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Article

Membrane adaptations and cellular responses of *Sulfolobus acidocaldarius* to the allylamine terbinafine

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Abstract: Cellular membranes are essential for compartmentalization, maintenance of membrane permeability and fluidity in all three domains of life. Archaea belong to the third domain of life and have a distinct phospholipid composition. Membrane lipids of archaea are ether-linked molecules, specifically dialkyl glycerol diethers (DGDs) and glycerol dialkyl glycerol tetraethers (GDGTs). Terbinafine has been proposed as an inhibitor of GDGT biosynthesis in Archaea based on radiolabel incorporation studies. Here, we have studied the early cellular responses of the archaeon Sulfolobus acidocaldarius to terbinafine whose membrane is mostly composed of the membrane-spanning GDGTs. Depletion of GDGTs and an accompanying accumulation of DGDs upon treatment with terbinafine was growth phase and dose dependent. Additionally, a major shift in the saturation of caldariellaquinones was observed, resulting in the accumulation of unsaturated molecules. Transcriptomic data indicated that terbinafine primarily targets the respiratory complex along with genes involved in motility, fatty acid metabolism and GDGT cyclization. Combined, these findings suggest that respiratory stress is the preliminary response of S.acidocaldarius to terbinafine while targeting multiple proteins involved in isoprenoid biosynthesis and saturation.

Keywords: GDGT; archaea; membrane; Sulfolobus; isoprenoids; caldariellaquinone; terbinafine

1. Introduction

Crenarchaeal membranes are highly dynamic as their composition is dependent on environmental influences like nutrient availability, pH and temperature. Ether-linked archaeal lipids have been studied extensively due to their unique chemical properties rendering membranes more robust with reduced proton permeability. In recent years, the lipid biosynthesis pathway in archaea has been well characterized. It commences with the isoprenoid building blocks – isopentenyl pyrophosphate (IPP) or dimethylallyl pyrophosphate (DMAPP) from the alternate or classical mevalonate pathway [1]. *Sulfolobales* utilize the classic mevalonate pathway (MVA) for this step [2]. IPP and DMAPP undergo sequential condensation reactions by geranylgeranyl pyrophosphate (GGPP) synthase to form GGPP [1]. The glycerol-1-phosphate (G1P) backbone is synthesized by G1P dehydrogenase (*EgsA*) in *Sulfolobus acidocaldarius*. GGPP is further processed by geranylgeranyl glycerol phosphate (GGGP) synthase (GGGPS) to form GGGP with a glycerol-1-phosphate backbone [1]. The formation of the diether 2,3-O-geranylgeranylglyceryl diphosphate (DGGGP) is catalyzed by DGGGP synthase (DGGGPS). CDP Archaeol synthase or *CarS* then activates DGGGP through cytidine triphosphate (CTP) to produce CDP-Archaeol [3]. In the next steps, the cytidine diphosphate (CDP) head group in replaced by polar head groups such as calditol, glycerol or dihexose. Geranylgeranyl reductase (GGR) is responsible for the hydrogenation of the unsaturated DGGGP to yield an archaeol [4]. The substrate specificity for GGR remains unclear, however it has been demonstrated that *CarS* is specific for the unsaturated substrate [5]

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suggesting that saturation is a downstream process. In archaea, tetraether lipids can be formed by a tail-to-tail condensation of two diether lipids. The molecules termed glycerol dialkyl glycerol tetraethers (GDGTs) span the entire membrane. Recently, the enzyme involved in this process was identified, *Tes*, which belongs to the family of radical S-adenosylmethionine (rSAM) [6] that catalyze reactions through the formation of free radicals. GDGTs can incorporate up to 8 cyclopentane rings in their structure. Two GDGT ring synthases – *GrsA* and *GrsB* were identified in *S. acidocaldarius* [7]. These proteins also belong to rSAM family of proteins. *GrsA* and *GrsB* introduce rings at the C-7 or C-3 position in GDGTs [7]. The calditol or nonnitol headgroup in GDGTs is synthesized by *Cds*, another rSAM enzyme to yield glycerol dialkyl nonnitol tetraether (GDNT) [8]. Membranes of *S. acidocaldarius* predominately consist of monolayer forming GDGTs and GDNTs have low abundance [9] [10]. Inositol-phosphate dialkyl glycerol diether (IP-DGD) is the most abundant dialkyl glycerol diether lipid (DGD) [10]. The membrane composition of *S. acidocaldarius* is modulated by environmental factors such as growth phase, growth rate, temperature, pH and starvation [7] [10] [11] [12]. The common adaptations include altering the ratio of DGDs to GDGTs and the incorporation of cyclopentane rings in tetraether lipids. These adaptations lead to reduction in the permeability of crenarchaeal membranes with higher temperatures. Various headgroups can be found in GDGT lipids of *Sulfolobus* including : inositol-phosphate, monohexose, dihexose,calditol or nonnitol [10] [13]. The calditol headgroup has an adaptive function for survival in acidic environments [8]. Apart from ether-linked lipids, crenarchaeal membranes contain other isoprenoids like respiratory quinones. The abundance of these quinones is correlated with pH, temperature and salinity in archaea [14]. Essentially, quinones with short-acyl chains are correlated with thermophiles while the presence of long-acyl chains are correlated with salinity[14]. Based on these correlations, quinones have been proposed as putative membrane regulators of archaea [14]. The respiratory quinones of *S. acidocaldarius* include caldariellaquinones (CQs) and sulfoquinones (SQs) [15]. Biochemical studies have recognized CQs as a pool of reducing factors for the cytochromes in the electron transport chain [16] [17] [18].

Radiolabeling assays with the crenarcheaote *Thermoplasma acidophilum* have indicated the accumulation of DGDs upon treatment with terbinafine, a squalene epoxidase inhibitor in fungi. It was suggested that this compound inhibits the tetraether synthase, now identified as *Tes* [19]. This effect was not observed in *Halobacterium salinarum* which has a membrane composition of ~100% diether lipids [19]. Terbinafine targets squalene epoxidase in fungi, which is a part of the ergosterol biosynthesis pathway [20]. Squalene epoxidase is a FAD-dependent enzyme. However, the exact mode of action of terbinafine remains unclear. Molecular dynamics simulations have indicated that the lipophilic part of terbinafine binds to the central cavity of squalene epoxidase and induces conformational changes which blocks the substrate from entering the binding site [20]. Archaea do not synthesize sterols, and thus the exact target(s) for terbinafine remain elusive. Further, the high concentrations of terbinafine required for growth inhibition suggest that the inhibitory mechanism might be more complex than originally anticipated. In this study, the impact of terbinafine on the lipidome of *S. acidocaldarius* was determined as well as transcriptomics analysis was conducted to gain insights into the early cellular responses of the crenarchaeote to terbinafine.

2. Results

2.1. *Terbinafine causes the accumulation of DGDs and depletion of DGTs in S. acidocaldarius membranes*

The Sulfolobus acidocaldarius MW001 strain was grown in the presence of various concentrations of terbinafine in brock medium at 75 °C, pH 3.0 with aeration.

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Terbinafine inhibits the growth of S. acidocaldarius in a concentration-dependent manner (Fig. 1). The lipidome of MW001 strain was analyzed at exponential and stationary phase of growth in the absence and presence of terbinafine. Accumulation of inositol phosphate dialkyl glycerol diether lipid (IP-DGD) was observed in exponential as well as stationary phase cells (Fig. 2). IP-DGD was the only diether lipid which showed consistent accumulation across growth phases. Levels of 2,3-O-geranylgeranylglyceryl diphosphate (DGGGP) and archaetidic acid (AA) were slightly elevated during the exponential phase, however, they were depleted in the stationary phase (Fig.2). A reduction in levels of all abundant DGTs : di-hexose inositol phosphate DGT, penta-hexose inositol phosphate DGT and di-hexose DGT was observed only in the stationary phase (Fig. 3b). Interestingly, this relative reduction in levels of DGTs was not observed in the exponential growth phase (Fig.3a). The lipidome of S.acidocaldarius was examined for GDGTs with various cyclopentane rings (0-8). A slight decrease in the levels of GDGT-1 to 3 was observed in the exponential phase of growth with terbinafine (Supplementary S1). Meanwhile, GDGT-1 to 6 had slightly decreased levels in the stationary phase of growth with terbinafine (Supplementary S1).

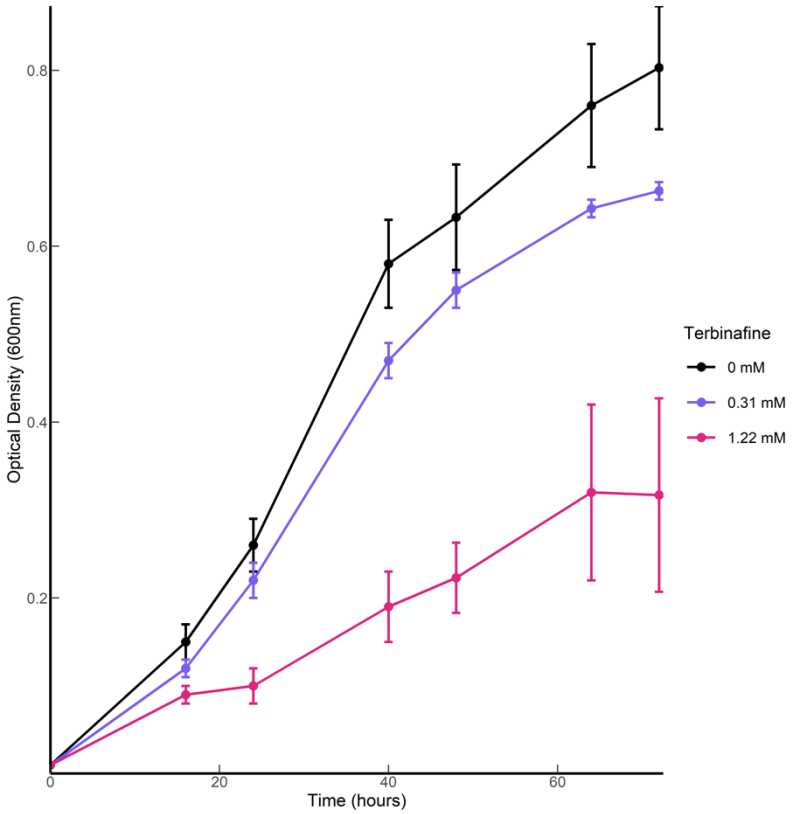

Figure 1. Growth curve of *S. acidocaldarius* MW001 with terbinafine: Error bars represent standard error of the mean.

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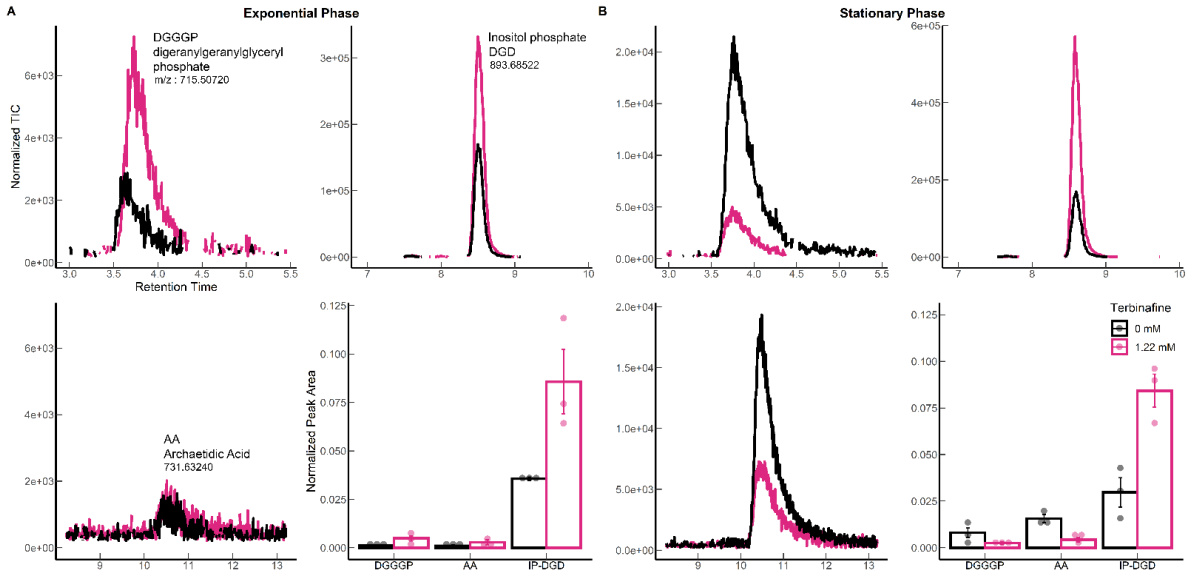

Figure 2. Influence of terbinafine on the bilayer forming membrane lipids (DGDs) of *S.acidocaldarius* : Representative extracted ion chromatograms and relative quantification of DGGGP (digeranylgeranylglyceryl phosphate) , AA (archaetidic acid), IP-DGD (inositol phosphate dialkyl glycerol diether) in (A) exponential phase and (B) stationary phase of growth. Error bars represent standard error of mean. Dots represent biological replicates.

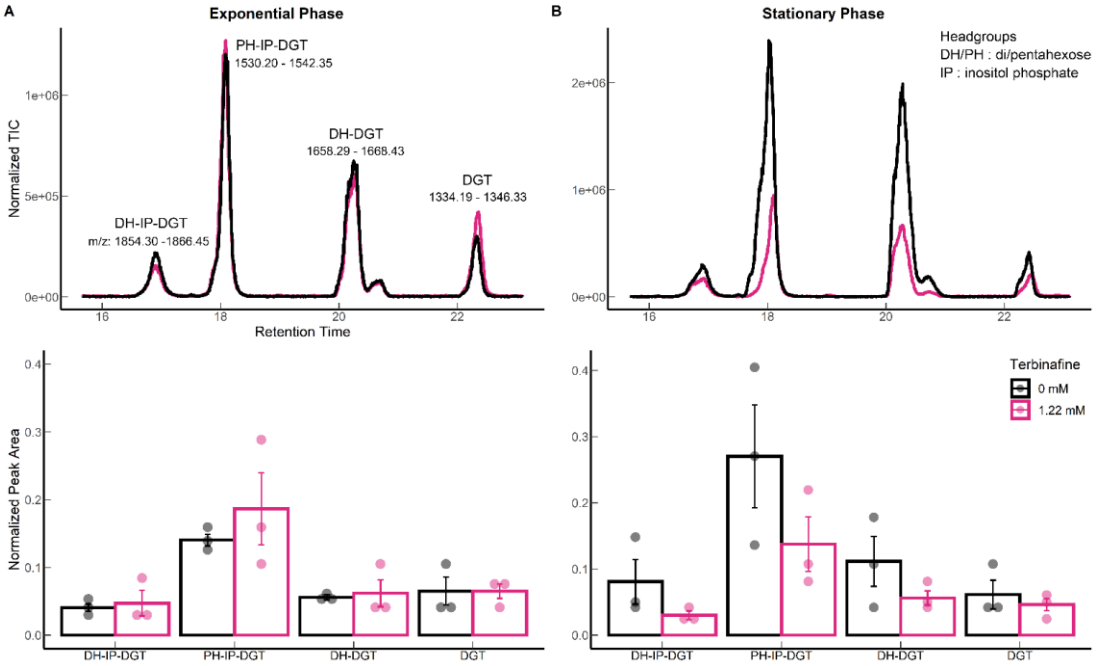

Figure 3. Influence of terbinafine on the monolayer forming membrane lipids (DGTs) of *S.acidocaldarius* : Representative extracted ion chromatograms and relative quantification of tetraether lipids in (A) exponential phase and (B) stationary phase of growth. Error bars represent standard error of mean. The peak areas also include the cyclopentane ring containing species (0-6) for each tetraether lipid. Dots represent biological replicates.

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2.2. *Terbinafine disrupts the respiratory complex in S. acidocaldarius*

To examine the cellular responses of S. acidocaldarius on terbinafine-induced growth inhibition, the transcriptomic response of MW001 was evaluated after three hours of growth with terbinafine at concentrations of 0.31 mM and 1.22 mM. At 0.31 mM of terbinafine, only five genes were differentially expressed (p-adjusted < 0.05) (Supplementary S2). Meanwhile, 260 genes were differentially expressed at 1.22 mM of terbinafine. These genes were mapped to the arCOG database. The density plot (Fig. 4a) illustrates the categorization of these genes based on arCOGs. Most of the differentially expressed genes (p-adjusted value < 0.05) were mapped to the arCOG pathway 'Energy production and conversion' as illustrated by the column graph (Fig.4b). S. acidocaldarius is a strict aerobe which conserves energy by a proton driven chemiosmotic gradient [21] . Transcript levels of sdhA , sdhC and nuoD were elevated which are a part of the SDH and NADH complexes responsible for reducing quinones [22] [16] (Fig.5). This respiratory chain has three terminal oxidase complexes : SoxABCDL, SoxEFGHIM and DoxBCE [23]. Transcript levels of soxA-C, soxG-I and doxB-C were also elevated (Fig. 5). The ATP synthase complex consists of 9 subunits : AtpA-I [17]. The transcript levels of atpA and atpB were elevated (Fig. 5).

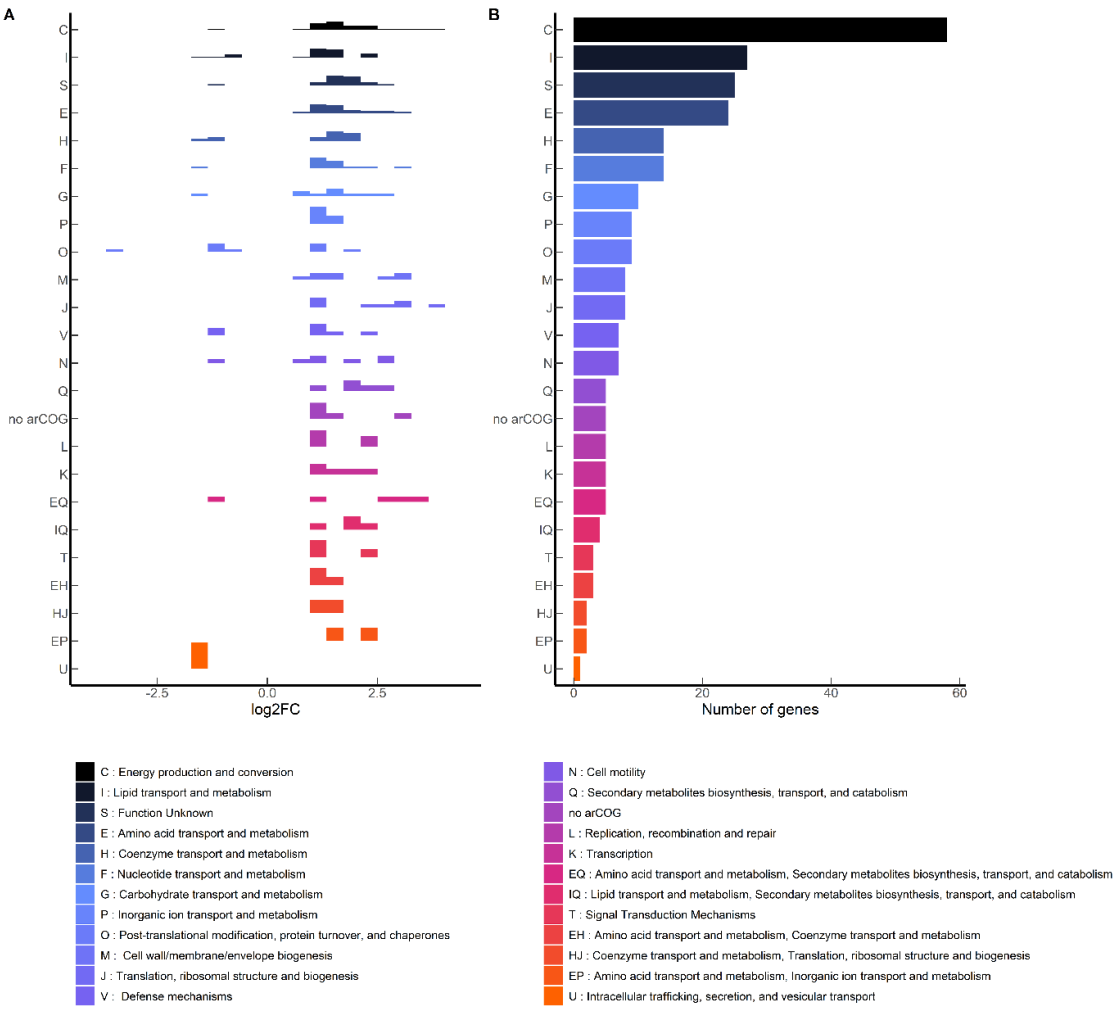

Figure 4. arCOG mapping of significantly affected MW001 genes with terbinafine (p-adjusted < 0.05): (A) Density plot illustrating log2 fold change distribution of genes, (B) Tally of genes mapping to the arCOG categories

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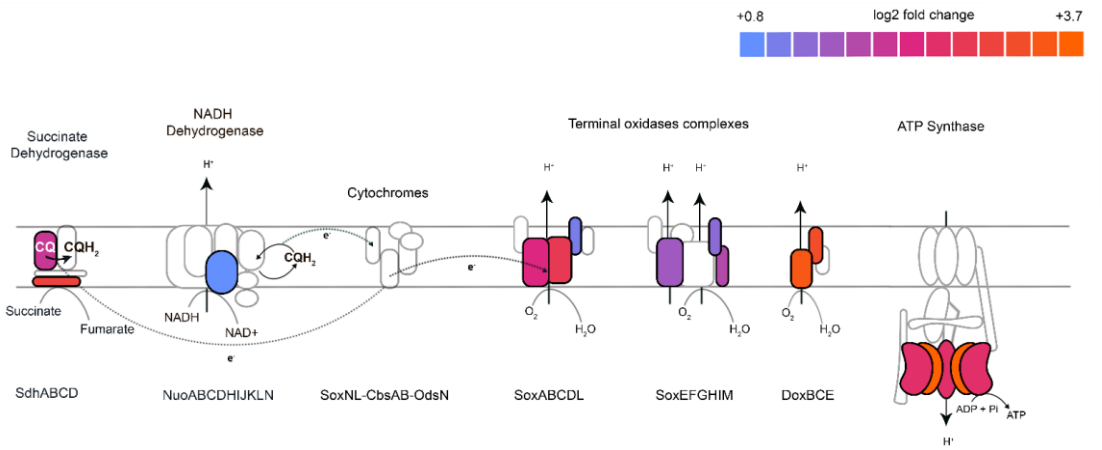

Figure 5. Effect of terbinafine on the respiratory complex of MW001: Colors represent log2 fold change values. No colors indicate unaffected significant transcript levels. All log2 fold change values are $p < 0.05$.

2.3. *A shift in the saturation levels of caldariellaquinone*

Caldariellaquinones (CQ) act as electron and proton carriers in the respiratory chain of thermoacidophiles [23] [21]. Sulfolobus predominantly synthesizes two classes of benzothiophenquinones : sulfoquinones and CQ [24]. S. solfataricus alters its quinone composition depending on temperature, carbon source and availability of oxygen [24]. The total quinone content increases with more aeration [24]. They are found only in saturated forms in crenarchaeotes [15]. Solely saturated caldariellaquinone (CQH) was detected in the lipid extracts of S. acidocaldarius without terbinafine (Fig. 6). Meanwhile, mostly unsaturated caldariellaquinones (CQ 6:1, CQ 6:2 & CQ 6:3) were observed in the exponential and stationary phase lipid extracts after growth with terbinafine (Fig. 6). A menaquinone (MK-7) prenyltransferase from Archaeoglobus fulgidus was shown to alter MK-7 saturation profile in E.coli [25], this enzyme has a homolog WP_011278262 (saci_1431) in S. acidocaldarius with 29% sequence identity. The transcript levels of this gene were unaffected (data not shown). Not much is known about the biosynthetic pathway of CQs in Sulfolobus. Geranylgeranyl pyrophosphate (GGPP) is a proposed common precursor of isoprenoids such as CQs and membrane lipids [26]. Transcript levels of GGGPS were unaffected (Fig.7) and GGPP could not be detected in the lipid extracts of S. acidocadalrius.

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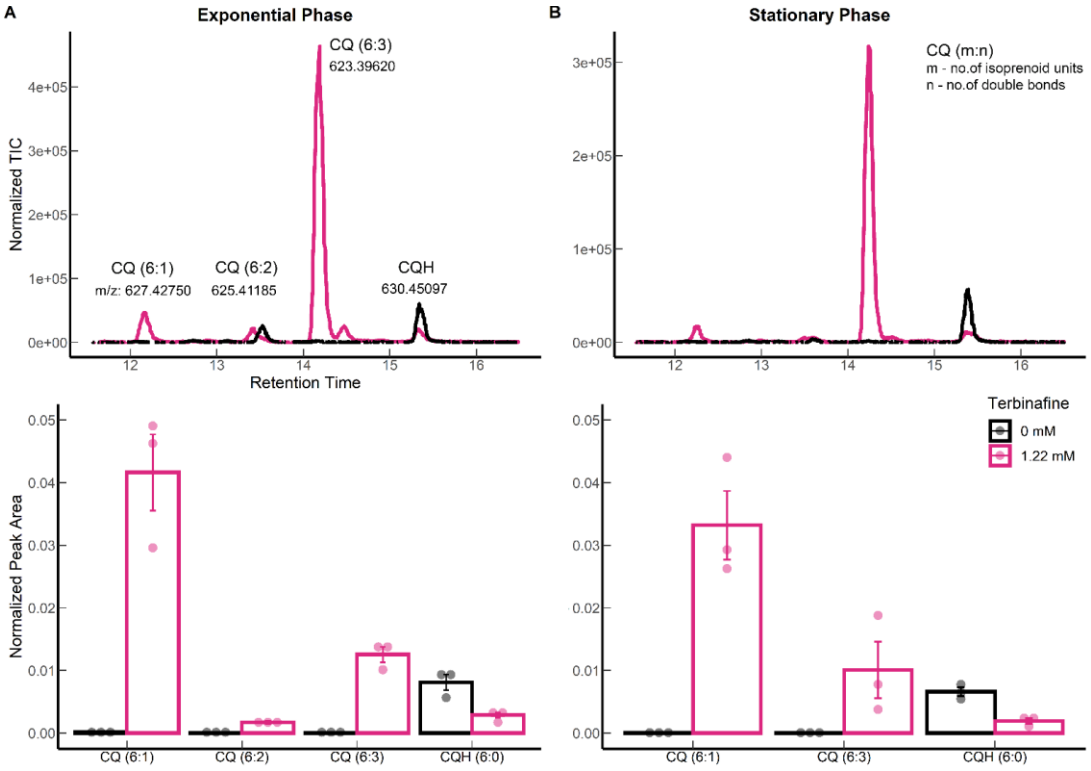

Figure 6. Altered saturation of caldariellaquinone (CQ) under the influence of terbinafine: (A) Representative extracted ion chromatogram (top) and relative quantification of peak areas (bottom) in the exponential phase of growth in S.acidocaldarius and (B) stationary phase. Error bars represent standard error of mean. Dots represent biological replicates.

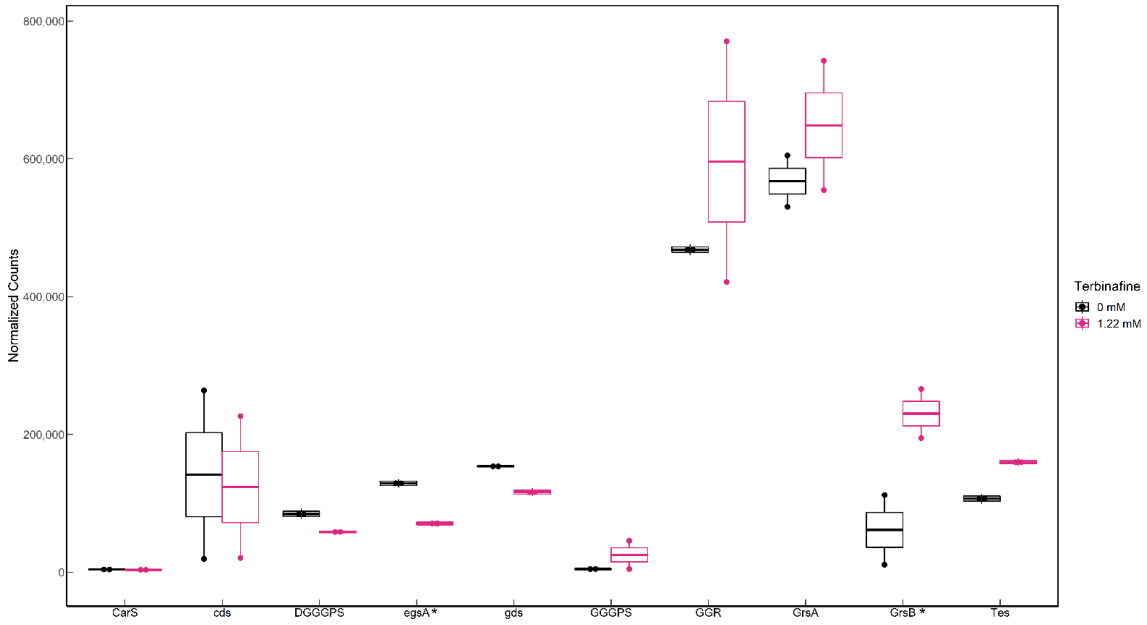

Figure 7. Transcriptomic response of lipid biosynthesis genes in MW001 to terbinafine: Significantly affected genes ($p < 0.05$) have been indicated with an asterisk (*) on the x-axis. Counts have been normalized for sequencing depth using mean of ratios method by DESeq2. *cds* : saci_1498 (calditol synthase), *carS* : Saci_0897 (CDP-archaeol synthase), DGGGPS : Saci_1565 (Digeranylgeranylglycerol phosphate synthase), *egsA* : Saci_0640 (Glycerol-1-phosphate dehydrogenase), *gds* : Saci_0092 (geranylgeranylpyrophosphate synthase), GGGPS : Saci_0728

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(Geranylgeranylglycerolphosphate synthase), GGR : Saci_0986 (geranylgeranyl reductase), *grsA* : Saci_1585 (GDGT cyclization A), *grsB* : Saci_0240 (GDGT cyclization B), *Tes* : Saci_0703 (Tetraether synthetase)

2.4 *Influence of terbinafine on the expression of phospholipid biosynthesis genes*

RNA sequencing data indicated that terbinafine caused an elevation in saci_0240 (grsB) transcript levels which has been identified as a GDGT cyclization enzyme in S. acidocaldarius [7] (Fig.6). However, the cyclization of GDGTs was not significantly affected. A growth phase dependent decrease was observed in the levels of GDGT-1 to 6 (Supplementary S3) . It is likely that this could be a response of the membrane to energy availability and not necessarily due to the elevated transcript level of grsB [27]. Transcriptomic data also indicated significant depleted levels of egsA (saci_0640), responsible for the NAD(P)H- dependent reduction of dihydroxyacetonephosphate (DHAP) to produce glycerol-1-phosphate backbone for archaeal lipids (Fig.7). Interestingly, terbinafine did not significantly affect the transcript levels of saci_0703 (Fig.7) which has been recently identified as tetraether lipid synthetase (Tes) [6].

2.5 *Cellular motility*

Motility in S. acidocaldarius is an ATP–dependent physiological process mediated by the archaellum [28] [29]. It is a rotating-type IV pilus which consists of seven proteins (flaBFGHIJX) transcriptionally regulated by arnR, arnR1 and arnB [28] [30] [31]. The flaB promoter is induced by environmental stressors like nitrogen starvation [32]. Both flaF and flaG are conserved components of the archaellum localized in the membrane [28] . Meanwhile, flaH and flaI are predicted cytoplasmic components. The transcript levels of flaB, flaF, flaG, flaH, arnR (log2 fold change : +1.062) were elevated while of flaG was reduced in the presence of terbinafine (Supplementary S4).

3. Discussion

The lipid membranes of thermoacidophilic organisms are highly adaptive owing to the extreme environments these organisms thrive in [7] [33] [8] [27] [34] [10]. Terbinafine is a squalene epoxidase inhibitor in fungi, and has been proposed as a tetraether lipid biosynthesis inhibitor in archaea based on radiolabeled [^{13}C]mevanolic acid incorporation studies [35]. However, the concentrations needed for inhibition are substantially higher than that required in eukaryotes, i.e., submillimolar versus micromolar, respectively. Therefore, it remains uncertain if terbinafine is a specific inhibitor of tetraether lipid biosynthesis or whether it has more global inhibitory effects on cells. Hence, the cellular response of *S. acidocaldarius* to the compound was studied. The inhibitory effect of terbinafine on growth and GDGT biosynthesis is consistent with the study in *Thermoplasma acidophilum* [36]. However, from the transcriptomics analysis it appears that a major target for growth inhibition is respiration. Apart from altering the distribution of GDGTs over IP-DGD, the precursor for GDGT biosynthesis, terbinafine inhibition also leads to a shift of the CQs to unsaturated species and to a smaller extent a reduced level of cyclization of the GDGTs.

Terbinafine inhibits squalene epoxidase in fungi [20]. Squalene epoxidase is involved in biosynthesis of ergosterols, a membrane regulator responsible for fluidity and permeability of membranes [37]. In bacteria, hopanoids serve a similar function [38]. Both sterols and hopanoids can induce ordered phases in the lipid membrane [14]. Sterols or hopanoids have not been reported in archaea. Possible candidates for these functions in archaea could be polyterpenes : carotenoids, polyprenols, quinones and polyisoprenoids [14]. Therefore, it is likely that the isoprenoids like CQs belong to the primary targets of terbinafine in archaea. The shift towards unsaturated CQs species starts occurring in the exponential phase and continues in the stationary phase. Respiratory quinones in archaea are proposed to serve as membrane regulators [14]. Hyperthermophiles in particular, harbor quinones with short acyl chains like CQs and these presumably aid in membrane packing and improvement of lipid chain order [14]. It is difficult to assess the significance of saturated CQs to *S. acidocaldarius* as

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little is known about these isoprenoids. Unsaturated CQs could influence fluidity of the membrane as well as the ordered phases of the membrane. So far, the distribution of CQs is known to be restricted to *Sulfolobales* [14] [24] [15] [39]. The only known quinone prenyltransferase is from *Archaeglobus fulgidus* which solely synthesizes menaquinones [25] [15]. This class of enzymes is orthologous to archaeal GGRs and their multiplicity in genomes indicates that such enzymes could be responsible for the saturation of other isoprenoids such as CQs [25]. *S. acidocaldarius* harbors five such uncharacterized homologs to GGR which are clustered into arCOG00570.

The Fad_{Rsa} cluster has predicted acyl-CoA dehydrogenases, 3-hydroxylacyl-CoA-dehydrogenase and acetyl-CoA acetyltransferase in *S.acidocaldarius* which are involved in the synthesis of acetoacetyl-CoA, a precursor of the alternate mevalonate pathway in archaea [40] [41]. The altered transcript levels of these genes may indicate a potential bottleneck in the synthesis of the isoprenoid building blocks – IPP and DMAPP. However, the transcript levels of most genes involved in the ether lipid biosynthesis pathway remained unaffected by terbinafine inhibition while the precursor GGPP could not be detected in the lipidome.

Our study confirms the growth phase dependent depletion of DGTs in presence of terbinafine for *S. acidocaldarius* as previously observed for *Thermoplasma acidophilum* [36]. Significant DGTs depletion was observed only in stationary phase cells, indicating that the alteration of the lipidome upon terbinafine inhibition is a slow event. Additionally, the transcript levels of the recently identified tetraether lipid synthetase Tes was not affected after three hours of growth with terbinafine. Taken together, these findings suggest that the depletion of GDGTs is possibly a secondary effect of terbinafine on the lipidome. IP-DGD is the predominant fully saturated diether in the membrane of *Sulfolobus* and its amounts are consistent across growth phases [10] [42]. Unsaturated archaetidylglycerol (AG) has been proposed as a precursor for tetraether lipids in *Thermplasma acidophilum* based on radiolabeling assays [43]. Interestingly, IP-DGD was the only diether lipid that increased in level upon the addition of terbinafine. The discovery of *Tes* has not resolved the question as to whether the precursor molecule needs to be saturated or (partially) unsaturated although it is assumed that the former is the case.

The archaeal membrane maintains metabolic processes through energy-transducing processes like transfer of electrons/protons or ATPase-dependent translocation of ions [44] . The altered transcript levels of the Fad_{Rsa} cluster, respiratory chain complex, *grsB* and the archaellum indicate that terbinafine incites a multitude of responses in *S. acidocaldarius* related to energy metabolism. The synthesis of tetraether lipids and the formation of cyclopentane rings are key adaptations which regulate its rigidity and homeostasis [45] [46] [47] [44]. A membrane formed with higher levels of IP-DGD, unsaturated CQs and lower levels of tetraether lipids might be less rigid and have higher basal permeability. The presence of unsaturated CQs in the membrane along with the upregulation of genes involved in the respiratory complex indicate that the flow of electrons is disrupted. Further, the altered expression of the F1F0-ATPase subunits suggests a possible problem with the synthesis of ATP in the cells. A possible explanation for these membrane adaptations and transcriptome response to terbinafine is that the organism adjusts to this respiratory stress by re-directing its metabolic energy for increased motility through the archaellum and not investing its net reducing power in the saturation of CQs or synthesizing complex GDGTs. However, a proteomics analysis and an assessment of the impact of terbinafine on the respiratory capabilities of the organism may further support this conclusion.

This study describes adaptation of archaeal membranes in the presence of terbinafine. Terbinafine has a multitude of effects in *S. acidocaldarius*, one of them being the altered ratio of DGD:GDGT in the membrane. Considering the effects on the respiratory chain and saturation of CQs, terbinafine appears as an indirect inhibitor of GDGT biosynthesis. The role of other isoprenoids in archaeal membrane architecture and their regulatory mechanisms remain elusive. Furthermore, the knowledge of quorum sensing in *S. acidocaldarius* is limited to a lactonase and the sensors responsible for membrane adaptations are not known [48].Therefore, present studies on archaeal membrane adaptations rely on the correlation of the changes in lipid species with the environments inhabited by these organisms.

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CQs have not been quantified in the *Sulfolobus* membrane so far, making it difficult to speculate on any possible structural role of these isoprenoids. This is the first study which shows an allylamine drug like terbinafine affecting the saturation profile of a respiratory quinone - CQ in the membrane of *S. acidocaldarius.* This drug incites respiratory stress in the organism, causing a redirection of its metabolism towards energy conservation and motility. This could possibly explain the reduced levels of complex phospholipids like DGTs in the membrane.

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4. Materials and Methods

4.1 Strains and Growth Conditions

Sulfolobus acidocaldarius MW001 was grown at 75°C by shaking at 120 rpm in Brock medium, pH 3 supplemented with 0.1% NZ-amine, 0.2% dextrin and 10 µg/mL uracil. As indicated, various amounts of a stock solution containing 250 mg/mL terbinafine hydrochloride (Sigma-Aldrich Chemie NV,final concentrations of 0.31, 0.61 and 1.22 mM) dissolved in DMSO was added such that its concentration did not exceed 0.16%. Growth curves were generated from 5 mL cultures (in biological triplicates) and growth was followed in time at $OD_{600\,nm}$ up to 72 hours. The initial inoculums were at an OD_{600nm} of 0.01.

4.2 RNA Isolation, Sequencing and Transcriptome Analysis

For RNA isolation, MW001 cultures (10 mL) were grown in duplicate until exponential phase ($OD_{600\,nm}$ of 0.3) and then subjected to terbinafine or DMSO treatment for 3 hours. Cultures were harvested (Allegra X-R benchtop cooled centrifuge, 3000 rcf, 20 mins, 4° C), and cells were processed further for isolating RNA using the Trizol method [33]. The obtained RNA was purified using ethanol precipitation. Ribosomal RNA depletion, library preparation, quantification and RNA sequencing was performed by GenomeScan BV, Leiden. Ribosomal RNA was depleted using the NEBNext rRNA depletion kit (New England BioLabs Inc.) for bacteria. Sequencing was performed with paired end reads of 150 bp. The sequenced reads were aligned against the genome of *S. acidocaldarius* DSM639 (RefSeq ID: NC_007181.1) using STAR [49]. These reads were visualized using multiQC [50]. Analysis of differential gene expression was performed using DESeq2 [51]. All p-values were adjusted to 0.05 for significance.

4.3 Lipid extraction and analyses

MW001 cells were inoculated in 200 mL of brock medium supplemented with either 0.16% DMSO or 1.22 mM terbinafine in biological triplicates. Cultures were harvested by centrifugation (3000xg, 4°C, 20 mins) at exponential and stationary phases of growth. The pellets were washed with fresh brock medium, centrifuged again, flash frozen in liquid nitrogen and freeze-dried at 0.07 mbar, -55 °C for 48 hours. Freeze-dried biomass (10 mg) was processed for lipid extraction through a modified acidic Bligh and Dyer method [52] using 0.1M HCl. Di-eoleoylphosphatidylglycerol (DOPG5 µg) was added as an internal standard. The obtained chloroform fraction was evaporated under N_2 stream to form a lipid film, re-extracted with chloroform and dried again. This step was repeated twice with 1:2 chloroform-methanol and finally with methanol. The obtained lipid film was dissolved in methanol (0.25 mg/mL) for UHPLC-MS analysis. Analysis was performed on an Accela1250 UHPLC system (Thermo Fisher Scientific) coupled to a Thermo Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with an ESI ion-source in negative mode. *Sulfolobus* lipids and quinones were separated using an Acquity UPLC CSH C18 column (2.1 × 5 mm, 1.7 µm) with an eluent flow rate of 300 µL/min at 55° C. A scan range of m/z 125–2500 was used in the full MS mode. Voltage parameters were as follows: 3kV (spray), -75 V (capillary), -190 V (tube lens) and -46 V (Skimmer). Eluent A was MilliQ:MeCN (40:60) and Eluent B was MilliQ:MeCB:1-BuOH (0.5:10:90), both containing 5 mM ammonium formate [53]. A linear elution gradient was used: 55/45 eluent A/B for 2.5 minutes; 10/90 eluent A/B from 2.5 – 24.5 mins; returning to 55/45 eluent A/B for 25-33 mins. Peak areas were integrated and calculated with the genesis algorithm

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by Xcalibur software (ThermoFisher) at 5 ppm. Relative quantification was performed using DOPG as an internal standard. Extracted ion chromatograms were visualized using xcms and MSNbase packages in R [54] [55].

Supplementary Materials: The supporting information can be downloaded at: www.mdpi.com/xxx/s1

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Data Availability Statement: Transcriptomics data used in this study can be found at : (insert link)

Conflicts of Interest: The authors declare no conflict of interest.

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