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

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CDP-2,3-di-*O*-geranylgeranyl-*sn*-glycerol:L-serine *O*-archaeetidyltransferase (archaeetidylserine synthase) activity in cell extracts of *Methanothermobacter thermautotrophicus* cells was characterized. The enzyme catalyzed the formation of unsaturated archaeetidylserine from CDP-unsaturated archaeol and L-serine. The identity of the reaction products was confirmed by thin-layer chromatography, fast atom bombardment-mass spectrum analysis, and chemical degradation. The enzyme showed maximal activity in the presence of 10 mM Mn²⁺ and 1% Triton X-100. Among various synthetic substrate analogs, both enantiomers of CDP-unsaturated archaeols with ether-linked geranylgeranyl chains and CDP-saturated archaeol with ether-linked phytanyl chains were similarly active toward the archaeetidylserine synthase. The activity on the ester analog of the substrate was two to three times higher than that on the corresponding ether-type substrate. The activity of D-serine with the enzyme was 30% of that observed for L-serine. A trace amount of an acid-labile, unsaturated archaeetidylserine intermediate was detected in the cells by a pulse-labeling experiment. A gene (MT1027) in *M. thermautotrophicus* genome annotated as the gene encoding phosphatidylserine synthase was found to be homologous to *Bacillus subtilis* *pssA* but not to *Escherichia coli* *pssA*. The substrate specificity of phosphatidylserine synthase from *B. subtilis* was quite similar to that observed for the *M. thermautotrophicus* archaeetidylserine synthase, while the *E. coli* enzyme had a strong preference for CDP-1,2-diacyl-*sn*-glycerol. It was concluded that *M. thermautotrophicus* archaeetidylserine synthase belongs to subclass II phosphatidylserine synthase (*B. subtilis* type) on the basis of not only homology but also substrate specificity and some enzymatic properties. The possibility that a gene encoding the subclass II phosphatidylserine synthase might be transferred from a bacterium to an ancestor of methanogens is discussed.

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 archaeetidic 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, *Methanothermobacter marburgensis* (formerly *Methanobacterium thermoautotrophicum* 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

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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 *Archaea* (Fig. 1, reaction 5), we began to look for an enzyme capable of synthesizing serine phospholipid in *M. thermautotrophicus* using the well-known bacterial pathway. We found such an activity (14), which catalyzed the formation of archaetidylserine from CDP-archaeol and L-serine (archaetidylserine synthase, e.g., CDP-2,3-di-*O*-geranylgeranyl-*sn*-glycerol: L-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 archaetidylserine 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 archaetidylserine synthase and bacterial or eukaryal phosphatidylserine synthases. Because homologies of the gene encoding phosphatidylserine synthase (*pssA*) in *Bacillus subtilis*, but not in *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 *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 archaetidylserine synthase in *M. thermautotrophicus* 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. *M. thermautotrophicus* ΔH (DSM 1053) and *E. coli* DSM 1649 were grown as previously described (14). *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-diacylglycerol. 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 archaetidic 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) and CDP-diacylglycerols (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). L-α-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 [³H]CDP-unsaturated archaeol. [Cytosine-5-³H]CDP-unsaturated archaeol was enzymatically synthesized as described in the previous paper (14) except that 15 μCi of [5-³H]CTP (0.625 Ci/mol) 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-5-³H]CDP-unsaturated archaeol was purified by acidic-alkaline partitioning (14).

TLC. Thin-layer chromatography (TLC) gel was developed on a Silica Gel 60 plate (Merck) 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 archaetidylserine was isolated from *M. thermautotrophicus* cells as previously described (17). Water-soluble products were developed on a thin-layer cellulose plate (Merck 5716) with phenol-water (100:38). 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 ³H-labeled lipids and type BAS-MS for ³²P- and ³³P-labeled lipids.

Preparation of cell-free homogenates. Frozen *M. thermautotrophicus* cells (about 20 g wet weight) 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 × g) for 10 min. The homogenate was stored at -20°C until further use. The membrane fraction was obtained by centrifugation at 100,000 × g for 2 h. The pelleted membrane fraction was resuspended in buffer M. Crude cell extracts of *B. subtilis* and *E. coli* were prepared as previously described (5, 6).

Enzyme assay. The complete assay mixture (final volume, 0.2 ml) for archaetidylserine synthase of *M. thermautotrophicus* contained 0.5 mM CDP-archaeol, 10 mM [3-³H]L-serine (1.25 Ci/mol; Amersham Pharmacia Biotech), the cell-free homogenate of *M. thermautotrophicus* (568 μg of protein), 0.125 M 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 ³H material was separated from water-soluble components by phase partitioning, and radioactivity was counted. In the case of determination of stereospecificity to serine of archaetidylserine synthase, [3-³H]L-serine was replaced by nonradioactive D- or 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 acetone 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 archaetidylserine was visualized by spraying with acid molybdate reagent, and the phosphorus content of the spot scraped off from the plate was determined (1). The activity of archaetidylserine synthase was calculated based on the formation of archaetidylserine over 30 min measured by phosphate determination. The phosphorus content of the spot corresponding to archaetidylserine in a control experiment without D- or L-serine was subtracted from the total phosphorus of archaetidylserine determined for the TLC spot.

Phosphatidylserine synthase activity was measured in *B. subtilis* and *E. coli* as previously described (5, 6). Protein content was determined by the bicinchoninic acid method (22).

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 L-serine replaced L-[³H]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 *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-5-³H] CDP-archaeol (290,000 dpm) was reacted with unlabeled L-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 archaetidylserine in *M. thermautotrophicus* cells. *M. thermautotrophicus* was grown successively twice in 50 ml of low-phosphate medium (17) containing 100 μCi of ³³P_i (5 Ci/mol) under a pressurized atmosphere of H₂ + CO₂ + H₂S (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 ³³P_i was inoculated with 5 ml of the last subculture. At the early logarithmic phase of growth (after incubation for 7 h), 2 mCi of ³²P_i 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 archaetidylserine was purified from the total lipid by two-dimensional TLC. The isolated archaetidylserine was treated with 5% HCl-methanol at 80°C for 1 h to degrade allyl ether archaetidylserine. The degradation products were partitioned into chloroform-soluble and water-soluble fractions. ³²P and ³³P radioactivities in the chloroform-soluble products, aqueous products, and the untreated archaetidylserine 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 *M. thermautotrophicus* catalyzed the conversion of [^3H]serine into chloroform-extractable ^3H -labeled material in the presence of CDP-2,3-di-*O*-geranylgeranyl-*sn*-glycerol. The chloroform-soluble ^3H -labeled product of the archaetidylserine synthase reaction showed a single spot comigrating with authentic archaetidylserine 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*-geranylgeranyl-*sn*-glycerol, CDP-2,3-di-*O*-phytanyl-*sn*-glycerol, and CDP-*rac*-di-*O*-oleoyl-glycerol were used instead of CDP-2,3-di-*O*-geranylgeranyl-*sn*-glycerol, identical results were obtained. While it might be expected that archaetidylethanolamine is formed from archaetidylserine by a decarboxylation reaction, as suggested by an *in vivo* pulse-chase experiment (17), no spot corresponding to archaetidylethanolamine was detected on the TLC plate after the archaetidylserine synthase reaction.

The reaction product from CDP-2,3-di-*O*-geranylgeranyl-*sn*-glycerol was also chemically and mass-spectrometrically analyzed. The lipid product enzymatically prepared from CDP-2,3-di-*O*-geranylgeranyl-*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 ($\text{M} + \text{Na} + \text{H}^+$), m/z 762 ($\text{M} - \text{serine} + 2\text{Na} + \text{H}^+$), and m/z 784 ($\text{M} - \text{serine} + 3\text{Na}^+$), which were consistent with the structure of archaetidylserine with geranylgeranyl 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-di-*O*-geranylgeranyl-*sn*-glycero-1-phosphoserine (unsaturated archaetidylserine), 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 *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 archaetidyl group (e.g., archaetidyltriton X-100) and was not further analyzed.

Properties of archaetidylserine 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 archaetidylserine 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. Archaetidylserine synthase activity was roughly linear with protein con-

FIG. 2. Conversion of [^3H]serine to lipid catalyzed by *M. thermautotrophicus* homogenate incubated with CDP-2,3-di-*O*-geranylgeranyl glycerol in the presence of 10 mM MnCl_2 and 1% Triton X-100 at 60°C , pH 8.5. AS, archaetidylserine.

centration under the assay conditions (data not shown). The effect of nonionic detergent Triton X-100 on archaetidylserine synthase activity is shown in Fig. 3A. Triton X-100 was required for archaetidylserine 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 *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 *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 archaetidylserine 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 archaetidylserine 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-diacylglycerol (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. Archaetidylserine synthase of *M. thermautotrophicus* showed similar activities when either enantiomer of CDP-archaeol possessing geranylgeranyl chains (substrates 1 and 2) was used as a substrate. The substrate analogs with sat-

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.

urated ones (substrate 3) or straight hydrocarbon chains (substrate 4) showed slightly lower activities, but the activity range was between less than $\pm 50\%$ 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

TABLE 1. Substrate specificities of archaetidylserine synthase and phosphatidylserine synthase

Substrate no.	Lipid substrate			% Relative activity (mean $\pm$ SE) ^a		
	Compound	Hydrocarbon	GP backbone	<i>M. thermoautotrophicus</i> ^b	<i>B. subtilis</i> ^c	<i>E. coli</i> ^c
1	CDP-unsaturated ArOH	Geranylgeranyl	G-1-P	100	80 $\pm$ 7	2 $\pm$ 0
2	CDP-unsaturated ArOH	Geranylgeranyl	G-3-P	149 $\pm$ 13	91 $\pm$ 0	8 $\pm$ 6
3	CDP-saturated ArOH	Phytanyl	G-1-P	86 $\pm$ 3	120 $\pm$ 2	1 $\pm$ 0
4	CDP-ArOH	Oleoyl ^d	G-3-P	64 $\pm$ 1	102 $\pm$ 5	41 $\pm$ 3
5	CDP-diacylglycerol	Oleoyl ^e	G-1-P	280 $\pm$ 33	143 $\pm$ 7	17 $\pm$ 1
6	CDP-diacylglycerol	Fatty acid from lecithin	G-3-P	199 $\pm$ 20	100	100

^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 [$CH_3(CH_2)_7CH=CH(CH_2)_8-$].

^e 9-*cis*-Octadecenyl [$CH_3(CH_2)_7CH=CH(CH_2)_7CO-$].

activity for D-serine was $32\% \pm 6\%$ ($n = 2$) of what was observed with L-serine.

Substrate specificity of bacterial phosphatidylserine synthase. Because archaetidylserine 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 archaetidylserine 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 archaetidylserine 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-dioleoyl-*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 archaetidylserine in *M. thermotrophicus* cells. In order to elucidate whether unsaturated (or allyl ether-type) archaetidylserine 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 archaetidylserine 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. thermotrophicus* was continuously labeled with $^{33}\text{P}_i$ and pulse-labeled with $^{32}\text{P}_i$ for 10 min in the presence of $^{33}\text{P}_i$. In this experiment, ^{32}P in archaetidylserine must represent the amount of mature archaetidylserine (the final product of the biosynthetic pathway) with saturated hydrocarbons, while ^{32}P must express the amount of newly synthesized archaetidylserine. Labeled archaetidylserine was purified from the total lipid of the cells. The purified archaetidylserine was decomposed with 5% HCl-methanol at 80°C for 1 h. The ratio of radioactivities of ^{32}P versus ^{33}P ($^{32}\text{P}/^{33}\text{P}$) of chloroform-soluble products and water-soluble products after acid treatment of archaetidylserine was compared to that of untreated archaetidylserine. The $^{32}\text{P}/^{33}\text{P}$ ratio (1.26) of untreated archaetidylserine 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 archaetidylserine. These results suggest that only a

trace amount of newly synthesized allyl ether-type archaetidylserine is present in the cells. Almost all archaetidylserines have fully saturated hydrocarbon chains, as shown by chemical analysis (17).

DISCUSSION

The results described above confirm the presence of archaetidylserine synthase in *M. thermotrophicus* homogenate. The enzyme catalyzed the transfer of an archaetidyl group from CDP-archaeol to serine. When CDP-unsaturated archaeol was used as a substrate, the product was identified as archaetidylserine 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 archaetyltriton 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.

Archaetidylserine 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 archaetidylserine 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. Archaetidylserine synthase preferred L-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 archaetidylserine synthase is known to belong to subclass II (12). A gene (MT1027) homologous to the *B. subtilis* phosphatidylserine synthase gene (*psaA*) has been identified in the *M. thermotrophicus* genome (21). The gene MT1027 was annotated as phosphatidylserine synthase. The amino acid sequence data of phosphatidylserine synthase of *B. subtilis* (P39823), *M. thermotrophicus* (A69004 = MT1027), and *Methanocaldococcus jannaschii* (Q58609 = MJ1212) were obtained at the NCBI site (www.ncbi.nlm.nih.gov). A multiple alignment of the

FIG. 5. Possible biosynthetic pathways of archaetidylserine from unsaturated archaetidic acid. CDP-archaeol synthase is known to be specific to unsaturated archaetidic acid (14). Because archaetidylserine synthase can react with both saturated and unsaturated CDP-archaeol, two pathways (reactions 1 + 2 and reactions 3 + 4) to form saturated archaetidylserine are possible. *, allyl ether bond.

lability of geranylgeranyl ethers. An *in vivo* pulse-label experiment with $^{32}\text{P}_i$ 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 archaeon *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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