CDP-2,3-Di-O-Geranylgeranyl-sn-Glycerol:1-L-Serine O-Archaetidyltransferase (Archaetidylserine Synthase) in the Methanogenic Archaeon * Methanothermobacter thermautotrophicus*

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Major polar lipids of Methanothermobacter thermautotrophicus (formerly Methanobacterium thermoautotrophicum [27]) ΔH have been reported to be diether- and tetraether-type phospholipids, glycolipids, and phosphoglycolipids containing L-serine, ethanolamine, myo-inositol, and β-D-glucosyl-(1-6)-β-D-glucose as polar head groups. Considering these polar lipid structures and other known structures of archaeal phospholipids, three common and specific characteristics of archaeal polar lipids are recognized. First, all the polar lipids consist of ether linkages between glycerophosphate (GP) and hydrocarbon chains; second, the hydrocarbon chains are exclusively isoprenoid alcohols; and third, the most exclusive feature of archaeal phospholipids is the stereoconfiguration of the GP backbone. The GP backbone of phospholipid in the Archaea is sn-glycerol-1-phosphate (G-1-P), which is the enantiomer of its bacterial and eucaryotic counterparts (10). The mechanism by which these specific characteristics are formed is now partly elucidated through in vitro studies of polar lipid biosynthesis in Archaea, which have already revealed four enzymatic reactions and their substrate specificities (14, 16, 28, 29) (Fig. 1). Dihydroxyacetone phosphate, which is an intermediate of glycolysis and gluconeogenesis, is the starting substrate of polar lipid biosynthesis (Fig. 1). Dihydroxyacetone phosphate is first reduced exclusively to G-1-P by G-1-P dehydrogenase (Fig. 1, reaction 1). The activity of G-1-P dehydrogenase or the genes encoding G-1-P dehydrogenase have been detected in all archaeal species studied so far (18). In the next two steps, G-1-P is bound to isoprenoid hydrocarbons through two ether bonds at the sn-2 and -3 positions of the glycerol moiety (28, 29) (Fig. 1, reactions 2 and 3). The ether bond-forming enzymes are specific to G-1-P and geranylgeranyl pyrophosphate, so the product of these steps is 2,3-di-O-geranylgeranyl-sn-glycerol-1-phosphate (unsaturated archaeic acid). Genes encoding the first ether bond-forming enzyme are detected in seven species of archaeal genomes (23). The intermediate formed is then activated by CTP to form CDP-unsaturated archaeol by the action of CTP:2,3-di-O-geranylgeranyl-sn-glycerol-1-phosphate cytidyltransferase (14) (Fig. 1, reaction 4). All these studies on polar lipid biosynthesis in Archaea were carried out for M. thermautotrophicus and its closest relative, Methanobacterium marburgensis (formerly Methanobacterium thermautotrophicum strain Marburg [27]), and this pathway is analogous to the bacterial pathway of phospholipid biosynthesis except for the structural differences in stereostructure of GP, ether bonds, and hydrocarbon chains. CDP-diacylglycerol, which corresponds to CDP-unsaturated archaeol, plays a central role in the biosynthesis of a number of phospholipids in Bacteria (4). In order to prove the next reaction of polar lipid
FIG. 1. Enzymatically identified reactions of biosynthesis of polar lipids in Archaea. Reactions 1 to 4 have already been reported (14, 16, 28, 29). Reaction 5 is described in the present work. DHAP, dihydroxyacetone phosphate; PP, pyrophosphate.
biosynthesis in \( \textit{Archaea} \) (Fig. 1, reaction 5), we began to look for an enzyme capable of synthesizing serine phospholipid in \( \textit{M. thermotrophicus} \) using the well-known bacterial pathway. We found such an activity (14), which catalyzed the formation of archaeidylserine from CDP-archaeol and \( \text{t-serine (archaeidylserine synthase, e.g., CDP-2,3-di-O-geranylgeranyl-sn-glycerol: t-serine-O-archaetidyltransferase)} \) according to the reaction 5 shown in Fig. 1.

Studying archaeal polar lipid biosynthesis, in particular the step of the attachment of the polar head group, one may expect to encounter several questions concerning the specific archaeal lipid structures. One of the questions is how archaeidylserine synthase contributes to the establishment and selection of the specific features of archaeal polar lipid structure. The enzymes that catalyze reactions 1 to 3 in Fig. 1 are specific for G-1-P and its derivatives of the same stereoconfiguration. By contrast, CDP-archaeol synthase, which catalyzes reaction 4, does not recognize the structure of the GP backbone or the ether or ester bonds between GP and hydrocarbon chains but mainly targets a substrate possessing geranylgeranyl chains. The second question deals with the relationship between archaeidylserine synthase and bacterial or eukaryal phosphatidylserine synthases. Because homologies of the gene encoding phosphatidylserine synthase and bacterial or eukaryal phosphatidylserine synthases. Because homologies of the gene encoding phosphatidylserine synthase (\( \text{psa} \)) in \( \textit{Bacillus subtilis} \), but not in \( \textit{Escherichia coli} \), were found in several archaeal species whose whole-genome sequences are known, the enzymatic properties which cannot be inferred from their genome sequences should be compared with those of the analogous enzymes. This might give a clue to the origin of amino group-containing phospholipids in \( \textit{Archaea} \). The other problem is the exact sequence of polar group attachment and hydrogenation of unsaturated hydrocarbon chains, which contrasts with completely saturated chains in the final products.

The present work reports some properties and the substrate specificity of archaeidylserine synthase in \( \textit{M. thermotrophicus} \) and compares them to those of bacterial phosphatidylserine synthase. The nomenclature of archaeal lipids proposed by Nishihara and Koga (15) is used throughout this paper.

### MATERIALS AND METHODS

**Growth of microorganisms.** \( \textit{M. thermotrophicus} \) \( \Delta \text{H} \) (DSM 1053) and \( \text{E. coli} \) DSM 1649 were grown as previously described (14). \( \textit{B. subtilis} \) JCM 1465 was grown with shaking at 37°C for 6 h in a 3-liter Erlenmeyer flask in 800 ml of JCM medium 22 (Japan Collection of Microorganisms catalogue of strains, 1999) supplemented with 1% glucose.

**Chemical synthesis of CDP-archaeol and CDP-diaoylglycerol.** CDP-2,3-di-O-geranylgeranyl-sn-glycerol and CDP-2,3-di-O-phytanyl-sn-glycerol were chemically synthesized with cytidine 5′-monophosphomorpholidate from corresponding arachidic acid as previously described (9, 14). Other substrates, CDP-1,2-di-O-geranylgeranyl-sn-glycerol and CDP-1,2-di-O-oleyl-sn-glycerol (diether type), CDP-diaoylglycerols (diester type, CDP-2,3-di-O-oleoyl-sn-glycerol and CDP-1,2-di-O-acyl-sn-glycerol) were synthesized as previously described (9, 14). t-serine-phosphatidic acid from egg yolk lecithin (Nacalai, Kyoto, Japan) was used as a starting material for the synthesis of CDP-1,2-di-O-acyl-sn-glycerol.

**Enzymatic preparation of \( [\text{H}] \text{CDP-unsaturated archaeol.} \)** Cystenose-5′-3′\( \text{H}\)CDP-unsaturated archaeol was enzymatically synthesized as described in the previous paper (14) except that 15 μCi of [5′-3′\( \text{H}\)]CTP (0.625 Ci/mmol) was used in 1.2 ml of the reaction mixture. After incubation for 5 h, a chloroform-soluble fraction was obtained from the reaction mixture and then [cytosine-5′-3′\( \text{H}\)]CDP-unsaturated archaeol was purified by acidic–alkaline partitioning (14).

**TLC.** Thin-layer chromatography (TLC) gel was developed on a Silica Gel 60 plate (Merek) with the following solvents: solvent A, chloroform-methanol–7 M ammonia (60:35:8); solvent B, chloroform-methanol-acetic acid-water (80:30:15:5). Spots of amino group-containing lipids were visualized by spraying with ninhydrin reagent. Authentic archaeidylserine was isolated from \( \textit{M. thermotrophicus} \) cells as previously described (17). Water-soluble products were decontaminated on a thin-layer cellulose plate (Merek, 5716) with benzophenone (80:20). Standard CMP was purchased from Kohjin, Tokyo, Japan. Radioactive spots were recorded by a Fujifilm Fluor image analyzer (model FLA-2000) with an imaging plate: Fujifilm type BAS-TR for \( \text{H} \)-labeled lipids and type BAS-MS for \( \text{P} \)- and \( \text{P} \)-labeled lipids.

**Preparation of cell-free homogenates.** Frozen \( \textit{M. thermotrophicus} \) cells (about 20 g wet weight) were suspended in 25 ml of 10 mM Bicine buffer (pH 8.0) containing 1 mM MgCl2, 5 mM 2-mercaptoethanol (buffer M), and 1 mg of DNase I (Sigma) were passed through a French pressure cell operated at 1,400 kg/cm2. This process was repeated twice. Cell debris and unbroken cells were removed by centrifugation (10,000 \( \times \) g) for 10 min. The homogenate was stored at \( -20°C \) until further use. The membrane fraction was obtained by centrifugation at 100,000 \( \times \) g for 2 h. The pellet membrane fraction was resuspended in buffer M. Crude cell extracts of \( \textit{B. subtilis} \) and \( \text{E. coli} \) were prepared as previously described (5, 6).

**Enzyme assay.** The complete assay mixture (final volume, 0.2 ml) for archaeidylserine synthase of \( \textit{M. thermotrophicus} \) contained 0.5 mM CDP-archaeol, 10 mM [3′-\( \text{H} \)-serine (1.25 Ci/mol), Amersham Pharmacia Biotech), the cell-free homogenate of \( \textit{M. thermotrophicus} \) (568 μg of protein), Bicine buffer (pH 8.5), 1% Triton X-100, and 10 mM MnCl2. After incubation at 60°C for 10 min, the reaction was stopped by the addition of 1 ml of 0.1 M HCl in methanol, 2 ml of chloroform, and 3 ml of 1 M MgCl2. Chloroform-extractable \( \text{H} \)-material was separated from water-soluble components by phase partitioning, and radioactivity was counted. In the case of determination of stereospecificity to serine of archaeidylserine synthase, [3′-\( \text{H} \)-]serine was replaced by nonradioactive \( \text{D} \)-or \( \text{L} \)-serine in 0.8 ml of the reaction mixture. After incubation at 60°C for 30 min, phospholipid was precipitated by the addition of aceton to a chloroform-soluble fraction in order to remove Triton X-100 (14). The precipitate of phospholipids was developed on TLC with solvent B. A spot corresponding to archaeidylserine was visualized by spraying with acid molybdate reagent, and the phosphorus content of the spot was separated off from the plate was determined (15). The activity of archaeidylserine synthase was calculated based on the formation of archaeidylserine over 30 min measured by phosphate determination. The phosphorus content of the spot corresponding to archaeidylserine in a control experiment without \( \text{D} \)-or \( \text{L} \)-serine was subtracted from the total phosphorus of archaeidylserine determined for the TLC spot.

**Identification of the reaction product.** To obtain a large amount of the enzyme reaction product for structural analysis, 20 times more reactants were incubated for 30 min. Nonradioactive \( \text{t}-\text{[H]serine.} \) The product was extracted and purified by acetone precipitation and TLC as described above. The fast atom bombardment-mass spectrum of the product was recorded with a mass spectrometer (JEOL JMS DX-303) with a matrix of \( \text{m} \)-nitrobenzyl alcohol containing a small amount of NaI in a positive mode. The presence of an allyl ether linkage was checked by the lability to the treatment of 5% HCl-methanol at 80°C for 1 h. For the identification of water-soluble product, [cytosine-5′-\( \text{H} \)]CDP-archaeol (200,000 dpm) was reacted with unlabeled \( \text{t}-\text{[H]serine} \) in a standard reaction mixture. The water-soluble radioactive product was recovered in the aqueous phase after Bligh-Dyer partitioning and was developed by cellulose-TLC.

**Detection of allyl ether containing archaeidylserine in \( \textit{M. thermotrophicus} \) cells.** \( \textit{M. thermotrophicus} \) was grown successively twice in 50 ml of low-phosphate medium (17) containing 100 μCi of \( \text{P} \) (5 Ci/mol) under a pressurized atmosphere of \( \text{H}_2 + \text{CO}_2 + \text{H}_2\text{O} \) (78:22:0.2) in a 500-ml flask with shaking for 24 h. Finally, 50 ml of the same medium containing the same specific radioactivity of \( \text{P} \), was inoculated with 5 ml of the last subculture. At the early logarithmic phase of growth (after incubation for 7 h), 2 ml of \( \text{P} \) was added and the culture was allowed to continue to grow. After 10 min, cells were harvested and total lipid was extracted. The radioactive phospholipid corresponding to archaeidylserine was purified from the total lipid by two-dimensional TLC. The isolated archaeidylserine was treated with 5% HCl-methanol at 80°C for 1 h to degrade allyl ether archaeidylserine. The degradation products were partitioned into chloroform-soluble and water-soluble fractions, \( \text{P} \) and \( \text{P} \) radioactivities in the chloroform-soluble products, aqueous phase, and the untreated sample were counted using a liquid scintillation analyzer (Packard TRI-CARB 2700TR) with Aquasol-2 (Packard) as a scintillator.
RESULTS

Identification of the reaction product. A cell-free homogenate of \textit{M. thermautotrophicus} catalyzed the conversion of \[^3H\]serine into chloroform-extractable \[^3H\]labeled material in the presence of CDP-2,3-di-O-geranylerganyl-sn-glycerol. The chloroform-soluble \[^3H\]labeled product of the archaeidylserine synthase reaction showed a single spot comigrating with authentic archaeidylserine when chromatographed on a TLC plate with solvent A \((R_f = 0.33)\) and solvent B \((R_f = 0.43)\). When several kinds of substrate analogs, that is, CDP-1,2-di-O-geranylerganyl-sn-glycerol, CDP-2,3-di-O-phytanyl-sn-glycerol, and CDP-rac-di-O-oleyl-glycerol were used instead of CDP-2,3-di-O-geranylerganyl-sn-glycerol, identical results were obtained. While it might be expected that archaeidylethanolamine is formed from archaeidylserine by a decarboxylation reaction, as suggested by an in vivo pulse-chase experiment \((17)\), no spot corresponding to archaeidylethanolamine was detected on the TLC plate after the archaeidylserine synthase reaction.

The reaction product from CDP-2,3-di-O-geranylerganyl-sn-glycerol was also chemically and mass-spectrometrically analyzed. The lipid product enzymatically prepared from CDP-2,3-di-O-geranylerganyl-sn-glycerol was purified by TLC with solvent B. The fast-atom bombardment mass spectrum of the lipid product gave signals of \(m/z\) 803 (M\(^+\)), \(m/z\) 827 (M + Na + H\(^+\)), \(m/z\) 762 (M - serine + 2Na + H\(^+\)), and \(m/z\) 784 (M - serine + 3Na\(^+\)), which were consistent with the structure of archaeidylserine with geranylerganyl groups as hydrocarbon chains. The presence of allylic ether linkages was also suggested by the acid lability. The product of the enzyme reaction was completely degraded by treatment with 5\% HCl-methanol at 80\°C for 1 h. These results suggest that the product from CDP-unsaturated archaeol most likely is \(2,3\text{-di-O-geranylerganyl-sn-glycero-1-phosphoserine}\) (unsaturated archaetylserine), even though the individual components and the stereostructure of the product were not completely determined. We also analyzed the water-soluble product of the reaction with [cytosine-5-\[^3H\]]CDP-archaeol and unlabeled l-serine as substrates. One radioactive spot \((R_f = 0.33)\) was found that cochromatographed with standard CMP on a cellulose TLC plate.

A nonradioactive by-product was detected on the TLC with an \(R_f\) of 0.19. The compound was positively stained with acid molybdate reagent on the TLC plate. For an analogous reaction, Walton and Goldfine \((26)\) reported that phosphatidyltransferase from \textit{Clostridium butyricum} catalyzed the transfer of the phosphatidyl moiety of phospholipid to Triton X-100, and in vitro formation of phosphatidyltriton was observed. Therefore, it was assumed to be a Triton X-100 adduct of an archaetyl group (e.g., archaetyltriton X-100) and was not further analyzed.

Properties of archaeidylserine synthase. Under the assay conditions used, radioactivity was incorporated into a chloroform-soluble fraction almost linearly for 30 min, and then the rate gradually slowed (Fig. 2). Approximately 30 nmol of archaeidylserine was formed when 100 nmol of CDP-archaeol was incubated in the reaction mixture for 1 h (Fig. 2). In routine assays, the incubation time was 10 min. Archaeidylserine synthase activity was roughly linear with protein content under the assay conditions (data not shown). The effect of nonionic detergent Triton X-100 on archaeidylserine synthase activity is shown in Fig. 3A. Triton X-100 was required for archaeidylserine synthase activity and maximum activity was obtained at a concentration of 1%. The enzyme activity was stimulated by the addition of Mn\(^{2+}\), with maximum activities occurring at concentrations of 5 mM or more. The addition of Mg\(^{2+}\) had much less effect on the enzyme activity (Fig. 3B). These results show that the enzyme activity was dependent on addition of Triton X-100 and Mn\(^{2+}\) ion. The enzyme did not require the addition of K\(^+\) ion. The enzyme activity was lowered to about 70\% of its maximum at 0.6 M K\(^+\), which corresponds to the intracellular concentration found in \textit{M. thermautotrophicus} \((20)\) (Fig. 3C). Maximal enzyme activity was observed at pH 8.0 to 8.5 (Bicine buffer) (data not shown) and at 60\°C (Fig. 3D). The membrane fraction and cell supernatant, respectively, contained 61 and 32\% of the total activity found in the cell-free homogenate of \textit{M. thermautotrophicus}. Specific activities of cell-free homogenates, membrane fraction and cell supernatant were 64, 146, and 19 nmol/h/mg of protein, respectively. Unfractionated cell-free homogenate was usually used for further studies.

Substrate specificity of archaeidylserine synthase. One of the major questions concerning archaeal lipid biosynthesis is how and at which step the specific structural characteristics of archaeal lipids are formed. The substrate stereospecificity of archaeidylserine synthase was, therefore, examined using a variety of chemically synthesized substrate analogs listed in Table 1. The list includes CDP-archaeol (substrates 1 to 4)/CDP-diaicylglycerol (substrates 5 and 6) analogs with both stereoisomers of the GP backbone, ether and ester bonds between GP and hydrocarbons, unsaturated and saturated isoprenoid hydrocarbon chains, and straight-chain hydrocarbons. Archaeidylserine synthase of \textit{M. thermautotrophicus} showed similar activities when either enantiomer of CDP-archaeol possessing geranylerganyl chains (substrates 1 and 2) was used as a substrate. The substrate analogs with sat-
urated ones (substrate 3) or straight hydrocarbon chains (substrate 4) showed slightly lower activities, but the activity range was between less than 50% of the activity on the natural substrate (substrate 1). Interestingly, when ester-lipids containing straight aliphatic chains (substrates 5 and 6) were used, activities were two to three times higher than those observed with an ether-type substrate (substrate 1) (Table 1). The stereospecificity of archaetidylserine to L- or D-serine was determined using nonradioactive substrates. Archaetidylserine synthase preferred L-serine to D-serine. The relative

![Image](https://via.placeholder.com/150)

**FIG. 3.** Effects of Triton X-100 concentration (A), Mn$^{2+}$ and Mg$^{2+}$ concentration (B), K$^{+}$ concentration (C), and temperature (D) on archaetidylserine (AS) synthase activity. The conditions of the experiments were the same as described in Materials and Methods with CDP-2,3-di-O-geranylgeranyl glycerol except that the K$^+$ concentration was 0.5 M for panels A and B, and the reaction was performed at 55°C for panels A, B, and C.

**TABLE 1.** Substrate specificities of archaetidylserine synthase and phosphatidylserine synthase

<table>
<thead>
<tr>
<th>Substrate no.</th>
<th>Compound</th>
<th>Lipid substrate</th>
<th>% Relative activity (mean ± SE)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hydrocarbon</td>
<td>GP backbone</td>
</tr>
<tr>
<td>1</td>
<td>CDP-unsaturated ArOH</td>
<td>Geranylgeranyl</td>
<td>G-1-P</td>
</tr>
<tr>
<td>2</td>
<td>CDP-unsaturated ArOH</td>
<td>Geranylgeranyl</td>
<td>G-3-P</td>
</tr>
<tr>
<td>3</td>
<td>CDP-saturated ArOH</td>
<td>Phytanyl</td>
<td>G-1-P</td>
</tr>
<tr>
<td>4</td>
<td>CDP-ArOH</td>
<td>Oleyl$^e$</td>
<td>G-3-P</td>
</tr>
<tr>
<td>5</td>
<td>CDP-diacylglycerol</td>
<td>Oleyl$^e$</td>
<td>G-1-P</td>
</tr>
<tr>
<td>6</td>
<td>CDP-diacylglycerol</td>
<td>Fatty acid from lecithin</td>
<td>G-3-P</td>
</tr>
</tbody>
</table>

$^a$ Data are from duplicate assays.

$^b$ Activities of the *M. thermoautotrophicus* enzyme with various substrates were expressed relative to the activity observed with substrate 1 (100%).

$^c$ Activities of the *B. subtilis* or *E. coli* enzyme with various substrates were expressed relative to the activity observed with substrate 6 (100%).

$^d$ 9-cis-Octadecenyl [CH$_3$(CH$_2$)$_7$CH=CH(CH$_3$)$_2$].

$^e$ 9-cis-Octadecenoyl [CH$_3$(CH$_2$)$_7$CH=CH(CH$_2$)$_2$CO$^-$.]
activity for d-serine was 32% ± 6% (n = 2) of what was observed with t-serine.

**Substrate specificity of bacterial phosphatidylserine synthase.** Because archaealidylserine synthase is known to be homologous to phosphatidylserine synthase from *B. subtilis* (12), the substrate specificities of phosphatidylserine synthase from *B. subtilis* and another type of phosphatidylserine synthase from *E. coli* were compared to that of archaealidylserine synthase (Table 1). *B. subtilis* phosphatidylserine synthase showed almost similar activities in every case when the substrates listed in Table 1 were used. That is, the enzyme did not discriminate between the stereostructures of the GP backbone, ether or ester linkage, and hydrocarbon chains of the analogs of CDP-archaeol. The substrate specificity of the *B. subtilis* enzyme was quite similar to that of the methanogen's archaealidylserine synthase described above. By contrast, *E. coli* phosphatidylserine synthase appeared to distinguish between such differences in the substrate structures. A drastic decrease in activity was observed when the mirror image isomer (CDP-2,3-diacyl-sn-glycerol [Table 1, substrate 5]) of the natural substrate (substrate 6) or the ether analogs with isoprenoid chain (substrates 1 to 3) were incubated with *E. coli* cell extracts. An ether-type substrate with natural GP stereostructure and straight-chain hydrocarbons (CDP-1,2-dioleyl-sn-glycerol [substrate 4]) revealed low but significant activity (41%) when compared with the ester-type natural substrate (substrate 6). In other words, substitution of ester linkages in the substrate structure showed a significant effect on the activity of *E. coli* phosphatidylserine synthase. Thus, a difference between phosphatidylserine synthases from both bacterial species was demonstrated also in substrate specificity.

**Intracellular occurrence of allyl ether-type archaealidylserine in *M. thermautotrophicus* cells.** In order to elucidate whether unsaturated (or allyl ether-type) archaealidylserine is really formed in the cells, we tried to detect it on the basis of the acid lability of allyl ether lipids. An allyl ether bond is degraded with 5% HCl-methanol at 80°C for 1 h (14). On the other hand, saturated archaealidylserine is stable to acid treatment because nonallyl ether bonds are not readily hydrolyzed and the phosphodiester bond cannot be hydrolyzed by cyclic phosphodiester formation due to the lack of a free hydroxyl group on serine residues (11). *M. thermautotrophicus* was continuously labeled with 32P, and pulse-labeled with 33P, for 10 min in the presence of 32P. In this experiment, 32P in archaealidylserine must represent the amount of mature archaealidylserine (the final product of the biosynthetic pathway) with saturated hydrocarbons, while 33P must express the amount of newly synthesized archaealidylserine. Labeled archaealidylserine was purified from the total lipid of the cells. The purified archaealidylserine was decomposed with 5% HCl-methanol at 80°C for 1 h. The ratio of radioactivities of 32P versus 33P (32P/33P) of chloroform-soluble products and water-soluble products after acid treatment of archaealidylserine was compared to that of untreated archaealidylserine. The 32P/33P ratio (1.26) of untreated archaealidylserine decreased by 17% to 1.04 in chloroform-soluble products (acid-stable lipids) after acid treatment. On the other hand, the ratio (2.17) in the aqueous fraction after acid treatment (acid-labile degradation products) was 1.7 times higher than the ratio (1.26) observed in untreated archaealidylserine. These results suggest that only a trace amount of newly synthesized allyl ether-type archaealidylserine is present in the cells. Almost all archaealidylserines have fully saturated hydrocarbon chains, as shown by chemical analysis (17).

**DISCUSSION**

The results described above confirm the presence of archaealidylserine synthase in *M. thermautotrophicus* homologate. The enzyme catalyzed the transfer of an archaeidyl group from CDP-archaeol to serine. When CDP-unsaturated archaeol was used as a substrate, the product was identified as archaealidylserine with geranylgeranyl chains. The water-soluble product of the reaction was identified as CMP. The stoichiometry could not be established because Triton X-100 interfered with the quantitative recovery of lipids from the reaction mixture and because of the formation of by-products such as archaeidyltriton X-100. The amount of lipid (30 nmol) synthesized during the enzyme reaction for 1 h was much more than the amount of endogenous allyl ether-type phospholipid (at most 0.17 nmol, see reference 14) in the enzyme source (the cell-free homogenate, 568 µg of protein, containing 36 nmol of total phospholipid [15]), excluding the possibility that the putative substrate might simply stimulate incorporation of radioactive serine into an endogenous allyl ether-type lipid.

Archaealidylserine synthase showed a loose specificity for CDP-archaeol analogs; that is, the enzyme is able to utilize CDP-archaeol analogs with both enantiomers of the GP backbone, ester and ether linkages, and unsaturated and saturated isoprenoid and straight-chain fatty acid. This means that archaealidylserine synthase is not involved in establishing the specific features of archaeal polar lipid structures. According to knowledge obtained so far, the first three enzymes in the biosynthesis pathway of the archaeal polar lipid appear to play a central role in the formation of the specific features of archaeal polar lipid structures, while the ensuing steps do not. Archaealidylserine synthase preferred t-serine over d-serine. Serine stereospecificity of phosphatidylserine synthase in *Bacteria* has not been reported.

There are two genetically distinct subclasses of phosphatidylserine synthase. The enzymes classified in subclass I are distributed in gram-negative bacteria (e.g., *E. coli*). The subclass II enzymes have widespread distribution in gram-positive bacteria (e.g., *B. subtilis*), yeast, and *Archaea* (*Methanococcus jannaschii*) (12). It is known that there are some different properties between *E. coli*-type phosphatidylserine synthase (subclass I) and *B. subtilis* phosphatidylserine synthase (subclass II). For example, their intracellular localization, divalent cation requirement, and reaction mechanisms are different (7). In addition, the enzymes from *E. coli* and *B. subtilis* show no sequence homology (19). An *M. jannaschii* gene encoding archaealidylserine synthase is known to belong to subclass II (12). A gene (MT1027) homologous to the *B. subtilis* phosphatidylserine synthase gene (pssA) has been identified in the *M. thermautotrophicus* genome (21). The gene MT1027 was annotated as phosphatidylserine synthase in the amino acid sequence data of phosphatidylserine synthase of *B. subtilis* (P39823), *M. thermautotrophicus* (A69004 = MT1027), and *Methanocaldococcus jannaschii* (Q58609 = MJ1212) were obtained at the NCBI site (www.ncbi.nlm.nih.gov). A multiple alignment of the
three sequences was constructed with an alignment software, CLUSTAL W 1.7 (Fig. 4). Although the archaeaetyl-
dylserine synthase characterized in this work has not been cloned and sequenced, it is most likely that MT1027 codes for
archaetylserine synthase. The deduced amino acid sequence of
MT1027 showed significant homology with B. subtilis phospho-
dydylserine synthase (23.1% identity and 52.6% similarity),
whereas MT1027 showed no homology with that of the E. coli
enzyme. In addition to finding homology in the primary
structures, this work also demonstrated similarities in some
biochemical properties (requirements of Triton X-100 and Mn
ion) of archaeal and Bacillus enzymes. Substrate specificity is
the other characteristic used for comparison of archaeal archaetyl-
dylserine synthase with subclass I and II phosphatidyl-
dylin synthases. We investigated lipid substrate specificity
of archaeaetylserine synthase from M. thermautotrophicus
and phosphatidylserine synthases from B. subtilis and E. coli.
The data described in this paper confirm the results presented
by Carman and Dowhan on the lipid substrate stereoisomer specific-
ity of phosphatidylserine synthase from E. coli (3); they
showed that the purified enzyme was specific for the sn-glyc-
o-3-phosphate (G-3-P) isomer of the liponucleotide and did
not recognize the G-1-P isomer in a kinetic study using CDP-
diphosphatidylyl-sn-glycerol and CDP-1,2-diphosphatidyl-
-sn-geranyl as substrates. In addition, our work showed that E. coli
phosphatidylserine synthase recognized ester bonds between
GP and the hydrocarbon chains and nonbranched hydrocarbon
chain. On the other hand, B. subtilis phosphatidylserine synthase
revealed loose substrate specificity like M. thermautotro-
phicus archaeaetylserine synthase. Therefore, we conclude that
archaeal archaeaetylserine synthase is a member of the Bacil-
lus-type phosphatidylserine synthase family (subclass II) not
only on the basis of amino acid sequence homology but also
from the enzymatic properties including substrate specificity.

It may be inferred from our results and other reports that
archaeal archaeaetylserine synthase and Bacillus phosphatidyl-
syne synthase originated from a common ancestral enzyme. Be-
cause amino group-containing phospholipids are confined only
to methanogens and some related Euryarchaeota among the
Archaea (11, 24) and widely distributed in bacteria (8), it is
speculated that the gene encoding the ancestral enzyme was
transferred from a gram-positive bacterium possessing subclass
II phosphatidylserine synthase to a group including methano-
gens after differentiation from other Archaea groups. A symbi-
tic relationship between methanogens and some kind of
hydrolytic fermentative bacteria via interspecies hydrogen
transfer in anaerobic environments (2, 25) would give an in-
creasing chance to exchange genes. This speculation is sup-
ported by the fact that the archaeaetylserine synthase activities
of ester lipids are twice (or more) those of ether lipids. Bacillus
phosphatidylserine synthase can catalyze the formation of ar-
chaetylserine from CDP-archaeol and L-serine (Table 1, sub-
cate I). It could be also speculated that, when the pssA
gene was transferred, the loose substrate specificity of the subclass
II phosphatidylserine synthase would be the most adequate to
catalyze reaction 5 (Fig. 1) in the biosynthesis of the enantio-
meric other phospholipid in an archaeon, if substrate specific-
ity at that time was the same as at present. The subclass I
(E. coli-type) phosphatidylserine synthase could not do this
 task because of the strict specificity. Although the intracellular
sodium (K\(^+\)) concentration (0.6 M) in M. thermautotrophicus
is not optimal for activity, the enzyme is still active at the intracellular
K\(^+\) concentration. This is in contrast to CDP-archaeol syn-
thesis, which shows no homology with bacterial enzymes and
exhibits maximum activity in the presence of 0.5 M K\(^+\) (14).
This fact is consistent with the speculation that the gene en-
coding archaeaetylserine synthase was transferred from Bacte-
ria to Archaea.

Although the three preceding enzymes involved in the ether
bond formation (Fig. 1, reactions 2 and 3) and the activation of
the intermediate by CDP of phospholipid biosynthesis (Fig. 1,
reaction 4) have been shown to be specific to geranylgeranyl
chains, archaeaetylserine synthase (Fig. 1, reaction 5) has not.
Archaeaetylserine synthase reacted with substrates with both
saturated and unsaturated isoprenoid chains. The cellular po-
lar lipids have fully saturated hydrocarbon chains. Therefore,
there should be steps of hydrogenation (saturation) of gera-
nylgeranyl chains somewhere before (reaction 3 in Fig. 5) or
after (reaction 2 in Fig. 5) the step of archaetylserine forma-
tion. The exact sequence is not known. In order to obtain a
clue to clarify this problem, the presence of an unsaturated
archaetylserine intermediate was surveyed based on the acid

FIG. 4. Multiple alignment of phosphatidylserine synthase and relatives. Abbreviations: Mja, M. jannaschii (201 amino acids); Mth, M. ther-
mautotrophicus (233 amino acids); Bsu, B. subtilis (177 amino acids).
lability of geranylgeranyl ethers. An in vivo pulse-label experiment with $^{32}$P revealed the intracellular presence of newly synthesized acid-labile (probably allyl ether-bonded) archaetidylserine. This result is consistent with the work of Moldoveanu and Kates (13), in which they demonstrated the presence of acid-labile unsaturated ether intermediates of phospholipid biosynthesis in the extremely halophilic archaebacter $Halobacterium cutirubrum$ by pulse-labeling and chase experiments. The detection of an acid-labile archaetidylserine intermediate indicates that at least an allyl ether-bonded archaetidylserine intermediate is really present in the cells which are involved in phospholipid biosynthesis. The presence of an allyl ether serine-containing intermediate suggests that the biosynthetic pathway via reactions 1 and 2 in Fig. 5 is probably operated in the cells, although the possibility of another pathway via reactions 3 and 4 is not excluded because archaetidylserine synthase did react with saturated CDP-archaeol and the intracellular absence of saturated CDP-archaeol has not been excluded.

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**REFERENCES**


