Novel polar lipid composition of *Clostridium innocuum* as the basis for an assessment of its taxonomic status

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The extractable polar lipids of *Clostridium innocuum* have been shown to consist of glycosyldiradylglycerols, phospholipids and phosphoglycolipids. The major glycosyldiradylglycerols are D-Glcp(α 1-3)radyl₂Gro and D-Galp(α 1-2)D-Glcp(α 1-3)radyl₂Gro. Both glycolipids have some 1-O-(alk-1-enyl)-2-O-acyl species, in addition to diacyl species. The phospholipids include bisphosphatidylglycerol (cardiolipin), lysocardiolipin and phosphatidylglycerol (PG). In addition, several novel lipids have been found, including a PG acetal of cardiolipin plasmalogen, smaller amounts of a lyso form of this lipid, a PG acetal of PG plasmalogen, and two phosphoglycolipids, which represent 65% of total polar lipids. The latter have been identified as 2'-amino-1',3'-dihydroxypropane-3'-P-6-D-Galp(α 1-2)D-Glcp(α 1-3)radyl₂Gro and a derivative of this lipid containing an acyl chain esterified to O-6 of the glucopyranosyl ring. Based on rRNA sequence data, *C. innocuum* is considered to be a relative of the mycoplasmas. Its unique lipid composition permits an assessment of the taxonomic status of *C. innocuum*, since the lipid amphiphiles display marked differences from those of *Acholeplasma laidlawii*.

Keywords: *Clostridium innocuum*, polar lipids, chemotaxonomy, plasmalogen, phosphoglycolipid

INTRODUCTION

The genus *Clostridium* has long been recognized as one of great metabolic diversity and of considerable divergence of its DNA guanine plus cytosine (G + C) content (Cato *et al.*, 1986). The rRNA studies of Johnson & Francis (1975) led to a division of this genus into four main groupings: homology groups I and II, which contained species with 22–29 mol % G + C; group III, containing species with 26–33 mol % G + C, which did not belong in the other groups; and group IV, containing species with 41–45 mol % G + C. These divisions were further supported by the work of Stackebrandt & Woese (1981) and their colleagues, who catalogued the 16S rRNA of a wide variety of bacteria. Their work led to the following conclusion: 'The low G + C Gram-positive bacteria are seen to be structured largely in terms of the clostridia. Far from being simply a genus, *Clostridium* appears to be a major phylogenetic unit, deeper (more ancient) even than the entire group of high G + C Gram-positive bacteria.' They also noted that the true mycoplasmas, i.e. the genera

Mycoplasma and *Acholeplasma*, appear to be offshoots of the clostridia. Two clostridial species, *C. ramosum* and *C. innocuum*, were considered to be specific relatives of the mycoplasmas. A separate examination of clostridial phylogenetic groupings was based on the structure of 5S rRNA. A limited group of clostridia was studied, and they were separated into three clusters. Again the relationship of *C. innocuum* to *Acholeplasma* was noted (Dams *et al.*, 1987).

The lipids of clostridia, with the exception of the apolar chains, have not been extensively examined. Most of the available information concerns a limited group of saccharolytic, butyric-acid-producing species including: *Clostridium beijerinckii* ATCC 6015, (formerly *C. butyricum*), *C. butyricum*, *C. acetobutylicum* and *C. saccharoperbutyl-acetonicum* (see Goldfine, 1993; O'Leary & Wilkinson, 1988, and references therein). In addition, limited information is available on *C. pasteurianum* and *C. thermocellum* (Goldfine, 1993; O'Leary & Wilkinson, 1988). We have begun to examine the polar lipids and glycoconjugates of clostridia with a view to testing the value of lipid compositional data in the chemotaxonomy of this diverse group of organisms. It has been apparent for at

Abbreviations: 1D, one-dimensional; 2D, two-dimensional; PG, phosphatidylglycerol; CL, cardiolipin (bisphosphatidylglycerol).

least two decades that the study of lipid composition provides significant data for bacterial taxonomy. Lipids show group-to-group and even species-to-species variations (Lechevalier & Lechevalier, 1988). In view of the suggested phylogenetic relationship between *C. innocuum* and *Mycoplasma* and *Acholeplasma*, we have begun with a study of the lipids of *C. innocuum*.

METHODS

Materials. Reinforced clostridial medium (RCM) was obtained from Unipath. Glass-distilled solvents, silica gel 100 and precoated TLC plates were from Merck. DEAE-cellulose was obtained from Whatman or Merck. Lipid standards were purchased from Sigma. D-GlcP(α 1-3)acyl₂Gro, D-GalP(α 1-2)D-GlcP(α 1-3)acyl₂Gro and D-GalP(α 1-2), acyl-6-D-GlcP(α 1-3)acyl₂Gro were available from previous preparations (Nakano & Fischer, 1977). The glycerol assay kit was purchased from Boehringer Mannheim; 'Purpald' was from Aldrich. Membrane preparations isolated from *Haemophilus parainfluenzae* were generously provided by Fang-Hua Lee.

Growth of cells, and extraction and separation of lipids. *C. innocuum* ATCC 14501 was grown on RCM in anaerobic culture overnight at 37 °C to a Klett₆₀₀ reading > 200 and pH approximately 5.5. The cells were harvested using a Millipore Pellicon cassette concentrator and the wet cell paste was lyophilized. For glycolipid separation, freeze-dried cells were suspended in 0.1 M sodium acetate buffer pH 4.7 (1 g in 10 ml) and disintegrated with glass beads in a Braun disintegrator as described previously by Fischer *et al.* (1983). Lipids were extracted by a modified Bligh-Dyer procedure (Kates, 1986).

For phospholipid separation the lyophilized cells were suspended in water (1:4, w/v) and extracted three times with 6 vols chloroform/methanol (1:1, v/v), the extract was evaporated almost to dryness, and nonlipid contaminants were removed by partition against 0.05 M NaCl. Phospholipids were separated from neutral lipids and glycosyldiradylglycerols by acetone precipitation, followed by chromatography on DEAE-cellulose acetate (Johnston & Goldfine, 1988). Purification of individual lipids was by preparative TLC.

Thin-layer chromatography. The following TLC solvents (by vol.) were used on silica gel plates except where noted: A, chloroform/methanol/water, 65:25:4; B, chloroform/methanol/25% ammonia/water, 60:30:3:3; C and C', chloroform/acetone/methanol/acetic acid/water, 5:2:1:1:0.5 or 8:2:1:1:0.4; D, chloroform/methanol, 98:2; E, chloroform/methanol/7 M ammonium hydroxide, 60:35:5; F, chloroform/methanol/acetic acid/water, 8:1.8:1.2:0.5; G, chloroform/methanol/acetic acid, 65:25:8; H, light petroleum (b.p. 30–60 °C)/diethyl ether/acetic acid, 70:30:1; I, on cellulose TLC plates, first dimension: 3.8 mM EDTA, 0.7 M ammonium bicarbonate in 90 mM ammonium hydroxide/67% ethanol; second dimension: isobutyric acid/water/conc. ammonia, 66:33:1 (Short *et al.*, 1969); J, on cellulose TLC plates, ethanol/1 M ammonium acetate, pH 7.5, 6.5:3.5; K, propan-1-ol/ethyl acetate/water, 7:2:2.

Detection reagents were as follows: phospholipids, molybdenum blue spray reagent (Sigma); glycolipids and phosphoglycolipids, α -naphthol (Vioque, 1984); neutral lipids, Rhodamine B; and for glycerol phosphate esters, the molybdate spray of Hanes and Isherwood (Dawson, 1967).

Chemical assays. Organic phosphate was measured by the method of Bartlett (1959), vinyl ethers by iodine uptake (Gottfried & Rapport, 1962) and by the *p*-nitrophenylhydrazone

assay (Wittenberg *et al.*, 1956), and acyl esters by the hydroxamate assay (Kates, 1986). Glycerol was assayed enzymically (Nägele *et al.*, 1985; Eggstein & Kuhlmann, 1974), and sugars by an anthrone procedure (Yamamoto & Rouser, 1970). Fatty acids (Behr *et al.*, 1992), D-galactose (Beutler, 1984) and D-glucose (Kunst *et al.*, 1984), were assayed as described in the respective references. Protein was measured by the Lowry procedure.

Glycolipid analysis. For compositional analysis, glycolipids were hydrolysed in 2 M HCl (100 °C, 2.5 h), and after cooling, fatty acids were extracted with light petroleum/chloroform (4:1, v/v). Mild deacylation was performed as described by Kates (1986). Glycosylglycerols were methylated by the procedure of Ciucanu & Kerek (1984). The methylation products were hydrolysed in 2 M HCl (100 °C, 2.5 h). After drying *in vacuo*, the partially methylated monosaccharides were reduced with NaBH₄, peracetylated and analysed by GLC-MS (Lindberg, 1972). For treatment with CrO₃ of peracetylated glycosylglycerols, the procedure of Laine & Renkonen (1975) was followed except that resistant monosaccharide constituents were released by hydrolysis with 2 M HCl (100 °C, 2.5 h).

GLC and GLC-MS were carried out as described previously (Behr *et al.*, 1992). Fatty acids and fatty aldehydes were released by acid hydrolysis (2 M HCl, 100 °C, 2.5 h), extracted with light petroleum/chloroform (3:1, v/v) and methylated with 10% (w/v) methanolic BCl₃ (80 °C, 10 min). Unsaturated fatty acid methyl esters were identified on GLC by co-chromatography with standards (methyl esters of *cis*-9 or *trans*-9-hexadecenoate, *cis*-9, *trans*-9-, *cis*-11-, and *trans*-11-octadecenoate) on two fused silica capillary columns, HP-5 (5% diphenyl/95% dimethylpolysiloxane, 25 m, internal diameter 0.32 mm, film thickness 0.33 μ m) and DB 225 (50% cyanopropylmethyl/50% methylphenyl-polysiloxane, 30 m, internal diameter 0.24 mm, film thickness 0.25 μ m), which were run isothermally at 180 and 150 °C, respectively. *cis*-Isomers emerged ahead of the respective *trans*-isomers from the apolar column (HP-5); on the polar column (DB 225) the order was reversed.

Glycerophospholipid analysis. The lipid class composition of the acetone-insoluble lipids was determined by triplicate two-dimensional (2D) TLC in solvent system E followed by solvent system F on 10 $\times$ 10 cm silica gel 60 plates. The lipids were stained with iodine, scraped into acid-washed tubes and digested for phosphate analysis (Goldfine *et al.*, 1993). Minor lipids were combined as indicated. Purified phospholipids (0.5–2.0 mg) were hydrolysed in 1 ml chloroform/methanol (1:1, v/v) plus 0.5 ml 2 M HCl. The mixture was vortex mixed and incubated at 55 °C for 3 min (Johnston & Goldfine, 1988), cooled on ice and neutralized by addition of approximately 0.7 ml 1.5 M ammonium hydroxide. After extraction by the method of Bligh & Dyer (1959), the acid-stable lipids were deacylated by alkaline methanolysis (Kates, 1986).

NMR spectra were obtained with a Bruker AM-500 spectrometer. Samples were dissolved in deuterated chloroform/methanol (2:1, v/v) and spectra were recorded as previously described (Johnston & Goldfine, 1988).

RESULTS

Lipid content

One gram of lyophilized cells contained 250 mg protein and 37 mg total lipid. The lipid consisted of phospholipids plus phosphoglycolipids (0.66 μ mol lipid P per mg total lipid) and glycolipids plus phosphoglycolipids (1.5 μ mol lipid hexose per mg total lipid). The vinyl ether content

Table 1. Apolar chain compositions of the total lipids and of glycolipids I and II of *C. innocuum*

The data, in mol %, represent the means of two experiments with a reproducibility of $\pm 5\%$ or better. ND, Not detected (see text).

Component	Total lipid	Glycolipid I	Glycolipid II
Dodecanoate	3.2	4.9	5.3
Tetradecenoate*	1.6	1.5	2.1
Tetradecanoate	9.9	10.2	10.5
Hexadecenoate*	5.2	6.0	4.7
<i>cis</i> -9-Hexadecenoate	7.9	9.6	10.9
Hexadecanoate	26.0	19.5	26.1
<i>cis</i> -9-Octadecenoate	13.1	25.2	19.7
<i>cis</i> -11-Octadecenoate	3.7	6.9	6.7
Octadecanoate	14.3	16.2	14.0
Hexadecanal	9.2	ND	ND
Octadecanal	5.9	ND	ND

* Not further characterized.

Fig. 1. 2D TLC of the polar lipids of *C. innocuum*. The solvent for the first dimension was solvent system A and that for the second dimension was a modified form of solvent system F with chloroform/methanol/acetic acid/water (80:14:10:3, by vol.). The lipids were stained by treatment with phosphomolybdic acid and charring. The directions of chromatography are indicated by arrows. Numbering of the compounds is according to Table 2. Compound 3 (n.d.) was not detected in the chromatogram shown, but could be seen when more sample was applied.

was $0.2 \mu\text{mol mg}^{-1}$. The fatty acyl and acid-labile ether chain composition is given in Table 1. Fig. 1 shows a chromatogram of the polar lipids. Relative abundances

Table 2. Membrane polar lipid composition of *C. innocuum*

No.*	Lipid	Percentage of total lipid P†	Mol %‡
1	Glycolipid I		5.1
2	Glycolipid		
3	Glycolipid II		ND
4	PG acetal of CL plasmalogen	5.6 ± 0.1	2.4
5	Biphosphatidylglycerol (cardiolipin)	26.1 ± 1.9	16.3
6	Phosphatidylglycerol	1.0 ± 0.5	1.3
7	Lyso form of PG acetal of CL plasmalogen and PG acetal of plasmenylglycerol	2.6 ± 0.6	1.2
8	Lysocardiolipin	2.8 ± 0.3	1.9
9	Phosphoglycolipid	0.9	1.1
10	Phosphoglycolipid III	11.9 ± 4.3	14.8
11	Phosphoglycolipid II	3.6 ± 0.3	4.4
12	Phosphoglycolipid I	41.2 ± 3.4	51.3

* Refers to Fig. 1.

† Where errors (SEM) of the mean are given, these data represent the means of separate determinations carried out in Erlangen and Philadelphia.

‡ ND, Not determined.

and identifications of the individual components are summarized in Table 2. There were three glycolipids, five phospholipids, and four phosphoglycolipids. The latter were identified by their staining with 1-naphthol/ H_2SO_4 and a molybdenum blue spray reagent. The most abundant component was phosphoglycolipid I (51 mol %), followed by biphosphatidylglycerol (16 mol %). The comparatively large spot of the latter on TLC (Fig. 1) was probably caused by microheterogeneity owing to the presence of four hydrocarbon chains.

Glyceroglycolipids

When the crude lipid extract was chromatographed on a column of DEAE-cellulose, as described previously (Fischer, 1977), two glycolipids (I, II) emerged from the column on elution with chloroform/methanol 97:3 and 9:1 (v/v), respectively. On TLC, glycolipid I (Fig. 1, spot 1) co-chromatographed with $\text{D-Glc}p(\alpha 1-3)\text{acyl}_2\text{Gro}$ in solvents A, B, and C'. Chromatography in solvent C' excluded the presence of $\text{D-Galp}(\alpha 1-2)\text{acyl-6-D-Glc}p(\alpha 1-3)\text{acyl}_2\text{Gro}$ (Fischer, 1984). Glycolipid II (Fig. 1, spot 3) co-chromatographed with $\text{D-Galp}(\alpha 1-2)\text{D-Glc}p(\alpha 1-3)\text{acyl}_2\text{Gro}$ in solvents A–C. On analysis glycolipid I gave D-glucose, glycerol, and fatty acids in molar ratios of 1:1:2. Glycolipid II contained in addition D-galactose in equimolar ratio to glucose and glycerol. The fatty acid composition of each glycolipid is listed in Table 1. Long-chain aldehydes were not recovered from the acid hydrolysate. It should be noted, however, that 2D TLC in solvent E with acid hydrolysis (Viswanathan *et al.*, 1968)

showed that fractions of these glycolipids were hydrolysed releasing aldehydes, which were detected at the solvent front by 'Purpald' reagent (Rahn & Schlenk, 1973). Glycosyldiacylglycerol was not hydrolysed by this method. Aldehydes can be lost by condensation with available hydroxyl groups on acid hydrolysis (Johnston & Goldfine, 1988). Analysis of glycolipid I gave glycerol:ester:vinyl ether molar ratios of 1:1.8:0.19. Proton NMR of glycolipid I showed a multiplet at 5.8–5.9 p.p.m., which is characteristic of the vinyl ether grouping.

On methylation analysis (Ciucanu & Kerek, 1984), glycolipid I yielded 2,3,4,6-tetra-O-methylglucose and glycolipid II gave approximately equimolar amounts of 2,3,4,6-tetra-O-methylgalactose and 3,4,6-tri-O-methylglucose, which were identified as alditol acetates by GLC-MS (Lindberg, 1972). Both glycolipids survived treatment with CrO_3 (Laine & Renkonen, 1975) as was shown by TLC in solvent D and by the unchanged molar ratios of the constituent sugar components to glycerol. Since all sugars were in the pyranose form, the resistance to oxidation with CrO_3 indicates the α -configuration. Taking further into account that glyceroglycolipids are biosynthetically derived from 1,2-di-O-acylglycerol, we propose for glycolipid I the structure $\text{D-Glcp}(\alpha 1-3)\text{radyl}_2\text{Gro}$, and for glycolipid II, $\text{D-Galp}(\alpha 1-2)\text{D-Glcp}(\alpha 1-3)\text{radyl}_2\text{Gro}$.

Glycerophospholipids

Bisphosphatidylglycerol (cardiolipin). Cardiolipin (CL; Fig. 1, spot 5) was identified as the most abundant phospholipid, representing approximately 25% of total lipid P. It was eluted from DEAE-cellulose acetate by 0.2% ammonium acetate in chloroform/methanol (2:1, v/v), and purified by preparative TLC in solvent system G. 1D and 2D TLC with authentic samples gave inconsistent results, presumably due to differences in lipid aliphatic chain compositions and counteractions between the bacterial lipids and standards. The product of successive mild acid and mild alkaline hydrolysis co-chromatographed with GroPGroPGro in solvent systems I, J and K. Acetolysis (Coulon-Morelec *et al.*, 1960) gave a water-soluble product which co-chromatographed with authentic glycerol 1,3-bisphosphate in solvent system J, and a lipid product consisting of mono- and diacylglycerol, which were identified by TLC with authentic standards in solvent system H. The presence of mono-acylglycerol was consistent with significant amounts of acid-labile lipid (plasmalogens). Analysis of the intact lipid gave a phosphorus:(ester + vinyl ether):glycerol ratio of 2:4:3. Of the aliphatic chains, 16% were linked by a vinyl ether bond. The parent lipid was also hydrolysed with a CL-specific phospholipase D in the membrane fraction of *Haemophilus parainfluenzae* (Ono & White, 1970). The products of hydrolysis were identified as phosphatidylglycerol (PG) and phosphatidic acid by 2D TLC in solvent system E followed by F. Proton NMR of the parent lipid showed the presence of vinyl ethers and a secondary hydroxyl group, but no primary hydroxyl in the polar head group.

Lysocardioliipin. Lysocardioliipin (Fig. 1, spot 8) was a minor component of freshly extracted *C. innocuum* lipids which increased upon storage of lipid extracts. It co-chromatographed with PG in neutral and acidic TLC systems but could be separated from PG in solvent system E. ^{32}P -labelled lysocardioliipin co-chromatographed with lysocardioliipin prepared from *C. butyricum* CL (Goldfine *et al.*, 1982) by acid hydrolysis. Deacylated lysocardioliipin chromatographed as GroPGroPGro on 2D TLC in solvent system I. The water-soluble product of acetolysis was a mixture of glycerol 1,3-bisphosphate, glycerol 1-phosphate, and a presumptive cyclic phosphate. An authentic sample of lysocardioliipin gave the same products. Acid hydrolysis of the presumptive cyclic phosphate produced a mixture of glycerol 1,3- and 1,2-bisphosphates.

PG. This lipid (Fig. 1, spot 6) comprised 1% of the total lipid P. 2D TLC in solvent systems E followed by F, or E followed by G, showed a minor spot corresponding in mobility to authentic PG. A fraction eluted from a preparative silicic acid column by chloroform/methanol (4:1, v/v) contained a phospholipid which co-chromatographed upon 2D TLC in solvent systems E followed by F with ^{32}P PG isolated from labelled *Escherichia coli* phospholipids (Goldfine *et al.*, 1993).

PG acetal of the plasmalogen form of CL. This lipid (Fig. 1, spot 4), which represented 5.6% of total lipid P, has not been previously identified and a complete description of its structural characterization will be published elsewhere. The structure was established by chemical analyses, analysis of the acid and alkaline hydrolysis products of the native and hydrogenated compounds, the products of treatment with a cardiolipin-specific phospholipase D, and 1D and 2D ^1H -NMR. It is related to the previously characterized PG acetal of the plasmalogen form of phosphatidylethanolamine (Johnston & Goldfine, 1988). A lyso form of this lipid is also present in which one aliphatic chain is absent. It represents 2 to 4% of total lipid P (Fig. 1, spot 7).

PG acetal of the plasmalogen form of PG. This phospholipid (Fig. 1, spot 7) represents < 1% of total lipid P. The structure of this compound is based on an analysis of its acid hydrolysis products. A description of its characterization will be published elsewhere.

Phosphoglycolipids

A unique feature of the lipid pattern is the presence of two major and two minor phosphoglycolipids, which together represent 55–60% of the lipid phosphorus. Analysis of the two major components (Fig. 1, spots 10 and 12) revealed that they are derived from the membrane glycolipid $\text{D-Galp}(\alpha 1-2)\text{D-Glcp}(\alpha 1-3)\text{radyl}_2\text{Gro}$. Both compounds carry a 2-amino-1(3)-dihydroxypropane 3(1)phosphate residue attached to O-6 of the galactopyranosyl residue. The less polar component (Fig. 1, spot 10) is esterified with a third fatty acid at O-6 of the glucopyranosyl residue. The complete structures have been established by chemical analyses, NMR studies (^1H , ^{13}C , 1D and 2D) and by fast atom bombardment mass

Table 3. Comparison of lipid amphiphile composition of *C. innocuum* and *Acholeplasma laidlawii*

<i>C. innocuum</i>	<i>A. laidlawii</i>	Reference(s)
D-GlcP(α 1-3)radyl ₂ Gro*	D-GlcP(α 1-3)acyl ₂ Gro	Shaw <i>et al.</i> (1968); Wieslander & Rilfors (1977)
D-GalP(α 1-2)D-GlcP(α 1-3)radyl ₂ Gro*	D-GlcP(α 1-2)D-GlcP(α 1-3)acyl ₂ Gro	Shaw <i>et al.</i> (1968); Wieslander & Rilfors (1977)
Bisphosphatidylglycerol (cardiolipin)	Cardiolipin	Smith (1972)
Phosphatidylglycerol	Phosphatidylglycerol	Smith (1972); Wieslander & Rilfors (1977)
2'-Amino-1',3'-dihydroxypropane-3'-P-6- D-GalP(α 1-2)D-GlcP(α 1-3)radyl ₂ Gro*	sn-Gro-3-P-6[D-GlcP(α 1-2) D-GlcP(α 1-3)]acyl ₂ Gro	Wieslander & Rilfors (1977); Shaw <i>et al.</i> (1972)
Poly(glycosylglycerophospho)-lipoteichoic acid†	Lipoglycan (Glc, Man, deoxyhexosamine, GlcN, GalN)	Smith (1977)

* Contains some 1-O-(alk-1-enyl)-2-O-acyl species.

† W. Fischer, unpublished.

spectroscopy. These data will be described in detail elsewhere.

DISCUSSION

Examination of the polar lipids of *C. innocuum* has revealed several unusual features including: (a) the presence of novel PG acetals of plasmalogens, (b) the absence of common amino lipids, and (c) a high content of phosphoglycolipids. The presence of ether lipids in clostridia is well known (Goldfine & Hagen, 1972; Goldfine, 1993). The major types have been previously characterized as plasmalogens or glycerol acetals of plasmalogens. In this study we report that the predominant ether lipids of *C. innocuum* consist of plasmalogens and PG acetals of plasmalogens, a recently characterized class of ether lipids in which the primary hydroxyl group of PG forms an acetal with the C-1 of the sn-1 ether chain of a plasmalogen. The first member of this class of lipids was found in *C. butyricum* and shown to be a PG acetal of plasmenylethanolamine (Johnston & Goldfine, 1988). Subsequent studies have examined the precursor-product relationships of this lipid with the corresponding plasmalogen (MacDonald & Goldfine, 1990).

Aminophospholipids such as phosphatidylethanolamine, phosphatidyl-N-monomethylethanolamine, and their respective plasmalogens, are major phospholipids of the saccharolytic, butyric-acid-producing clostridia including *C. butyricum*, *C. beijerinckii*, *C. acetobutylicum*, and related species (Johnston & Goldfine, 1983). There is a division of Gram-positive bacteria between the aminolipid-containing, spore-forming genus *Bacillus* and the non-sporeforming genera, *Streptococcus* and *Lactobacillus* (Goldfine, 1982) which mirrors differences in their 16S rRNA sequences. These sequences have been used to construct a bacterial phylogeny, in which a division of the Gram-positive tree into two major trunks was proposed (Stackebrandt & Woese, 1981). One trunk includes the high G + C (≥ 55 mol %) 'actinomyces' group, whose members have a distinctive lipid composition (Goldfine, 1982), and another major trunk, the low G + C (≤ 50 mol %) 'clostridia', which diverge to form bacillus,

lactobacillus and streptococcus groups, and four groups of clostridia.

In this phylogeny, which is supported by 5S rRNA sequence analyses (Dams *et al.*, 1987; Rogers *et al.*, 1985), *Mycoplasma* and *Acholeplasma* are considered to be offshoots of the low G + C Gram-positive branch, and two clostridia, *C. innocuum* and *C. ramosum*, are thought to be specific relatives of the mycoplasmas (Mollicutes). Table 3 compares some of the major features of the polar lipids of *A. laidlawii*, as an example of Mollicutes, with those we have found in *C. innocuum*. In common with other Gram-positive bacteria both species have CL, PG, glycosyl-diradylglycerols and phosphoglycolipids. *A. laidlawii* contains in addition D- and L-alanyl PG (Koostra & Smith, 1969) and phosphatidylhexosyl diacylglycerol (Smith, 1972). The monoglycosyl diacylglycerol in both organisms is D-GlcP(α 1-3)radyl₂Gro. In *A. laidlawii* the glycolipids are diacyl, while those in *C. innocuum* have some vinyl-ether-linked chains. The diglycosyl diacylglycerols differ in having as the terminal sugar α -D-galactose in *C. innocuum* and α -D-glucose in *A. laidlawii*. D-GalP(α 1-2)D-GlcP(α 1-3)acyl₂Gro has previously been found in other Gram-positive bacteria such as lactobacilli and *Streptococcus pneumoniae* (Kates, 1990). A major component of the polar lipids of *C. innocuum* is a novel derivative of the diglycosyl diradylglycerol in which 2-amino-1,3-dihydroxypropane 3-phosphate is substituted on O-6 of the terminal galactose. This structure differs from the sn-glycero-3-phosphoglycolipid found in *A. laidlawii*. It should be noted here that these 'aminoglycerophosphoglycolipids', in contrast to the glycerophosphoglycolipids of other Gram-positive bacteria (Fischer, 1990), are not related to lipoteichoic acid and therefore serve exclusively as membrane components. A further difference between *C. innocuum* and *A. laidlawii* is the presence of a poly(glycosylglycerophospho)-lipoteichoic acid in the former and a lipoglycan in the latter species (Table 3).

Based on lipid amphiphile composition, it appears that *C. innocuum* is no more closely related to Mollicutes than it is to other Gram-positive bacteria such as *Streptococcus* and *Lactobacillus*. It is of interest that a more recent proposal

for a hierarchical structure for the Gram-positive bacteria groups *C. innocuum* and *C. ramosum* in the same family with *Lactobacillus cateniforme*, *Erysipelothrix* and *Streptococcus pleomorphicus* (Cato & Stackebrandt, 1989). There is little detailed information on the lipid amphiphiles of these non-clostridial species (O'Leary & Wilkinson, 1988).

Much more work needs to be done on the lipids of clostridia before a coherent chemotaxonomic picture emerges. This ancient group of bacteria will probably be divided into different taxa as more information on macromolecular structures is obtained (Cato & Stackebrandt, 1989). Recent work has shown the utility of analysis of the apolar chains in identification of clostridia (Ghanem *et al.*, 1991), but with the exception of five species of *Clostridium*, definitive data on the polar lipids is not available (O'Leary & Wilkinson, 1988). Given the wide variation in phospholipid and glycoconjugate structure and composition among Gram-positive species, the value of information on clostridial complex lipids for chemotaxonomy is evident.

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