Identification of two glycosylated components of *Mycoplasma penetrans*: a surface-exposed capsular polysaccharide and a glycolipid fraction

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Among the wall-less mycoplasmas only a few species have been identified with a capsule at their cell surface. *Mycoplasma penetrans* is a recently identified mycoplasma with unique morphology, isolated from HIV-infected patients. Using transmission electron microscopy, it was found that *M. penetrans* is surrounded by capsular material 11 nm (strain GTU-54-6A1) to 30 nm (strain HF-2) thick, which can be stained with ruthenium red and labelled with cationized ferritin. The polysaccharide composition of this capsule was indicated by its staining with periodic acid-thiocarbohydrazide silver proteinate and the abolition of ruthenium red staining of the cell surface by neuraminidase treatment. In addition, proteinase K treatment of the *M. penetrans* cells resulted in removal of the capsule, suggesting that polypeptides may contribute in anchoring it to the membrane or in its stability. Two different types of glycosylated material were detected in mycoplasma extracts by SDS-PAGE and periodic acid-Schiff staining. The first component was a high-molecular-mass material, which was heat- and proteinase-K-labile and which probably constitutes the capsular polymer. The other component was a low-molecular-mass glycolipid fraction, which was proteinase-K-, heat- and EDTA-resistant. The identification of a capsule at the *M. penetrans* cell surface is of particular interest for a mycoplasma which has been shown to adhere to various host cells and to penetrate into their intracellular compartments. The capsule may have significance in the pathogenesis of disease associated with infection by this organism.

Keywords: *Mollicutes*, *Mycoplasma penetrans*, capsule, polysaccharide, glycolipid

INTRODUCTION

A very wide range of bacteria, including *Escherichia coli*, *Klebsiella* spp., *Pseudomonas* spp., *Haemophilus* spp., *Salmonella* spp., *Bacillus* spp., clostridia, pneumococci, streptococci and staphylococci, produce polysaccharide capsules (Kasper, 1986; Whitfield, 1988). The capsule usually consists of high-molecular-mass exopolysaccharides containing acidic residues. These polymers are exposed at, and attached to, the extra-

cellular surface of the bacteria. Due to their anionic properties, they appear as electron-dense material in transmission electron micrographs following ruthenium red (RR) treatment. Capsules have been implicated in bacterial virulence, cell adhesion and invasion (Moxon & Kroll, 1990), and also in resistance to complement-mediated lysis and phagocytosis (Cross, 1990). Bacterial capsules are often poor immunogens if not combined with Freund's adjuvant and are thought to induce a T-cell-independent immune response (Kasper, 1986).

A few species of the Class *Mollicutes* (trivial name, mycoplasmas), including *Mycoplasma gallisepticum* (Tajima *et al.*, 1982), *M. meleagridis* (Green & Hanson, 1973), *M. mycoides* subsp. *mycoides* (Buttery & Plackett, 1960), *M. dispar* (Howard & Gourlay, 1974),

Abbreviations: GLF, glycolipid fraction; HMM, high-molecular-mass material; PAS, periodic acid-Schiff reagent; PATAg, periodic acid-thiocarbohydrazide silver proteinate; PBMC, peripheral blood mononuclear cells; RR, ruthenium red.

M. hominis (Furness *et al.*, 1976), *M. hyopneumoniae* (Tajima & Yagihashi, 1982), *M. pneumoniae* (Wilson & Collier, 1976), *M. pulmonis* (Taylor-Robinson *et al.*, 1981) and *Ureaplasma urealyticum* (Robertson & Smook, 1976), possess extramembraneous RR-stainable capsule material. A lipoglycan, not necessarily revealed by RR staining, has also been described in several mycoplasma species (Smith, 1992). The biochemical composition of the capsular polymers of only a few mycoplasma species has been determined. The capsules from *M. mycoides* subsp. *mycoides*, *M. mycoides* subsp. *capri* and *M. dispar* were identified as a polymer of 1,6- β -linked galactose residues (Buttery, 1970), a polyglucan (Jones *et al.*, 1965) and a polymer of galacturonic acid (Rosenbusch & Minion, 1992), respectively. By analogy with similar surface material from other bacterial species, mycoplasma capsules were suggested to be associated with virulence and the ability of these species to evade the host immune response and phagocytosis (Marshall *et al.*, 1995; Rosenbusch & Minion, 1992). Indeed, the ability of *M. hyopneumoniae* to produce pulmonary lesions in pigs was lost when capsule thickness was reduced following passaging *in vitro* (Tajima & Yagihashi, 1982). In addition, it was shown for *M. dispar* (Almeida *et al.*, 1992) that there is a correlation between the presence of capsule and the resistance of mycoplasma cells to phagocytosis.

Mycoplasma penetrans was only recently isolated, initially from HIV-infected patients, and has unique morphology (Lo *et al.*, 1991). It is cytopathic and capable of adhesion to, and invasion of, mammalian epithelial cells (Andreev *et al.*, 1995; Giron *et al.*, 1996; Lo *et al.*, 1993). It is associated with HIV infection, at least among homosexual patients (Grau *et al.*, 1995; Wang *et al.*, 1993). In addition, Sasaki *et al.* (1995) showed that *M. penetrans* is capable of inducing the *in vitro* proliferation of CD4⁺ and CD8⁺ T lymphocytes from HIV-seronegative and -seropositive subjects. Finally, different mycoplasma species, including *M. penetrans*, have been proposed to act as co-factors during HIV infection, accelerating the evolution of this retroviral disease (Blanchard *et al.*, 1997; Blanchard & Montagnier, 1994; Brenner *et al.*, 1996).

The aim of this study was to examine the non-proteinaceous cell surface components of *M. penetrans* in order to gain a greater understanding of the components involved in its interaction with host cells.

METHODS

Mycoplasma strains and culture. *M. penetrans* strain GTU-54-6A1 (type strain), originally isolated by S.-C. Lo (Armed Forces Institute of Pathology, Bethesda, MD, USA), was obtained from J. G. Tully (National Institute of Allergy and Infectious Diseases, Frederick, MD, USA) and was cultivated in SP-4 medium (Tully *et al.*, 1977) supplemented with 10% (v/v) foetal calf serum (Gibco-BRL). For some experiments, [¹⁴C]palmitic acid (Amersham) or [³²P]H₃PO₄ (Amersham) was added to the medium [9.25×10^4 Bq (ml culture medium)⁻¹]. Another strain of *M. penetrans* (strain HF-2; provided by A. Yañez, University of Puebla, Puebla, Mexico) was also used in some experiments and was cultivated as

indicated above for the type strain. Strain HF-2 was isolated from a tracheal aspirate sample collected from a patient with a primary antiphospholipid syndrome (A. Yañez and others, unpublished).

Experimental *M. penetrans* infection of cultured human peripheral blood mononuclear cells (PBMC). PBMC were isolated from the fresh blood of a healthy donor by centrifugation over Ficoll-Paque Plus (Pharmacia). PBMC were cultured for 24 h in RPMI 1640 supplemented with 10% heat-inactivated foetal calf serum, 2 mM L-glutamine and 10 μ g phytohaemagglutinin A (PHA) ml⁻¹ at 37 °C in a humidified atmosphere containing 5% CO₂. The PBMC were then transferred to the same medium without PHA and *M. penetrans* was added at a multiplicity of infection of about 10 colour changing units per cell. After 24 h of co-culture, the cells were processed for electron microscopy as described below.

Glycolipid fraction (GLF) isolation. GLF was prepared as follows: mycoplasma cells were recovered by centrifugation (12000 g, 20 min, 4 °C) and washed twice in PBS (0.145 M sodium chloride, 0.15 M sodium phosphate). Proteinase K was added to the mycoplasma cell suspension [1 μ g proteinase K (2.5 μ g protein)⁻¹] and the mixture was incubated for 2 h at 56 °C. Proteinase K was then inactivated by heating for 15 min at 95 °C. The sample was then dialysed (1:1000) against PBS (or water for preparations being analysed by gas chromatography) for 48 h at 4 °C in 12–14 kDa cut-off dialysis tubing (Spectrum Medical Industries), with several changes of the dialysis buffer. The absence of protein was verified for each preparation of GLF by SDS-PAGE and Coomassie-blue staining.

Transmission electron microscopy. Mycoplasmas, or mycoplasma-infected PBMC, were harvested by centrifugation (8000 g, 15 min, 4 °C), washed twice in 0.1 vol. PBS and fixed for 1 h at room temperature in 0.1 M cacodylate buffer containing 0.075% RR and 3% (v/v) glutaraldehyde. The pellets were then washed three times in 0.1 M cacodylate buffer containing 0.075% RR and post-fixed for 2 h at room temperature in 0.1 M cacodylate buffer containing 0.075% RR and 1% (v/v) osmium tetroxide (Sigma). The pellets were washed three times in water and dehydrated through a graded ethanol series. The resulting pellets were embedded in Epon 812 resin. In some experiments the mycoplasma cells were treated for 30 min at 37 °C with either proteinase K [2 μ g proteinase K (μ g protein)⁻¹] or *Vibrio cholerae* neuraminidase [0.1 U (120 μ g protein)⁻¹] prior to fixation.

For ferritin labelling, the mycoplasma pellets were fixed for 1 h in 0.1 M cacodylate buffer containing 2.5% glutaraldehyde and then incubated in a 1 mg ml⁻¹ cationized ferritin solution (Sigma) for 1 h at room temperature. The pellets were then washed three times in 0.1 M cacodylate buffer and post-fixed by incubation for 1 h at room temperature in a 0.1 M cacodylate buffer containing 2% osmium tetroxide. Finally, samples were washed, dehydrated and embedded as described above.

For periodic acid-thiocarbohydrazide silver proteinate (PATAg) staining, the procedure described by Thiery (1967) was followed. Briefly, ultrathin sections of mycoplasma cells mounted on gold grids were treated for 25 min with 1% periodic acid and then rinsed three times in distilled water. The grids were incubated in 20% (v/v) acetic acid containing 0.2% thiocarbohydrazide for 36 h and then incubated in a series of decreasing concentrations of acetic acid followed by three washes in water. Finally, the grids were floated on 1% silver proteinate (Fluka Biochemica) for 30 min in the dark and then rinsed three times with water. In control sections, the

periodic acid treatment was omitted and the samples were incubated in 10% (v/v) H_2O_2 for 20 min and then processed as described above.

Thin sections were examined with a JEOL 1200EX 2 electron microscope.

Electrophoresis and staining procedures. SDS-PAGE was performed through a 4% acrylamide/bisacrylamide (29:1, w/w) stacking gel, and a 17% acrylamide/bisacrylamide (29:1, w/w) separating gel according to the method of Laemmli (1970). For higher resolution of glycoconjugates, Tricine-SDS-PAGE was used as described by Lesse *et al.* (1990). The *M. penetrans* cells or extracts were solubilized in the SDS-PAGE sample cocktail and loaded on the gels after heating for 5 min at 95 °C. This heating treatment was omitted in some experiments as indicated. Triton X-114 extraction was performed as described by Ferris *et al.* (1995).

Glycoconjugates in gels were stained with the periodic acid-Schiff reagent (PAS) as described by Gerard (1990). The gels were then washed successively in 7% acetic acid for 24 h and in 10% acetic acid/40% methanol for 2 h and then stained with Coomassie blue R250. Apparent molecular masses were determined by comparison with the electrophoretic mobility of low-molecular-mass polypeptide markers (Pharmacia Biotech). Autoradiography was performed using Hyperfilm-MP (Amersham), and a phosphorimager radioanalytical imaging system (Molecular Dynamics) was used to quantify the radioactive signals.

Quantification of sugar in GLF and protein in cellular extracts. Sugars from *M. penetrans* GLF obtained after the dialysis step were determined using a modification of the procedure described by Kirkeby *et al.* (1992). Briefly, GLF was oxidized by adding 0.1 vol. 0.02% periodic acid/7% acetic acid and incubating for 2 h at 37 °C; 0.1 vol. Schiff reagent was then added and the solution incubated for 30 min at room temperature. The A_{550} was measured and compared to a standard glycogen solution. This procedure did not allow the detection of mono- or disaccharides (Kirkeby *et al.*, 1992). The results were expressed as μg glycogen equivalents ml^{-1} .

The protein concentration of cell extracts was measured using the Micro BCA Protein Assay Reagent kit (Pierce).

Determination of GLF sugar composition using GLC. The sugar composition of *M. penetrans* GLF was determined by GLC analysis. Monosaccharides were released from freeze-dried GLF by methanolysis and hydrolysis with mesoinositol as the internal standard. Methanolysis was performed by incubating in 0.5 M HCl in anhydrous methanol (total volume: 0.5 ml) for 24 h at 80 °C. The pH was adjusted to 6.0 by adding silver carbonate. The free amine groups of amine-containing sugars were *N*-acetylated by adding 20 μl acetic anhydride and incubating overnight at room temperature in the dark. The samples were centrifuged, the methanol phase evaporated under a nitrogen stream and the dried residue trimethylsilylated in 100 μl 50% (v/v) pyridine/*N,O*-bis(trimethylsilyl)trifluoroacetamide for 2 h at room temperature according to Kamerling *et al.* (1975). Hydrolysis was performed by incubating with 0.5 ml 4 M trifluoroacetic acid for 4 h at 100 °C. After evaporation of the reagent under vacuum, the monosaccharides were reduced by treatment with 1 mg NaBH_4 in 500 μl 0.1 M NH_4OH for 2 h at room temperature. The excess NaBH_4 was eliminated by increasing the pH to 6.0 by addition of acetic acid and the borate salts were removed by co-distillation with methanol using a rotary evaporator. The dried residue was acetylated by 50 μl pyridine, 200 μl acetic acid overnight at room temperature. The reagents were removed under a nitrogen stream in the presence of toluene

and the residual alditol acetates were dissolved in methanol according to Sadawardeker *et al.* (1965). GLC was performed on a Delsi 200 instrument with a flame ionization detector using a capillary column (25 m $\times$ 0.2 mm) filled with DB-1; gas vector: helium, 0.8 bar (80 kPa); column temperature: 120–240 °C at 2 °C min^{-1} .

RESULTS

Identification of a capsule at the *M. penetrans* surface

RR staining or binding of cationized ferritin revealed the presence of an 11–13 nm thick layer uniformly coating the outer surface of the *M. penetrans* plasma membrane (Fig. 1a, b). It should be noted that this material also covered membrane blebs, structures previously described by others (Giron *et al.*, 1996), which seemed to be released from the mycoplasma cell surface (Fig. 1b). Although the thickness of this layer or capsule was similar in different preparations of the *M. penetrans* type strain, it was twice as thick (25–30 nm) in strain HF-2 (Fig. 2b). The RR staining and the labelling with cationized ferritin indicated that the capsule was composed of anionic polymers. In addition, the polysaccharide composition of this material was demonstrated by two independent experiments: (i) the capsule could not be stained with RR after treatment of the cells with neuraminidase (Fig. 2e) and (ii) the capsule was stained with the PATAg reagent, which is specific for polysaccharides (Fig. 2f). Although these results indicated that the capsule contained polysaccharides, they did not exclude the possibility that other components played a role in anchoring it to the membrane or in its stability. Indeed, proteinase K treatment of the mycoplasma cells resulted in the disappearance of the capsule (Fig. 2d), whereas it was not affected when the cells were incubated under the same conditions (37 °C, 30 min) without protease (Fig. 2c). The *M. penetrans* capsule could also be detected by RR staining when the mycoplasmas were incubated with eukaryotic cells and was found to occupy the space between the two plasma membranes (Fig. 2a).

Detection of *M. penetrans* glycoconjugates

To identify the compound(s) stained during transmission electron microscopy by the PATAg reagent, various extracts of *M. penetrans* were analysed by Tricine-SDS-PAGE and polysaccharides identified with PAS (Fig. 3). A high-molecular-mass PAS-stainable material (HMM) from a whole-cell extract was found in the stacking gel (Fig. 3, lane 5). This material was heat-labile as demonstrated by its disappearance when the whole-cell SDS extract was heated before loading (5 min at 95 °C; Fig. 3, lane 1). In addition, the HMM could not be detected in the gel after proteinase K treatment of the cells (Fig. 3, lane 3). Concomitant with this disappearance, some PAS-stainable material, which did not migrate into the gel, was detected at the bottom of the well. In a control experiment, using the same conditions of incubation (30 min, 37 °C), the HMM did not disappear when the protease was omitted (Fig. 3, lane 2).

Fig. 1. Ultrastructural analysis of *M. penetrans* cells. Capsular material on the surface of *M. penetrans* cells (strain GTU-54-6A1) revealed using RR staining (a) or cationized ferritin labelling (b). Arrows indicate the capsule. Bars, 150 nm.

Fig. 2. *M. penetrans* capsule thickness in the presence of eukaryotic cells, in different strains and effect of proteinase K or neuraminidase treatment. (a–e) Samples stained with RR. (a) *M. penetrans* (type strain) co-cultivated with human PBMC. (b) *M. penetrans* strain HF-2 cultivated in SP-4 medium. (c–e) The *M. penetrans* strain GTU-54-6A1 capsule could not be stained with RR after proteinase K (d) or neuraminidase (e) treatment of the cells, whereas it remained attached to the cells when the mycoplasmas were incubated under the same conditions but without the enzyme (c). (f) The *M. penetrans* (type strain) capsule could also be stained with the PATAg reagent. Arrowheads indicate the capsule. Bars, 100 nm.

In contrast to the protease treatment, neuraminidase treatment of the cells did not affect the HMM (Fig. 3, lane 4). EDTA had the same effect on HMM as

proteinase K treatment (Fig. 3, lane 6). Finally, HMM was not detected in the aqueous phase after hot phenol/water extraction, unlike classical lipoglycans

Fig. 3. Identification of HMM from *M. penetrans* cells. SDS-PAGE was performed using Tricine buffer and the glycoconjugates were detected by staining with PAS. *M. penetrans* cells were solubilized in the SDS-PAGE sample cocktail and loaded after heating for 5 min at 95 °C (lane 1). This heating treatment was omitted for the other samples but the mycoplasma cells were subjected to various treatments prior to solubilization in the SDS-PAGE sample cocktail and loading on the gel: incubation for 30 min at 37 °C without added enzyme (lane 2) or with added proteinase K (lane 3) or neuramidase (lane 4), incubation for 20 min at room temperature without (lane 5) or with (lane 6) 2 mM EDTA. The position of molecular mass markers is indicated on the left of the figure and the interface between the stacking and resolving gels is indicated by the double arrowheads. The HMM and GLF are indicated with brackets.

(Smith, 1992), and was not stained by Coomassie blue or radiolabelled after culture of the mycoplasmas in [^{14}C]palmitate-containing medium (data not shown). In addition to the HMM, a 1–6 kDa band was consistently detected in all the *M. penetrans* extracts tested (Fig. 3, all lanes). This glycoconjugated material was further studied (see below).

Identification of a glycolipid fraction from *M. penetrans*

In preliminary experiments using a standard procedure for lipoglycan purification (Smith, 1985, 1992) no glycoconjugate could be extracted from *M. penetrans* cells with hot phenol after removal of lipids. All the glycosylated material was found in the methanol/chloroform phase used for removal of lipids (data not shown). Another method combining proteinase K digestion with dialysis, previously described for *M. dispar* (Almeida & Rosenbusch, 1991), was tested (see Methods). The preparation obtained following this protocol was analysed by SDS-PAGE and a 1–6 kDa band was detected after PAS staining (Fig. 4). In this preparation, there was also a minor band (11–12 kDa) (Fig. 4a, lane 5) which was not found in most of the

Fig. 4. Electrophoretic analysis of a *M. penetrans* glycolipid fraction. SDS-PAGE was performed on a 17% polyacrylamide gel. Glycoconjugates were stained with PAS (a) and proteins were subsequently stained with Coomassie blue (b). In (b), the bands correspond to the combination of both detection methods. The samples loaded were creatinase (lane 1), transferrin as a glycoprotein control (lane 2), SP-4 medium (lane 3), *M. penetrans* whole-cell lysate (lane 4), proteinase-K-digested and dialysed *M. penetrans* whole-cell lysate (lane 5) and the detergent phase of the Triton X-114 extract of *M. penetrans* cells (lane 6). Incorporation of palmitic acid and phosphate in GLF was revealed after cultivation of mycoplasmas with [^{14}C]palmitic acid (c) or [^{32}P]H $_3$ PO $_4$ (d). In (c) and (d), proteinase-K-digested and dialysed *M. penetrans* whole-cell lysates were separated and radioactivity was detected by autoradiography.

preparations of *M. penetrans* cells. The 1–6 kDa band was also detected in the *M. penetrans* whole-cell lysates (Fig. 4a, lane 4) but not in the culture medium (Fig. 4a, lane 3). The 1–6 kDa band and the 11–12 kDa band were not stained with Coomassie blue, suggesting that they did not contain protein (Fig. 4b, lanes 4 and 5).

To detect covalently linked lipids and phosphate groups in the PAS-stained materials, mycoplasmas were cul-

tivated in the presence of [$1\text{-}^{14}\text{C}$]palmitic acid or [^{32}P]pyrophosphate and the proteinase-K-treated cell lysate was analysed by SDS-PAGE. The 1–6 kDa material incorporated both palmitate and phosphate (Fig. 4c, d).

M. penetrans cells labelled with [$1\text{-}^{14}\text{C}$]palmitic acid were extracted with Triton X-114 and cell components phase-partitioned. The detergent phase contained the 1–6 kDa material as assessed by PAS staining (Fig. 4a, lane 6). Quantitative analysis of the radioactivity associated with the 1–6 kDa GLF revealed that 95% of this material partitioned in the detergent phase (data not shown).

Analysis of the sugar composition of *M. penetrans* GLF by GLC

The sugar composition of GLF was determined by GLC and was found to be Man:Glc:GlcNAc:GalNAc in a ratio of 1:6:1:2. This analysis was performed on two independent preparations of GLF and the results obtained were almost identical. No galactose residues were detected. No attempt was made to detect sulfated, phosphorylated or other modified sugars. The sugar composition of a second *M. penetrans* strain (HF-2) was the same.

DISCUSSION

Electron microscopy using RR staining and cationized ferritin labelling revealed a polyanionic material covering *M. penetrans* cells, including the tip structure believed to mediate cytoadherence (Fig. 1). This 11–13 nm capsule was thinner than similar cell surface structures from other mycoplasma species: 20 nm for *M. gallisepticum* (Tajima *et al.*, 1982), 40 nm for *M. hyopneumoniae* (Tajima & Yagihashi, 1982), 30 nm for *M. mycoides* subsp. *mycoides* (Jones *et al.*, 1965) and 24 nm for *M. dispar* (Howard & Gourlay, 1974). The *M. penetrans* capsule was not lost during centrifugation or washing and was therefore not considered a slime (Whitfield, 1988). The capsule was thicker on strain HF-2 than on the type strain of *M. penetrans* (Fig. 2) indicating that capsule thickness varied among different strains. Capsule synthesis may be regulated, as was reported for other mycoplasma species, including *M. dispar* (Almeida & Rosenbusch, 1991) for which inducible capsule synthesis was demonstrated *in vitro* in the presence of a soluble factor secreted by bovine lung fibroblasts. Capsule thickness has been associated with virulence in *M. hyopneumoniae* (Tajima & Yagihashi, 1982) and *M. gallisepticum* (Tajima *et al.*, 1982). In addition, it has been suggested that capsules of *M. meleagridis* (Green & Hanson, 1973), *M. dispar* (Howard & Gourlay, 1974) and *M. pneumoniae* (Wilson & Collier, 1976) play a role in the attachment of bacteria to host cells. Ultrastructural examination indicated an intimate interaction between the capsule of *M. penetrans* and the surface glycoconjugates of the infected eukaryotic cell (Fig. 2a). This interaction suggests a role for the *M. penetrans* capsule in attachment and this possibility

is supported by the demonstration that neuraminidase or sodium periodate treatment of the *M. penetrans* cells reduced attachment (Giron *et al.*, 1996). Since the *M. penetrans* capsule was stained with the PATAg reagent and could not be RR-stained after neuraminidase treatment, we concluded that the capsule was composed, at least partially, of polysaccharides. Furthermore, as the *V. cholerae* neuraminidase is known to hydrolyse terminal neuraminic acids from polysaccharides (with a specificity for the bonds 2,3- α ->2,6- α ->2,8- α -), the RR staining and cationized ferritin labelling of the *M. penetrans* capsule are probably due to the presence of acidic (anionic) groups at the extremity of the capsular polysaccharide molecules. This result is still consistent with the inability of neuraminidase treatment to remove the HMM seen on SDS-PAGE gels (Fig. 3), as this neuraminidase treatment only hydrolyses terminal neuraminic acids without necessarily affecting polysaccharide chain integrity. Furthermore, the fact that proteinase K treatment of the cells also removed the capsule, without otherwise altering cell morphology, suggested that polypeptides might be involved in the anchoring or stabilization of this material.

To identify the polysaccharide components of the capsule, we looked for glycosylated components by SDS-PAGE and PAS staining. Two types of glycosylated materials of different size were identified (Figs 3, 4). The *M. penetrans* GLF is probably inserted into the plasma membrane as it was shown to possess a lipid moiety. This component is too small to account for the thickness of the capsule, which is more likely to be composed of larger glycoconjugates such as the HMM. Since the capsule was also removed from the cells following similar treatments to those which disrupted the HMM (Fig. 2), the HMM is most likely to be the polysaccharide from this capsule. In addition, the HMM was sensitive to EDTA, which suggests that divalent cations are involved in the maintenance of its structure. Furthermore, since the *M. penetrans* capsule is not a slime, the HMM is probably anchored to the plasma membrane by interactions with components (lipid moieties including glycolipids and polypeptides) which remain to be identified.

The GLF components detected by SDS-PAGE were resistant to proteinase K, partitioned in methanol and in the Triton X-114 detergent phase and contained palmitic acid and phosphate (Fig. 4). This GLF was totally recovered using a proteinase K-dialysis method. When analysed by gas liquid chromatography, it was found to contain Man:Glc:GlcNAc:GalNAc in a ratio of 1:6:1:2 for both strain GTU-54-6A1 and HF-2. The polymeric nature of sugar residues in GLF was supported by the fact that this material was detected by the method of Kirkeby *et al.* (1992), which does not detect mono- or disaccharides. The glucose residues may enable the purification of *M. penetrans* GLF by concanavalin A affinity chromatography. Preliminary mass spectrometry data indicated that the GLF contained components ranging from 700 to 1700 Da. A major peak for a 1430 Da component was found, which is consistent

with a diglyceride linked to five or six hexose residues. Further investigations will be necessary to determine the chemical structure of GLF. In *M. dispar* (Almeida & Rosenbusch, 1991), a glycosylated component similar to *M. penetrans* GLF was also detected by SDS-PAGE and PAS staining in proteinase-K-digested and dialysed cells. This *M. dispar* material appeared as a single 4 kDa band in 12% polyacrylamide gels. Using electrophoretic conditions with higher resolution for low-molecular-mass glycoconjugates (Lesse *et al.*, 1990), the *M. penetrans* GLF was resolved into two or three bands, which suggested that it contained distinct glycolipids. The GLF was also found to be labelled with radioactive phosphate (Fig. 4d). Within the *Mollicutes*, the presence of covalently linked phosphate residues in glycolipids has only been reported in some *Acholeplasma* species (Smith, 1992) and in *M. fermentans* for the phosphocholine-containing glyco-glycerophospholipids (Matsuda *et al.*, 1995, 1997; Zähringer *et al.*, 1997; Ben-Menachem *et al.*, 1997). Our results showing the presence of phosphate groups in the GLF suggest that this component could consist of such phosphoglycolipids. However, we cannot exclude the possibility that the GLF is heterogeneous and could correspond to a mixture of different moieties including non-phosphorylated glycolipids, i.e. glycosylacylglycerols, polyterpenol glycosides and/or acylated sugars, co-migrating with phosphorylated polar lipids. In addition, the GLF partitioned with lipoproteins in the detergent phase of the Triton X-114 extract (this study; Ferris *et al.*, 1995). Therefore, it remains to be determined whether the GLF contributed to the reported immuno-stimulant activities of this extract (Feng & Lo, 1994; Rawadi & Roman-Roman, 1996). Determination of the specific role of the *M. penetrans* capsule in virulence and cytoadherence will require the development of capsule-defective mutants.

In this study, we reported for the first time that the *M. penetrans* membrane is surrounded by a capsule composed, at least in part, of polysaccharides. We also identified another glycoconjugate fraction from this mycoplasma. The identification of such cell surface components is of particular interest for *M. penetrans*, which has been shown to adhere to various host cells and to penetrate into their cytoplasmic compartments. Our findings have potential implications for explaining the chronicity of *M. penetrans* infections because the capsule might contribute to evasion of the host immune response and phagocytosis.

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