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Gene deletions leading to a reduction in the number of cyclopentane rings in *Sulfolobus acidocaldarius* tetraether lipids

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ABSTRACT

The cell membrane of (hyper)thermophilic archaea, including the thermoacidophile *Sulfolobus acidocaldarius*, incorporate dibiphytanylglycerol tetraether lipids. The hydrophobic cores of such tetraether lipids can include up to eight cyclopentane rings. Presently, nothing is known of the biosynthesis of these rings. In the present study, a series of *S. acidocaldarius* mutants deleted of genes currently annotated as encoding proteins involved in sugar/polysaccharide processing were generated and their glycolipids were considered. Whereas the glycerol-dialkyl-glycerol tetraether core of a *S. acidocaldarius* tetraether glycolipid considered here mostly includes four cyclopentane rings, in cells where the *Saci_0421* or *Saci_1201* genes had been deleted, species containing zero, two or four cyclopentane rings were observed. At the same time, in cells lacking *Saci_0201*, *Saci_0275*, *Saci_1101*, *Saci_1249* or *Saci_1706*, lipids containing mostly four cyclopentane rings were detected. Although *Saci_0421* and *Saci_1201* are not found in proximity to other genes putatively involved in lipid biosynthesis, homologues of these sequences exist in other Archaea where cyclopentane-containing tetraether lipids are found. Thus, Saci_0421 and Saci_1201 represent the first proteins described that somehow contribute to the appearance of cyclopentane rings in the core moiety of the *S. acidocaldarius* glycolipid considered here.

INTRODUCTION

The lipids that comprise biological membranes serve to distinguish Archaea from Eukarya and Bacteria. In eukaryal and bacterial membranes, phospholipids essentially comprise fatty acid side chains linked to a 1,2-sn-glycerol-3-phosphate backbone via ester bonds. In contrast, archaeal phospholipids contain isoprenoid hydrocarbon side chains linked to a 2,3-sn-glycerol-1-phosphate backbone via ether bonds (Koga and Morii 2007; Villanueva et al. 2014). While many Archaea organize such lipids, mainly based on a diphytanylglycerol diether (archaeol) hydrophobic core yet presenting different head groups, into a bilayer structure, (hyper)thermophilic archaea contain membranes that are based on varying ratios of such lipids and dibiphytanylglycerol tetraether lipids. In dibiphytanylglycerol tetraether lipids, two 40 carbon-long isoprenoid chains are ether-linked to glycerol backbones at each end or to a glycerol or a calditol group at either end, which in turn, can present different head groups (Chong 2010; Chong et al. 2012). These hydrophobic cores of tetraether lipids, i.e., caldarchaeol (or glycerol-dialkyl-glycerol tetraether, GDGT) and calditoglycerocaldarchaeol (or glycerol-dialkyl-nonitol tetraether, GDNT), can include up to eight cyclopentane rings (Chong 2010; Chong et al. 2012).

To understand the importance of cyclopentane rings in the hydrophobic cores of tetraether lipids, both *in vivo* and *in vitro* strategies have been adopted. Studies with various strains have revealed that the number of cyclopentane rings increases as growth temperature rises but decreases as medium pH becomes more acidic (Chong 2010; Boyd et al 2011; Oger and Cario 2013; Jensen et al. 2015). Further insight into the importance of the cyclopentane rings has come from biophysical analysis of liposomes based on

tetraether lipids. Differential scanning calorimetry and pressure perturbation calorimetry studies revealed changes in the thermodynamic properties of such liposomes as a function of whether or not the tetraether lipids contained cyclopentane rings (Chong et al. 2005). Specifically, the presence of cyclopentane rings was proposed to make the membrane tighter and more rigid. Molecular dynamics simulation studies support this concept (Gabriel and Chong 2000). Others, however, failed to see reduced membrane leakiness as the number of cyclopentane rings increased (Koyanagi et al. 2016). Cryo-transmission electron microscopy and small angle X-ray scattering studies on synthetic tetraether lipids containing cyclopentane rings have shown that the stereochemistry of cyclopentane rings with the biphytanyl chains of tetraether lipids can affect the shape of multilamellar vesicles composed of such lipids (Jacquemet et al. 2011; Jacquemet et al. 2012). At the same time, the position of the cyclopentane ring apparently affects hydration properties, lyotropic liquid crystalline behavior and membrane organization of vesicles comprising tetraether lipids (Brard et al. 2004).

While advances into understanding the functions of the cyclopentane rings of GDGT- and GDNT-based tetraether lipids have been made, virtually nothing is known of the steps used to generate these moieties. In the present study, genes affecting the formation of cyclopentane rings in a GDGT-based *Sulfolobus acidocaldarius* glycolipid were identified for the first time.

MATERIALS AND METHODS

Strains and growth

S. acidocaldarius (MW001) (Wagner et al. 2012) and the same strain deleted of various genes were grown at 75°C in Brock's medium (Brock et al. 1972), pH-adjusted to 3 using sulphuric acid, supplemented with 0.1% (w/v) NZ-amine, 0.2% (w/v) dextrin and 10 µg/ml uracil, under constant shaking.

Construction of deletion plasmids

Marker-less deletion mutants of *Saci_0201, Saci_0275, Saci_0421, Saci_1101, Saci_1201, Saci_1249* and *Saci_1706* were obtained in background strain *S. acidocaldarius* MW001, as previously described (Wagner et al. 2009). To construct the gene deletion plasmids pSVA1270, pSVA1231, pSVA1256, pSVA1228, pSVA1238, pSVA1239, and pSVA1254, respectively containing the up- and downstream regions of *Saci_0201, Sac_0275, Saci_421, Saci_1101, Saci_1201, Saci_1249,* and *Saci_1706,* 800–1000 bp of the sequences found up- and downstream of each gene were PCR amplified. At the 5´-ends of the upstream forward primer and the downstream reverse primer, *Apa*I and *Bam*HI restriction sites were introduced, respectively. The upstream reverse primers and the downstream forward primers were designed to each incorporate 15 bp of the reverse complement strand of the other primer, resulting in a 30 bp overlapping stretch. All up- and downstream fragments were fused by overlapping PCR, using the 3′-ends of the up- and downstream fragments as primers. The primers used to generate the deletion strains are listed in Supplementary Table 1. The overlapping PCR fragments were purified and digested with *Apa*I and *Bam*HI and ligated in the pre-digested plasmid

pSVA407, containing *pyrEF* (Wagner et al. 2009). The deletion plasmids obtained (listed in Supplementary Table 2) were transformed into *Escherichia coli* DH5α and selected on LB plates containing 50 mg/ml ampicillin. The accuracy of the plasmids was ascertained by sequencing. To avoid restriction in *S. acidocaldarius*, the plasmids were methylated by transformation in *E. coli* ER1821.

Transformation and selection of S. acidocaldarius deletion mutants

Generation of competent cells was performed based on the protocol of Kurosawa and Grogan as previously described (Kurosawa and Grogan, 2005). Methylated pSVA1270, pSVA1231, pSVA1256, pSVA1228, pSVA1238, pSVA1239 or oSVA1254 (400–600 ng) were added to a 50 µl aliquot of competent MW001 cells and incubated for 5 min on ice, before transformation in a 1 mm gap electroporation cuvette at 1250 V, 1000 Ω, 25 mF using a Biorad Gene Pulser II (Biorad, Hercules CA). Directly after transformation 50 µl of a 2x concentrated recovery solution (1% sucrose, 20 mM β-alanine, 10 mM $MgSO_4$, 20 mM malate buffer, pH 4.5) were added to the sample, which was incubated at 75°C for 30 min under mild shaking conditions (150 rpm). Before plating, the sample was mixed with 100 µl of heated 2x concentrated recovery solution and twice, 100 µl were spread onto gelrite plates containing Brock medium supplemented with 0.1% NZ-amine and 0.1% dextrin. After incubation for 5–7 days at 75°C, large brownish colonies were used to inoculate 50 ml of Brock medium containing 0.1% NZ-amine and 0.1% dextrin, which were incubated for 3 days of 78°C. After confirming the presence of the integrated plasmid by PCR, each culture was grown in Brock medium supplemented with 0.1% NZ-amine and 0.1% dextrin until an OD of 0.4. To confirm gene deletion, 40 µl aliquots were spread onto

selection plates supplemented with 0.1% NZ-amine, 0.1% dextrin and 10 mg/ml uracil, and incubated for 5–7 days at 78°C. Newly formed colonies were streaked out on new selection plates to ensure that they were formed from single colonies, before each was screened for the absence or presence of the deleted genes by PCR.

S. acidocaldarius lipid extraction

S. acidocaldarius lipid extraction was performed essentially as described previously (Murae et al. 2001). Briefly, a solution (2 ml) of $CHCl_3$:MetOH (2:1, v/v) was added to a *S. acidocaldarius* cell pellet (~ 800 µl). The pellets were manually homogenized with a glass homogenizer and sonicated in a Elmasonic bath sonicator for 30 minutes at room temperature. The homogenate was centrifuged for 10 min at 10,000 g at 4˚C. The supernatant was removed and transferred into a fresh 15 ml Falcon tube. A solution (2 ml) of $CHCl_3$:MetOH (1:2, v/v) was added to the pellet, which was homogenized in a glass homogenizer, sonicated for 30 minutes and centrifuged for 10 min at 10,000 g at 4˚C. The supernatant was removed and transferred to the tube containing the previous supernatant. The second set of homogenization, sonication, centrifugation and removal of supernatant steps was repeated two more times. The combined supernatants were then filtered through a 0.22 µm syringe PVDF filter (Merck Millipore) and evaporated to dryness under a stream of N_2.

High performance liquid chromatography-electrospray ionization mass spectrometry (HPLC-ESI MS) analysis of the S. acidocaldarius lipid extract

Normal phase HPLC-ESI MS of the *S. acidocaldarius* lipid extract was performed using an Agilent 1200 Quaternary LC system coupled to a high resolution TripleTOF5600 mass spectrometer (Sciex, Framingham, MA). Chromatographic separation was performed on an Ascentis Silica HPLC column, 5 μm, 25 cm x 2.1 mm (Sigma-Aldrich, St. Louis, MO). Elution was achieved with mobile phase A, consisting of chloroform/methanol/aqueous ammonium hydroxide (800:195:5, v/v/v), mobile phase B, consisting of chloroform/methanol/water/aqueous ammonium hydroxide (600:340:50:5, v/v/v/v) and mobile phase C, consisting of chloroform/methanol/water/aqueous ammonium hydroxide (450:450:95:5, v/v/v/v), over a 40 min-long run, performed as follows: 100% mobile phase A was held isocratically for 2 min and then linearly increased to 100% mobile phase B over 14 min and held at 100% B for 11 min. The mobile phase composition was then changed to 100% mobile phase C over 3 min and held at 100% C for 3 min, and finally returned to 100% A over 0.5 min and held at 100% A for 5 min. The LC eluent (with a total flow rate of 300 μl/min) was introduced into the ESI source of the high resolution TF5600 mass spectrometer, with MS settings as follows: Ion spray voltage (IS) = -4500 V, Curtain gas (CUR) = 20 psi, Ion source gas 1 (GS1) = 20 psi, De-clustering potential (DP) = -55 V, and Focusing Potential (FP) = -150 V. Samples were analyzed in negative-ion mode, with the full-scan spectra being collected in the *m/z* 300-2000 range. Nitrogen was used as the collision gas (collision energy = 40 eV) for tandem mass spectrometry (MS/MS) experiments. Data acquisition and analysis were performed using Analyst TF1.5 software (Sciex, Framingham, MA).

RESULTS

Deletion of Saci_0421 and Saci_1201 leads to decreased numbers of cyclopentane rings

As part of ongoing efforts aimed at defining novel components of the pathway for protein N-glycosylation in *S. acidocaldarius*, a number of genes encoding products suspected of contributing to this post-translational modification (*Saci_0201*, *Saci_0275*, *Saci_0421*, *Saci_1101*, *Saci_1201*, *Saci_1249* and *Saci_1706*) were deleted. LC-ESI MS analysis of FlaB and SlaA, two reporter glycoproteins previously shown to be modified by an N-linked hexasaccharide (Peyfoon et al. 2010; Guan et al. 2016), as well as the dolichol pyrophosphate carrier upon which this glycan is assembled (Guan et al. 2016), revealed patterns of glycosylation in the deletion strains identical to what was seen for these same molecules isolated from the parent strain (not shown). Given that distinct genes contribute to protein glycosylation and lipid glycosylation in the halophilic archaea *Haloferax volcanii* (Naparstek, Vinagradov and Eichler 2010), efforts next focused on assessing whether the absence of *Saci_0201*, *Saci_0275*, *Saci_0421*, *Saci_1101*, *Saci_1201*, *Saci_1249* or *Saci_1706* had any effect on *S. acidocaldarius* glycolipids.

The *S. acidocaldarius* membrane contains tetraether glycolipids based on GDGT presenting phospho-*myo*-inositol attached to glycerol at one end of the molecule and *β*-D-galactosyl-D-glucose attached to the glycerol at the other end (De Rosa, Gambacorta and Nicolaus 1983) (Fig 1A). Accordingly, when a total *S. acidocaldarius* lipid extract from the MW001 parent strain was assessed by LC-ESI MS, a species with a $[M-H]^-$ monoisotopic ion peak at *m/z* 1858.425, corresponding to this glycolipid (calculated mass

1858.37 Da; error 30 ppm), was detected (Fig 1B). Analysis of the product spectrum obtained upon MS/MS analysis of the doubly charged $[M-H+Cl]^{2-}$ ion at *m/z* 946.784 showed peaks consistent with this species (Fig 1C). Furthermore, the monoisotopic ion peaks at *m/z* 1696.32 and 1534.27 are consistent with the $[M-H]^-$ ions of precursors or derivatives of the trisaccharide-containing glycolipid modified by either two or one hexoses, respectively (calculated masses 1696.37 and 1534.37Da, with the *m/z* 1696.32 species containing two hexoses, and the *m/z* 1534.27 species containing only one hexose), were also observed (not shown). The masses of the different variants of the glycolipid detected are consistent with the presence of four cyclopentane rings, with two assumed to be in each phytanyl chain.

When lipid extracts prepared from *S. acidocaldarius* strains deleted of *Saci_0275*, *Saci_1101* or *Saci_1249* were similarly assessed, $[M-H]^-$ monoisotopic ion peaks at *m/z* 1858.378, 1858.376 and 1858.373, respectively, were observed (Fig 1D). Similar peaks were also detected in the lipid extracts of strains lacking *Saci_0201* or *Saci_1706* (not shown). In addition, precursors or derivatives of the trisaccharide-containing glycolipid modified by either two or one hexoses were also seen in these deletion strains (not shown). It would thus appear that these mutants contain the same trisaccharide-bearing glycolipid as found in the parent strain.

At the same time, closer examination of the LC-ESI MS profiles obtained for two of the mutant strains, Δ*Saci_0421* and Δ*Saci_1201*, revealed additional peaks not seen in the profiles of the other strains considered above (Fig 2A; compare with Fig 1B, D). These

peaks, showing incremental 2 Da increases, represent species possessing fewer degrees of unsaturation, and are consistent with variants of the trisaccharide-charged glycolipid containing fewer than four cyclopentane rings, as supported by isotopic distribution simulations (Fig 2B). Simulation of the isotopic distribution of the glycolipid containing four, two and zero cyclopentane rings shows that the expected profiles resemble what was observed in the Δ*Saci_0421* and Δ*Saci_1201* profiles (compare Fig 2A and Fig 2B). It is unlikely that these additional peaks reflect an expansion of the isotopic distribution of the $[M-H]^-$ monoisotopic ion peak associated with the trisacccharide-charged glycolipid observed at *m/z* 1858.37 due to a higher amount of this species in the Δ*Saci_0421* and Δ*Saci_1201* lipid extracts as the intensity of this peak from these species was considerably lower than that of the same peak from the parent strain and the Δ*Saci_0275*, Δ*Saci_1101* and Δ*Saci_1249* mutants (compare peak intensities in Fig 1B and 1D with those in Fig 2A). Moreover, the major peak in the profile from the Δ*Saci_1201* sample was not observed at *m/z* 1858.37 but rather at *m/z* 1862.39. Moreover, additional peaks in the LC-ESI MS profiles of the di- and monosaccharide-charged precursors/derivatives of the complete trisaccharide-charged glycolipid, again representing species presenting fewer degrees of unsaturation, were also observed in the Δ*Saci_0421* and Δ*Saci_1201* mutant strains but not in the profiles of the other strains considered (not shown).

The possibility that the extra peaks seen in the LC-ESI MS profiles of the Δ*Saci_0421* and Δ*Saci_1201* strains represent variants of the trisaccharide-charged glycolipid (and its di- and monosaccharide-charged precursors/derivatives) containing fewer than four

cyclopentane rings was more directly considered. Specifically, the $[M-H+Cl]^{2-}$ ion peak observed at *m/z* 950.70 in the total lipid extract from the Δ*Saci_0421* species was subjected to MS/MS analysis. The product spectrum of this species was consistent with chlorine adduct of the trihexose-charged glycolipid lacking cyclopentane rings (Fig 2C). Therefore, it appears that Saci_0421 (and Saci_1201) contributes to the appearance of cyclopentane rings in the polyisoprene chains of the GDGT moiety of the *S. acidocaldarius* glycolipid considered here, and possibly in other tetraether lipids in this species.

Finally, given the reported increase in cyclopentane ring content of *S. acidocaldarius* tetraether lipids as the growth temperature rises (De Rosa et al. 1980), mutants in which cyclopentane ring content is compromised would be expected to grow less well at elevated temperatures than would the parent strain. No differences in the growth rates of the *ΔSaci_0421* and *ΔSaci_1201* mutants and the parent strain were observed when the cells were grown at either standard (75°C) or elevated (80°C) growth temperatures.

Homologues of Saci_0421 and Saci_1201 are found in other (hyper)thermophiles

To determine whether genes in the vicinity of *Saci_0421* (currently annotated as encoding a dolichyl-phosphate-mannose-protein mannosyltransferase termed Agl1, given its putative role in N-glycosylation (Meyer et al. 2011)) and/or *Saci_1201* (currently currently annotated as encoding a glycogen synthase) might also encode proteins putatively involved in cyclopentane ring, GDGT or tetraether lipid assembly, the putative products of the twelve open reading frames lying upstream and downstream of each gene

were considered (Supplementary Table 3). *Saci_0422* (*agl2*), *Saci_0423* (*agl3*) and *Saci_0424* (*agl4*) are predicted to encode proteins that participate in N-glycosylation in *S. acidocaldarius*, with experimental proof for the role of Saci_0423 (Agl3) as a sulfoquinovose synthase having been provided (Meyer et al. 2011). *Saci_0422* (*agl2*) and *Saci_0424* (*agl4*) are predicted to encode a dTDP-glucose pyrophosphorylase and a glucokinase, respectively (Meyer et al. 2011). Other annotated genes in this region and in the region surrounding *Saci_1201* are currently predicted to encode proteins serving various roles, and many are listed as encoding conserved or hypothetical proteins. As such, it would appear that neither *Saci_0421* nor *Saci_1201* belong to an operon or gene cluster involved in tetraether lipid biogenesis.

Finally, efforts were aimed at determining whether homologues of Saci_0421 and/or Saci_1201 are found in other Archaea where cyclopentane-containing tetraether lipids are found. BLAST searches using Saci_0421 as query detected the presence of homologues in various species (Supplementary Table 4). Although all are (hyper)thermophiles, the presence of tetraether lipids containing cyclopentane rings has been demonstrated in only a few of these species other than *Sulfolobus acidocaldarius* (De Rosa et al. 1980), such as *Thermoproteus tenax* (Thurl and Schafer 1988), *Sulfolobus solfataricus* (De Rosa, Gambacorta and Gliozzi 1986) and *Pyrococcus horikoshii* (Sugai et al. 2000). At the same time, no Saci_0421 homologues were detected in other species, such as *Thermoplasma acidophilum* or *Archaeoglobus fulgidus*, where cyclopentane-containing tetraether lipids have been described (Shimida et al. 2002; Lai, Springstead and Monbouquette 2008). When Saci_1201 served as query in a BLAST search, homologues

were also detected in a variety of species (Supplementary Table 5). Again, those species containing the homologous sequences are all (hyper)thermophiles, although this list was not identical to that of species containing Saci_0421 homologues.

DISCUSSION

As the numbers of archaeal genome sequences and strains for which genetic tools are available grow, a clearer picture of archaeal biochemistry and those aspects unique to this life form is emerging. Still, much remains to be clarified. In the case of the tetraether lipids that comprise the membranes of (hyper)thermophilic archaea, many biosynthetic steps either remain as predictions, such the presumed coupling of two archaeol lipids to generate GDGT (Koga and Morii 2007; Villanueva et al. 2014), or completely undefined, such as the steps leading to the appearance of cyclopentane rings in each of the phytanyl chains of such lipids. In the present report, the deletion of specific genes was shown for the first time to affect cyclopentane ring formation in a *S. acidocaldarius* glycolipid. Specifically, the deletion of *Saci_0421* and *Saci_1201*, currently annotated as encoding a dolichyl-phosphate-mannose-protein mannosyltransferase and a glycogen synthase, respectively, led to the formation of a GDGT moiety within a trihexose-bearing glycolipid with reduced numbers or even lacking cyclopentane rings. In the presence of these genes, both in the parent strain and in a series of other *S. acidocaldarius* deletion strains, the GDGT moiety largely contained four cyclopentane rings under the growth conditions employed here.

It was previously reported that the number of cyclopentane rings in GDGT is affected not only by growth temperature and pH but also by stirring of the cell cultures when growing and by the method used for lipid extraction (Uda et al. 2001). Since the various *S. acidocaldarius* strains considered here were all similarly grown and processed, it is unlikely that the observed effects of *Saci_0421* and *Saci_1201* deletion on glycolipid

cyclopentane ring content reflect growth- or preparation-related effects. Instead, it would seem that the observed effects of deleting these genes on cyclopentane content are biologically relevant. Based on what is known of tetraether lipid biosynthesis (Koga and Morii 2007; Villanueva et al. 2014), two scenarios leading to the appearance of cyclopentane rings within a GDGT (or GDNT) hydrophobic core can be envisaged. In the first, internal cyclization of saturated phytanyl chains would occur. Alternatively, cyclopentane rings would be present in the prenyl groups being added to the growing chain. However, reports of the number of cyclopentane rings changing as a function of growth temperature and growth phase (Chong 2010; Oger and Cario 2013; Jensen et al. 2015 and references therein) are difficult to reconcile with this second scenario, unless substantial lipid turnover occurs. It should be noted that no change in cyclopentane ring content was seen when the growth temperature was raised from 75 to 80°C in the present study; this could be related to the particular growth conditions employed. It is even less clear how enzymes thought to be involved in sugar/polysaccharide processing and/or assembly, such as Saci_0421 or Saci_1201 (Cardona et al. 2001), could contribute to the appearance of cyclopentane rings. While it is possible that they somehow contribute to the cyclization process presumably involved in cyclopentane ring biogenesis, one can also imagine that these proteins instead modify some other aspect of the membrane, with the observed drop in ring numbers being an indirect effect resulting from the loss of the predicted functions of Saci_0421 or Saci_1201.

The identification of tetraether lipids represents yet another example of how the study of Archaea has expanded our appreciation of the diverse solutions Nature provides to a

given challenge. Indeed, different versions of tetraether lipids isolated from a range of archaeal species that thrive in a variety of environments and that present distinct chemical modifications affecting the functions of such lipids, have been described (Damsté et al. 2002; Knappy et al. 2011). Understanding of the biosynthetic pathways involved in generating such variability is, however, lacking. The present study represents a step towards addressing this gap.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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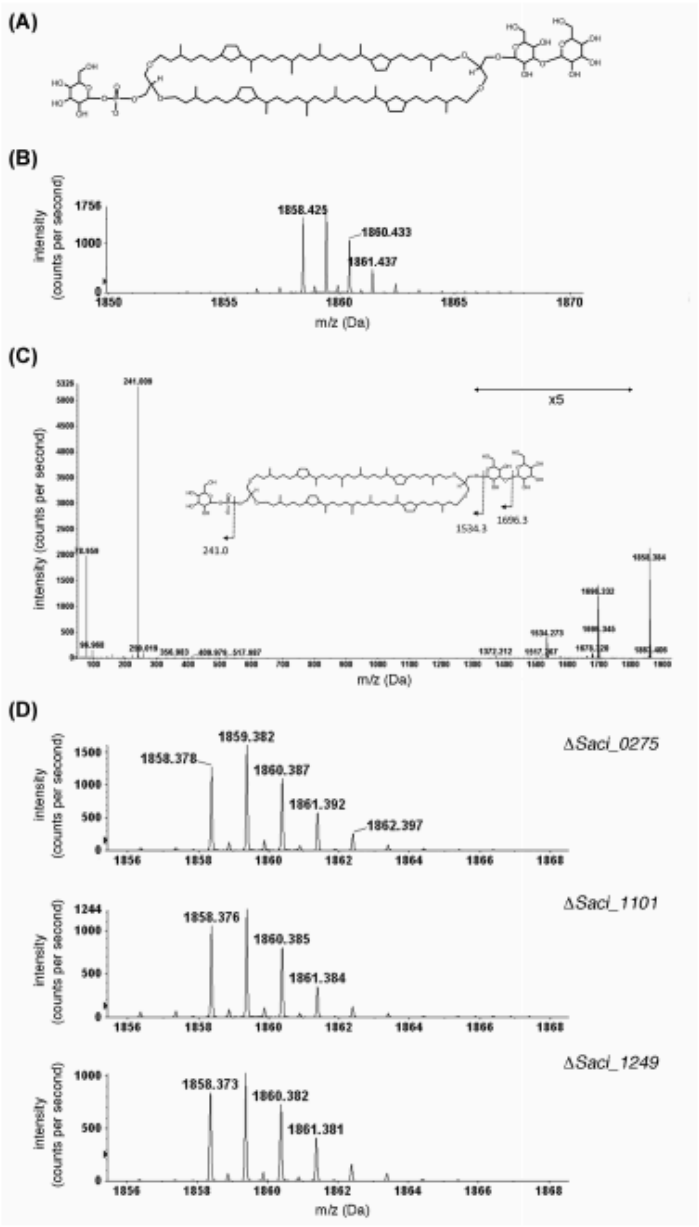

Figure 1. Detection of a *S. acidocaldarius* glycolipid containing four cyclopentane rings. (A) Schematic depiction of a glycolipid based on GDGT and modified by a phosphate group and three hexoses and including four cyclopentane rings evenly distributed between the two phytanyl chains. (B) Sulfolobus acidocaldarius cells present a $[M\text{-}H]^-$ monoisotopic ion peak at m/z 1858.425 corresponding to the glycolipid (calculated mass 1858.37 Da). (C) MS/MS spectrum of the $[M\text{-}H + Cl^-]^{2-}$ ion at m/z 946.784 (Cl^- chloride ion). The inset schematically represents the fragmentation scheme. The arrows indicating ×5 reflect magnification of the ion peaks in the corresponding region of the m/z values on the graph. (D) *Sulfolobus acidocaldarius* ΔSaci_0275, ΔSaci_1101 and ΔSaci_1249 present a $[M\text{-}H]^-$ monoisotopic ion peak at m/z 1858.37 corresponding to the glycolipid depicted in panel A.

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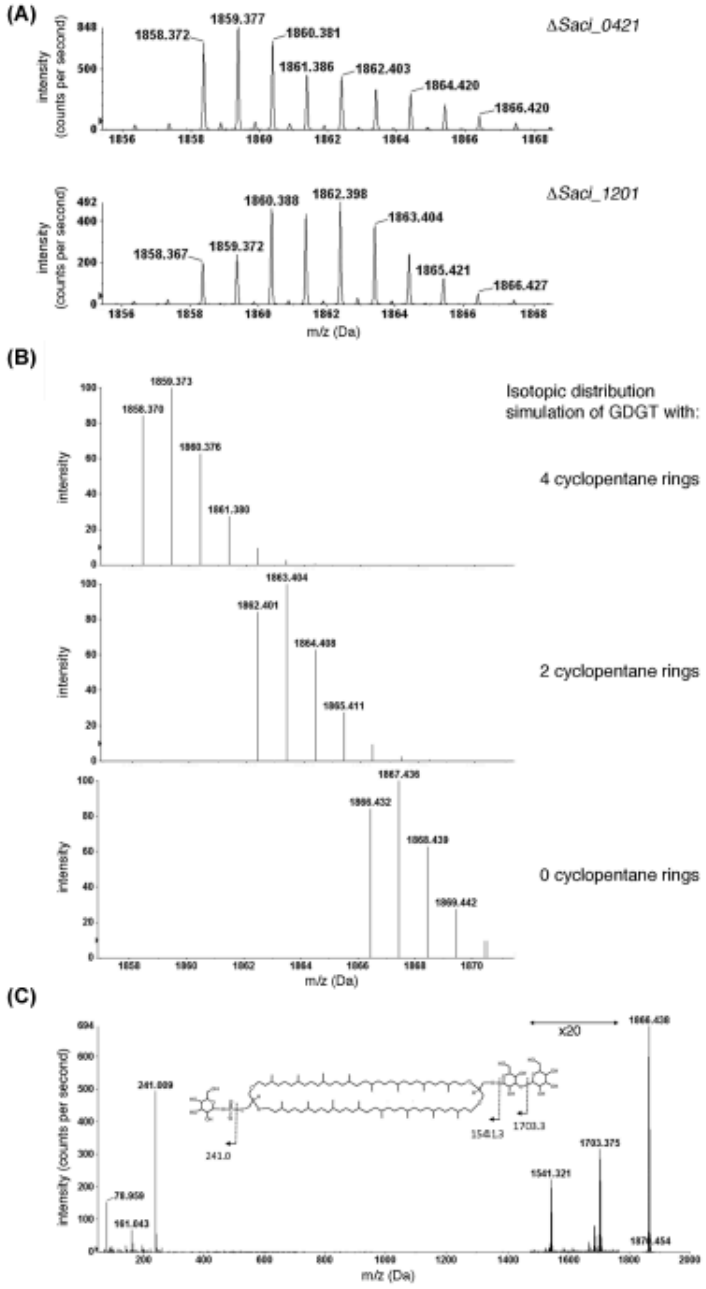

Figure 2. The same glycolipid in S. acidocaldarius ΔSaci_0421 and ΔSaci_1201 cells contains 0–4 cyclopentane rings. (A) Sulfolobus acidocaldarius ΔSaci_0421 and ΔSaci_1201 present a $[M\text{-}H]^-$ monoisotopic ion profile containing additional peaks not seen in parent strain, ~~and~~ ΔSaci_0275, ΔSaci_1101 or ΔSaci_1249 cells. (B) Simulation of the isotopic distribution of the glycolipid containing four, two and zero cyclopentane rings, as indicated. (C) MS/MS spectrum of the $[M\text{-}H + Cl^-]^{2-}$ ion at m/z 950.70. The inset schematically represents the fragmentation scheme. The arrows indicating ×20 reflect magnification of the ion peaks in the corresponding region of the m/z values on the graph.

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