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ORIGINAL PAPER

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Characterization of the precursor of tetraether lipid biosynthesis in the thermoacidophilic archaeon *Thermoplasma acidophilum*

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Abstract Polar lipid biosynthesis in the thermoacidophilic archaeon *Thermoplasma acidophilum* was analyzed using terbinafine, an inhibitor of tetraether lipid biosynthesis. Cells of *T. acidophilum* were labeled with [^{14}C]mevalonic acid, and their lipids were extracted and analyzed by two-dimensional thin-layer chromatography. Lipids labeled with [^{14}C]mevalonic acid, [^{14}C]glycerol, and [^{32}P]orthophosphoric acid were extracted and hydrolyzed under different conditions to determine the structure of polar lipids. The polar lipids were estimated to be archaetidylglycerol, glycerophosphatidylcaldarchaetidylglycerol, caldarchaetidylglycerol, and β-L-gulopyranosylcaldarchaetidylglycerol, the main polar lipid of *T. acidophilum*. Pulse and chase experiments with terbinafine revealed that one tetraether lipid molecule is synthesized by head-to-head condensation of two molecules of archaetidylglycerol and that a sugar group of tetraether phosphoglycolipid is expected to attach to the tetraether lipid core after head-to-head condensation in *T. acidophilum*. A precursor accumulated in the presence of terbinafine with a fast-atom-bombardment mass spectrometry peak m/z 806 was compatible with archaetidylglycerol. The relative height of the peak m/z 806 decreased after removal of the inhibitor. The results suggest that most of the precursor, archaetidylglycerol, is in fully saturated form.

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Introduction

Core structures of membrane lipids in Archaea are different from those in Bacteria and Eukarya. Membrane lipids in Archaea are composed of ether lipids containing saturated isopranyl chains, archaeol, and caldarchaeol. Archaeol is composed of two isopranoid groups with 20 or 25 carbon atoms attached to one glycerol molecule. Caldarchaeol is composed of two isopranyl groups with 40 carbon atoms attached to two glycerol molecules (Koga et al. 1993). A lipid containing archaeol or caldarchaeol as its core lipid is a diether lipid or tetraether lipid, respectively (see Fig. 1).

Thermoplasma acidophilum is a thermoacidophilic archaeon that grows optimally at pH 1–2 and at 55–59 °C. About 90% of the membrane lipids of this organism are tetraether lipid and the remainder is diether lipid (Langworthy et al. 1972). Approximately one-half of the polar ether lipids are β-L-gulopyranosylcaldarchaetidylglycerol, known as the main polar lipid (MPL) in *T. acidophilum* (Swain et al. 1997). The other half of the polar lipids have been extensively analyzed, and their structures were previously reported as two phospholipids (diether and tetraether lipid), seven glycolipids (tetraether lipid having mono- or oligosaccharides with gulose [Gul] or glucose and zero to three mannose groups as the sugar moiety), and eight phosphoglycolipids (tetraether lipids having glycerophosphate as the phosphoester moiety and gulose or glucose and zero to three mannose groups as the sugar moiety) (Shimada et al. 2002; Uda et al. 1999, 2000b).

The biosynthetic pathway of polar ether lipids has been analyzed by pulse and chase experiments using radiolabeled compounds in *Halobacterium cutirubrum* (Moldoveanu and Kates 1988) and *Methanobacterium*

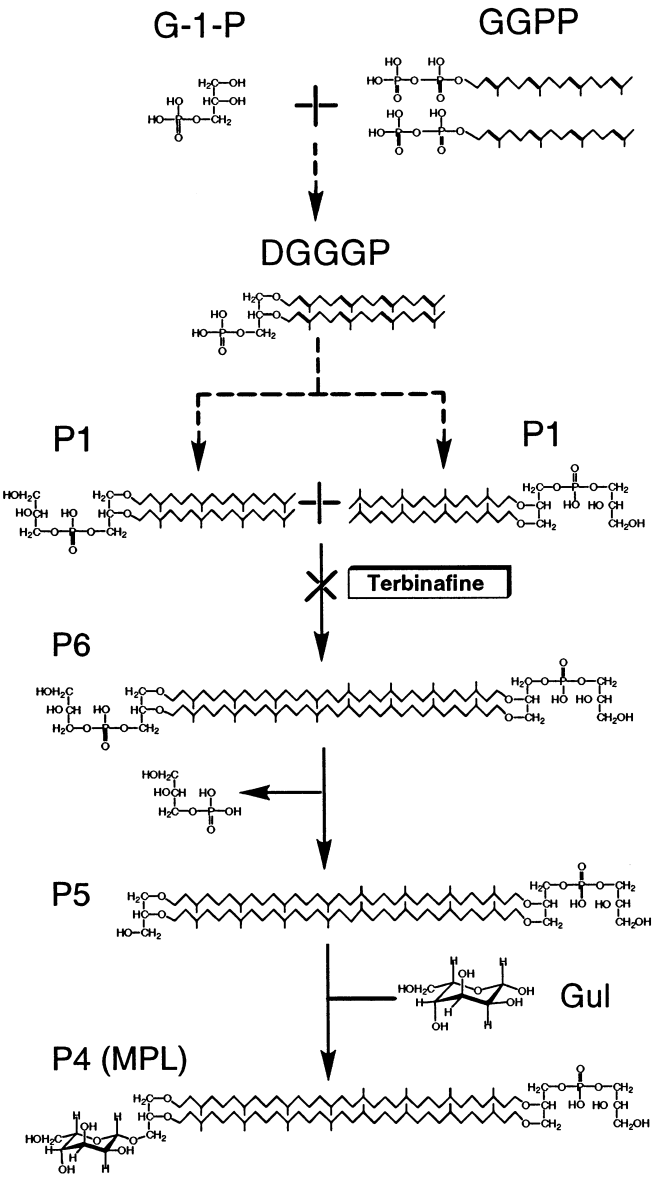

Fig. 1 Biosynthetic pathway of tetraether lipids in *Thermoplasma acidophilum* proposed based on the results of this and previous studies (Kon et al. 2002; Shimada et al. 2002; Zhang and Poulter 1993). See Introduction for formation of DGGGP. The formation of P6 from P1 is considered to be inhibited by terbinafine (see Results and Discussion). P1 is a diether lipid, and P4, P5, and P6 are tetraether lipids. *P1* Archaetidylglycerol, *P6* glycerophosphatidylcaldarchaetidylglycerol, *P5* caldarchaetidylglycerol, *P4* β-L-gulopyranosylcaldarchaetidylglycerol (*MPL*: main polar lipid)

thermoautotrophicum (Nishihara et al. 1989). The former report suggested that phospholipids and glycolipids were synthesized from a common precursor, unsaturated archaetidic acid as intermediate for all phospholipids, and saturation of hydrophobic moieties of all polar lipids occurs after each polar head group is attached to the core. The latter report suggested that one molecule of a tetraether lipid is synthesized from two molecules of corresponding diether lipids without changes of polar groups.

Enzymes or genes in the lipid biosynthetic pathway in Archaea have been reported: isoprenyl synthesis from mevalonic acid to geranylgeranyl pyrophosphate (GGPP) via isopentenyl pyrophosphate (Chen and Poulter 1993; Ohnuma et al. 1994; Tachibana 1994); ether bond formation between GGPP and *sn*-glycero-1-phosphate (G-1-P) (Chen et al. 1993; Soderberg et al. 2001; Zhang and Poulter 1993), which produces geranylgeranylglyceryl phosphate and then digeranylgeranylglyceryl phosphate (DGGGP); and modification of a polar group from a simple phosphate group to an ethanolamine group via cytidine 5′-diphosphate (CDP) addition (Morii et al. 2000).

After the production of DGGGP, several steps are required to form tetraether lipids including MPL: (1) saturation of isoprenyl chains, (2) head-to-head condensation to form the tetraether core moiety, and (3) modification of a polar group from a simple phosphate group to a sugar and/or a modified phosphoric ester group. The order of these steps is not yet clear. However, the enzymes involved in ether bond formation and CDP addition require the fully unsaturated form of C_{20} isoprenyl alcohol (Morii et al. 2000; Zhang and Poulter 1993). Therefore, saturation of double bonds is expected to occur following the CDP addition to the DGGGP.

Recently, we found that tetraether lipid biosynthesis in *T. acidophilum* is inhibited by terbinafine (Kon et al. 2002), an allylamine that is a known antifungal compound inhibiting squalene epoxidase (Ryder 1984). The results suggested that the precursor of tetraether lipid is not DGGGP but rather a modified diether lipid, because (1) inhibition of the synthesis of tetraether lipids and simultaneous accumulation of diether lipids were detected in the presence of terbinafine, (2) accumulated diether lipid contained a modified polar head group (not a simple phosphate group), and (3) double bonds in hydrophobic moieties were not detected by high-performance thin-layer chromatography (HPTLC) analysis (Kon et al. 2002). Despite this knowledge, the detailed structure of accumulated diether lipid and modification of the polar head group from the diether lipid to MPL remain to be elucidated. In the present study, we analyzed the structure of the precursor of tetraether lipids using radiolabeled compounds and mass analysis, and we propose a tetraether lipid biosynthetic pathway in *T. acidophilum*.

Materials and methods

Microorganisms and culture conditions

Thermoplasma acidophilum HO-62 (Yasuda et al. 1995) was grown without shaking at 56 °C in a medium described previously (Kon et al. 2002). The mid-log-phase culture (about 64-h incubation) was used for labeling experiments. *Halobacterium halobium* L-33 was grown with shaking at 37 °C for 3 days in a medium described previously (Kon et al. 2002).

Labeling experiment

A 5-ml culture of *T. acidophilum* was incubated for 15 to 120 min in the presence of 5 μCi of RS-[2-^{14}C]mevalonic acid dibenzylethylenediamine salt (NEN Life Science Products, Boston, Mass.) at

56 °C. In pulse-labeling and chase experiments, cells incubated for 60 min with [^{14}C]mevalonic acid and 0.6 mg/ml terbinafine were washed twice and resuspended in fresh medium without the inhibitor and radiolabeled substrate and then incubated for 0–120 min at 56 °C. For phospholipid labeling, *T. acidophilum* cells were incubated for 60 min at 56 °C in 5 ml of a phosphate-free medium supplemented with 0.1 mCi of [^{32}P]orthophosphoric acid (NEN Life Science Products). For glycerol labeling, cells were concentrated to 100 times for efficient labeling. The cells of *T. acidophilum* in 5-ml culture were harvested by centrifugation at 8,000 *g* for 5 min. The resulting precipitated cells were resuspended in 50 μl of fresh medium and incubated at 56 °C in the presence of 0.1 μCi of [^{14}C(U)]glycerol (NEN Life Science Products).

Lipid extraction

An aliquot of the above-treated culture was immediately centrifuged at 1,650 *g* for 20 min using a swing rotor, and the cells obtained were washed twice with distilled water (adjusted to pH 2.0 with H_2SO_4) to remove the radiolabeled substrate. Lipid was extracted from the cells by the method of Bligh and Dyer (1959) as described (Kon et al. 2002). The unextracted lipids were re-extracted three times with chloroform. All chloroform fractions were pooled and dried under a stream of nitrogen gas. The total residual lipids were dissolved in 0.1 ml of chloroform-methanol (2:1, v/v) and used for the following analyses. Radioactivity of lipid extracts and recovered components (see below) was estimated using a liquid scintillation counter LSC-1000 (Aloka, Tokyo, Japan) with scintillation cocktail Scintisol AL-1 or Scintisol 500 (triton/toluene type for aqueous samples) (Dojindo, Kumamoto, Japan). Respective radioactivities of ^{14}C and ^{32}P in double-labeled samples were estimated using the double-counting program of the above liquid scintillation counter.

Thin-layer chromatography

Thin-layer chromatography (TLC) was carried out on Silica Gel 60 plates (Merck, Darmstadt, Germany) that were activated by incubation at 180 °C for 1 h before use. For two-dimensional TLC (2-D TLC), the solvent systems used were chloroform-methanol-water (65:25:2, v/v) for vertical direction (first dimension) and chloroform-methanol-acetic acid-water (100:20:12:2.5, v/v) for horizontal direction (second dimension). For TLC of the hydrophobic moiety after acid methanolysis, the solvent system used was chloroform-diethyl ether (9:1, v/v). For three-step TLC analysis, solvent systems were chloroform-methanol-water (65:25:0.5, v/v), chloroform-methanol (9:1, v/v), and hexane-diethyl ether (5:5, v/v) for first, second, and third development, respectively. After each development, plates were completely dried before the next development of the three-step TLC. Lipid spots on TLC plates were detected by spraying the plates with sulfuric acid solution (30% H_2SO_4, 5% HCHO) and charring at 150 °C for 30 min or, alternatively, by detecting radioactivity using a Bio Imaging Analyzer BAS2000 (Fuji Film, Tokyo, Japan) with an imaging plate.

Chemical analysis of ether lipids

Lipids were recovered from TLC spots by sequential elution of silica gel with mixtures of 2:1 then 1:9 (v/v) chloroform-methanol and filtration of the eluate using a glass filter (Whatman GF/B; Whatman, Kent, UK). Ether core lipids were prepared by acid methanolysis (Langworthy 1982) of the total lipid or lipids extracted from TLC spots as described (Kon et al. 2002). The phosphoric ester group of the phospholipids was removed by alkaline hydrolysis (Kushwaha et al. 1981) with 0.5 ml of 1 M NaOH in methanol-water (9:1, v/v) at 100 °C for 3 h. After addition of 0.45 ml chloroform and 0.42 ml water, the mixture was shaken vigorously. The chloroform phase was recovered after centrifugation. The unextracted lipids in the methanol-water phase were extracted with 0.45 ml chloroform three times, and the chloroform fractions were pooled. The extracted hydrolysates were separated using a three-step TLC system, and incorporation of radioactivity was estimated by a liquid scintillation counter.

Standard samples

Standard samples of archaeol and caldarchaeol were prepared from [^{14}C]mevalonic acid-labeled cells of *H. halobium* and *T. acidophilum*, respectively, at the stationary phase.

Mass spectrometry

Total lipid was extracted from *T. acidophilum* cells incubated for 60 min in the presence of terbinafine (0.6 mg/ml), and the cells were washed and incubated for another 120 min in the absence of the inhibitor. Total-lipid samples were analyzed by fast-atom-bombardment mass spectrometry (FAB-MS) carried out using a mass spectrometer (Autospec-E, Micromass, Manchester, UK) in a negative-ion mode using triethanol amine-*m*-nitrobenzyl alcohol (2:1, v/v) as a matrix.

Results

Effect of terbinafine on lipid biosynthesis in *Thermoplasma acidophilum*

Thermoplasma acidophilum cells were incubated for 15 min in the presence of [^{14}C]mevalonic acid. Total lipid was extracted from the cells and analyzed using 2-D TLC (Fig. 2a). Radioactive spots detected at the center and the solvent front of the 2-D TLC plate were named P1 and P2, respectively, although the latter was faint in

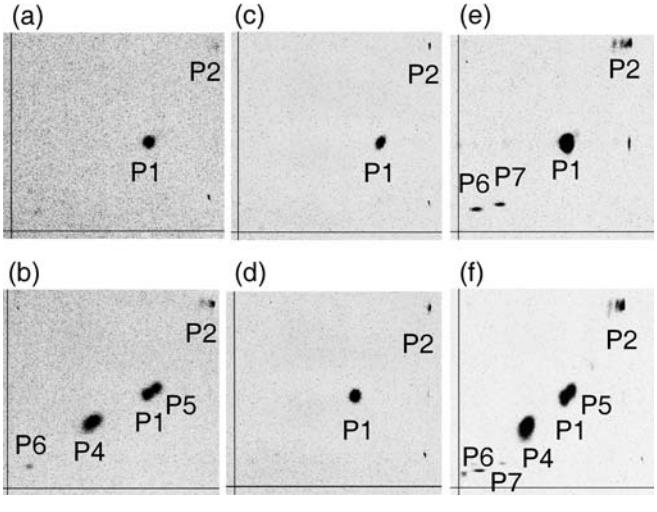

Fig. 2a–f Autoradiogram of the total lipid separated by 2-D TLC. *T. acidophilum* cells were incubated with 5 μCi [^{14}C]mevalonic acid, with or without terbinafine, at 56 °C. Lipids extracted from *T. acidophilum* cells after 15 min (**a, c**) and 120 min (**b, d**) incubation with 1.0 mg/ml (**c, d**) or without (**a, b**) terbinafine are shown. Results after 60 min incubation with 0.6 mg/ml terbinafine followed by a 0-min (**e**) or 120-min (**f**) chase period without inhibitor and mevalonic acid are also presented

Fig. 2a. After a 120-min incubation, several additional spots, P4, P5, and P6 were detected (Fig. 2b). In the presence of terbinafine, only P1 and P2 were detected even after the 120-min incubation (Figs. 2c,d).

To investigate the relationship among these spots, total lipid of *T. acidophilum* was pulse labeled and chased. The cells were incubated in the presence of [^{14}C]mevalonic acid and terbinafine for 60 min before being washed and resuspended in fresh medium without terbinafine and the labeled compound. They were incubated for either 0 or 120 min. In our previous study, we showed that inhibition of growth and tetraether lipid biosynthesis in *T. acidophilum* by terbinafine is reversible (Kon et al. 2002). Four spots, P1, P2, P6, and P7, were detected from the sample prepared after the 0-min chase period. The intensities of P6 and P7 varied between experiments, even after careful preparation of the preculture. The radioactivity of P1 corresponded to 78% of the activity associated with the total lipid recovered for this experimental condition (Fig. 2e). P2 was composed of several components that migrated as neutral lipids at the solvent front. The 2-D TLC of the lipids extracted after a 120-min chase period is shown in Fig. 2f.

A time course of radioactivity for each spot during this chase experiment is shown in Fig. 3. Low radioactivity in P4 was detected after 15 min, and it increased with further incubation. The P4 spot detected on the 2-D TLC plate showed the same mobility as the MPL in *T. acidophilum* (Swain et al. 1997). The radioactivity of P1 markedly decreased during the incubation period, while that of P4 significantly increased. In addition, a gradual increase in the radioactivity of P2 and a rapid decrease in that of P7 were observed. The radioactivity of P5 and P6 showed peaks at 90 min and 45 min, respectively, and decreased thereafter. The pattern of radioactive spots after a 120-min incubation in the chase experiment (Fig. 2f) was similar to that shown in Fig. 2b (120-min labeling without inhibitor).

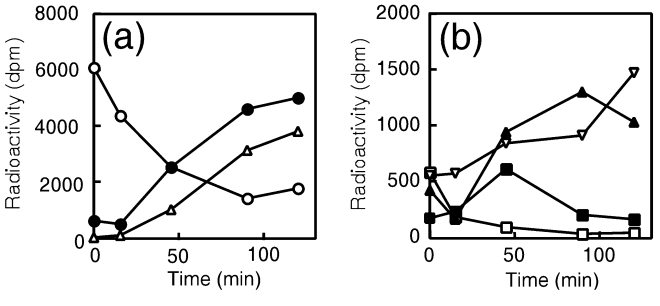

Fig. 3a, b Time course of radioactivity of 2-D TLC spots in chase experiment (see Fig. 2f). *T. acidophilum* cells were incubated with 5 μCi [^{14}C]mevalonic acid in the presence of 0.6 mg/ml terbinafine for 60 min. The cells were washed and resuspended in medium without terbinafine and mevalonic acid, and the samples were taken for analysis after the incubation times indicated. **a**: ○ P1, △ P4, ● total of tetraether lipids (P4, P5, and P6). **b**: ▽ P2, ▲ P5, ■ P6, □ P7

Determination of core lipid structure by acid methanolysis

For the analysis of core lipids, the radioactive compound was extracted from each spot of the 2-D TLC plates in the pulse-labeling and chase experiments. The extracted lipids were treated by acid methanolysis and the core lipids were then analyzed by TLC. The core lipid of P1 recovered after a 0-min chase period showed the same R_f value as that of archaeol (Fig. 4a, lane 3). The core lipid of P1 recovered after a 120-min chase period was also found to be an archaeol (lane 4). However, caldarchaeol was also detected in the P1 methanolysate, particularly in that recovered from the sample after a 120-min chase period (lane 4). This can be ascribed to contamination by P5 that migrated to a position close to P1.

Low radioactivity was detected after acid methanolysis of P2 from the sample after the 0-min chase period (lane 5). P2 contains several neutral lipids, and may include mevalonic acid derivatives (such as squalene or quinone) that have no archaeal ether core lipid and are degraded by acid methanolysis (Langworthy 1982; Langworthy et al. 1972). However, archaeol and caldarchaeol were detected after acid methanolysis of P2 recovered after a 120-min chase period (lane 6). When analyzed by three-step TLC, P2 was found to contain free archaeol and caldarchaeol (data not shown).

The core lipid of P4, P5, and P6 was caldarchaeol (Fig. 4a, lanes 7, 8, and 9, respectively). Minor spots with a mobility slightly higher than that of caldarchaeol were detected in P4 and P5 after treatment (Fig. 4a, lanes 7 and 8). These spots were expected to be trialkyl-type tetraether lipids that have been reported to be present in core lipids of *T. acidophilum* (Uda et al. 2000a). Alternatively, the spot between archaeol and caldarchaeol (lanes 2 and 6) might be methoxylated

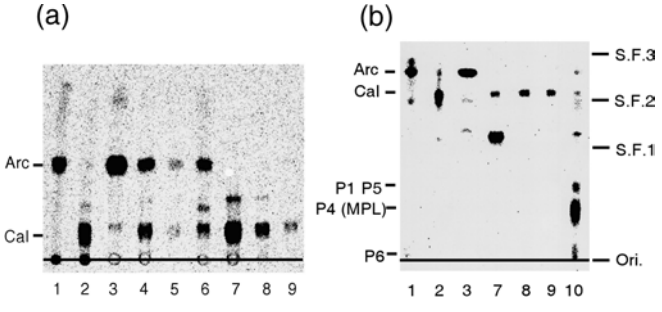

Fig. 4 Autoradiograms of the TLC plate after **a** acid methanolysis and **b** three-step TLC plate after alkaline hydrolysis. Two-D TLC spots of the sample after a 0-min (*lane 3 and 5*) or 120-min (*lanes 4, 6–9*) incubation without the inhibitor in the chase experiment as shown in Fig. 2e, f were extracted and analyzed by TLC after **a** acid methanolysis or **b** alkaline hydrolysis. *Lane 1* Archaeol (2,3-di-*O*-phytanyl-*sn*-glycerol, Arc), *lane 2* caldarchaeol (tetra-*O*-di-(biphytanyl)-diglycerol, Cal), *lanes 3 and 4* P1, *lanes 5 and 6* P2, *lane 7* P4, *lane 8* P5, *lane 9* P6, *lane 10* total lipid of *T. acidophilum* not subjected to hydrolysis. *Ori.* Origin, *S.F. 1, 2, and 3* solvent front in first, second, and third development of three-step TLC, respectively

hydroxy archaeol, which is derived from the acid methanolysis treatment of hydroxy archaeol (Ekiel and Sprott 1992). Swain et al. (1997) have reported that *T. acidophilum* contains a small amount of hydroxy archaeol. In the core structure of P6, no cyclopentane ring was detected using HPTLC (data not shown), which is able to separate the core lipids with different numbers of cyclopentane rings (Trincone et al. 1988).

The core lipid of P7 could not be analyzed because of its low radioactivity. A small amount of P7 that accumulated in the presence of terbinafine was decreased significantly after the removal of the inhibitor. Accordingly, P7 may also be a precursor in ether lipid biosynthesis. However, P7 cannot be the sole precursor in the biosynthesis of tetraether lipids, because the radioactivity of P7 was only about 10% of that of P1 or less in the presence of terbinafine.

Estimation of polar head groups by alkaline hydrolysis

While both phosphoric ester and sugar groups are removed from polar ether lipids by acid methanolysis treatment, only phosphoric ester groups are removed by alkaline hydrolysis treatment (Kushwaha et al. 1981). Accordingly, a combination of alkaline hydrolysis and acid methanolysis allows for estimation of the type of polar head groups of the polar lipids. The hydrolysate of the compounds recovered from 2-D TLC spots was analyzed by three-step TLC. Archaeol was detected from P1 after alkaline hydrolysis (Fig. 4b, lane 3). Therefore, the polar head group of P1 is expected to have the phosphoric ester bond but no sugar directly attached to glycerol of the core lipid.

Most of the alkaline hydrolysate of P4 migrated more slowly than caldarchaeol, although it migrated faster than P4 itself (Fig. 4b, lane 7). TLC analysis of the tetraether lipid that had only a sugar group (gulose) as a polar head on one side showed mobility between caldarchaeol and MPL (Shimada et al. 2002; Uda et al. 1999). Accordingly, P4 is expected to be a glycophospholipid with a phosphate group and a sugar group on each side of the core lipid. These results are consistent with the assignment of P4 as MPL.

Compounds extracted from P5 and P6 migrated at the same position as that of caldarchaeol after alkaline hydrolysis. Therefore, P5 and P6 are expected to be caldarchaeol to which no sugar group but one or two phosphate groups are attached.

Estimation of the number of phosphoric ester groups

All spots except P2 (i.e., P1, P4, P5, P6, and P7) were labeled with [^{32}P]orthophosphate (data not shown, see Table 1). This result is compatible with the result of alkaline hydrolysis, suggesting that P1, P4, P5, and P6 contain lipids with a phosphoric ester group.

Table 1 Relative number of phosphoric ester groups in each 2-D TLC spot of *Thermoplasma acidophilum* polar lipids double labeled with [^{14}C]mevalonic acid and [^{32}P]orthophosphoric acid

		Radioactivity (dpm)		^{32}P/^{14}C	Relative number of phosphoric ester group [b]
		^{14}C	^{32}P		
P1	Ex1[a]	1,282	258	0.201	1.8
	Ex2	700	132	0.189	2.0
P4 (MPL)	Ex1	3,015	342	0.113	(1.0)
	Ex2	2,504	240	0.096	(1.0)
P5	Ex1	1,298	203	0.156	1.4
	Ex2	430	39	0.092	1.0
P6	Ex1	495	97	0.196	1.7
	Ex2	560	101	0.180	1.9

[a] Upper and lower lines of datasets show results of two independent experiments

[b] Relative number of phosphoric ester groups per caldarchaeol molecule was estimated based on the ^{32}P/^{14}C ratio of each spot by normalizing against the ratio in P4 (MPL) in each experiment

To estimate the number of phosphoric ester groups in each spot, *T. acidophilum* cells were incubated in the presence of [^{14}C]mevalonic acid and [^{32}P]orthophosphate. The relative ratio of ^{32}P to ^{14}C activities of each spot was estimated and normalized against that of P4 (MPL), which has one phosphoric ester group per caldarchaeol molecule, in each set of experiments (Table 1). P1 showed a ^{32}P/^{14}C ratio twice that of P4 (MPL), which has a caldarchaeol core. The core lipid of P1 was determined to be an archaeol in the above experiments. Accordingly, P1 is expected to have one phosphoric ester group per archaeol molecule. P5 and P6 were estimated to have one and two phosphoric ester groups per caldarchaeol molecule, respectively.

Incorporation of [^{14}C]glycerol

Among the phospholipids reported in *T. acidophilum,* glycerophosphate is the only polar head group that contains phosphate (Langworthy et al. 1972; Shimada et al. 2002; Uda et al. 2000a). Other types of phosphate-containing lipids such as ether lipid analogues of phosphatidylinositol, phosphatidylcholine, and phosphatidylglycerophosphate have not been reported. Accordingly, the spots with a phosphoric ester group (P1, P4, P5, and P6) are expected to have a glycerophosphate as a polar head group. To confirm the presence of a glycerol in the polar head group of lipids recovered from 2D-TLC, *T. acidophilum* was grown in the presence of [^{14}C]glycerol, and phosphoric ester groups of the lipids in 2-D TLC spots were removed by alkaline hydrolysis. The ratio of the radioactivity associated with the polar head group (aqueous phase) versus that of the hydrophobic cores (organic phase) was nearly 1:1 in P1 and P6 (Table 2). This result supports the notion that the polar head groups in P1 and P6 are glycerophosphate. The ratios for P4 were approximately between 3:1 and 6:1. This ratio is higher than the theoretical ratio 2:1 in P4.

Table 2 Radio activities of alkaline hydrolysates of lipid spots P1, P4, and P6 from *T. acidophilum* cells incubated with [^{14}C] glycerol

		Radioactivity (dpm)		Ratio of radioactivity Organic phase/ Aqueous phase
		Organic phase	Aqueous phase	
P1	Experiment1	280	277	1.0
	Experiment2	159	141	1.1
P4	Experiment1	490	92	5.3
	Experiment2	269	91	3.0
P6	Experiment1	232	235	1.0
	Experiment2	173	174	1.0

P4 (MPL) contains a sugar moiety per molecule, while P1 and P6 have no sugar group. The sugar moiety of the polar head group in P4 may also be labeled with [^{14}C]glycerol after passing through the metabolic pathway. P5 could not be analyzed because of the spot's low labeling efficiency and contamination by P1. However, P5 migrated at the same position as the authentic cald-archaetidylglycerol in 2-D TLC (Shimada et al. 2002).

These results suggest the following: (1) P1 is a diether lipid that has glycerophosphate as the polar head group; (2) P4 is a tetraether lipid that has a sugar and a glycerophosphate on each side; (3) P5 is a tetraether lipid that has a glycerophosphate on only one side while the other side remains free; and (4) P6 is a tetraether lipid with two glycerophosphate groups on each side.

Structure of tetraether lipid precursor, P1

In the labeling experiment, the polar head group of P1 was determined to be glycerophosphate. To confirm this, P1 was extracted from the 2-D TLC plate and was compared with the lipids extracted from *Halobacterium halobium*. When radiolabeled lipids were extracted from *H. halobium* and analyzed by 2-D TLC, two spots were detected in the polar-lipid region. The spot that migrated near the origin on the TLC plate was expected to be archaetidylglycerophosphate (PGP). The other spot that migrated at the center of the TLC plate was expected to be archaetidylglycerol (PG) (Fig. 5) according to Ross and Grant's (1985) results of 2-D TLC. The total lipid of *H. halobium* and the P1 prepared from *T. acidophilum* cells were mixed and developed by 2-D TLC. P1 lipid migrated to a position indistinguishable from that of PG of *H. halobium* (Fig. 5).

Mass spectral analyses

In our previous study (Kon et al. 2002), double bonds in the hydrophobic core moiety of accumulated diether lipid were examined by HPTLC analysis. However, that method may not be suitable for detection of all double bonds. In the present study, in order to determine the structure of the hydrophobic core moiety of P1, mass spectrum of the total lipid was examined. The total lipids from cells incubated in the presence of terbinafine (0.6 mg/ml) and from cells after a chase period in the absence of the inhibitor were analyzed by FAB-MS. The mass spectrum of the total polar lipid extracted from the cells incubated with terbinafine is shown in Fig. 6a. An ion peak at m/z 806 corresponds to the saturated form of archaetidylglycerol. Minor ion peaks at m/z 804 and 802 correspond to archaetidylglycerol with one or two double bonds or cyclopentane rings. The signals at m/z from 1,606 to 1,618 correspond to MPL with different numbers of cyclopentane rings (Shimada et al. 2002; Swain et al. 1997). The peak at m/z 1,448 could not be identified. The same peaks were detected from the total lipid prepared from the cells incubated for another 120 min in the absence of terbinafine (Fig. 6b).

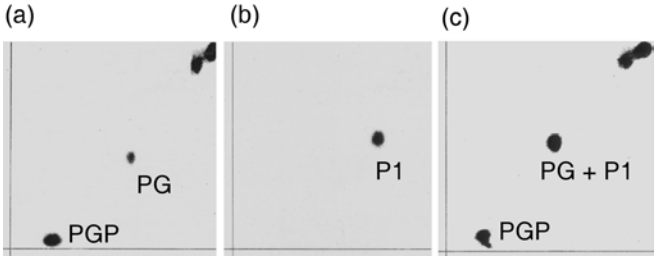

Fig. 5 Autoradiogram of 2-D TLC plates showing **a** total lipid of *Halobacterium halobium*; **b** compound extracted from P1 spot in the pulse-labeling experiment similar to that shown in Fig. 2e, and **c** mixture of total lipid of *H. halobium* and the extracted P1. *PG* Archaetidylglycerol, *PGP* archaetidylglycerophosphate

The relative intensity of the peak at m/z 806 (archaetidylglycerol) normalized against the sum of the intensities of the MPL peaks from m/z 1,606 to 1,618 (indicated by asterisks in Fig. 6) was $11.2 \pm 1.4\%$ in the lipid extracted with a 0-min chase period and only $3.8 \pm 1.3\%$ after a 120-min chase period (Fig. 6b and Table 3). The peak at m/z 806 corresponded to precursor P1, a fully saturated form of archaetidylglycerol. The height of the peak corresponding to archaetidylglycerol with one (m/z 804) or two (m/z 802) double bonds or cyclopentane rings did not decrease significantly after a 120-min incubation without the inhibitor (Table 3).

Discussion

In a previous report where we mainly analyzed hydrophobic groups (Kon et al. 2002), we found that tetraether lipid biosynthesis is reversibly inhibited by

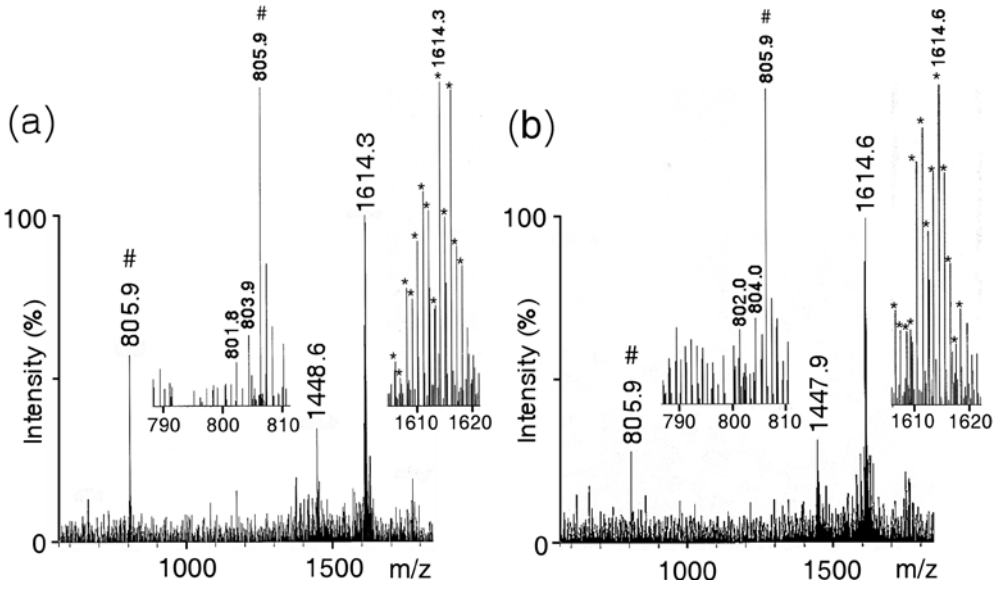

Fig. 6a, b FAB-MS spectra of total lipid extracted from *T. acidophilum*. *T. acidophilum* cells were incubated with 0.6 mg/ml terbinafine for 60 min. The cells were washed and resuspended in the medium without the inhibitor for **a** 0 min or **b** 120 min. Total lipid was extracted from the cells and analyzed with FAB-MS as described in Materials and Methods. Expanded spectra are shown on top. *Pound signs* (#) indicate the signals corresponding to P1, fully saturated form of archaetidylglycerol. *Asterisks* indicate the signals that were used to obtain the sum of the peak intensities of MPL (*m*/*z* from 1,606 to 1,618)

Table 3 Relative intensities of FAB-MS (negative-ion mode) peaks of lipids of *T. acidophilum* cells incubated for 0 or 120 min after removal of terbinafine

Lipid	*m*/*z* [M-H]$^-$	Relative intensity (%)	
		0 min	120 min
MPL[a]	1,606–1,618	100	100
P1	806	11.2 ± 1.4	3.8 ± 1.3
	804	2.0 ± 0.4	1.5 ± 0.06
	802	1.3 ± 0.14	0.9 ± 0.04

[a] Relative intensity of MPL is the sum of intensities of peaks from *m*/*z* 1606 to 1618 (indicated by asterisks in Fig. 6) and was used for normalization. Data are shown as mean ± SD of triplicate experiments

terbinafine and that the precursor diether lipid accumulated in the presence of terbinafine is almost fully saturated. In this study, we analyzed polar head groups of the precursor of tetraether lipid biosynthesis. We separated several radioactive spots in 2-D TLC plates and analyzed the structure of the lipids in these spots. In another previous report, we also determined the structures of most polar lipids present in *Thermoplasma acidophilum* (Shimada et al. 2002). By comparing the structural analysis of the radiolabeled lipids with the structures reported previously, compounds in each spot were identified as: P1, archaetidylglycerol; P4, β-L-gulopyranosylcaldarchaetidylglycerol (MPL); P5, caldarchaetidylglycerol; and P6, glycerophosphatidylcaldarchaetidylglycerol. From the time course results shown in Fig. 3b, the biosynthetic pathway of tetraether lipids was estimated and presented in Fig. 1. Archaetidylglycerol (P1) was the only precursor-diether lipid to have accumulated in the presence of terbinafine in *T. acidophilum*. There were no other candidate precursors accumulated and detected in large amounts. Accordingly, the major precursor of tetraether lipid biosynthesis is expected to be archaetidylglycerol (P1).

Filled circles in Fig. 3a show the sum of radioactivities of tetraether lipids (P4, P5, and P6). This increase was comparable to the decrease in radioactivity of P1. The result suggests that one molecule of tetraether lipid is synthesized from two molecules of archaetidylglycerol (P1). The time course data in Fig. 3 and the structural analyses suggested the following: (1) glycerophosphatidylcaldarchaetidylglycerol (P6) seems to be synthesized from P1; (2) removal of one glycerophosphate residue from P6 produces caldarchaetidylglycerol (P5); and (3) addition of a sugar group to P5 finally produces MPL, β-L-gulopyranosylcaldarchaetidylglycerol.

Sugar residues are expected to attach to the lipid core after tetraether lipid core synthesis. Though several tetraether glycolipids were detected, no diether glycolipid has been previously detected in *T. acidophilum* by structural analysis (Shimada et al. 2002; Swain et al. 1997; Uda et al. 1999, 2000b). This fact is consistent with our biosynthesis model, in which tetraether is formed from archaetidylglycerol and the sugar moiety is attached later.

P6 (glycerophosphatidylcaldarchaetidylglycerol), the tetraether lipid with glycerophosphate groups on both sides, was detected as an intermediate. This lipid had not been detected in our earlier comprehensive analysis of the lipids of *T. acidophilum* (Shimada et al. 2002). P6 may not exist stably in stationary-phase biosynthesis of tetraether lipids. On the other hand, some of the polar lipids reported in *T. acidophilum* (e.g., Shimada et al. 2002; Uda et al. 1999, 2000b) were not detected in this study and are not involved in the above-mentioned biosynthetic pathway. These lipids are probably synthesized through a minor pathway, which was not detected in this experiment, or are converted from MPL through a subsequent modification process. It is known that the thermoacidophilic archaeon *Sulfolobus acidocaldarius* N-8 has a polar lipid with one or two inositolphosphate groups at each side of the tetraether core lipid (Sugai et al. 1996). Sugai et al. (1996) suggested that tetraether lipids are synthesized by the

condensation of two archaetidylinositol molecules in *S. acidocaldarius* N-8. Their model is consistent with ours, in the sense that both models presume the conjugation reaction between two molecules of phosphatidyl diether lipid. In contrast, Nishihara et al. (1989) proposed the heptad hypothesis, in which one molecule of a tetraether lipid is synthesized from two molecules of corresponding diether lipids without changes of the polar lipid group, based on the polar lipid analysis of *Methanobacterium thermoautotrophicum*. Thermoacidophiles and methanogens may have different routes for tetraether phosphoglycolipid biosynthesis. However, the biosynthetic pathway was proposed based on the structural analysis of the polar lipids in previous studies. It is necessary to analyze directly the tetraether lipid biosynthetic route in other Archaea.

Mechanisms of head-to-head linkage reactions of tetraether lipid synthesis are not clear and have been the topic of lengthy discussion. Double bonds in hydrophobic moieties are expected to contribute to the reaction of methyl ends, although this idea is not based on any direct result (De Rosa et al. 1983). In our previous study, the hydrophobic moiety of the precursor lipid was analyzed by HPTLC, and only a few double bonds in the precursor diether lipid were found (Kon et al. 2002). In our present study, a large portion of the precursor of tetraether lipid biosynthesis was fully saturated, as can be seen in the main peak at m/z 806 in Fig. 6. The relative intensity of the peak (m/z 806) was markedly reduced upon removal of the inhibitor, while the peaks with one (m/z 804) and two (m/z 802) double bonds and/or cyclopentane rings were small and did not change significantly. These results indicate that most of the P1 that accumulated in the presence of terbinafine is fully saturated and suggest that it is used as the precursor for formation of tetraether lipid, at least indirectly.

There are at least three possible interpretations of our analytical results of the precursor lipid. The mechanisms involved in head-to-head condensation reaction of diether lipid can be one of the following. Eguchi et al. (2000) suggested the occurrence of double-bond migration in the hydrophobic moiety and proposed the mechanism of a new C–C bond-forming reaction involving double bonds at methyl ends in the thermophilic methanogens *Methanococcus jannaschii* and *Methanobacterium thermoautotrophicum*. Even if we did not detect a large amount of or subsequent large decrease in unsaturated precursor lipids, it is impossible to deny that double bonds are saturated or modified during our experimental procedures despite the careful analysis of the lipids. Alternatively, saturation and unsaturation reactions may be reversible, and a double bond may be reintroduced to P1 before the condensation reaction. In both cases, a mechanism similar to that proposed by Eguchi et al. (2000) may take place in *T. acidophilum*. Other types of reactions, such as those involving radicals, may participate in the head-to-head condensation processes of the saturated form of hydrophobic moiety. Further analysis of the tetraether lipid synthesis reaction is necessary to determine which of the three possible mechanisms actually takes place.

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