LIPIDS AND LIPOPROTEINS:

CTP:2,3-di-*O*-geranylgeranyl-*sn*-glycero-1-phosphate Cytidyltransferase in the Methanogenic Archaeon *Methanothermobacter thermoautotrophicus*

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CTP:2,3-di-*O*-geranylgeranyl-*sn*-glycero-1-phosphate Cytidyltransferase in the Methanogenic Archaeon *Methanothermobacter thermoautotrophicus**

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CDP-2,3-di-*O*-geranylgeranyl-*sn*-glycerol synthase (CDP-archaeol synthase) activity was discovered in the membrane fraction of the methanoarchaeon *Methanothermobacter thermoautotrophicus* cells. It catalyzed the formation of CDP-2,3-di-*O*-geranylgeranyl-*sn*-glycerol from CTP and 2,3-di-*O*-geranylgeranyl-*sn*-glycero-1-phosphate (unsaturated archaetidic acid). The identity of the reaction product was confirmed by thin layer chromatography, fast atom bombardment-mass spectroscopy, chemical analysis, and by UV spectroscopy. One mole of the product was formed from approximately 1 mol of each of the reactants. The enzyme showed maximal activity at pH 8.5 and 55 °C in the presence of Mg^{2+} and K^+ ions. By *in vivo* pulse labeling of phospholipids with $^{32}P_i$, CDP-archaeol was found to be an intracellular intermediate. A cell-free homogenate of *M. thermoautotrophicus*, when incubated with L-serine, converted the product of CDP-archaeol synthase reaction to a product with the same chromatographic mobility as archaetidylserine. It was concluded from these results that both CDP-archaeol and CDP-archaeol synthase were involved in cellular phospholipid biosynthesis. Among various synthetic substrate analogs, both enantiomers of unsaturated archaetidic acid possessing geranylgeranyl chains showed similar levels of activity, while archaetidic acid with saturated or monounsaturated isoprenoid or straight chains was a poor substrate, despite having the same stereostructure as the fully active substrate. The ester analogs with geranylgeranioyl chains showed significant activities. These results suggest that the enzyme dose not recognize ether or ester bonds between glycerophosphate and hydrocarbon chains nor the stereostructure of the glycerophosphate backbone but mainly targets substrates with geranylgeranyl chains.

To our knowledge, 100 or more polar lipids have been identified from 25 species of Archaea (LipidBank for Web, available via the World Wide Web). Because all the archaeal phospholipids consist of ether linkages between glycerophosphate (GP)[1] and isoprenoid alcohols, they are usually referred to as "ether lipids." Another remarkable feature of archaeal phospholipids is the stereoconfiguration of the GP backbone. The GP backbone of phospholipid in Archaea is *sn*-glycero-1-phosphate (G-1-P), which is the enantiomer of its bacterial and eucaryal counterparts (1). So far, no exception to this arrangement in the stereostructure of GP has been found, and remains the most fundamental distinguishing characteristic of the organisms in each of these domains (2). *In vitro* studies of polar lipid biosynthesis in Archaea have been carried out since 1990, and the enzymes catalyzing the first three steps have been identified (Refs. 3–6, Fig. 1). The last intermediate (2,3-di-*O*-geranylgeranyl-G-1-P = 2,3-GG-GP ether = unsaturated archaetidic acid) corresponds to phosphatidic acid (the fatty acyl ester form of the phospholipid intermediate) in phospholipid biosynthesis of *Escherichia coli*, which is then activated by CTP to become CDP-diacylglycerol by the action of CTP-phosphatidate cytidyltransferase (EC 2.7.7.41) (7). CDP-diacylglycerol plays a central role in the biosynthesis of a number of phospholipids in bacteria (8). Although 2,3-GG-GP ether is the analogous intermediate of polar lipid biosynthesis in Archaea, its hydrocarbon chains are still highly unsaturated unlike the final products in which the chains are completely saturated. Therefore, one may reasonably expect to encounter hydrogenation, nucleotide activation, and polar group attachment in the archaeal polar lipid biosynthetic pathway, but their exact sequence of events is not known. In order to elucidate this pathway of polar lipid biosynthesis in Archaea, we began by looking for an enzyme capable of activating the unsaturated archaetidic acids by CTP. We found such activity in the methanogenic archaeon *Methanothermobacter thermoautotrophicus* (formerly *Methanobacterium thermoautotrophicum*, Ref. 9), which catalyzed the formation of CDP-di-*O*-geranylgeranylglycerol (CDP-unsaturated archaeol) from CTP and 2,3-GG-GP ether (CTP:2,3-GG-GP ether cytidyltransferase, *e.g.* CDP-di-*O*-geranylgeranylglycerol synthase or CDP-archaeol synthase) according to Reaction 4, shown in Fig. 1.

The enzymes catalyzing steps 1–3 in Fig. 1 appear to be specific for G-1-P and derivatives of the same stereoconfigura-

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[1] The abbreviations used are: GP, glycerophosphate; G-1-P, *sn*-glycero-1-phosphate; G-3-P, *sn*-glycero-3-phosphate; 1,2-GG-GP ether, 1,2-di-*O*-geranylgeranyl-*sn*-glycero-3-phosphate; 2,3-GG-GP ether, 2,3-di-*O*-geranylgeranyl-*sn*-glycero-1-phosphate; 2,3-phyta-GP ether, 2,3-di-O-phytanyl-*sn*-glycero-1-phosphate; 2,3-phyto-GP ether, 2,3-di-*O*-phytyl-*sn*-glycero-1-phosphate; 2,3-ole-GP ether, 2,3-di-*O*-oleyl-*sn*-glycero-1-phosphate; 1,2-GG-GP ester, 1,2-di-*O*-geranylgeranioyl-*sn*-glycero-3-phosphate; 2,3-GG-GP ester, 2,3-di-*O*-geranylgeranioyl-*sn*-glycero-1-phosphate; *rac*-GG-GP ester, 1,2-di-*O*-geranylgeranioyl-*rac*-glycero-3-phosphate; 2,3-ole-GP ester, 2,3-di-*O*-oleoyl-*sn*-glycero-1-phosphate; *rac*-ole-GP ester, 1,2-di-*O*-oleoyl-*rac*-glycero-3-phosphate; FAB-MS, fast atom bombardment-mass spectrum; TLC, thin layer chromatography; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine.

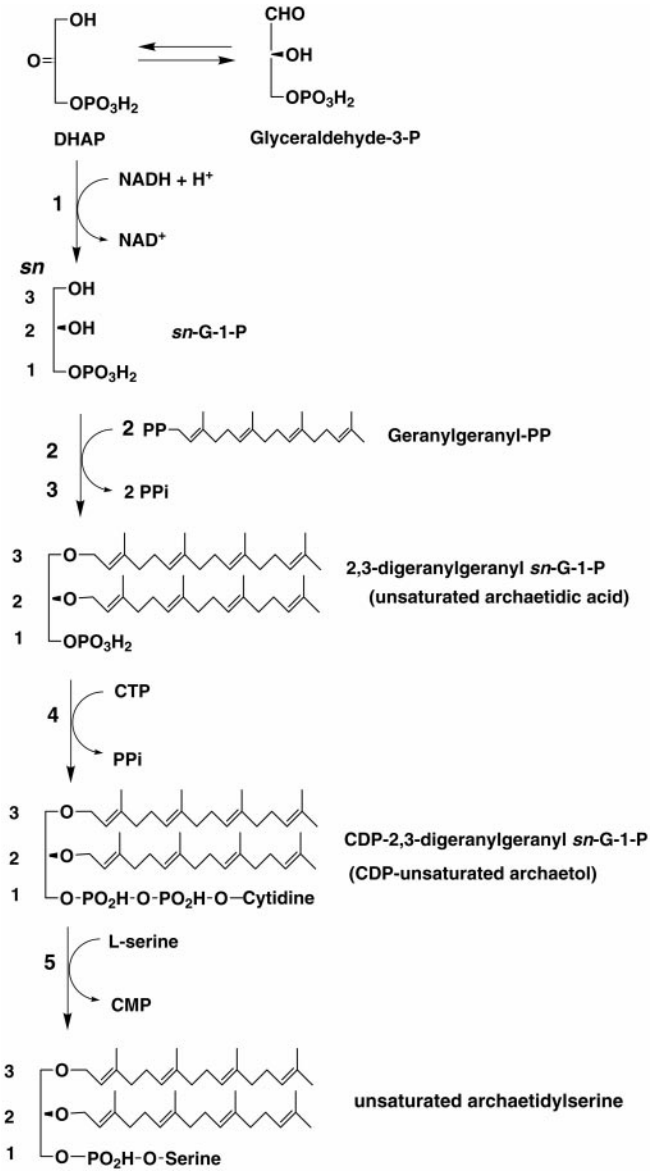

FIG. 1. **Enzymatically identified reactions of biosynthesis of polar lipids in Archaea.** *Reactions 1–3* have already been reported (3–6), and *Reactions 4* and *5* are described in the present paper. *PPi*, pyrophosphate; *DHAP*, dihydroxyacetonephosphate.

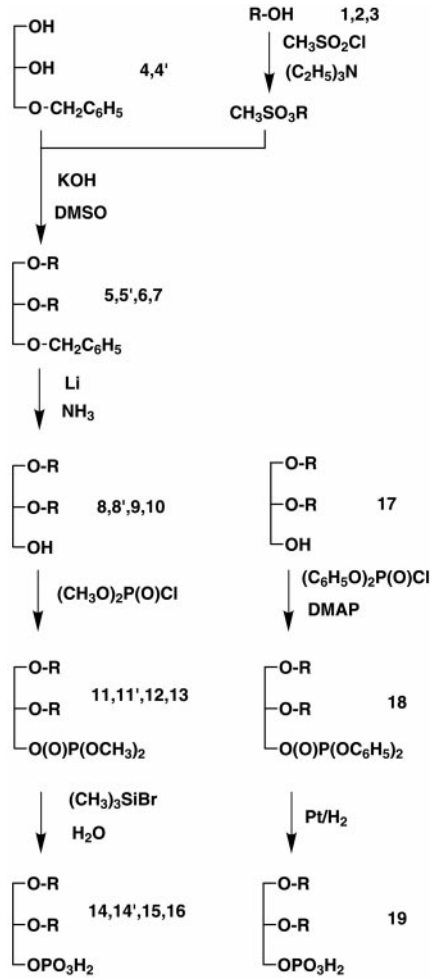

FIG. 2. **Synthesis of unsaturated and saturated archaetidic acids.** *R* represents a geranylgeranyl (***1***, ***5***, ***5′***, ***8***, ***8′***, ***11***, ***11′***, ***14***, ***14′***), phytyl (***2***, ***6***, ***9***, ***12***, ***15***), oleyl (***3***, ***7***, ***10***, ***13***, ***16***), or phytanyl (***17***, ***18***, ***19***) group. ***4*** and ***5–16***, 1-*O*-benzyl-*sn*-glycerol and its derivatives, respectively; ***4′***, ***5′***, ***8′***, ***11′***, and ***14′***, 3-*O*-benzyl-*sn*-glycerol and its derivatives, respectively. ***17***, ***18***, ***19***, 2,3-di-*O*-phytanyl-*sn*-glycerol and its derivatives, respectively. *DMSO*, dimethyl sulfoxide; *DMAP*, 4-(dimethylamino)pyridine.

tion. The next question is whether this enzyme is also specific for archaeal lipids with G-1-P stereostructure possessing ether bonds.

The present paper reports, for the first time, the characterization of CDP-archaeol synthase in *M. thermoautotrophicus* and comments upon the biosynthetic significance of the enzyme. The nomenclature of archaeal lipids proposed by Nishihara *et al.* (10) is used throughout this text.

EXPERIMENTAL PROCEDURES

Growth of Microorganisms—M. thermoautotrophicus ΔH (DSM 1053) was grown at 65 °C in a 15-liter fermentor (Mituwa, Japan) containing 5 liters of medium 2 (11) or in a 50-liter fermentor (Marubishi Model MSJ-N2) containing 33 liters of the same medium. Cells were harvested at log phase. *Halobacterium salinarum* (JCM 8981) was grown in 1 liter of DSM medium 97 (DSM Catalogue of Strains 1989) with shaking at 37 °C for 2 days. *E. coli* (JCM 1649) was grown in 1 liter of JCM medium 12 (JCM Catalogue of Strains 1999) with shaking at 37 °C for 6 h.

Thin Layer Chromatography (TLC)—TLC was carried out on a Silica Gel 60 plate (Merck) with the following solvents: solvent A, chloroform, methanol, and 7 M ammonia (60:35:8); solvent B, chloroform, methanol, acetic acid, and water (80:30:15:5). Phospholipid spots were visualized by spraying acid molybdate reagent. The radioactive spots were recorded by a Fujifilm FLA-2000 fluor image analyzer with an imaging plate (Fujifilm type BAS-TR for ^{3}H-labeled lipids and type BAS-MS for ^{14}C- and ^{32}P-labeled lipids).

Chemical Synthesis of Archaetidic Acids—The chemical syntheses of various archaetidic acids are summarized in Fig. 2. Unsaturated archaetidic acid and its analogs (**14**, **14′**, **15**, and **16**) were synthesized from geranylgeraniol (**1**, for the systematic name, see Table III), phytol (**2**, Funakoshi), or oleyl alcohol (**3**, Kanto Chemical Co.) and either 1-*O*-benzyl-*sn*-glycerol (**4**) or 3-*O*-benzyl-*sn*-glycerol (**4′**) (Funakoshi) as described (5, 12, 13). De-methylation conditions with trimethylsilyl bromide of **11**, **11′**, **12**, and **13** were modified to 0 °C for 2 min because these compounds are labile under the original conditions (13). The final products (**14**, **14′**, **15**, and **16**), were purified by TLC using solvent A. Because of the lability of these allyl ether bond-containing compounds, the overall yield was always 6% or less. The intermediate products (**8** and **8′**), di-*O*-geranylgeranyl glycerols, were confirmed by FAB-MS of the intact compounds and their fully hydrogenated derivatives. $[\alpha]_D$ of **8** and **8′** were +9.6° and −9.7°, respectively, which confirmed their stereostructure (10). The identities of two of the final products, 2,3-GG-GP ether (**14**) and 1,2-di-*O*-geranylgeranyl-*sn*-glycero-3-phosphate (**14′**, 1,2-GG-GP ether), were confirmed by their molecular ions, m/z 740 $(M + Na + H)^+$ and m/z 715 $(M - H)^-$ respectively, as revealed by positive and negative FAB-MS. The phosphate group was assigned to the *sn*-1 position based upon the formation of digeranylgeranylglycerol from 2,3-GG-GP ether by the action of *E. coli* alkaline phosphatase (Sigma). FAB-MS analysis of 2,3-di-*O*-phytyl-*sn*-glycero-1-phosphate (**15**, 2,3-phyto-GP ether) and 2,3-di-*O*-oleyl-*sn*-glycero-1-phosphate (**16**,

2,3-ole-GP ether) revealed m/z 752 ((M + Na + H)$^+$ and m/z 695 ((M + Na)$^+$), respectively.

Saturated archaetidic acid with phytanyl chains, 2,3-di-*O*-phytanyl-*sn*-glycero-1-phosphate (**19**, 2,3-phyta-GP ether) was prepared by phosphorylation of 2,3-di-*O*-phytanyl-*sn*-glycerol (**17**, archaeol), with diphenylphosphoryl chloride (Tokyo Kasei, Japan) prepared from total lipid extract of *H. salinarum* cells (14). The phenyl groups were removed by catalytic hydrogenolysis (15). Authentic archaetidic acid (16), obtained from *M. thermoautotrophicus*, was co-chromatographed with product **19** on one-dimensional TLC plates using solvents A (R_F 0.41) and B (R_F 0.73), and product **19** was also confirmed by m/z 733 (M + H)$^+$ of FAB-MS.

Chemical Synthesis of Phosphatidic Acids—Several kinds of phosphatidic acids (diacyl glycerophosphate) were synthesized by acylation of G-1-P prepared as described (14). *sn*-Glycero-3-phosphate (G-3-P, Nacalai Tesque, Japan), or *rac*-glycerophosphate (*rac*-GP, Nacalai Tesque) with geranylgeranioic anhydride or oleoic anhydride prepared with *N,N′*-dicyclohexylcarbodiimide from the corresponding carboxylic acids as described (17). Geranylgeranioic acid was prepared from geranylgeraniol as described (18, 19). The products (phosphatidic acids) were purified by CM52 carboxymethyl cellulose (Whatman) column chromatography and one-dimensional TLC with solvent A. The purified products were identified by co-chromatography with di-*O*-oleoyl-*rac*-GP (*rac*-ole-GP ester, Funakoshi) using solvent A (R_F 0.40) and solvent B (R_F 0.66), FAB-MS (m/z 743 (M − H)$^-$) in the case of 2,3-di-*O*-geranylgeranioyl-G-1-P (2,3-GG-GP ester), 1,2-di-*O*-geranylgeranioyl-G-3-P (1,2-GG-GP ester), m/z 765 (M + Na − 2H)$^-$ in the case of di-*O*-geranylgeranioyl-*rac*-GP (*rac*-GG-GP ester), and m/z 699 (M − H)$^-$ for 2,3-di-*O*-oleoyl-G-1-P (2,3-ole-GP ester) and *rac*-ole-GP ester, and mild alkaline methanolysis (20) which hydrolyzed ester bonds but not ether analogs.

Chemical Synthesis of CDP-archaeols—The method of Kates (21) was used with cytidine 5′-monophosphomorpholidate to synthesize CDP-2,3-di-*O*-geranylgeranyl glycerol (CDP-unsaturated archaeol) and CDP-2,3-di-*O*-phytanyl glycerol (CDP-saturated archaeol) from 2,3-GG-GP ether and 2,3-phyta-GP ether. They were comigrated with the authentic CDP-dioleoylglycerol (Funakoshi) on TLC plates with solvents A (R_F 0.37) and B (R_F. 0.40). Positive FAB-MS analysis of the synthesized CDP-unsaturated archaeol revealed m/z 739 (M − CMP + Na + H)$^+$, m/z 1021 (M)$^+$, m/z 1043 (M − H + Na)$^+$ and m/z 1065 (M − 2H − 2Na)$^+$. CDP-saturated archaeol prepared as above showed m/z 731 (M − CMP)$^-$ and m/z 1036 (M − H)$^-$ when analyzed by negative FAB-MS, which confirmed their structures.

Preparation of Cell-free Homogenates—Frozen *M. thermoautotrophicus* or *E. coli* cells (equivalent to about 1.5 g, dry weight) were suspended in 10 ml of 0.2 M potassium phosphate buffer (pH 6.5) containing 1 mg of DNase (Sigma) and then passed through a French pressure cell operated at 1,400 kg/cm^2. This process was repeated twice. In the case of the archaetidylserine synthase assay, 5 mM dithiothreitol was included prior to cell disruption. Cell debris and unbroken cells were removed by low speed centrifugation. The membrane fraction was obtained by centrifugation at 100,000 × *g* for 2 h. The pelleted membrane fraction was re-suspended in water (*M. thermoautotrophicus*) or in 0.1 M potassium phosphate buffer (pH 7.4) (*E. coli*) and stored at −20 °C until further use.

Enzyme Assay—(*a*) For CDP-archaeol synthase, the complete assay mixture (final volume, 0.2 ml) contained the membrane fraction of *M. thermoautotrophicus* (200 μg of protein), 0.16 M Bicine buffer (Dojin Laboratories, Kumamoto, Japan), pH 8.5, 0.5 M KCl, 1.5 mM $MgCl_2$, 2 mM lipid substrate, and 2 mM [5-^{3}H]CTP (PerkinElmer Life Sciences, 6.25 Ci/mol). After incubation at 55 °C for 2 h, the reaction was stopped by the addition of 1 ml of 0.1 M HCl in methanol. Chloroform-extractable ^{3}H material was then counted.

(*b*) CDP-diacylglycerol synthase of *E. coli* was assayed as described by Sparrow (22).

(*c*) For archaetidylserine synthase, the complete assay mixture (final volume 0.2 ml) contained 0.125 mM CDP-archaeol, 10 mM L-[3-^{14}C]serine (Amersham Pharmacia Biotech, 1.25 Ci/mol), the cell-free homogenate of *M. thermoautotrophicus* (726 μg of protein), 0.125 M Bicine buffer (pH 8.0) containing 1% Triton X-100, 0.5 M KCl, and 10 mM $MnCl_2$. After incubation at 55 °C for 30 min, reaction was stopped by the addition of 1 ml of 0.1 M HCl in methanol. Chloroform-extractable ^{14}C material was then counted.

Preparative Enzymatic Synthesis of CDP-archaeol—To obtain a fairly large amount of the enzyme reaction product for structural analysis, CDP-archaeol was enzymatically synthesized with 7.5 mg of 2,3-GG-GP ether, 4.8 mg of unlabeled CTP, and *M. thermoautotrophicus* membrane fraction (4.4 mg of protein). These reactants were incubated in the same buffer as the CDP-archaeol synthase assay (final volume, 5 ml) for 22 h. Since CDP-archaeol is soluble in an alkaline aqueous phase but soluble in the chloroform phase only under neutral or acidic conditions, the product was first extracted using the Bligh and Dyer solvent (23) under an acidic condition followed by extraction into an alkaline aqueous layer. After removal of the lower organic layer, the aqueous solution was acidified slightly with diluted HCl and re-extracted by the Bligh and Dyer method (acidic-alkaline partitioning). Most cellular phospholipids remained in the chloroform fraction while highly acidic lipids, such as CDP-archaeol and archaetidylserine, were extracted into the aqueous phase under alkaline conditions. The product was finally purified by TLC with solvent B.

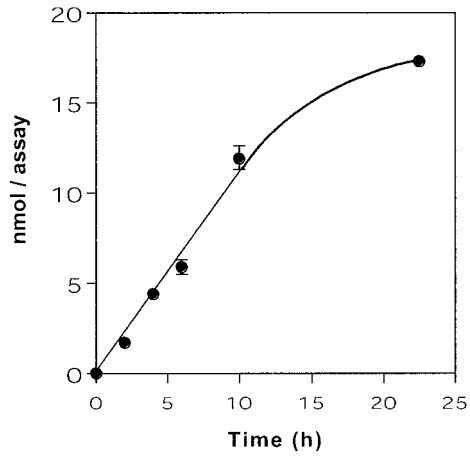

FIG. 3. **Conversion of [^{3}H]CTP to lipid catalyzed by *M. thermoautotrophicus* membrane fraction incubated with 2,3-GG-GP ether in the presence of 0.5 M K^+ and 2 mM Mg^{2+} at 55 °C.** Data are the average of duplicate assays ± S.E.

Analytical Methods and Degradation Procedure of CDP-archaeol—Phosphate (24) and protein (25) were determined as described previously. CDP-archaeol was hydrolyzed with 5% HCl-methanol at 80 °C for 1 h. Equal volumes of chloroform and water were added to the reaction mixture, and the two layers were separated. A portion of the aqueous product was dried under a stream of nitrogen. The resulting residue was dissolved in 0.01 M HCl. UV spectra (220–320 nm) of this solution were recorded by a Shimadzu UV-2200A spectrophotometer at pH 2 and 13. Cytidine was determined by the measurement of A_{280} at pH 2 (ϵ = 12800).

Physical Measurements—FAB-MS was recorded with a mass spectrometer (JEOL JMS DX-303) with a matrix of *m*-nitrobenzyl alcohol in a positive and/or a negative mode. In the case of CDP-unsaturated archaeol, the matrix contained a small amount of NaI. Optical rotation was measured at room temperature at 589 nm with an automatic digital polarimeter (PM-201, Photal, Japan). Radioactivity was counted using a liquid scintillation spectrometer (Aloka LSC-3500E, Japan) with Aquasol-2 (Packard) as scintillator.

Detection of CDP-archaeol in M. thermoautotrophicus Cells—At the midlog phase of growth of *M. thermoautotrophicus* grown in 50 ml of the low phosphate medium (26), 1 mCi of [^{32}P]P_i (Amersham Pharmacia Biotech, 50 Ci/mol) was added and the culture was allowed to continue to grow. After 60 min the cells were harvested, and unlabeled CDP-unsaturated archaeol was added (60 μg/10 mg cells). Total lipid was extracted, and the CDP-archaeol-containing fraction was roughly purified by the acidic-alkaline partitioning method described above. A quarter of the final acidic chloroform fraction was developed on two-dimensional TLC with solvent A twice in the first direction and with solvent B once in the second direction. A radioactive spot that was superimposable with the molybdate-positive spot of carrier CDP-archaeol was detected among a number of radioactive spots on the TLC.

RESULTS

CDP-archaeol Synthase Activity—A cell-free homogenate of *M. thermoautotrophicus* catalyzed the conversion of [^{3}H]CTP to chloroform-extractable material. Under assay conditions, incorporation of radioactivity into a lipid form continued almost linearly for at least 10 h, and then gradually slowed down (Fig. 3). In routine assays, the incubation time was 2 h. The activity was entirely dependent on the lipid substrate (2,3-GG-GP

TABLE I
Incorporation of [^{3}H]CTP into lipid by the membrane fraction of M. thermoautotrophicus

The complete reaction mixture contained the membrane fraction, 2 mM 2,3-GG-GP ether, and 2 mM [5-^{3}H]CTP as described under "Experimental Procedures." After incubation at 55 °C for 2 h, the reaction was stopped and ^{3}H in chloroform-extractable material was counted.

Reaction mixture	[^{3}H]CTP incorporated into lipid
	nmol
Complete	3.6
− 2,3-GG-GP ether	0.01
− Membrane fraction	0.08
− Membrane fraction + heated membrane fraction[a]	0.09
− $MgCl_2$	0.00
− KCl	0.14

[a] The membrane fraction was heated in a boiling water bath for 30 min.

ether) and required the addition of Mg^{2+} and K^+ ions (Table I). Maximal enzyme activity was observed at pH 8.5 and 55 °C (Fig. 4, *A* and *B*) when 0.5 M K^+ and 1–2 mM Mg^{2+} were added (Fig. 4, *C* and *D*). Under these conditions, radioactivity recovered in chloroform fraction after incubation was proportional to the amount of added membrane fraction protein (0–220 μg) (data not shown). Dithiothreitol showed no effect on the activity. Enzyme activity was inhibited 96% by 0.1% Triton X-100. The membrane fraction contained 92% of total activity found in the cell-free homogenate of *M. thermoautotrophicus*. The membrane fraction was able to be stored for several months at −20 °C without significant loss of activity. Freezing and thawing caused a small loss (5–10%) of activity.

Identification of the Product as CDP-archaeol—The ^{3}H-labeled product in the chloroform extract of the reaction mixture was chromatographed on TLC. Most of the applied radioactivity (90%) was recovered in a single spot, which co-migrated with authentic CDP-1,2-dioleoyl-*rac*-glycerol with solvents A (R_F 0.37) and B (R_F 0.39), respectively.

The reaction product was also chemically and mass-spectrometrically analyzed. For this purpose 26 times more substrate (2,3-GG-GP ether, 7.5 mg) was used for the prolonged reaction with unlabeled CTP, and 1.2 mg of the purified product was obtained. The FAB-mass spectrum of the product gave signals of m/z 739 $(M - CMP + Na + H)^+$, m/z 1021 $(M)^+$, m/z 1043 $(M - H + Na)^+$, and m/z 1065 $(M - 2H + 2Na)^+$ in the positive ion mode. The product was completely hydrolyzed by 5% HCl-methanol at 80 °C for 2 h without forming any phosphate-containing, chloroform-soluble materials. This is probably because the core lipid portion of CDP-archaeol is acid-labile. Water-soluble materials of the hydrolysate were extracted using the Bligh and Dyer solvent. UV spectra (220–320 nm) of the aqueous fraction showed typical spectra of cytidine at pH 2 and 13. The molar ratio of cytidine and total phosphate in the aqueous fraction was 1:2.1 (Table II). When chemically synthesized CDP-unsaturated archaeol (M_r = 1021) was treated as above, identical results were obtained. However, when CDP-saturated archaeol was hydrolyzed under the same conditions, a chloroform-soluble phosphate-containing compound, chromatographically identical to archaetidic acid, was formed. In this case, the ratio of cytidine, water-soluble phosphate, and chloroform-soluble phosphate was 1:1.20:1.17 (Table II). These results suggest that enzymatically synthesized CDP-archaeol still contains two acid-labile allyl ether bonds but no acid-stable ether bonds. In other words, it possesses two geranylgeranyl ether bonds.

Stoichiometry of the Enzyme Reaction—2,3-GG-GP ether (1.14 mg) was incubated with CTP and the membrane fraction of *M. thermoautotrophicus* at 55 °C for 15 h. Before and after the incubation, 2,3-GG-GP ether and CTP were measured by phosphate determination after separation by TLC of the chloroform-soluble fraction and UV measurement of the aqueous fraction, respectively. CDP-archaeol was determined by phosphate determination after separation by TLC of the chloroform-soluble fraction and UV measurement of the aqueous fraction after hydrolysis of the chloroform-soluble fraction. The decrease in mass of the reactants was 49 nmol (2,3-GG-GP ether) and 46 nmol (CTP). There was a concomitant increase of the product of CDP-archaeol. This was determined to be 38 nmol by spectrophotometric measurement or 43 nmol by phosphate determination. These results establish the stoichiometry of the reaction.

Substrate Specificity of CDP-archaeol Synthase—Archaeal glycerolipids are fundamentally characterized by the G-1-P stereostructure of their backbone. Substrate stereospecificity of the enzymes involved in polar lipid biosynthesis is therefore of special interest. To examine this issue, we synthesized a variety of substrate analogs with ether or ester bonds between GP and both unsaturated and saturated hydrocarbons, and with both stereoisomers of the GP backbone. Table III shows relative activities of the enzyme in the presence of 2 mM of each of the substrate analogs. Both enantiomers of archaetidic acid possessing geranylgeranyl chains (2,3-GG-GP ether and 1,2-GG-GP ether) showed similar activities as a substrate for the enzyme. In contrast to the stereostructure of GP backbone, unsaturation of the hydrocarbon chains of these substrates was critical; archaetidic acid with saturated (2,3-phyta-GP ether) or monounsaturated (2,3-phyto-GP ether) isoprenoid chains of the same carbon number were poor substrates as were analogs with monounsaturated straight chains (2,3-ole-GP ether), even though they had the same stereostructure as the fully active natural substrate (2,3-GG-GP ether). As to the ester analogs of the substrate, the GP esters with geranylgeranioyl chains again showed high activities. In this case, the 1,2-GG analog showed a half-maximal activity. The activities to the ester analogs of the substrate with oleoyl chains were also in low levels. These results suggest that the enzyme dose not recognize ether or ester bonds between GP and hydrocarbon chains nor the stereostructure of the GP backbone but mainly targets substrates with geranylgeranyl chains.

Substrate specificity of *E. coli* CDP-diacylglycerol synthase for the stereostructure and ester or ether linkage has not been reported previously. The substrate specificity of *E. coli* CDP-diacylglycerol synthase was investigated in order to form a baseline against which the substrate specificity of CDP-archaeol synthase could be compared. The synthetic enantiomeric phosphatidic acid (2,3-ole-GP ester) was a poor substrate for *E. coli* CDP-diacylglycerol synthase. Both enantiomers of archaetidic acid (ether type) with oleyl chains showed similarly low activities when introduced as substrates. These results indicate that *E. coli* CDP-diacylglycerol synthase recognizes ester bonds between GP and the hydrocarbon chain as well as the stereostructure of the GP backbone.

Conversion of CDP-archaeol to Archaetidylserine—In order to confirm that CDP-archaeol could be an actual intermediate metabolite of phospholipid biosynthesis in this Archaeon, we investigated the ability of a cell free extract of *M. thermoautotrophicus* to catalyze the conversion of CDP-archaeol to its likely next state, archaetidylserine. Cell-free homogenate of *M. thermoautotrophicus* was found to catalyze the incorporation of [^{14}C]serine into the chloroform-extractable fraction in the presence of chemically synthesized CDP-archaeol. The incorporation of [^{14}C]serine continued linearly for 30 min. The activity was completely dependent on the CDP-archaeol and the homo-

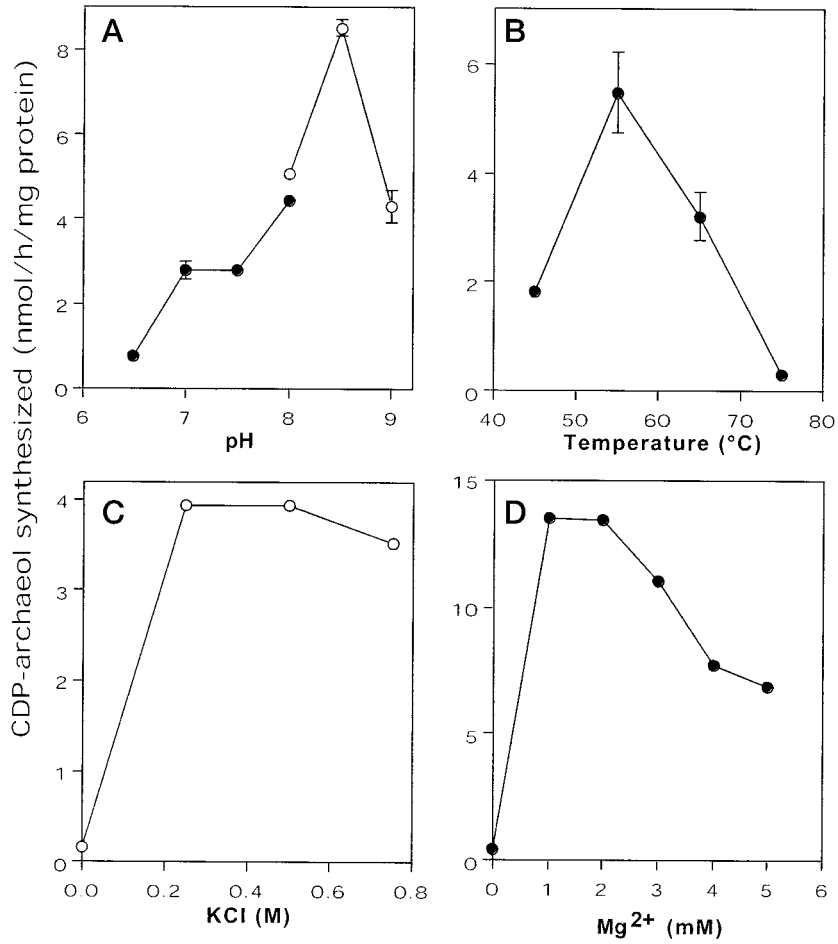

FIG. 4. **Effects of pH (*A*), temperature (*B*), K^+ concentration (*C*), and Mg^{2+} concentration (*D*) on CDP-archaeol synthase activity.** The conditions of the experiments were the same as described in Table I except that Mg^{2+} concentration was 4 mM in *A* and *C* and at pH 8 in *C*. In *panel A*, imidazole (pH 6.5–8.0, ●) and Bicine (pH 8.0–9.0, ○) buffers were used. Data in *A* and *B* are the average of duplicate assays ± S.E.

TABLE II
Hydrolysis products of CDP-archaeols

CDP-archaeols were hydrolyzed in 5% HCl-methanol at 80 °C for 1 h. The products were partitioned between aqueous and chloroform layers using the Bligh and Dyer solvent.

CDP-archaeol	Cytidine	Phosphate	
		Water-soluble	Chloroform-soluble
Chemically synthesized saturated	1	1.20	1.17
Chemically synthesized unsaturated	1	1.96	ND[a]
Enzymatically synthesized	1	2.10	ND

[a] Not determined because no phosphate-positive material was detected on TLC.

genate from *M. thermoautotrophicus* (Table IV). When developed on one-dimensional TLC with solvent B, the labeled lipid material migrated as a single spot, chromatographically identical with archaetidylserine. Under these conditions, 30% of CDP-archaeol was converted to archaetidylserine. Enzymatically synthesized CDP-archaeol was also converted to chloroform-soluble material at a significant but slightly lower rate compared with that of chemically synthesized CDP-unsaturated archaeol.

Presence of CDP-archaeol in M. thermoautotrophicus Cells—Total lipid extract of *M. thermoautotrophicus* was chromatographed using two-dimensional TLC in an attempt to detect an acid-labile phospholipid species co-migrating with synthetic CDP-archaeol. However the effort was unsuccessful, as only trace amounts would be present, if at all, and several large spots were distributed around the location of the reference spot on the TLC plate. [^{32}P]CDP-archaeol was concentrated from a total lipid extract of *M. thermoautotrophicus* cells pulse-labeled in $^{32}P_i$-containing medium using the acidic-alkaline partitioning method. This treatment removed significant amounts, but not all, of phospholipids such as diglucosylcardarchaetidylethanolamine, which interferes with the detection of CDP-archaeol. To improve resolution of the pertinent spots on the two-dimensional TLC, the TLC was developed twice in the first direction using the same solvent. The solvent was allowed to dry between the first and second developments. In this way we detected a tiny ^{32}P-spot superimposed on the spot of carrier CDP-unsaturated archaeol detected by means of acid molybdate spray (Fig. 5).

The ^{32}P-phospholipid (8202 cpm) corresponding to CDP-archaeol spot detected by I_2 vapor was scraped off from the TLC plate and extracted from the silica gel with Bligh-Dyer solvent containing 0.2 M HCl. The extracted ^{32}P-lipid (and carrier CDP-archaeol) was incubated at 55 °C for 30 min with L-serine, cell-free homogenate of *M. thermoautotrophicus*, and Bicine buffer with the composition as described for the archaetidylserine synthase assay. After the incubation, a chloroform-soluble fraction was obtained and Triton X-100 was removed by acetone precipitation of phospholipid. A significant amount of radioactivity, most of which were impurities, was removed during this process. The reaction product was developed on TLC with solvent B. A tiny ^{32}P-spot (144 cpm) cochromatographed with archaetidylserine was detected. As CDP-archaeol and archaetidylserine contain two and one phosphate groups in their molecules, respectively, and it is assumed that 30% of CDP-archaeol is converted to archaetidylserine under the conditions, roughly 10% of the ^{32}P-phospholipid recovered from the TLC plate should be [^{32}P]CDP-archaeol.

TABLE III
Substrate specificity of CDP-archaeol synthase and CDP-diacylglycerol synthase
The assay conditions were the same as in Table I except for the substitution of 2,3-GG-GP ether by other lipid substrates.

Lipid substrate				Relative activity[a]	
Compound	Hydrocarbon	Double bond	GP backbone	CDP-archaeol synthase (*M. thermoautotrophicus*)	CDP-DG synthase (*E. coli*)
				%	
2,3-GG-GP ether	Geranylgeranyl[b]	4	G-1-P	100	0.8 ± 0.1
1,2-GG-GP ether	Geranylgeranyl	4	G-3-P	94.0 ± 5.8	0.1 ± 0.0
2,3-Phyta-GP ether	Phytanyl[c]	0	G-1-P	3.1 ± 0.2	
2,3-Phyto-GP ether	Phytyl[d]	1	G-1-P	0.0 ± 0.0	
2,3-Ole-GP ether	Oleyl[e]	1	G-1-P	0.0 ± 0.0	7.8 ± 0.3
1,2-Ole-GP ether	Oleyl	1	G-3-P		7.8 ± 0.2
2,3-GG-GP ester	Geranylgeranioyl[f]	4	G-1-P	122.0 ± 13	
1,2-GG-GP ester	Geranylgeranioyl	4	G-3-P	49.8 ± 3.8	
rac-GG-GP ester	Geranylgeranioyl	4	*rac*-GP	81.4 ± 0.7	
2,3-Ole-GP ester	Oleoyl[g]	1	G-1-P	12.9 ± 0.7	2.6 ± 0.5
1,2-Ole-GP ester	Oleoyl	1	G-3-P		100
rac-Ole-GP ester	Oleoyl	1	*rac*-GP	3.0 ± 1.1	

[a] Data are the average of duplicate assays ± S.E.
[b] 3,7,11,15-Tetramethylhexadec-2*E*,6*E*,10*E*,14*E*-tetraenyl.
[c] 3*R*,7*R*,11*R*,15-Tetramethylhexadecanyl.
[d] 3,7*R*,11*R*,15-Tetramethylhexadec-2*E*-enyl.
[e] (*Z*)-Octadec-9-enyl.
[f] 3,7,11,15-Tetramethylhexadec-2*E*,6*E*,10*E*,14*E*-tetraenoyl.
[g] (*Z*)-Octadec-9-enoyl.

TABLE IV
Incorporation of [^{14}C]serine into lipid by homogenate of M. thermoautotrophicus
The complete reaction mixture contained CDP-unsaturated archaeol (CDP-2,3-di-*O*-geranylgeranyl glycerol), L-[3-^{14}C]serine, and the cell homogenate as described under "Experimental Procedures." After incubation at 55 °C for 30 min, reaction was stopped, and chloroform-extractable ^{14}C-labeled material was counted.

Reaction mixture	[^{14}C]Serine incorporated into lipid
	nmol
Complete	7.4
− Homogenate	0.0
− CDP-unsaturated archaeol	0.0
− CDP-unsaturated archaeol + enzymatically synthesized CDP-archaeol	5.7
− CDP-unsaturated archaeol + CDP-2,3-di-*O*-phytanyl glycerol	4.9

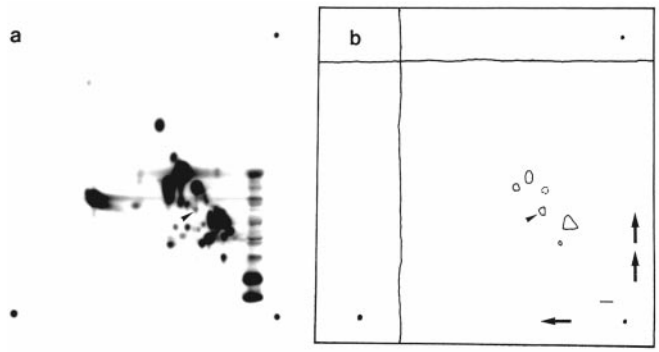

FIG. 5. **Presence of CDP-archaeol in the cells of *M. thermoautotrophicus*.** Cells of *M. thermoautotrophicus* pulse-labeled with $^{32}P_i$ for 1 h, and labeled lipids were roughly concentrated as described under "Experimental Procedures." Two-dimensional TLC was carried out with CDP-unsaturated archaeol as a carrier lipid. The chromatogram was recorded by autoradiography (*a*) for ^{32}P-spots and by acid molybdate spraying (*b*) for phospholipids. The radioactive spot marked with an *arrowhead* on the chromatogram (*a*) was completely superimposed with the acid molybdate-positive spot (marked with an *arrowhead*) on the chromatogram (*b*).

DISCUSSION

The enzyme activity of CDP- archaeol synthase was found in the membrane fraction of *M. thermoautotrophicus*. The enzyme catalyzes the reaction analogous to CDP-diacylglycerol that plays the central role in phospholipid biosynthesis in bacteria. Because it is a newly described enzyme activity, the details of the reaction should be established unambiguously. The product of CDP-archaeol synthase reaction was identified as CDP-di-*O*-geranylgeranylglycerol (CDP-unsaturated archaeol) based on the following observations. (*a*) Most of the radioactivity in the chloroform-soluble extract of the reaction products was recovered in a single spot on TLC, which co-migrated with chemically synthesized CDP-archaeol. (*b*) The product contained cytidine and phosphate in a molar ratio of 1:2. (*c*) The mild acid hydrolysis of the product yielded two water-soluble phosphate groups but the same treatment of CDP-saturated archaeol yielded one water-soluble phosphate group and one chloroform-soluble archaetidic acid, suggesting that the core lipid portion of CDP-archaeol is composed of acid-labile allyl ether bonds. (*d*) The molecular weight of the product coincided with that of CDP- di-*O*-geranylgeranylglycerol. The difference between the total molecular weight of CDP-unsaturated archaeol and the molecular weight of cytidine plus two phosphate groups is consistent with the molecular weight of digeranylgeranylglycerol. These results strongly support the theory that this enzyme activity catalyzes the conversion of unsaturated archaetidic acid and CTP to CDP-archaeol. The stoichiometry of this reaction was also established. Since this is the first report of CDP-archaeol synthase activity, it was necessary to address a concern that the putative substrates added to the reaction might simply stimulate incorporation of CTP into an endogenous lipid. When 7.5 mg of 2,3-GG-GP ether was incubated with the membrane fraction (equivalent to 4.4 mg of protein) of *M. thermoautotrophicus* cells, 1.2 mg of CDP-unsaturated archaeol was synthesized. Although the membrane fraction contained 2 mg of endogenous lipid, up to 0.47% of it was allyl ether type phospholipids determined by a newly developed method of reductive cleavage of ether bonds and GLC analysis of the resultant hydrocarbon (data not shown), *i.e.* the unsaturated lipid in this membrane fraction amounted to no more than 0.0094 mg. Since the hydrocarbon groups of the product were composed of geranylgeranyl chains, far more unsaturated product was produced from the substrate than from the endog-

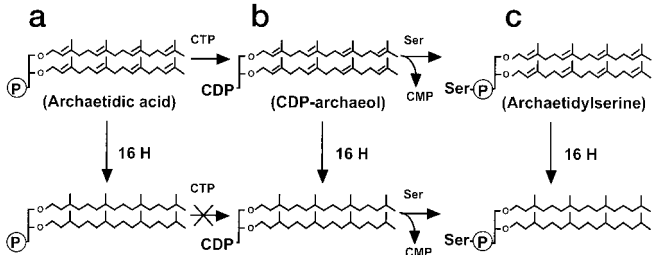

FIG. 6. **Three possibilities (*a*, *b*, and *c*) of hydrogenation of phospholipid hydrocarbon chains during phospholipid biosynthesis in Archaea.**

enous lipid present in the membrane fraction. This means that CTP was actually incorporated into the added 2,3-GG-GP ether.

It has been shown that it is possible to convert enzymatically synthesized CDP-archaeol to archaetidylserine *in vitro*. In addition, the fact that CDP-archaeol, the product of the CDP-archaeol synthase reaction, was detected in trace amounts in *M. thermoautotrophicus* cells argues in favor of this compound being involved in cellular phospholipid metabolism. CDP-archaeol could not be detected in total lipid of the cells by spraying phospholipid detection reagent on a TLC plate, but could be detected by intense labeling with $^{32}P_i$ for short periods. Detection of the small spot of CDP-archaeol among 20 or more much larger spots derived from other cellular membrane phospholipids on TLC was near impossible using conventional TLC methods. However, we recorded some success when we first reduced the effects of interfering lipids by means of the acidic-alkaline partitioning technique. The identity as CDP-archaeol of a part of the tiny ^{32}P-spot detected by superimposition on a TLC with chemically synthesized CDP-archaeol was further confirmed by its conversion to archaetidylserine by the action of cell-free homogenate. Together, these two lines of evidence make a compelling argument in favor of a role for CDP-archaeol as a biosynthetic intermediate in phospholipid metabolism in Archaea.

Although intermediate steps other than Reactions 1–5 (Fig. 1) in the phospholipid biosynthetic pathway in Archaea have not been definitively reported, CDP-archaeol could be one of the most important precursors for phospholipid biosynthesis in Archaea based on analogy with the bacterial pathway. After CDP-archaeol is synthesized in this pathway, two major issues continue to pose problems; saturation of the hydrocarbon chains must occur before or after the attachment of polar groups, and the formation of tetra-ether polar lipids. With regard to the former, three possibilities may be considered. Hydrogenation could occur (*a*) at GG-GP ether (archaetidic acid), (*b*) at CDP-archaeol, or (*c*) after attachment of a polar group (Fig. 6). Although the hydrogenation step has not been identified, it could occur at step *b* or *c* since CDP-archaeol synthase was found to be specific for unsaturated lipid.

One of the most remarkable features of archaeal phospholipids is the stereoconfiguration of G-1-P backbone. This work, therefore, focused on stereospecificity of the lipid substrate for *M. thermoautotrophicus* CDP-archaeol synthase. Surprisingly, the CDP-archaeol synthase recognized neither the stereochemical structure of a glycerophosphate backbone nor the linkage between glycerol and hydrocarbon groups (ester or ether linkage). It can be concluded that the stereostructure of archaeal polar lipids is established prior to or at the generation of unsaturated archaetidic acid (just before the CDP-archaeol synthase step). It should be noted that a geranylgeranyl chain contains four double bonds in the alkyl group. It is most likely that CDP-archaeol synthase is specific for archaetidic acid or phosphatidic acid with geranylgeranyl chains. However, the possibility has not been excluded that the substrate specificity might indicate the presence of two or more enzymes with different specificities since the enzyme has not yet been purified. Another possibility worth considering is that the preference of this enzyme for polyunsaturated hydrocarbon chains may be due to difficulty in dispersing the saturated compound in the reaction mixture as described by Sparrow *et al.* (27). If this is the sole reason of the low activity of CDP-archaeol synthase with the saturated substrate, the activity, although low, ought to be detected in significant amounts. Table III shows, however, that the activity with the saturated substrate was negligible. This suggests that problems associated with the dispersion are unlikely to be the reason for the low activity of CDP archaeol with saturated substrate.

By contrast, CDP-diacylglycerol synthase from *E. coli* recognized both G-3-P backbone and ester linkages. The complete genomic sequence of *M. thermoautotrophicus* has been reported (28) and functional annotation assigned. However, these do not reveal the presence of the gene encoding CDP-diacylglycerol synthase. Archaeal CDP-archaeol synthase might be quite different in its primary structure from bacterial or eucaryotic CDP-diacylglycerol synthase.

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