by chromatography of the saponification products; they represented only a small percentage (0–5%) of dried corynebacterial mass (Table 3), compared to mycobacteria (roughly 10% in the case of *M. tuberculosis*; Jackson *et al.*, 1999). Except for *C. amycolatum*, the various strains contained corynomycolates. It has to be noted that the different strains labelled *C. xerosis* differed one from another in terms of the chain length of their corynomycolates; while the type strain of the species (ATCC 373) and strain ATCC 7711, a true *C. xerosis* (Funke *et al.*, 1996), produced C₃₄ and C₃₆ corynomycolates, the other strains elaborated

Fig. 5. Freeze-fractured and deep-etched preparations of corynebacterial strains grown on BHI-containing agar plates (a–f) and cell envelope outermost lipid material (g). (a) *C. glutamicum* CGL2025 (PS2⁺); (b) *C. diphtheriae* (strain C8.(–) Tox⁺); (c) *C. pseudodiphtheriticum* (strain Breuillaud); (d) *C. xerosis* (ATCC 7711); (e) *C. xerosis* (ATCC 373^T); (f) *C. amycolatum* (ATCC 49368^T). Note the absence of the ordered surface layer on all the strains examined. Depending on the strains, however, a fracture plane is seen either in the cell wall fracture plane (2) close to the bacterial surface (1) in (a)–(d) or in the plasma membrane (3) in (e) and (f). (g) Crude octylglucoside extract recovered by centrifugation, extensively dialysed and then pelleted at 200 000 g. Note the fracture plane that occurs within the homogeneous smooth vesicles and indicates that the material analysed spontaneously forms liposomes. Bars, 500 nm (a–f); 400 nm (g).

Fig. 6. Distribution and sublocalization of the low-molecular-mass porin in corynebacteria and functional analysis of the cell wall porin of *C. diphtheriae* (strain C8, (–) Tox[–]). (a) Culture filtrates (CF), surface-exposed (S), cell wall (CW) and plasma membrane (PM) material of various strains were analysed by Western blotting using polyclonal antibodies directed against the low-molecular-mass porin from *C. glutamicum*. Positions of size markers are shown on the right. (b) Histogram of the probability $P(G)$ of the occurrence of a given conductivity unit observed with membranes formed of phosphatidylcholine/phosphatidylserine (molar ratio 4:1)/*n*-decane in the presence of the detergent-solubilized cell wall extract of *C. diphtheriae*. $P(G)$ 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, pH 6.0; the applied potential was 20 mV at 20 °C. The mean single-channel conductance was 2.3 nS for 148 single-channel events. (c) Single-channel recording of a PC:PS (molar ratio 4:1)/*n*-decane membrane in the presence of 10 μ l the cell wall fraction of *C. diphtheriae* treated with 1% Genapol. The aqueous phase contained 1 M KCl, pH 6.0. The applied membrane potential was 20 mV and the temperature was 20 °C.

C_{30} and C_{32} chains (Table 3). Furthermore, comparative data from the various strains by biochemical tests (API CORYNE) and fatty acid compositional analysis (data not shown) demonstrated that the other ATCC strains were misidentified and did not correspond to strains of *C. xerosis*, in agreement with the heterogeneity of this species (Coyle *et al.*, 1993; Funke *et al.*, 1996).

Examination of the various corynebacterial strains by freeze-etch electron microscopy demonstrated that all strains of *C. amycolatum* exhibited a fracture plane only within the plasma membrane (Fig. 5f). In corynomycolate-containing strains, however, no correlation was found between the occurrence of a fracture plane within the cell wall and the content in cell-wall-associated corynomycolates (Table 3). This was particularly true for the type strain of *C. xerosis*, which exhibited a relatively high cell-wall-associated corynomycolate content, but most frequently exhibited a unique fracture plane within the plasma membrane (Fig. 5e); a small percentage of these cells (10%) may, however, show a

fracture plane within the cell wall (Fig. 5d). This observation was independent of the origin of the strain (the strain was obtained from three different culture collections), the batches, the carbon source used for the bacterial growth and the various growth phases of the strain. Conversely, the *csp1*-disrupted mutant of *C. glutamicum*, which contains only 0.5% of covalently linked corynomycolates, exhibited a cell wall fracture plane superimposable on that of its parent (Fig. 5a). In contrast, in the representative strains of the other species, as well as the other strain of *C. xerosis* (ATCC 7711), the fracture plane propagated exclusively within their cell wall (Fig. 5, Table 3). These data indicated that the occurrence of a fracture plane within the cell was not attributable to the cell-wall-linked corynomycolate alone.

Non-covalently associated cell envelope lipids

To explain the occurrence of the cell wall fracture plane in strains exhibiting a very low cell-wall-associated

Table 3. Lipid analysis and occurrence of the cell wall fracture plane in corynebacteria

Bacteria were extracted with organic solvents to yield non-covalently bound lipids. The residues were saponified and the resulting cell wall-linked corynomycolic acids were extracted with diethyl ether and their percentage (relative to cell dry mass) was determined in two ways (see Methods). The values represent the means of three independent determinations. The chain lengths of corynomycolates were determined by GC-MS; the major chain length is shown in bold. When analysed by freeze-fracture electron microscopy, the fracture plane was observed within the cell wall (+) or the plasma membrane (−) of corynebacteria. While the type strain and strain ATCC 7711 correspond to true *C. xerosis* the others strains labelled '*C. xerosis*' are misidentified (see text).

Species	Non-covalently bound lipids (%)	Cell-wall-linked corynomycolic acids (%)	Major chain length	Fracture in the cell wall
<i>C. amycolatum</i> ATCC 49368 ^T	3.7 ± 0.1	0		—
<i>C. amycolatum</i> CIP 79.37	5.1 ± 0.1	0		—
<i>C. amycolatum</i> CIP 100836	5.3 ± 0.1	0		—
<i>C. amycolatum</i> CIP 103818	5.1 ± 0.1	0		—
<i>C. amycolatum</i> CIP 103819	4.1 ± 0.1	0		—
<i>C. amycolatum</i> CIP 103820	6.1 ± 0.1	0		—
<i>C. amycolatum</i> CIP 103821	5.3 ± 0.1	0		—
<i>C. xerosis</i> ATCC 373 ^T	3.2 ± 0.1	3.5 ± 0.1	34, 36	—*
' <i>C. xerosis</i> ' ATCC 7094	11.8 ± 0.2	1.9 ± 0.05	30, 32	+
' <i>C. xerosis</i> ' ATCC 7711	12.0 ± 0.2	4.8 ± 0.1	34, 36	+
' <i>C. xerosis</i> ' ATCC 9016	10.8 ± 0.2	1.3 ± 0.5	30, 32	+
<i>C. diphtheriae</i> C8r(−)Tox [−]	8.0 ± 0.1	2.5 ± 0.1	30, 32	+
<i>C. pseudodiphtheriticum</i> Breuillaud	5.8 ± 0.1	2.5 ± 0.1	32, 34	+
<i>C. glutamicum</i> CGL2005	7.0 ± 0.1	1.0 ± 0.5	32, 34 , 36	+
<i>C. glutamicum</i> CGL2022	7.0	0.5	32, 34 , 36	+

* 90% of the cells were devoid of the cell wall fracture plane.

corynomycolate content, we postulated that other lipids may replace corynomycoloyl residues in the inner monolayer of the outer membrane, based on the structural similarities between C_{32–36} corynomycolate-containing molecules, e.g. TDCM and TMCM (with two parallel C_{16–18} chains), and C_{16–18}-containing lipids such as phospholipids. Therefore, non-covalently associated lipids were extracted from bacterial cells grown with organic solvents and weighed (Table 3). Interestingly, strains of *C. amycolatum* and the type strain of *C. xerosis* contained 3–6% of extractable lipids whereas the other corynebacterial strains produced significantly more lipids. TLC analysis of these lipids showed that their composition was similar to that found in the extracellular and surface-exposed materials; TDCM, TMCM and phospholipids were the main substances detectable in all the strains, except *C. amycolatum*, where only phospholipids were observed. Compared to the outermost lipid materials, however, many more phospholipids were detected in bacterial lipids, as expected from their partiality for plasma membrane. It was thus concluded that the presence of significant amounts of extractable lipids, e.g. TDCM and TMCM, probably contributes to the occurrence of a cell wall fracture plane in corynebacteria.

To evaluate the possible contribution of non-covalently bound lipids in the cell wall permeability barrier, the outermost material of *C. glutamicum* was extracted

with octylglucoside; this method did not affect the integrity of bacterial cells, as judged by freeze-fracture microscopy (data not shown). The crude detergent extract, which contained mostly polysaccharides and lipids, was extensively dialysed to eliminate the detergent and to reconstitute organized structures; it was then centrifuged and the pellet was analysed by freeze-fracture electron microscopy (Fig. 5g). Homogeneous smooth vesicles whose diameter varied from 50 to 500 nm were observed throughout the samples analysed, indicating that the material examined spontaneously forms liposomes upon dialysis of the detergent; analysis of the lipid material by TLC showed that it primarily consisted of trehalose dicorynomycolates, with small amounts of phospholipid and trehalose monocorynomycolates. It follows then that the non-covalently bound lipids from the cell envelope of corynebacteria are strong candidates for participating in a membrane-like structure.

DISCUSSION

Corynebacteria, mycobacteria, nocardiae, rhodococci and closely related genera are members of a distinctive suprageneric actinomycete taxon which includes bacterial species of such biotechnological, medical, veterinary and environmental importance that it is crucial to understand fully their physiology and metabolism

(Sutcliffe, 1997). From the enormous work that has been devoted to the characterization of members of this taxon, it appeared that many of their distinctive properties were related to their unusual cell envelope composition and architecture, compared to those of other Gram-positive micro-organisms. Consequently, models of the mycobacterial envelope, consistent with their chemical and physical properties, have been proposed (Daffé & Draper, 1998; Liu *et al.*, 1995; Minnikin, 1982; Rastogi, 1991) and extrapolated to other members of the taxon (Minnikin & O'Donnell, 1984; Sutcliffe, 1997).

As far as the ultrastructural appearance is concerned, the cell envelope of corynebacteria is similar to that of mycobacteria, consisting of a typical plasma membrane bilayer, a thick electron-dense layer (EDL), an electron-transparent layer (ETL) and an outer layer (OL). The electron density of the EDL makes it likely that it contains the cell wall peptidoglycan, which possesses charged groups able to bind metallic stains used in electron microscopy. The EDL is surrounded by a thin ETL which is traditionally considered to consist of mycolic residues, based on the transparency of this layer to electrons; indeed, the occurrence of eumycoloyl, nocardomycoloyl, or corynomycoloyl residues covalently linked to the cell wall arabinogalactan of mycobacteria and related genera, on the one hand, and the effect of the removal of lipids from isolated mycobacterial cell walls, which causes the disappearance of the layer, on the other hand, support this interpretation. Surprisingly, however, this layer was seen in all corynebacterial species examined, including *C. amycolatum*, which is devoid of corynomycolates (Fig. 1); indeed, this observation could be explained by the replacement on the arabinogalactan of *C. amycolatum* of corynomycoloyl residues by normal-chain fatty acyl residues, i.e. C_{16-18} . This hypothesis was ruled out by the analysis of the purified cell walls of the bacterium, where no fatty acyl substituent could be detected, indicating that the ETL was not synonymous with a lipid layer. Furthermore, the thickness of this layer in corynebacteria is comparable to that measured in mycobacteria (6–7 nm). Assuming a linearity between the thickness of the hydrocarbon portion and the number of carbon atoms, the theoretical length of the C_{50-60} main alkyl chain, the so-called meromycolate chain, of eumycoloyl residues is estimated to be 6.5 nm (see Daffé & Draper, 1998) and that of corynomycoloyl residues (C_{16}) should be one-third of that observed in mycobacteria. Clearly, further studies are warranted to decipher the chemical nature of the ETL in corynebacteria and mycobacteria. The OL of the corynebacterial strains examined were heavily stained with ruthenium red, as previously seen for mycobacteria (Rastogi *et al.*, 1986) but in apparent conflict with a previous report (Rastogi *et al.*, 1984). Although it is not clear what bacterial components react with the dye, it is evident that these are not acidic polysaccharides as previously claimed (Rastogi *et al.*, 1984), primarily because the outermost carbohydrate constituents of corynebacteria (this study) and myco-

bacteria (Ortalo-Magné *et al.*, 1995; Lemassu *et al.*, 1996) were found almost exclusively to be neutral substances.

Consistent with the phylogenetic relatedness between mycobacteria and corynebacteria is the observation that the main structural features of the major polysaccharides of the two genera were very similar. No gross structural difference was found between the non-covalently associated cell envelope polysaccharides, i.e. the 110 kDa glucan, 13 kDa arabinomannan and lipoglycans, characterized in this study and those published for mycobacteria (Lemassu & Daffé, 1994; Lemassu *et al.*, 1996; Ortalo-Magné *et al.*, 1995; Hunter *et al.*, 1986); in addition, low-molecular-mass glucan and arabinomannan, not previously described in mycobacteria, were identified in corynebacteria. The backbone of the major cell wall polysaccharide, arabinogalactan, was also found to be held in common between mycobacteria (Daffé *et al.*, 1990, 1993) and corynebacteria (Abou-Zeid *et al.*, 1982; this study). In contrast to mycobacteria, however, while the mycobacterial arabinogalactans are almost exclusively composed of Ara and Gal residues (Daffé *et al.*, 1993), significant amounts of Man and Glc residues may be found covalently linked to arabinan and/or galactan segments, a situation encountered in other actinomycetes such as rhodococci and nocardiae (Daffé *et al.*, 1993). As in these latter genera, the glycosyl-linkage composition of arabinogalactan in corynebacteria may vary according to the species examined.

Although they are Gram-positive bacteria, corynebacteria and closely related micro-organisms share with Gram-negative bacteria the property of possessing in their envelopes an outer barrier that is distinct from the plasma membrane. While this additional barrier in Gram-negative micro-organisms is a typical bilayer of phospholipid and lipopolysaccharide, in mycobacteria and corynebacteria the cell-wall-linked mycolates and corynomycolates certainly contribute to this barrier, since the disruption of genes that code for mycoloyl-transferases causes a decrease in the amount of cell wall-bound mycolates and corynomycolates, and affects the permeability of the envelope of the mutants (Jackson *et al.*, 1999; Puech *et al.*, 2000). This outer permeability barrier also includes non-covalently linked lipids which are probably arranged to form a bilayer with the corynomycoloyl residues, as proposed for mycobacteria (Minnikin 1982; Rastogi, 1991; Liu *et al.*, 1995). As expected from these data, freeze-fractured samples of mycobacteria, corynebacteria and related genera showed that these organisms possess, in addition to the expected plasma membrane fracture, a major plane of weakness in their cell walls, close to the cell surface of mycobacteria (Chami *et al.*, 1995; Benedetti *et al.*, 1984). The present study extends this concept by showing that the various corynebacterial strains that are devoid of corynomycolates, i.e. *C. amycolatum*, exhibit only one fracture plane, which occurs in their plasma membrane, whereas the corynomycolate-containing strains exhibit a cell wall fracture plane, with the notable exception of the type strain of *C. xerosis*. Paradoxically, this strain

Fig. 7. A tentative model for the cell envelope of corynomycolate-containing corynebacteria, e.g. *C. glutamicum*. From the cytoplasmic to the external side of the bacteria the cell envelope is composed of a plasma membrane (PM), a complex wall that is seen in thin sections as an electron-dense layer (EDL) and an electron-transparent layer (ETL), and an outer layer (OL). The PM is a typical bilayer of proteins (dark rectangles and oval spots) and phospholipids (PL, empty oval symbols). The EDL consists of thick peptidoglycan (PG) covalently linked to the heteropolysaccharide arabinogalactan (AG); some of the arabinosyl termini of this polysaccharide are esterified by C_{32-36} corynomycolic acids (thin parallel bars). Because the amount of these fatty acids in most corynebacteria is not sufficient to cover the bacterial surface (see text), covalently bound corynomycoloyl residues are probably arranged to form with other non-covalently linked lipids, e.g. trehalose dicorynomycolates (TDCM, a pair of empty squares with two pairs of thin parallel bars), and trehalose monocorynomycolates (TMCM, a pair of empty squares with one pair of thin parallel bars), the inner leaflet of symmetric bilayer that represents a virtually outer barrier obstructing access of hydrophilic substances; the outer leaflet is composed of non-covalently linked lipids which assemble themselves into a monolayer. In addition, like that of Gram-negative bacteria, the cell envelope of corynebacteria contains proteins, including ones with pore-forming ability (P, grey squares) which are present in all the cell envelope compartments, except the PM. In freeze-fractured and deep-etched preparations of most corynomycolate-containing strains the major fracture plane (FP) is seen within the cell wall, presumably located between the two leaflets of the outer membrane (arrow); in strains devoid of corynomycolates and porins, e.g. *C. amycolatum*, the FP occurs within the PM (arrow). The different non-covalently linked lipids are also present in the OL, which consists primarily of polysaccharides and contains proteins.

possesses a relatively high content of covalently linked cell wall corynomycolates (Table 3) but most cells exhibit only a plasma membrane fracture plane, indi-

cating that the corynomycolate content is not sufficient to explain the occurrence of the cell wall fracture plane. Furthermore, in most corynebacterial species examined, notably the *csp1*-inactivated mutant of *C. glutamicum* (CGL2022), the amount of covalently linked cell wall corynomycolates (Table 3) is less than the $0.085 \mu\text{mol}$ of mycolic acids necessary for forming a monolayer which would cover the 230 cm^2 bacterial cell surface area (calculated for 1 mg of bacterial dry weight; see Nikaido *et al.*, 1995). To reconcile this fact with the occurrence of the second fracture plane in the cell envelope of most of the corynomycolate-containing species, we reasoned that other non-covalently linked lipids, e.g. trehalose dicorynomycolate, may interact with the covalently bound cell wall corynomycolates to form a monolayer which would cover the bacterial cell surface area. This assumption was based first on the structural analogy between C_{32-36} corynomycolate-containing molecules, whose two C_{16-18} chains would be involved in hydrophobic interactions in a monolayer model, and C_{16-18} -containing complex lipid compounds. In this respect, it has been shown that, like phospholipids, suspensions of synthetic trehalose dicorynomycolates exhibited well-defined transition phases and that these suspensions in a fluid state were clearly more active on mitochondria than in a gel state (Durand *et al.*, 1979). Secondly, and more importantly, we showed that trehalose dicorynomycolates are capable of forming bilayers. Consequently, a quantification of lipid extracts from the various corynebacterial strains was undertaken (Table 3); this led to the demonstration that strains exhibiting a second fracture plane contained more extractable lipids than those lacking the cell wall fracture plane. Based on this observation, we speculate (Fig. 7) that in most corynebacteria, non-covalently linked lipid molecules are involved in the monolayer that is traditionally composed of mycolates. Thus, although the cell envelopes of corynebacteria and mycobacteria have several chemical and physical properties in common, the two organisms may be different in terms of the intimate arrangement of their constituents.

The existence in mycobacteria, corynebacteria and related genera of an outer membrane diffusion barrier poses a vital problem to the bacterial cell, i.e. to obtain a supply of nutrients; like those of Gram-negative bacteria, the cell envelopes of some members of the *Mycobacterium* genus and related genera contain specialized non-specific pore-forming proteins (porins) which certainly facilitate the passage of small hydrophilic molecules through their outer membranes (Kartmann *et al.*, 1999; Lichtinger *et al.*, 1998, 1999; Rieß *et al.*, 1998; Mukhopadhyay *et al.*, 1997; Trias *et al.*, 1992; Trias & Benz, 1994; Senaratne *et al.*, 1998). To determine precisely the localization of these porins in the bacterial cell and their distribution in other members of the genus *Corynebacterium*, we investigated the occurrence of the low-molecular-mass porins recently characterized in *C. glutamicum* (Lichtinger *et al.*, 1998) in different cell fractions of various corynebacterial strains. We demonstrated that functionally active low-

molecular-mass porins occur in some corynebacterial species, e.g. *C. diphtheriae* and *C. xerosis* ATCC 7711, but not in all corynebacteria, e.g. *C. amycolatum* and the type strain of *C. xerosis* (ATCC 373). These peptides are found mainly in the cell wall fractions, as expected, but are also present in culture filtrates and the cell surfaces, but not in the plasma membranes, of the bacteria. It has to be noted that strains lacking porins, and virtually devoid of an outer permeability barrier, do not need these proteins for the uptake of nutrients since they present no obvious growth defect on complex media. Therefore, although strains of corynebacteria appear in numerical phenetic studies as clusters closely associated with each other, the present study clearly showed that, unlike mycobacteria, the genus *Corynebacterium* is heterogeneous in terms of both cell wall chemical composition and cell envelope organization.

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