

COMPLEX LIPIDS OF CALDARIELLA ACIDOPHILA, A THERMOACIDOPHILE ARCHAEBACTERIUM

MARIO DE ROSA*, AGATA GAMBACORTA*, BARBARA NICOLAUS* and JOHN D. BU'LOCK†

*Laboratorio per la Chimica M.I.B. del C.N.R., Via Toiano 2, Arco Felice, Napoli, Italy; †Department of Chemistry, University of Manchester, U.K.

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Abstract—Isoprenoid ether lipids are common to the 'urkingdom' archaeobacteria, within which members of the *Caldariella* group of extreme thermoacidophiles and some methanogenic species have lipids based on macrocyclic tetraethers containing two 16,16'-biphytanyl chains. As a step towards closer classification, the complex lipids of *Caldariella acidophila* have been more fully characterized.

INTRODUCTION

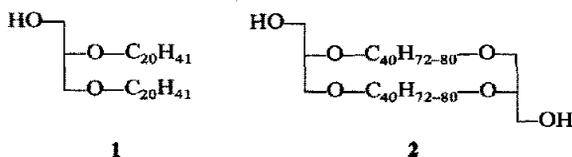
The *Caldariella* group of extreme thermoacidophilic organisms has been defined by us [1] as a form-habitat assembly including *Caldariella acidophila* [2, 3], the species with which our own studies have been mainly concerned, together with the (closely related) *Sulfolobus acidocaldarius* [4], 'ferrolobus' [5], and the somewhat more distant *Thermoplasma acidophila* [6]. The membrane lipids of *Caldariella*, *Sulfolobus*, 'ferrolobus' and *Thermoplasma* are all [7, 8] based upon 72-membered macrocyclic tetraethers [9, 10] with two hydrophobic C₄₀ chains having the 16,16'-biphytanyl skeleton, and this feature alone seemed sufficiently unusual to set the group apart from other prokaryotes.

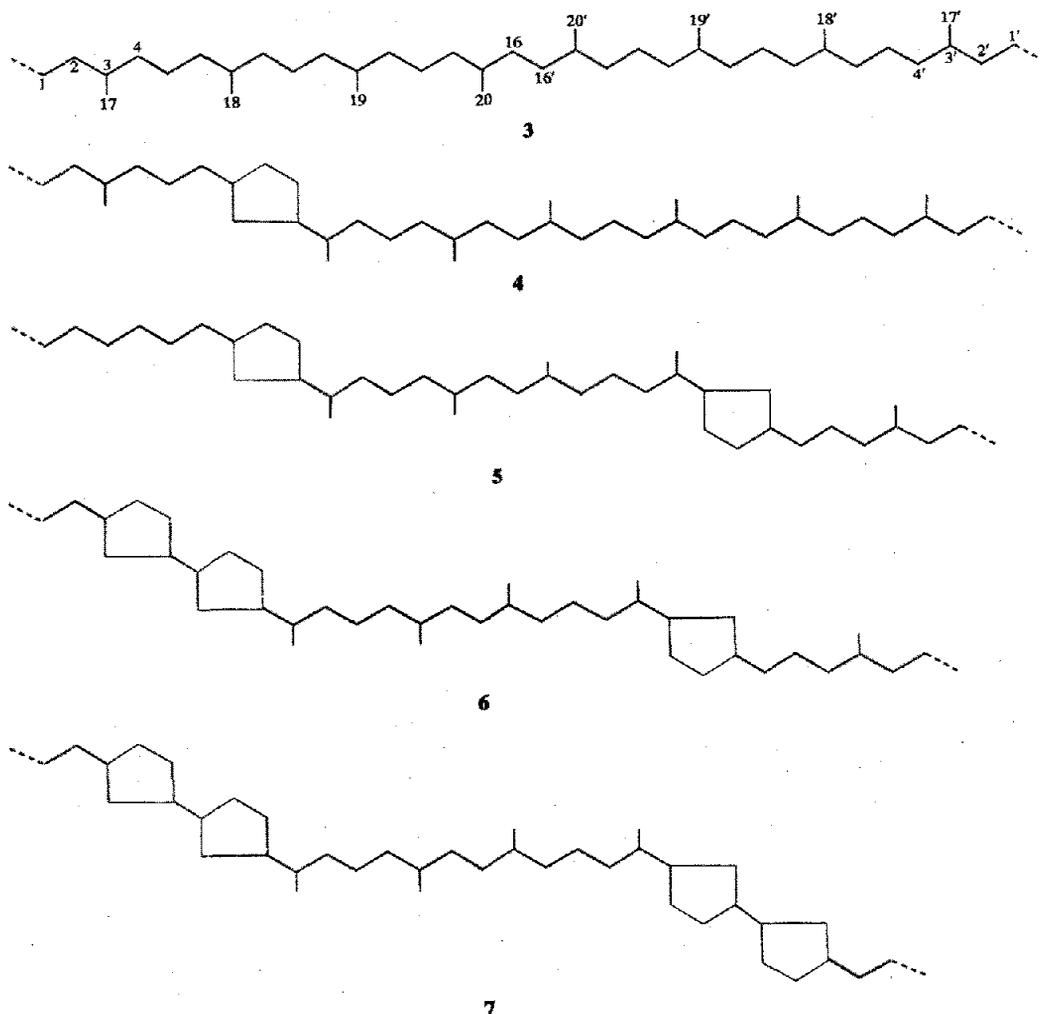
Recent studies have made this simple but perhaps puzzling situation at once more complex and to some extent more comprehensible. On the one hand, a major division or 'urkingdom' has been wholly set apart from the 'classical' bacteria and defined, on grounds which are indicative of very prolonged evolutionary separation, as the Archaeobacteria [11-13]. At present this division comprises the several groups [12] of methanogenic anaerobes, the extreme halophiles, and the *Caldariella* series of thermoacidophiles. It was already known [14] that the membrane lipids of the extreme halophiles were based on derivatives of diphytanyl glycerol diether (1), which provided the closest analogy for the tetraether lipids such as 2 found in the *Caldariella* series. Recently it has been reported [15] that the methanogens are also characterized by the predominance of ether lipids, and while some contain only diether lipids based on 1 others

contain both 1 and 2 [16, 17]. Thus across the considerable variety existing within the recognized archaeobacteria there emerges a spectrum of variations upon the basic ether lipid structure, in which more precise structural characterizations become significant; our present communications are concerned with these more specialized features in the particular case of *Caldariella acidophila*.

One kind of variation which is becoming apparent concerns the detailed structure of the biphytanyl chains in tetraether lipids such as 2. The simplest of these, 16,16'-biphytanyl itself (3), is found generally, but whereas it appears as the only C₄₀ component in the methanogens [16, 17] and the predominant one in *Thermoplasma*, it is a relatively minor one in *Sulfolobus* and, particularly, in *Caldariella* [18]. In these more thermophilic acidophiles, 3 is predominantly replaced by cyclized versions of the biphytanyl chain, of which we have characterized the 4 principal structures 4-7. We have earlier remarked [18] that the extent of these cyclizations seems to increase, within members of the *Caldariella* group, in relation to increasing temperature optima of different isolates, while in the accompanying communication this trend is investigated in relation to varying growth temperatures for a single strain of *C. acidophila*.

Another distinction is the presence of a third type of ether lipid (8), in which one of the two glycerol units present in 2 is modified into calditol, a unique branched-chain nonitol. We have characterized such lipids in *C. acidophila* [10], and some lipid components of *Sulfolobus* clearly have the same basic structure through the calditol moiety has been incorrectly described as a glycerol polyol ether [19]. From published chromatograms it is also possible that a polyol similar to calditol exists in some methanogens; *Methanosarcina barkeri*, a species in which only the diether lipids based on 1 were confirmed, also gave a periodate-reacting polar lipid component after acid hydrolysis [17].

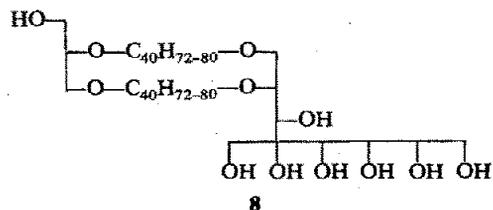




Further variety exists amongst the complex native lipids from which the parent ethers **1**, **2** and **8** are only obtained after acid hydrolysis. These have been partly characterized in *Thermoplasma* [20, 21] and in *Sulfolobus* [8, 19], between which there are considerable differences; unfortunately these characterizations were incomplete and, in particular, based on incorrect formulations of the parent ethers. In the present paper the complex lipids of *C. acidophila*, which are very similar to those of *Sulfolobus*, are formulated with more precision. Temperature-associated variations of the C₄₀ components of these lipids are dealt with in a subsequent communication.

RESULTS

Batch cultures of the MT-4 strain of *C. acidophila* were grown at temperatures between 75 and 89°. As



shown in Table 1, growth is most rapid at 85°, but the lipid content of the cells does not vary significantly over the range studied. The yield of extracted lipid, virtually all of which is recovered from the subsequent separation, was ca 8% of the cell wt, considerably higher than the values reported for *Sulfolobus*, by Langworthy *et al.* [8, 19], which range from 2.6 to ca 5%.

Group separation of the extracted lipids was carried

Table 1. Growth rate and lipid content of *Caldariella acidophila* grown at different temperatures

Growth temperature °	75	80	85	89
Specific growth rate μh^{-1}	0.069	0.092	0.112	0.036
Cell yield (lyophilized) g/l.	0.21	0.23	0.24	0.17
Extracted lipids as % dry cells	8.5	7.5	8.0	8.5

Table 2. Fractionation of *Caldariella acidophila* lipids on silica gel

Eluting solvent	CHCl ₃	CHCl ₃ -MeOH, 6:1	CHCl ₃ -MeOH-H ₂ O, 65:25:4		
Fraction (%)*	16	15	6	8	48
Identity	neutral lipids	glycolipids A, B	phospholipid A	sulpholipid	phospholipids B, C

*% refers to the total lipid extract chromatographed.

out by chromatography on silica gel; the composition of the lipid mixture did not vary significantly with temperature and is shown in Table 2, which also summarizes the chromatographic procedure. In comparison with the description of *Sulfolobus* lipids, the components are very similar, if not identical, but the ratio of intact phospholipids to glycolipids is considerably higher (3.6, compared to reported ratios of 0.3 [19] or 1.2-1.3 [8]). The lipid components were further separated and characterized as follows.

Neutral lipids

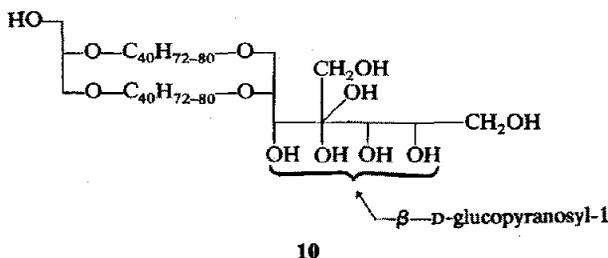
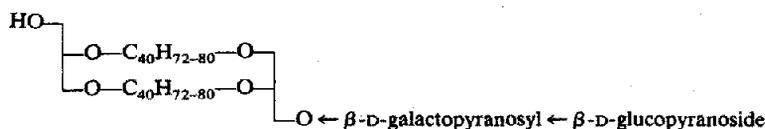
In addition to some 4% of the respiratory quinone of *Caldariella* characterized as benzo[b]thiophen-4,7-quinone [22, 23], and small amounts of the reduced glycerol tri(geranylgeranyl) triethers previously described [24], this fraction contains ca 90% of a very complex mixture of hydrocarbons, also reported in *Sulfolobus* [19]. Somewhat surprisingly, the ¹H NMR spectrum of this mixture shows that it contains a relatively low proportion of Me-CH groups (i.e. it is not isoprenoid in nature); MS and GLC data indicate a mixture of hydrocarbons in the C₃₀-C₄₀ range.

Glycolipids

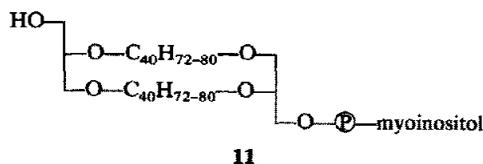
The glycolipid fraction was further resolved by TLC on boric acid-treated silica gel into two main components, glycolipids A and B, making up 4.5 and 10.5% of the total lipids respectively.

Glycolipid A on hydrolysis in HCl-methanol affords the diglycerol tetraether (2), galactose, and glucose, in equimolar amounts. It is presumably identical with the glucose→galactose→glycerol ether lipid already described from *Sulfolobus* [19]. The ¹³C NMR of this lipid shows one free glycerol -CH₂OH and signals of anomeric carbons consistent with a β stereochemistry of the glycosidic linkages. In accordance with the earlier description and our ¹³C NMR data the glycolipid A is formulated as 9, but no definitive information has been obtained on the location of the glucosidic linkage to the galactose.

Glycolipid B is also identical with the component from *Sulfolobus* described [19] as a glucosyl-polyol(ether)glycerol ether; on hydrolysis it afforded equimolar amounts of glucose and the calditol glycerol tetraether (8). The ¹³C NMR of this lipid shows unambiguously that it is a 1-β-glucopyranosyl derivative at a secondary OH of the calditol, so that this lipid is formulated as 10.



10



11

Phospholipid A

By strong acid treatment this gave in an equimolecular amount, the diglycerol tetraether (2) and an inositol phosphate. It is thus identical to the myo-inositol-containing 'polar lipid C' from *Sulfolobus* [19] and accordingly formulated as 11.

Sulpholipid

This is also identical to the 'polar lipid D' from *Sulfolobus* [19], and on hydrolysis in HCl-MeOH it gave the calditol glycerol tetraether (8), glucose and sulphate, in a ca 1:1:1 mol ratio. It is therefore an O-sulphate of 10, but there are no data to locate the sulphate linkage.

Phospholipids B and C

These two components are together presumed to constitute the 'polar lipid E' described from *Sulfolobus* [19] and we were also unable to resolve the mixture directly. Alkaline hydrolysis of the mixture gave the two glycolipids, 9 and 10, together with phosphate and an inositol, in a molar ratio of ca 1 (total tetraethers): 1:1. These two components are therefore formulated as phosphoinositol derivatives of glycolipids A (9) and B (10), respectively.

Calditol vs glycerol lipids

Phospholipid B constitutes over 42% of the total lipids of *C. acidophila* strain MT-4 and is therefore the major complex lipid component. Taking this together with glycolipid B (10) and the corresponding sulpholipid, the calditol glycerol tetraether lipids make

up over 61% of the total, and the diglycerol tetraethers less than 17%. In *Sulfolobus* grown heterotrophically, the proportion of what are now identified as the calditol lipids appears [8] to be significantly lower, ca 40% of the total, but in *Sulfolobus* grown autotrophically and in the obligate autotrophic 'ferrolobus' it is 60 and 53%, respectively [8].

DISCUSSION

The pattern of complex lipids in *C. acidophila* is similar, except for quantitative differences, to that reported in *Sulfolobus* grown heterotrophically [8, 19], supporting the view that despite the considerable difference in the G+C content of their DNA [1] these two species are closely related. Similarly the complex lipid composition of *Sulfolobus* grown autotrophically (on sulphur), and also of the very similar 'ferrolobus' which is an obligate autotroph, are quantitatively close to this pattern except for the additional presence of two minor glycolipids and a quite unusual acidic lipid (which appears to contain both sulphate and C-phosphonic acid groups [8] attached to a glycosylated calditol lipid).

The complex lipids of the fourth type of thermoacidophile now assigned to the archaeobacteria, *Thermoplasma*, are however, quite different [20]. They are based on the diglycerol tetraether (2) with no clear indication of the presence of calditol lipids, and have a predominance of glycerolphosphoryl derivatives; in terms of the revised tetraether structure (2) the principal complex lipid is to be formulated as glycerol-phosphoryl-glucosyl-2. The respiratory quinone of *Thermoplasma* appears to be a normal menaquinone [20] and not the thionaphthenequinone of *Caldariella*.

The complex lipids of *Halobacterium cutirubrum* have been studied in considerable detail by Kates and co-workers [14]; without reviewing the data exhaustively it is sufficient to say that they are based upon the simple glycerol diether (1) but in other respects, e.g. in the prevalence of glycerolphosphoryl lipids, they appear relatively closer to the *Thermoplasma* pattern than to *Caldariella* and *Sulfolobus*. The neutral lipids contain menaquinone [25] and substantial amounts of isoprenoid hydrocarbons including squalene and hydro-squalenes [15].

In view of the diversity of the thermoacidophiles and halophiles, a study of the complex lipids of the methanogens would be of considerable interest. However, the only data so far available [15-17] are that while in some methanogens the lipids are based only on the diether (1), in others the lipids are based upon both 1 and the tetraether 2, and that the neutral lipids contain a substantial proportion of squalenes and other isoprenoid hydrocarbons similar to those in *Halobacterium*. Thus the study of the complex lipids promises to throw considerable light upon the difficult question of differences and affinities within the separated 'urkingdom' of the archaeobacteria.

EXPERIMENTAL

Microorganism. The MT4 strain was grown as previously described [2] in 901 batches in a Terzano fermenter with slow

mechanical agitation, aeration at 2.5-3.0 l./min, and at the temp. indicated in Table 1. The culture vessel was inoculated with 8 l. of a logarithmic broth culture grown at the temp. of each expt. Growth was measured as A at 540 nm; cells were harvested in the late-exponential phase by continuous-flow centrifugation in an Alfa-Laval LAB. 102B-25 separator.

Extraction and fractionation of lipids. Dried cells were extracted (Soxhlet) for 48 hr with CHCl_3 -MeOH (1:1). After evap. the total lipid residue was resolved on a Si gel (Merck Kieselgel 70-230 mesh) column (3×40 cm) eluted with 600 ml CHCl_3 (neutral lipids), 600 ml CHCl_3 -MeOH (6:1) (glycolipids) and with 800 ml CHCl_3 -MeOH- H_2O (65:25:4) (polar lipids). The neutral lipid fraction was subjected to chromatography on Si gel in hexane with increasing proportions of Et_2O . Hexane eluted the hydrocarbon fraction, Et_2O -hexane (19:1) eluted benzo[b]thiophen-(4, 7)-quinone [22, 23] and Et_2O -hexane (4:1) eluted triethers [24]. Glycolipids were further separated into glycolipid A (9) and B (10) by prep.-TLC (Merck Kieselgel 60-F 254) washed with 0.1 N boric acid and activated by heating at 100° for 5 hr. The plates were eluted with CHCl_3 -MeOH- H_2O (65:25:4) and the products located with I_2 vapour. Chromatographic analysis by TLC on Si gel: the neutral lipids were eluted with hexane- Et_2O (9:1) or hexane- Et_2O (7:3) while polar lipids were eluted with CHCl_3 -MeOH- H_2O (65:54:4). Compounds were detected by exposure to I_2 vapour, by spraying with $\text{Ce}(\text{SO}_4)_2$ or with the Dittmer and Lester reagent [26] for phospholipids. In the above procedure the glycolipids A (9) and B (10) have R_f 0.80, the phospholipid A (11) R_f 0.45, the sulpholipid derivative of 10 R_f = 0.36, and phospholipids B and C, R_f 0.24.

Glycolipid A (9). Glycolipid A (9) was hydrolysed with 20 ml of dry methanol-HCl (1 N) at reflux for 5 hr. The reaction mixture, evapd under N_2 , was resolved on a Si gel column (1×8 cm); CHCl_3 - Et_2O (9:1) eluted tetraether (2) and CHCl_3 -MeOH (1:1) eluted glucose and galactose. The molar ratio between tetraether (2) and the carbohydrate mixture was determined gravimetrically. The carbohydrate mixture was analysed by GLC of the corresponding TMSi derivatives on a 20 m glass capillary column coated with OV 101 operating at 140°, and showed equimolecular amounts of glucose and galactose. The ^{13}C NMR spectrum of 9 showed, at low field, the characteristic signals of β -anomeric carbons (δ 103.4 and 103.9 for the glucopyranose and galactopyranose, respectively) and the signal of one glycerol CH_2OH at δ 62.5. In order to confirm the sequence of the sugars in the molecule, the mild hydrolysis procedure, previously described in ref. [19] was repeated.

Glycolipid B (10). Methanolysis of glycolipid B was performed as above. The hydrolysis products were resolved by chromatography on a Si gel column (1×8 cm); CHCl_3 -MeOH (19:1) eluted tetraethers (8) and CHCl_3 -MeOH (1:1 v/v) eluted glucose (identified by GLC of TMSi derivative). The tetraethers (8) and glucose were recovered in equimolecular amounts as estimated by the wt of the fractions. The ^{13}C NMR spectrum of glycolipid B (10) showed a signal at δ 103.4 for the β -anomeric carbon of glucopyranose and signals of three CH_2OH at the same chemical shifts as the parent tetraethers (8).

Phospholipid A (11). Vigorous hydrolysis (6 N HCl, reflux, 3 days) gave a mixture containing in equimolecular amounts the tetraethers (2), phosphate and inositol. The phosphate content was assayed on an aliquot of hydrolysis mixture according to ref. [27]; the remaining hydrolysate was dried under N_2 and chromatographed on a Si gel column (1×8 cm); CHCl_3 - Et_2O (9:1) eluted tetraethers (2) and CHCl_3 -

MeOH (1:1) eluted a polyol identified as an inositol according to ref. [19]. The ratio between the last two compounds was determined gravimetrically.

Sulpholipid derivative of glycolipid B. The methanolysis product of the sulpholipid, obtained as above, gave tetraethers (**8**) and glucose in a 1:1 molar ratio gravimetrically evaluated after chromatographic separation (as for the methanolysis of glycolipid B (**9**)). The sulphate content of the sulpholipid, by the barium chloranilate method [28] on the methanolysis product, was ca 1 mol of sulphate per mol of glycolipid.

Phospholipids B and C. The mixture of phospholipids B and C was hydrolysed with M NaOH at reflux for 3 hr. The hydrolysis mixture, after neutralization with 0.1 M HCl, was extracted with CHCl₃. The organic phase containing the glycolipid A (**9**) and B (**10**) was resolved by boric acid prep. TLC as described above, and the relative proportion of the two glycolipids was evaluated gravimetrically. The H₂O phase contained equimolecular amounts of phosphate (assayed as above) and inositol, estimated gravimetrically by prep. TLC of a lyophilized aliquot of the H₂O phase, eluting with CHCl₃-MeOH (1:1).

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