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Cold Adaptation in the Antarctic Archaeon *Methanococcoides burtonii* Involves Membrane Lipid Unsaturation

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Direct analysis of membrane lipids by liquid chromatography-electrospray mass spectrometry was used to demonstrate the role of unsaturation in ether lipids in the adaptation of *Methanococcoides burtonii* to low temperature. A proteomics approach using two-dimensional liquid chromatography-mass spectrometry was used to identify enzymes involved in lipid biosynthesis, and a pathway for lipid biosynthesis was reconstructed from the *M. burtonii* draft genome sequence. The major phospholipids were archaeol phosphatidylglycerol, archaeol phosphatidylinositol, hydroxyarchaeol phosphatidylglycerol, and hydroxyarchaeol phosphatidylinositol. All phospholipid classes contained a series of unsaturated analogues, with the degree of unsaturation dependent on phospholipid class. The proportion of unsaturated lipids from cells grown at 4°C was significantly higher than for cells grown at 23°C. 3-Hydroxy-3-methylglutaryl coenzyme A synthase, farnesyl diphosphate synthase, and geranylgeranyl diphosphate synthase were identified in the expressed proteome, and most genes involved in the mevalonate pathway and processes leading to the formation of phosphatidylinositol and phosphatidylglycerol were identified in the genome sequence. In addition, *M. burtonii* encodes CDP-inositol and CDP-glycerol transferases and a number of homologs of the plant geranylgeranyl reductase. It therefore appears that the unsaturation of lipids may be due to incomplete reduction of an archaeol precursor rather than to a desaturase mechanism. This study shows that cold adaptation in *M. burtonii* involves specific changes in membrane lipid unsaturation. It also demonstrates that global methods of analysis for lipids and proteomics linked to a draft genome sequence can be effectively combined to infer specific mechanisms of key biological processes.

Archaea are abundant in cold environments, where they represent a large proportion of the microbial biomass (8, 13, 16). *Methanococcoides burtonii* is a cold-adapted methanogen from Ace Lake, Antarctica, where it was isolated from permanently cold (1 to 2°C), methane-saturated water (10). *M. burtonii* has served as a model for examining cold adaptation, with useful insight gained through studies on comparative genomics (27), proteomics (11, 12), tRNA (22), gene regulation (17), intracellular solutes (37), and protein structure (29, 35, 36, 37).

One aspect of cold adaptation that has not been fully investigated in archaea is membrane lipid composition. Low temperature promotes the formation of a more rigid array of the lipid bilayer (gel phase) which impairs the function of biological membranes (4, 26). In bacteria and eucaryotes, a liquid crystalline state of the membrane may be maintained at low temperature by increasing the proportion of unsaturated fatty acids (26). Unsaturation may be achieved by desaturase enzymes which modify existing lipids or through de novo synthesis that incorporates unsaturated lipids directly into the membrane. While the former mechanism provides a potentially more rapid response to a sudden change in temperature and is characteristic of the cold shock response in *Bacillus subtilis* (1, 38) and cyanobacteria (33), de novo synthesis provides an appropriate mechanism for an organism adapted to a permanently cold environment.

Membrane lipids of archaea are characterized by unusual structural features which serve as useful biomarkers (3). In methanogens, the lipid core structure consists mainly of a diether structure (15), with macrocyclic (5) and hydroxy diether (9, 30) modifications. While unsaturated lipids from archaea have been previously reported (20), concerns with analytical methodologies limited the veracity of the reports. As a result of improvements in analytical approaches, such as electrospray/high-performance liquid chromatography-mass spectrometry (ES/HPLC-MS) of intact lipids, the presence of unsaturated diether lipids (UDLs) has been confirmed in archaea (23, 24).

As synthesis of UDLs potentially provides a mechanism of cold adaptation in archaea, we examined their presence in *M. burtonii*. We found a series of UDLs for the core lipids of archaeol and hydroxyarchaeol of the species phosphatidylglycerol and phosphatidylinositol. Unsaturated hydroxyarchaeol phospholipids have not previously been reported. To examine the effects of growth temperature on UDLs, we examined the lipid composition in cells grown at 4 and 23°C. The level of unsaturation increased at 4°C, demonstrating that *M. burtonii* regulates UDL synthesis. To identify genes involved in lipid

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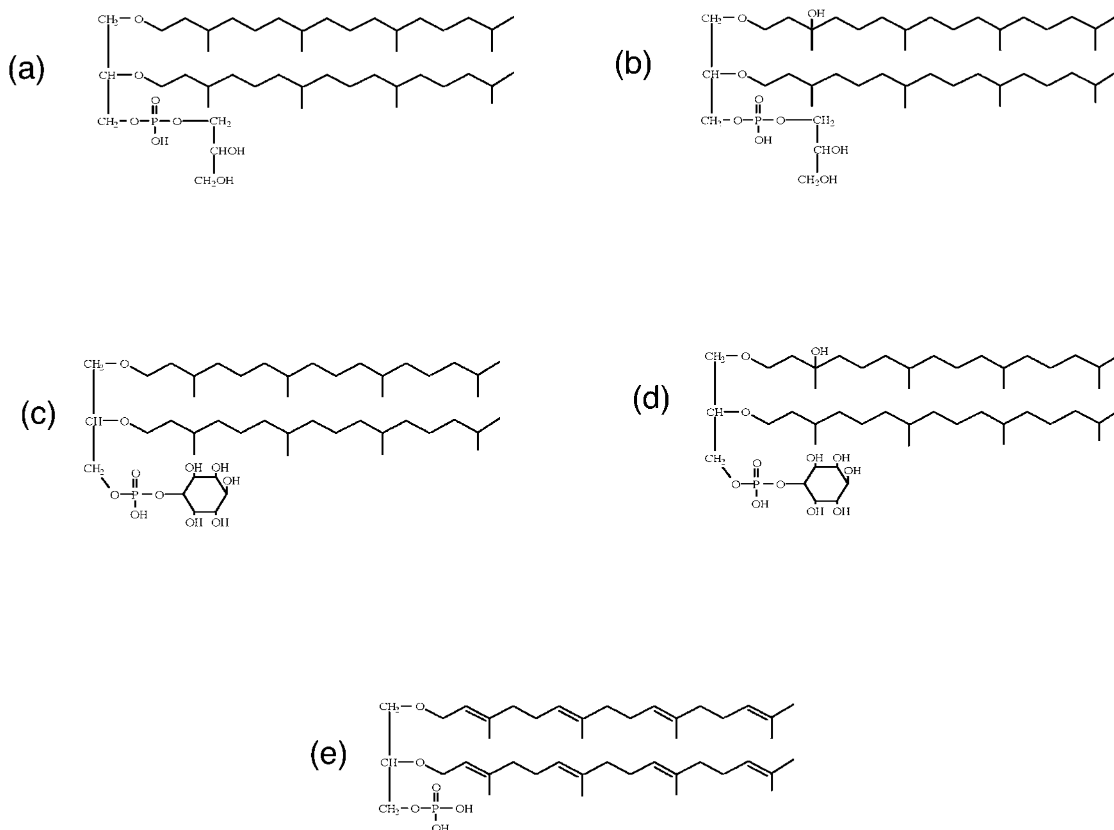

FIG. 1. Structures of archaeal phospholipids: (a) ArPG, (b) Ar_{OH}PG, (c) ArPI, (d) Ar_{OH}PI, and (e) digeranylgeranyl glycerol, the intermediate core diether lipid in the biosynthesis of diether lipids.

synthesis during growth at 4°C, we performed a proteome analysis using LC/LC-MS/MS. Knowledge of the expressed proteins and genes encoded on the draft genome enabled us to reconstruct the main lipid synthesis pathways and propose a mechanism for UDL formation.

MATERIALS AND METHODS

Organism and culture conditions. *M. burtonii* was grown as previously described (12). Multiple batches of cultures were harvested anaerobically at late logarithmic phase (absorbance of 0.25 at 600 nm) and centrifuged at 2,800 × *g* for 25 min at 4°C. Cell pellets were washed in half the original culture volume of sterile, anaerobic phosphate-buffered saline (pH 7.4) and centrifuged for 15 min at 2,800 × *g*. The combined pellet was resuspended in 1 ml of deionized water, frozen in a dry ice-ethanol bath, and lyophilized overnight.

Lipid extraction and isolation. Cell material was extracted using a modified Bligh and Dyer approach (2, 39). A single phase extraction ($CHCl_3$-methyl hydroxide-H_2O) was used to yield a total solvent extract. Fractionation of lipid classes was accomplished using silicic acid column chromatography. Briefly, the total solvent extract was applied to a 1-g column of silicic acid (preheated at 100°C for 1 h) and separated into hydrocarbon, neutral lipids, glycolipids, and polar lipids in a stepwise elution using 10 ml each of hexane, chloroform, acetone, and methanol (14). A portion of the polar lipid fraction was also subjected to a hydrogenation reaction. Briefly, hydrogen gas was bubbled through the polar lipid sample dissolved in chloroform prior to addition of platinum(IV) oxide catalyst. Hydrogen flow was continued for 3 h, and the solvent was replaced by evaporation.

Analysis of phospholipids by ES/HPLC-MS/MS. Analyses of the polar lipid fractions were undertaken by combined HPLC-MS using negative electrospray ionization (ES/HPLC-MS/MS). A Waters Alliance 2690 HPLC fitted with a Waters Nova-Pak C_{18} column (150 by 3.9 mm) was coupled to a Finnigan LCQ ion trap MS fitted with an electrospray source. An isocratic mobile phase of 95% methanol and 5% 0.1 M ammonium acetate at a flow rate of 0.8 ml/min was used. For initial analyses the range from *m/z* 700 to 2,000 was monitored. For subsequent analyses, to improve sensitivity, the range from *m/z* 700 to 1,200 was monitored, with "data dependent " MS/MS product ion scans alternating with normal scans. The MS/MS scans isolated the strongest ion observed in the *m/z* 700 to 1,200 range with an isolation window of 5 *m/z* units, applied a collision energy of 40%, and monitored all product ions down to the ion trap cutoff point of 25% of the *m/z* value of the isolated precursor ion. The capillary temperature was 275°C, sheath gas was 85 lb/in^2, auxiliary gas was 40 lb/in^2, and needle voltage was 4.8 kV.

Proteomic analysis by LC/LC-MS/MS. Protein extraction, digestion, and MS analysis were performed as previously described (12), with the following modifications. Total proteins from *M. burtonii* were digested with trypsin in a 1:100 (trypsin-protein) ratio overnight at 37°C. Digested peptides were separated by online strong cation exchange (SCX) and nano-C_{18} LC using an Ultimate HPLC, Switchos and Famos autosampler system (LC-Packings). Peptides (~500 ng) were dissolved in formic acid (0.1%, 25 μl) and loaded onto an SCX micro trap (1 by 8 mm; Michrom Bioresources). Peptides were eluted sequentially with 5, 10, 15, 20, 25, 30, 40, 50, 75, 150, 300, and 1,000 mM ammonium acetate (20 μl). The unbound load fraction and each salt step fraction were concentrated and desalted onto a micro-C_{18} precolumn (500 μm by 2 mm; Michrom Bioresources) with H_2O-CH_3CN (98:2, 0.1% formic acid, buffer A) at 20 μl/min. After a 10-min wash the precolumn was switched (Switchos) into line with an analytical column containing C_{18} RP silica (PEPMAP, 75 μm by 15 cm; LC-Packings) or a fritless C_{18} column (75 μm by ~12 cm). Peptides were eluted with a linear gradient of buffer A to H_2O-CH_3CN (40:60, 0.1% formic acid-buffer B) at 200 nl/min over 60 min. The column was connected via a fused silica capillary to a low-volume tee (Upchurch Scientific) where high voltage (2,300 V) was applied and a nanoelectrospray needle (New Objective) or fritless column outlet was positioned ~1 cm from the orifice of an API QStar Pulsar i hybrid tandem mass spectrometer (Applied Biosystems). The QStar was operated in information-dependent acquisition mode. A Tof MS survey scan was acquired (*m/z* 350 to 1,700, 0.5 s), and the two largest precursors (counts > 10) were sequentially selected by Q1 for MS-MS analysis (*m/z* 50 to 2,000, 2.5 s). A processing script generated data suitable for submission to the database search programs. Extracted spectra were

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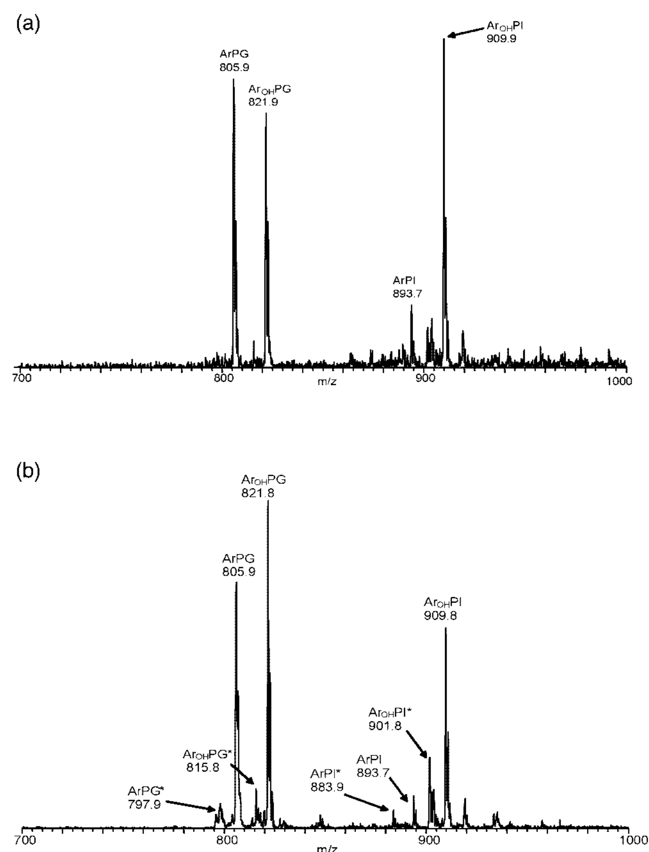

FIG. 2. Negative-ion electrospray mass spectra of *M. burtonii* polar lipids. Each spectrum was obtained by averaging the HPLC region between 3 and 35 min to give an overview. Lipids were prepared from cultures grown at 23°C (a) and 4°C (b). Major ions are labeled, and the most abundant level of unsaturation of a species is marked by an asterisk.

also analyzed by using DTASelect to simplify interpretation (34). CID spectra were analyzed by using SEQUEST software with the following parameters: peptide mass tolerance of 1.5 Da and strict trypsin enzyme digestion with the modification +16 methionine. Searches were performed on a local database of *M. burtonii* translated sequences obtained from http://www.jgi.doe.gov/JGI_microbial/html/. Proteins were considered identified if they matched set criteria. For SEQUEST the criteria were as follows: fragments were tryptic, the Xcorr score was >2 for $[M + 2H]^{2+}$, and a distinct ladder sequence was visible. For the peptides analyzed, a SEQUEST Xcorr score of >2 indicated identity. Mascot MS/MS ion search (Matrix Science) criteria were as follows: trypsin digestion allowing up to one missed cleavage, oxidation of methionine, peptide tolerance of 1.0 Da, and MS/MS tolerance of 0.8 Da. A Mascot score of >18 indicated identity. All $^{-}$SEQUEST and Mascot scores were manually verified.

Identification of lipid-related genes. Gene sequences used for similarity searches against the *M. burtonii* draft genome sequence (available at http://genome.ornl.gov/microbial/mbur/) were retrieved from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov) or from the genomes of *Methanosarcina mazei* (http://www.g2l.bio.uni-goettingen.de/mm/), *Methanosarcina acetivorans* (http://www.broad.mit.edu/annotation/microbes/methanosarcina), *Halobacterium* strain NRC-1 (http://zdna2.umbi.umd.edu/~haloweb/), or *Solfolubus sofataricus* (http://www-archbac.u-psud.fr/projects/sulfolobus). BLASTP searches were performed locally (http://psychro.bioinformatics.unsw.edu.au/genomes), and PSI-BLAST searches were performed via the National Center for Biotechnology Information server, using protein sequences translated from the *M. burtonii* draft genome (27). Proteins were considered to be homologous if the BLAST-P or PSI-BLAST scores were $<e^{-9}$. *M. burtonii* genes are described with contig number[lowem]gene number based on the Oak Ridge National Laboratories annotation (release date, 11 December 2003).

RESULTS

Composition of membrane phospholipids. The polar lipid fraction of *M. burtonii* cells was examined by ES/HPLC-MS/MS to identify the major phospholipid components. Diether hydroxy core lipids were identified, and tetraether lipids were absent, as confirmed by initial analyses in the range *m/z* 700 to 2,000. The major phospholipids (Fig. 1) archaeol phosphatidylglycerol (ArPG) $[M-H]^-$ at *m/z* 805.9, archaeol phosphatidylinositol (ArPI) $[M-H]^-$ at *m/z* 893.7, hydroxyarchaeol phosphatidylglycerol ($Ar_{OH}PG$) $[M-H]^-$ at *m/z* 821.8, and hydroxyarchaeol phosphatidylinositol ($Ar_{OH}PI$) $[M-H]^-$ at *m/z* 909.8 were identified from the ES/HPLC-MS/MS spectra (Fig. 2). Careful examination of the ES/HPLC-MS/MS spectra and chromatograms revealed a clear fingerprint of unsaturation, with the unsaturated analogues having progressively shorter retention times than the fully saturated species. A series of phospholipids differing by up to 10 Da less than the saturated species were separated by HPLC (Fig. 3). For the nonhydroxylated lipids (ArPG and ArPI), differences of 2 to 10 Da were observed, which can be attributed to 1 to 5 degrees of unsaturation (double bonds) within the phytenyl chains, respectively (Fig. 3a and b). For the hydroxylated lipids ($Ar_{OH}PG$ and $Ar_{OH}PI$), unsaturated phospholipid mass differences of 2, 4, 6, and 8 Da less than the saturated species were observed; however, a species differing by 10 Da was not observed (Fig. 3c and d). Similar patterns of unsaturation for the nonhydroxylated and hydroxylated lipids were observed from cultures grown at 23°C (Table 1).

MS/MS data supported the structural assignments, with an intense product ion observed for the loss of 74 Da ($C_3H_6O_2$) = (glycerol-water) (e.g., see Fig. 4a) for $Ar_{OH}PG$ and ArPG and a product ion for the loss of 162 Da ($C_6H_{10}O_5$) = (inositol-water) for $Ar_{OH}PI$ and ArPI. The MS/MS spectra also showed conservation of the unsaturation in the major product ion; Fig. 4b shows the MS/MS product ion spectra for the $[M-H]^-$ ions for ArPG with 5 degrees of unsaturation. The assignment of unsaturation rather than ring structures was supported by hydrogenation reactions of the polar lipid fractions. For example, hydrogenation resulted in the disappearance of the ArPG phospholipid series and sole detection of the fully saturated ArPG species (data not shown).

To examine the changes in phospholipid composition that occurred in response to growth temperature, the relative abundance of ArPG, ArPI, $Ar_{OH}PG$, and $Ar_{OH}PI$ were calculated from chromatograms (Table 1). Irrespective of the class of lipid, the level of unsaturation was always higher in cells grown at 4°C. The proportion of the nonhydroxylated phospholipid ArPI with the highest degree of unsaturation (five double bonds) was twofold higher in cells grown at 4°C. A similar fold increase was observed for the hydroxylated phospholipid $Ar_{OH}PI$, which also contained the highest degree of unsaturation (four double bonds). The only instances where the individual species of the phospholipids did not clearly reflect this thermal pattern was for ArPG with two double bonds and $Ar_{OH}PI$ with one and two double bonds.

Genes expressed in lipid biosynthesis. To identify genes involved in lipid biosynthesis that were expressed in cells growing at 4°C, cell extracts were analyzed by LC/LC-MS/MS and proteins were identified from the *M. burtonii* draft genome

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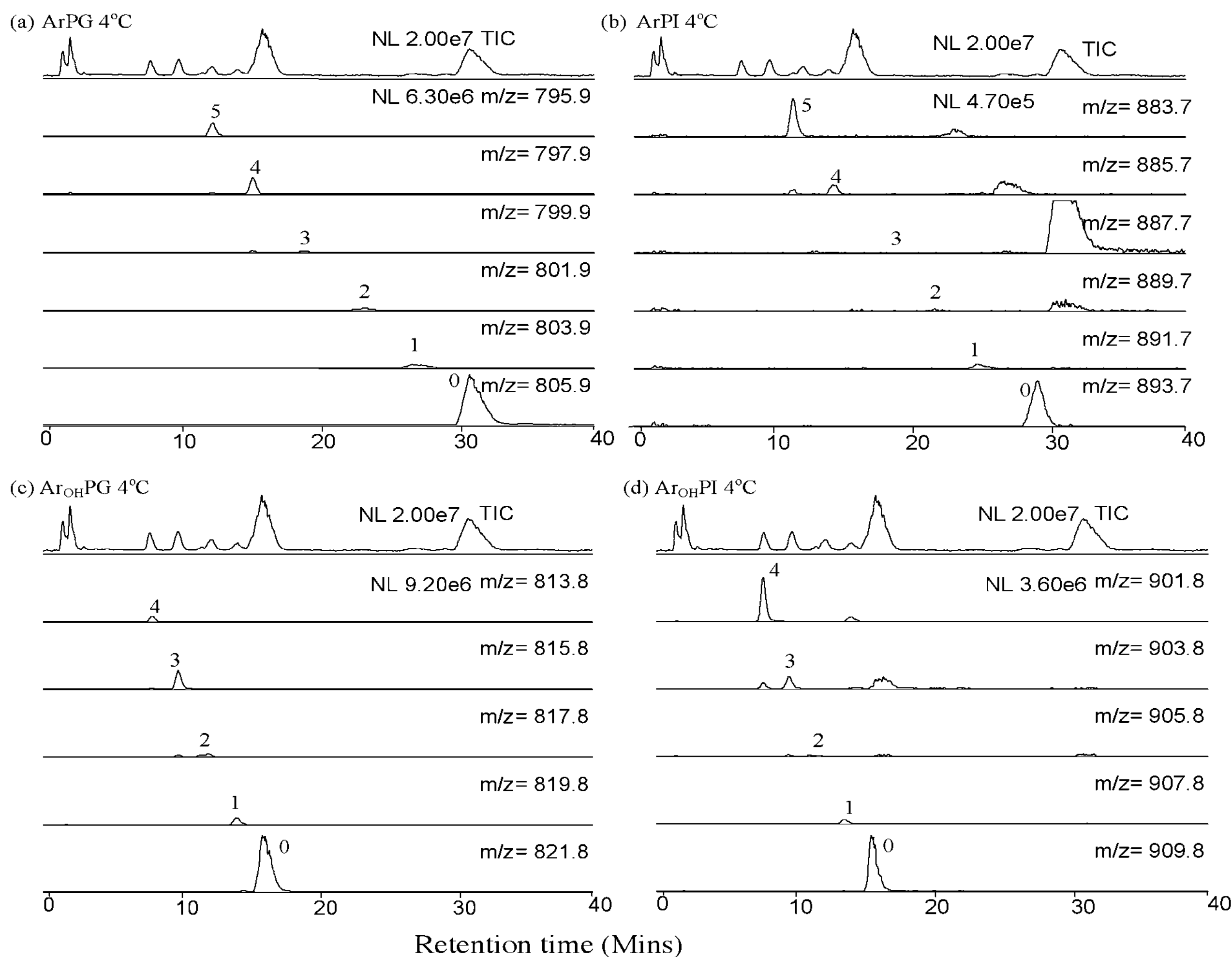

FIG. 3. HPLC-MS chromatograms of phospholipid species from *M. burtonii* grown at 4°C. Individual mass chromatograms for the $[M\text{-}H]^-$ ions for each degree of unsaturation are shown to the same scale for each lipid group. (a) ArPG, (b) ArPI, (c) $Ar_{OH}PG$, and (d) $Ar_{OH}PI$. NL, normalization level.

sequence. A number of proteins involved in the biosynthesis of lipids were identified, including acetoacetyl coenzyme A (CoA) thiolase, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase, geranylgeranyl diphosphate (GGPP) synthase, farnesyl diphosphate (FPP) synthase, and *myo*-inositol-1 phosphate synthase (Table 2).

Lipid biosynthesis genes present in the draft genome sequence. The genome sequence data were searched using BLAST-P and PSI-BLAST. With the exception of phosphomevalonate kinase and digeranylgeranylglyceryl phosphate (DGGGP) synthase, homologs of all genes involved in the mevalonate pathway and isoprenoid lipid synthesis were identified (Fig. 5). To identify genes possibly involved in archaeol phospholipid desaturation, fatty acid desaturase genes from *Mus musculus* and *Bacillus cereus* were searched against the genome data; however, no significant matches were found. Genes known to be involved in de novo synthesis were searched and a number of genes (70_2723, 68_1676, 57_641,

TABLE 1. Estimated relative percentages of unsaturated archeol phospholipids within lipid classes from *M. burtonii* grown at 4 and 23°C[a]

No. of double bonds	ArPG		ArPI		$Ar_{OH}PG$		$Ar_{OH}PI$	
	4°C	23°C	4°C	23°C	4°C	23°C	4°C	23°C
0	73	84	59	76	74	89	58	74
1	5.4	4.3	6.0	4.1	6.1	2.2	4.2	4.3
2	2.6	3.0	0.8	0	3.5	1.8	1.6	2.3
3	1.6	0.7	0	0	13	5.0	9.0	5.2
4	9.4	4.0	7.6	7.1	3.8	1.8	28	14
5	7.8	4.5	27	13	0	0	0	0

[a] Relative percentages were estimated from the peak areas of the single-ion current for each lipid (e.g., see Fig. 3), ignoring any response factor differences.

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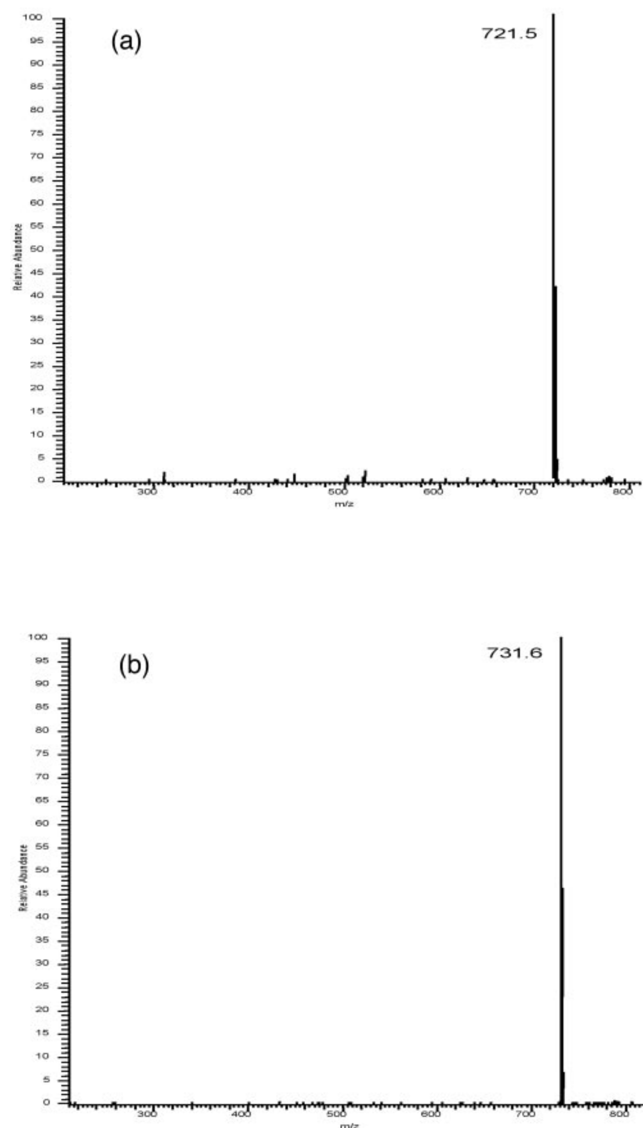

FIG. 4. MS/MS spectra of product ions from [M-H]$^-$ ion at *m/z* 795.8 for ArPG with five degrees of unsaturation (a) and product ions from [M-H]$^-$ ion at *m/z* 805.8 for fully saturated ArPG (b). The major ion results from the loss of 74 Da ($C_3H_6O_2$ = glycerol-water).

and 55_468) homologous to geranylgeranyl reductase genes from plants were found. In addition, genes encoding a CDP-inositol transferase (69_2036) and a CDP-glycerol transferase (70_2290) were identified. A number of the genes, including those for mevalonate kinase (48_216) and isopentenyl diphosphate isomerase (48_218) were arranged in gene clusters with their orientation and intergene spacing (<50 bp) indicative of operon structures (12).

DISCUSSION

Unsaturated phospholipids in archaea. This is the first study to address the role that membrane lipid unsaturation plays in cold adaptation in archaea. The discovery of UDLs in *M. burtonii* indicates that cold-adapted archaea are capable of controlling membrane fluidity by manipulating their lipid composition, a strategy adopted by bacteria adapting to the cold (26). An important finding was that the overall level of unsaturation was higher when cells were grown at 4°C than at 23°C. This was manifested through differential synthesis of hydroxylated and nonhydroxylated archaeol phospholipids varying in their degree of unsaturation. The greatest changes in abundance between cells grown at 4 and 23°C occurred for the phospholipids ArPG and Ar_{OH}PI, with the highest degree of unsaturation, five double bonds and four double bonds, respectively.

Thermal regulation of membrane lipid composition appears to be a form of microbial adaptation shared by archaea and bacteria. However, the mechanism by which this is achieved varies depending on the organism. For example, the degree of cyclization within the core lipids of *Sulfolobus solfataricus* increases with increasing environmental temperatures (6). Marine crenarchaeota have adapted to life at low temperature with the production of a novel tetraether lipid ("crenarchaeol") (30) and also regulate the degree of cyclization within core lipids over the temperature range 0 to 30°C (28). In bacteria, increasing unsaturation of phospholipid ester-linked fatty acids is a common mechanism of cold adaptation (25). The discovery of UDLs by ES/HPLC-MS/MS in the haloalkiphile *Natronobacterium magadii* and by independent methods in the hyperthermophile *Methanopyrus kandleri* illustrate that unsaturation is not restricted to ester-linked fatty acids of bac-

TABLE 2. Characteristics of *M. burtonii* proteins involved in lipid biosynthesis identified by LC/LC-MS/MS

Contig—gene	Gene product	Peptide(s) matched	Mascot score	SEQUEST Xcorr
48_170	*myo*-inositol-1 phosphate synthase	TEAVQSVLTER	60	3.2
		LFGDVPMNIELR	39	3.1
		GVGGILYSPSSYFMK	21	
		DGVSEHMVNYEDQYK	62	4.0
		LSVEDSPNSAGVVIDAIR	78	4.0
		RIPATEPESTEEDVTSILK	66	3.5
		TYQLNTGGNTDFLNMLNRDR	22	
		LDDHNIHIGPSDYVPWQQDNK	31	
65_1217	GGPP synthase	GIGEFLPISHPEELYR	46	4.2
66_1469	FPP synthase	RPIILLLASRICGK		2.52
69_2077	HMG-CoA synthase	FTGEPGYFK	23	
		HVTGAANGLMEK	29	
		MGTKPSDYDYAVFHQPNGK	19	
69_2078	Acetoacetyl-CoA thiolase	QLVEVTQQLR	26	
		DITTLDASVVAGK	37	
		NHQNGSMNPIAQYK	22	

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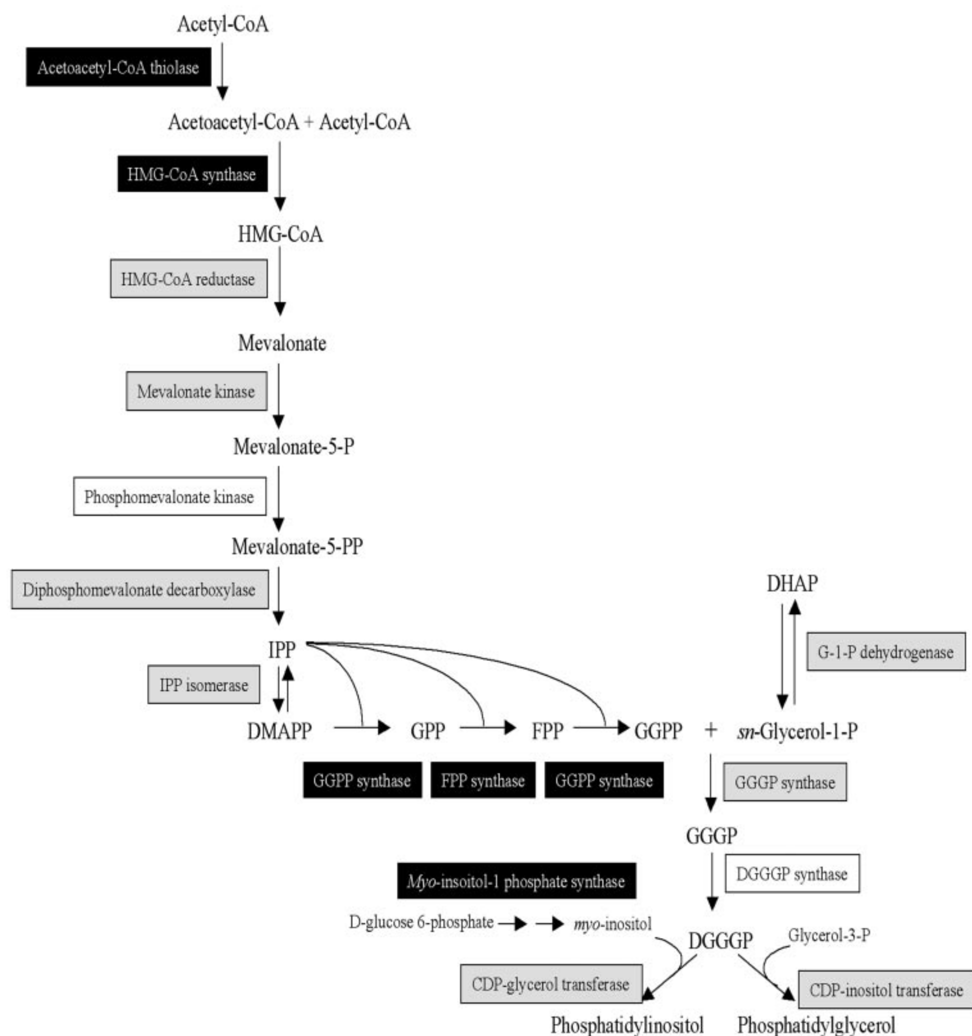

FIG. 5. Proposed pathway for the synthesis of C_{20}-C_{20} diether lipids in *M. burtonii*. Enzymes are boxed, and metabolites are shown without boxes. Shown are proteins detected from the expressed proteome (black boxes), gene products identified from the *M. burtonii* genome (grey boxes), and gene products in the pathway not identified in the draft genome (open boxes). Abbreviations used are as follows: 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), phosphate (P), isopentenyl diphosphate (IPP), dimethylallyl diphosphate (DMAPP), geranyl diphosphate (GPP), farnesyl diphosphate (FPP), geranylgeranyl diphosphate (GGPP), geranylgeranylglyceryl phosphate (GGGP), digeranylgeranylglyceryl phosphate (DGGGP), glycerol-1-phosphate (G-1-P). Gene identification numbers (contig number[lowem]gene number) are as follows: acetoacetyl-CoA thiolase (69_2078), HMG-CoA synthase (69_2077), HMG-CoA reductase (68_1665), mevalonate kinase (48_216), diphosphomevalonate decarboxylase (66_1376), IPP isomerase (48_218), GGPP synthase (65_1217), FPP synthase (66_1469), G-1-P dehydrogenase (68_1720), GGGP synthase (63_1054), *myo*-inositol-1 phosphate synthase (48_170), CDP-diacyl-G-3-P transferase (70_2290), CDP-diacyl-inositol transferase (69_2036).

teria but also occurs within ether-linked lipids of archaea (21, 23). Significantly, our studies with *M. burtonii* illustrate not only the presence of UDLs but also indicate that they play a central role in the cold adaptive response of this archaeon to low temperature.

Properties of membrane lipids in *M. burtonii*. The similarity in relative abundance of phospholipids with the highest degrees of unsaturation of the Ar series (four and five) and Ar_{OH} series (three and four) series (Table 1) may be an indication that synthesis of these nonhydroxylated and hydroxylated phospholipids are linked through a common biosynthetic hydration or dehydration step. The presence of a hydroxy group does not appear to affect the unsaturation pattern; however, it does in effect reduce the total number of possible sites where unsaturation can occur. The implication of this is that unsaturation may first occur at the C-2 position (relative to the glycerol backbone) on the isoprenol chain (Fig. 1). Subsequent unsaturation may occur via a regular process, proceeding away from the backbone in both chains.

The positions of the double bonds are not known, and isomers of unsaturation across the *sn*-2 and *sn*-3 phytanyl chains may be produced. An indication of isomeric forms are the broad peaks observed in the second and third degrees of unsaturation in the total ion current chromatogram for individual phospholipids (Fig. 3). In *Methanococcus jannaschii*, the formation of macrocyclic lipids has been proposed to involve the

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migration of double bonds along the isoprenoid chains of phospholipid intermediates (7). A similar mechanism may lead to the formation of unsaturated isomers in *M. burtonii*.

Hydroxyarchaeol, which has a hydroxyl group at the C-3 of the isoprenoid chain on the *sn*-3 position (Fig. 1), is thought to be unique to methanogens (9). The hydroxylation of the diether core lipid is likely to result in differences in the cytoplasmic membrane properties. The hydrophobic 3-hydroxyl group may either extend the head group of the polar lipid to cause local increased surface area and effectively shorten the lipid core or remain within the interior of the membrane bilayer to generate a hydrophilic pocket (32).

Lipid biosynthetic pathways in *M. burtonii*. The biosynthesis of lipids in archaea occurs by a complex pathway undertaken by a multienzyme, membrane-bound system (15). *Sulfolobus* is the only archaeon for which a complete mevalonate pathway has been identified for the synthesis of isoprenoid lipids (3). We identified HMG-CoA synthase, HMG-CoA reductase, mevalonate kinase, and a putative diphosphomevalonate decarboxylase in the draft genome of *M. burtonii* (Fig. 5). The identification of acetoacetyl-CoA thiolase and HMG-CoA synthase in the expressed proteome is a clear indication that the mevalonate pathway is likely to be functional and responsible for the synthesis of the lipid isoprenoid side chains.

In addition to the mevalonate pathway, genes involved in all but one step of lipid synthesis leading to the formation of ArPI and ArPG were identified (Fig. 5). The identification of FPP synthase and GGPP synthase in the expressed proteome illustrate their functions in the sequential condensation of the isoprenoid to a polyisoprenoid diphosphate.

It is possible that the phosphomevalonate kinase and DGGGP synthase are encoded in the genome but are not present in the draft coverage (presently $\sim$12$\times$). Alternatively, enzymes with these activities may be present but difficult to identify because they are encoded by novel genes. In this regard it is noteworthy that a significant proportion of the genes encoded by *M. burtonii* are hypothetical, and in excess of 100 of these are known to be expressed during growth at 4°C (N. F. W. Saunders et al., submitted for publication).

The isoprenoid chains of the core lipids synthesized via the mevalonate pathway produce a fully unsaturated hydrocarbon chain (15). Reduction of the double bonds occurs after linkage of the isoprenoid chains to the glycerol backbone (18, 19). In *M. burtonii*, the formation of fully or partially unsaturated phospholipids may involve CDP-inositol transferase (or CDP-glycerol transferase) and a reductase of unknown character (Fig. 5). In plants, the enzyme responsible for the reduction of the unsaturated geranylgeranyl diphosphate to phytyl diphosphate is geranylgeranyl reductase (31). Hence, a reductase responsible for the saturation of archaeol isoprenoid chains in *M. burtonii* may be homologous in part to plant geranylgeranyl reductase. *M. burtonii* encodes both the CDP-inositol and CDP-glycerol transferases and a number of homologs of plant geranylgeranyl reductase. In contrast, homologs of bacterial and eukaryotic desaturase genes do not appear to be present in *M. burtonii*. Available evidence therefore points to a scenario where double bonds are selectively retained in the isoprenoid chains of an archaeol phospholipid intermediate to produce the UDLs identified in this study. In addition, we have shown that the degree of unsaturation is specific for each phospholipid class (Table 1). This implies that the saturation process is affected by the nature of the phosphate-based portion of the molecule.

In view of the lipid biosynthesis apparatus being membrane bound, it is noteworthy that we were able to identify three of the key proteins involved. In a separate study focused on global proteomic analysis, 528 expressed proteins were identified in *M. burtonii*, including a number with predicted transmembrane domains (11). In the present study, we have shown that coupling ES/HPLC-MS/MS for lipid analysis to LC/LC-MS/MS for proteomic analysis is an effective means of combining distinct global analysis approaches to infer specific mechanisms of key biological processes in archaea.

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