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EXPERIMENTAL ARTICLES

Survival of a Novel Bacterium *Acidiphilium symbioticum* H8 under Thermal Stress

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Abstract—An acidophilic heterotrophic gram-negative *Acidiphilium symbioticum* strain H8 is an important bacterium possessing a high capacity for heavy metal binding. As these bacteria inhabit acidic mine regions they are subjected to occasional temperature stress; thus their membrane profile is very important. Cell morphology determined by scanning and transmission electron microscopy revealed the surface and internal adaptation of the organelles. Phosphatidyl ethanolamine, phosphatidyl inositol, and phosphatidic acid are the major phospholipids affected by growth temperature. The membrane fluidity in response to temperature stress was investigated by measuring fluorescence anisotropy using 1,6-diphenyl-1,3,5-hexatriene (DPH) as the probe. Significant changes in membrane fluidity revealed the transition temperature midpoints as 11 and 38°C. To maintain the optimum fluidity increasing the percentage of *cis*-vaccenic acid at suboptimal and palmitic acid at the upper cardinal temperature support the evidence of membrane remodeling in different growth temperatures. The protein profile from SDS-PAGE provided information about different polypeptide bands which may be responsible for the different metal binding abilities of the strain.

Keywords: *Acidiphilium symbioticum*, fatty acids, membrane fluidity, morphology, phospholipids, temperature stress

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Bacteria, being poikilotherms, are often exposed to various environmental hazards and the cell envelope is always in contact with the external environment. As bacteria cannot insulate themselves from unfavorable conditions, the membrane becomes vulnerable to physical and chemical perturbations (Bohuszewicz et al., 2016). Any extrinsic factor acting on the cell envelope will induce some changes in it imposing an adaptive response by the bacteria. To respond to the variations in the extracellular environment, bacteria use lipid diversity, altering their membranes to ensure that the cell envelope remains within an optimal state and continued functionality (Peng et al., 2017). Studying bacterial cytoplasmic membrane adaptations by fatty acid analysis provides information on membrane physiology in different environments. However, the variability in lipid composition and the complexity of lipid behavior that occurs in a heterogeneous bacterial membrane makes it difficult to characterize the impact of these changes in response to the stress conditions. Thus, fluorescence polarization offers a means of a quantitative measure of the relative changes in the fluidity of the bacterial cytoplasmic membrane under any growth condition (Mykytczuk et al., 2007). Different bacterial taxa have evolved strategies for maintaining a fluid membrane in response to environmental stresses (Murínová and Dercová, 2014). Temperature, the most widely studied perturbing factor in this respect, exerts a significant/considerable fluidizing effect above the optimal growth temperature. With increasing temperature, the membrane becomes more disordered, encouraging more acyl chains to undergo the gel-to-liquid crystalline transition and even assume non-lamellar phases. Researchers also presented a concurrent analysis of several bacterial species under a range of temperature conditions resulting in a trend of increased fluidity with increasing temperature for all bacteria (Mykytczuk et al., 2007). Significant changes in phospholipid composition in bacteria exposed to temperature and other various environmental stresses were extensively studied by Hasegawa et al. (1980) and Murínová and Dercová (2014). However, such changes are less commonly documented than changes observed in fatty acyl chain structure/length. Though the effects of various stress conditions on the structure and dynamic properties of biological membranes have been extensively studied over the last few decades, such reports are not available for the acidophilic heterotrophic *Acidiphilium* strain used in this study. This acidophile, originally isolated (Bhattacharya et al., 1991) from natural ore-leaching sites, is very often exposed to temperature fluctuations

and the membrane molecules adjust themselves in accordance with such environmental disturbances by changing their structures or molar ratios. The bacterium has already been shown to have the potential as a crucial aid in bioremediation (Chakravarty and Banerjee, 2012). Thus, in the present work, we have investigated the survival strategies of the *Acidiphilium symbioticum* H8 strain using some surface characteristics and changes in morphology in response to thermal stress.

MATERIALS AND METHODS

Chemicals. All chemicals and solvents used were of high analytical or HPLC grade and were obtained from Merck, Germany. Standard phospholipids, fatty acid methyl esters, and staining reagents, e.g., Schiff base, ninhydrin, and Molybdenum Blue were purchased from Sigma, United States. Media components for bacterial cultivation were purchased from DIFCO and Hi-Media, India.

Bacterial strain and growth conditions. An acidophilic heterotroph, *Acidiphilium symbioticum* strain H8, was previously isolated in our laboratory from a culture of *Acidithiobacillus ferrooxidans* of Indian mine origin (Bhattacharya et al., 1991). At 30°C the strain was routinely subcultured aerobically with 5% (vol/vol) inocula as stated by Bhattacharya et al. (1991). The growth experiments carried out by Mahapatra and Banerjee (1996) revealed that *Acidiphilium symbioticum* strain H8 could not grow below 21°C and above 42°C, having the optimum growth temperature of 30°C. The thermally stressed cells were therefore grown at 22 and 41°C, respectively, keeping other parameters the same. The cells were harvested at the late exponential phase (OD_{540} = ca. 0.65 with a corresponding cell count of ca. 6.0×10^8 mL^{-1}). The culture reached the late exponential stage in about 27, 23, and 34 h at the cultivation temperatures of 22, 30, and 41°C, respectively.

Membrane isolation. The plasma membranes of normal and temperature-stressed *Acidiphilium symbioticum* H8 cells were isolated according to Guiliani and Jerez (2000). In the supplementary information (S1), details of the procedure have been incorporated.

Preparation of lipid extracts and transesterification. Membrane lipids were extracted by the method of Bligh and Dyer (1959), with a few modifications. In the supplementary information (S1), details of the procedure have been incorporated.

Cell membrane preparation for protein content analysis. The protein content of the plasma membrane preparation of normal and stressed bacterial cells was estimated by the method of Lowry et al. (1951). Optical density was measured at 660 nm on a Shimadzu PharmaSpec UV-1700 Series spectrophotometer. SDS-PAGE, and staining procedures were carried out as described by Pal et al. (2010).

Scanning and transmission electron microscopy. Before and after thermal stress, the bacterial cells were prepared for scanning and transmission electron microscopy as described earlier (Chakravarty and Banerjee, 2012).

RESULTS AND DISCUSSION

Morphological changes of *Acidiphilium symbioticum* H8 in response to thermal stress. Morphological variation is the most visible component of biodiversity, and its change in a stressful environment is the most visible indicator of organismal adaptation. Many bacteria have been reported to adapt to temperature stresses through morphological changes (Neumann et al., 2005). The normal flora of acidic mines often experiences various stresses, especially temperature fluctuations. Changes in cell morphology in response to temperature stress observed in this study can indicate the activity of a protective mechanism of *Acidiphilium symbioticum* strain H8. At optimum growth temperature (30°C), the cells grew as aggregates of loosely packed coccobacilli or as singles with an average size of 1.0–1.3 × 0.4–0.7 μm. Cells having a smooth surface and a notch-like appearance at one end were evident from micrographs (Fig. 1a). Less morphological changes were observed upon exposure of the cells at 22°C. While the cells were slightly elongated, the rod shape was generally preserved and deformations of the cell surface were almost absent (Fig. 1b). SEM revealed aggregates of highly packed coccobacillus-type cells at this temperature. The cell morphology at suboptimal temperature with normal cell division supported the suggestion that at this temperature the strains could adapt initially to the stressful temperature of 22°C. However, when the growth temperature was 41°C, most of the cells had an irregular shape and the average size could not be precisely measured (Fig. 1c). The presence of some elongated cells in the culture suggested that this temperature might have hindered the completion of the binary fission process. The aggregated cells showed lesions with loss of characteristic coccobacillus shape. They were found to adhere to the substrate through their body surface as evident from Fig. 1c. SEM provided strong support that the elevated temperature was stressful for the strain, as evidenced by elongation and destruction of cellular structure in the exponential phase culture. Thus, this study clearly shows that 41°C was a hostile environment to the strain. The bacterial suicidal response hypothesis (Aldsworth et al., 1999) provides a potential explanation for the destruction of the cells and cessation of cell division in cultures at high growth temperatures or subject to any other stressful treatment imposed on an exponentially growing bacterial population.

Metal ions, especially Ca^{2+}, are reported to maintain the lipopolysaccharide assembly on the cell surface of *Escherichia coli* and such ions play an import-

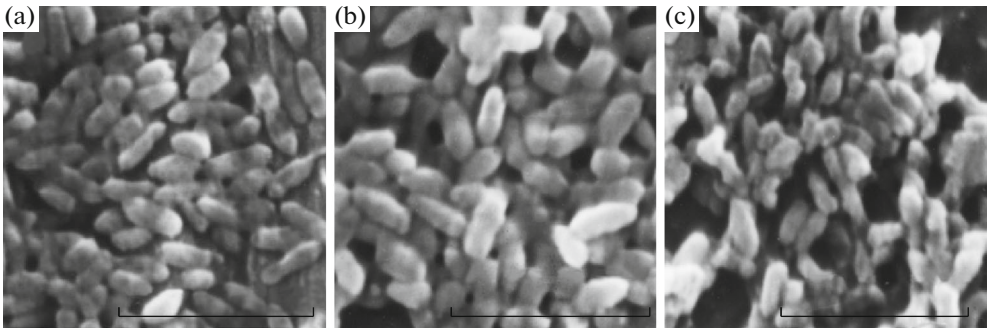

Fig. 1. SEM images of surface morphologies of *Acidiphilium symbioticum* H8 cells grown at different temperatures; (a) 30°C; (b) 22°C; (c) 41°C. The scale bar = 5 μm.

ant role in cellular metabolism through binding with a variety of proteins (Pidcock and Moore, 2001). At higher growth temperatures, bacterial calcium metabolism is hindered (Talaro, K.P. and Talaro, A., 1999), and thus the presence of extracellular polysaccharides were not observed in the *Acidiphilium symbioticum* H8 cells. Bacterial cell shape is determined by the composition of the cell envelope, where penicillin-binding proteins (PBPs) are present (Chakravarty and Banerjee, 2008). Bacterial cells under thermal stress dislodge cell-bound Ca^{2+}, thereby inactivating/modulating some of the functioning PBPs. This functional modification of normal PBPs' enzyme activity may activate other PBPs' that do not function in normal cells or may change the activity of normal enzymes; in either case, cells change their shape as evident when treated with various metal ions (Neumann et al., 2005; Chakravarty and Banerjee, 2008).

Negatively stained high resolution transmission electron microscopy of *Acidiphilium symbioticum* H8 cells showed that they were capsulated with an average (n = 100) length of 0.9–1.4 μm. Like all other gram-negative bacteria, the cells have two distinct layers on the outer surface. The entire outer surface was covered with extremely thin electron-dense, viscous, amorphous extracellular polysaccharide substances forming microfibrils or capsular filaments, which were intimately associated with the cell envelope (Fig. 2a). These might be cited as protective to the cells in toxic environments. Most of the cells possessed several randomly oriented intracellular electron-dense spherical granules with an average diameter of 150 nm (Fig. 2b). At 22°C, the cells were found mostly as coccobacilli like the normal ones; they were slightly shorter in size, with polyphosphate bodies present inside (Fig. 2b); but when grown at 41°C, they were in aggregated form with condensed cytoplasm and a rippled wavy membrane. In this case, cell dimensions became less and no distinct features were evident (Fig. 2c). At both elevated and suboptimal temperatures, polysaccharide bodies were not found.

A general view of the sections showed that the normal cells were bound by a well-defined plasma membrane and contained within a multilayered cell envelope with slight irregularities in the contour (Fig. 2d). Chemical analysis of the cell envelopes and plasmalemma appeared to be a logical course, especially in light of the high lipid content compared to the *Acidithiobacillus ferrooxidans* cells (Matlakowska et al., 2006). The plasma membrane appeared as a typical trilaminar unit membrane that expanded away from the cytoplasmic matrix. In dividing cells, the two layers of the cell surface appeared as trilaminar arches and almost oval shaped, which may be interpreted as the beginning of binary fission. With the progression of division, the area became elongated; subsequently, two nucleoids were formed with the separation of daughter cells through the septa formation. Some storage granules and lipid bodies were also found in the cells (Fig. 2d). The cytoplasm appeared dense and uniform with many dark granules—presumably ribonucleoprotein particles (RNP). A loose irregular network of fibrils of the nucleoid was observed almost in the centre of the cytoplasm. Large electron-dense bodies characteristic of volutin granules and thought to be composed of polymetaphosphate (Matsuzawa et al., 2000) have been frequently observed. Such bodies may well be the source of the hydrolyzable polyphosphate previously detected in *Acidithiobacillus thiooxidans* (Barker and Kornberg, 1954) and in *Acidithiobacillus ferrooxidans* (Lundgren et al., 1964). These polyphosphate bodies were also found to be membrane-associated in *Acidiphilium rubrum* as revealed by Matsuzawa et al. (2000).

A lot of variation in the cellular organization (viz. lack of cytoplasmic density, fragmenting ribosomes, and relatively low density of the nuclear material) was seen when the cells were grown under temperature stress which indicated a different physiological state (Figs. 2e, 2f). The cell envelope acts as a physical barrier between the cytoplasm and the stressful environment of the cell. A large accumulation of the middle layer, denser than the normal ones, was observed at 22°C (Fig. 2e). Nuclear material and other dense granules were found scattered throughout the cytoplasm along with heavily stained ribosomal aggregates.

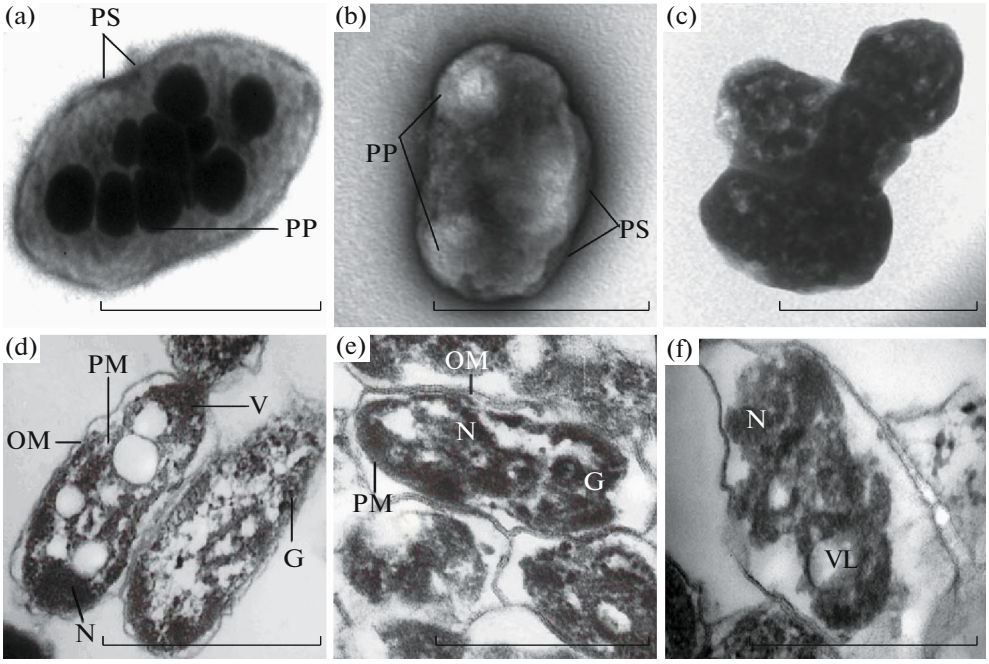

Fig. 2. TEM images of negatively stained *Acidiphilium symbioticum* H8 cells grown at different temperatures; (a), 30°C; (b), 22°C; (c), 41°C. Fine structures of (d), normal cells and thermal stressed cells grown at (e), 22°C and (f), 41°C. The scale bar = 1 μm. Cells showing outer (OM) & Plasma (PM) membrane, Granules (G), Nuclear material (N), Volutin particles (V), Polyphosphate bodies (PP), Polysaccharides (PS), Vesicles (VL).

The cell envelope appeared as a five-layer structure similar to gram-negative bacteria (Beveridge and Graham, 1991). At this temperature, granule formation from unknown origin was much higher than in the normal cells (Fig. 2e). At 41°C, the trilaminar structure of the membrane was not clearly evident. The plasma membrane of some cells remained well preserved, but the cell envelope appeared separated although still intact. The intermediate layer of the cell envelope was much thicker without any difference in density and the plasma membrane occasionally appeared to be folded into the cytoplasm (Fig. 2f). At higher temperatures the black granules of polyphosphate bodies were not evident. These granules were probably coagulated products of the high-density intracellular globules formed after cell disruption when grown at elevated temperatures (Itoh et al., 1998). X-ray diffraction pattern of such black bodies identified in *Acidiphilium rubrum* revealed the presence of Fe, Cr, and Ni, which confirms their storing nature. Surprisingly, no poly-β-hydroxybutyrate granules were found in *Acidiphilium symbioticum* H8 cells, contrary to what was revealed in *Acidiphilium rubrum* (Matsuzawa et al., 2000). The granules are mostly found in the nucleoplasm associated with the fine electron-dense fibrils interpreted as deoxyribonucleic acid material. High temperature exposure caused ultrastructural modifications (wall loosening and disruptions, cell leakage, cytoplasmic aggregation, and disappearance of extracellular polysaccharides), and such alterations were not observed in the bacteria exposed to suboptimal temperature. This finding suggests that the upper cardinal temperature is the crucial growth temperature of these bacteria. Temperature resulting in such morphological aberrations was previously reported by Beveridge and Graham (1991), Yasuda et al. (1995) and Young (2006).

Alterations in membrane phospholipid and fatty acid composition. Thin layer chromatography of the phospholipids (PLs) in different solvent systems together with their responses to the different spray reagents and comparison of their R_f values with those of the authentic standards revealed the presence of phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidyl ethanolamine (PE), phosphatidylcholine (PC), and a minor unknown component (relative mole fraction of less than 10%).

Lipids are regarded as a part of an effective adaptation system that reflects the changes in the extracellular environment (Denich et al., 2003). This adaptation allows the bacteria to survive unfavorable conditions. Variations in lipid composition enable the microorganisms to maintain membrane fluidity and functions in the face of environmental fluctuations. In the studied bacterial strain, 90–92% of phospholipids came from the membrane, and a remarkable change in the relative abundance of phospholipid composition of the strain was observed when it was grown at different temperatures (Fig. 3a). PE was the predominant lipid found in the cell membrane; its content slightly

decreased with increasing growth temperature, similar to *Geobacillus stearothermophilus* (Souza et al., 1974), *Priestia megaterium* (Eisenberg and Corner, 1973), and *Yersinia pseudotuberculosis* (Davydova et al., 2016). The relative percent level of PI was surprisingly increased (~40%) despite the decrease of PE (~15%) at the elevated temperature. The PC level was almost the same at 41°C, and a very small amount (less than 3%) was found under the suboptimal conditions. The PC loss was accompanied by the appearance of its *lyso*-form, lysophosphatidylcholine (LPC) when the growth temperature was set at 22°C. Although the temperature is known to affect the content and composition of microbial lipids and temperature-induced variations in lipid compositions are generally thought to be associated with the regulation of liquid-crystalline to gel phase transition temperature for the maintenance of the physiological state of the cell membrane, only a few reports are available on novel alterations in the amount of membrane lipids resulting from a change in growth temperature (Denich et al., 2003). Since it is evident that PE is associated with several cellular activities, its primary role in bacterial membranes is to spread out the negative charges caused by anionic membrane phospholipids over the cell membrane and it acts as a chaperone to help the membrane proteins to function properly (Bohuszewicz et al., 2016). Thus, PE distribution facilitates the change in bacterial shape, and a decrease in PE content at elevated temperatures leads to the loss of proper membrane structure in the *Acidiphilium symbioticum* H8 cells, which was also evident from the electron micrographs. Moreover, such a change will increase the propensity for forming non-bilayer phases, since PE is supposed to give the molecule a conical shape (Young, 2006). However, lowering of growth temperature will lower acyl chain fluidity and, therefore, the diameter of the lower dimension (i.e. distal to the head group) of the cone, so that it was more cylindrical and therefore more likely to form a lamellar (bilayer) phase as evident from the electron micrographs (Fig. 2b). Phosphatidic acid is the universal precursor required for the production of PLs. Though phosphatidyl glycerol (PG) is one of the main constituents of membrane phospholipids in gram-negative bacteria, the presence of PA instead of PG is the result of de novo biosynthesis, as explained by Yao and Rock (2013). Moreover, the presence of PC in the membrane of the *Acidiphilium symbioticum* H8 strain resembles that observed in *Acidithiobacillus ferrooxidans*, as revealed by Sohlenkamp and Geiger (2016). It may be due to the N-methylation pathway for PC synthesis from PE in prokaryotes (Sohlenkamp et al., 2003). Although the ability to produce PI is thought to be characteristic of eukaryotes, actinomycetes and a few δ-proteobacteria also exhibit this ability. The increase of PI concentration (~1.9 fold) at the elevated temperature may be due to a phosphatidyl inositol phosphate synthase (PIPs) enzyme, which catalyzes the reaction between inositol-1-phosphate and CDP-DAG, leading to the formation of phosphatidylinositol phosphate (PIP) (Sohlenkamp and Geiger, 2016). Recently, PI was also found in the thermophilic bacteria *Rhodothermus marinus* (Jorge et al., 2015). Thus, one can speculate that the phospholipid headgroup compositional change would counteract the effect of lowered temperature in terms of membrane fluidity and phase behavior. With increasing temperature, the lipid-to-protein ratio in the cell membrane was observed to decrease in *Acidiphilium symbioticum* H8 cells (data not shown). It may be due to higher protein content which may render the membrane more rigid and helps the organism to maintain its functionality at higher growth temperatures. When *Geobacillus stearothermophilus* was grown at different temperatures, such alterations were noted by Wisdom and Welker (1973). The ratio of zwitterionic (PE, PC) to anionic (PI, PA) phospholipids decreased by 14.76% when the bacteria were grown at 41°C. The increased amount of the anionic phospholipids or the negative charges on the membrane may be induced by thermal stress. This accumulation of acidic phospholipids might be a stabilization factor for the membrane lipid bilayer (Bakholdina et al., 2004). In this study, we observed that the phospholipid and fatty acid compositions of the acidophilic bacteria were significantly affected by growth temperature but we are still far from understanding the physiological significance and detailed molecular mechanism by which membrane phospholipids are generated.

The fatty acid composition of membrane phospholipids is the sum of complex factors maintaining the optimal viability of the cell under different environmental conditions. Bacteria have evolved mechanisms to control the formation of new fatty acids and modify the structure of existing fatty acids, thus allowing the cells to adjust membrane viscosity to match environmental requirements. This study also presents some unique observations regarding the previous reports on fatty acid compositions of various *Acidiphilium* species (Kisimoto et al., 1993; Wakao et al., 1994). Gas-liquid chromatography coupled with mass spectrometry revealed the presence of various saturated (SFA), unsaturated (UFA), and cyclic (CFA) fatty acids in the bacterial phospholipids. The major fatty acids present in these bacteria were $C_{12:0}$ (lauric), $C_{14:0}$ (myristic), $C_{16:0}$ (palmitic); $C_{18:0}$ (stearic); *cis*-$C_{16:1\delta9}$ (palmitoleic); *cis*-$C_{18:1\delta11}$ (*cis*-vaccenic); *cis*-$C_{20:1\delta11}$ (gondoic), *cis*-$C_{22:6\ \Delta4,7,10,13,16,19}$ (DHA), and a CFA, $\Delta C_{19cyc11}$ (listed in Table 1). Data presented in Table 1 show the total fatty acid spectrum as a function of temperature. The predominant fatty acids at all conditions of growth were $C_{16:0}$, $C_{18:0,}$ *cis*-$C_{16:1\Delta9}$; *cis*-$C_{18:1\Delta11}$; *cis*-$C_{20:1\Delta11}$, and $\Delta C_{19cyc11}$. Most SFA (78%) were accounted as palmitic acid at optimal growth temperature (30°C), whereas gondoic acid and DHA were the major UFAs (~88%) found in the strain when grown

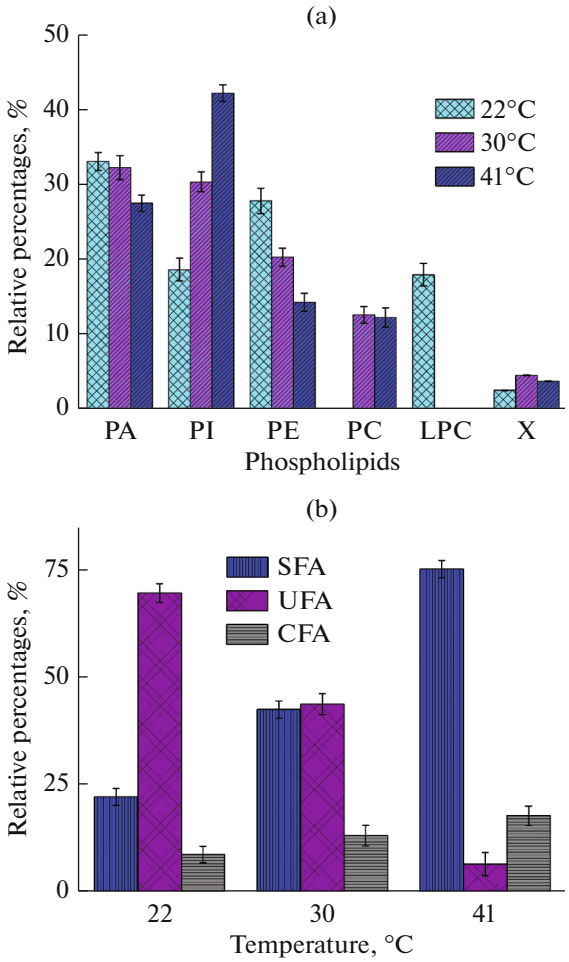

Fig. 3. (a) Relative percentages of phospholipids of the *Acidiphilium* cell grown at different temperatures. Data represent an average of five independent experiments. The error bars show the standard deviation. (b) Temperature effect on the fatty acid spectrum of the *Acidiphilium symbioticum* H8 cells when grown at different temperatures. Data represent an average of five independent experiments. The error bars show the standard deviation.

at that temperature. The relative percentages of $C_{16:0}$ and $\Delta C_{19cyc11}$ were significantly decreased when the cultivation temperature was decreased from 41 to 22°C. The relative amount of UFA (~90%) was decreased and compensated with increased SFA and CFA (Fig. 3b). DHA was the only polyunsaturated fatty acid (PUFA) found significantly when the *Acidiphilium symbioticum* strain H8 were grown at suboptimal temperature (Table 1). The degree of fatty acid unsaturation and fatty acid chain length has been found to increase in the cells at lower growth temperatures due to the increase in palmitoleic, *cis*-vaccenic and DHA and the decrease in palmitic acid. The CFA was found to increase approximately 2.0-fold at elevated temperature, whereas the ratio of UFA to SFA in the membrane increased from 0.09 to 3.11, when growth temperature was shifted from 41 to 22°C. Since individual fatty acids play an important role in indicating the degree of membrane remodeling, the sum of total fatty acids was used to determine overall changes in the membrane structure in response to thermal stress. It was found that the *Acidiphilium* strains had a major percentage of *cis*-$C_{18:1\Delta11}$ (Wichlacz et al., 1986); however, we found that *Acidiphilium symbioticum* H8 had relatively high percentages of $C_{16:0}$, *cis*-$C_{20:1\Delta11}$, and *cis*-$C_{22:6\Delta4,7,10,13,16,19}$ at optimal growth condition (Table 1). The most common response of gram-negative bacteria to decreasing growth temperature appeared to be an increase in the degree of fatty acid unsaturation and fatty acid chain length. Such a change typically occurs due to increasing biosynthesis of palmitoleic acid, *cis*-vaccenic acid, and the proportion of long-chain fatty acids like gondoic acid and DHA, accompanied by a decrease in palmitic acid. The similarity of *Acidiphilium symbioticum* H8 cells with *Pseudomonas aeruginosa* was also found when short-chain lauric acid ($C_{12:0}$) and biosynthesis of 16 carbon palmitoleic acid were increased at decreasing growth temperature (Suutari and Laakso, 1994). Interestingly, no hydroxy fatty acids were detected in the *Acidiphilium symbioticum* H8 strain under either condition, contrary to what was reported earlier for another *Acidiphilium* strain (Wichlacz et al., 1986). Under temperature stress, the alteration of relative percentages of UFA, CFA, and SFA was similar to that of other bacterial cells; i.e., with increasing temperature, the relative percentage of UFA was decreased, compensating for the increased amount of SFA to maintain the optimum membrane fluidity. At suboptimal temperatures, the increase in the degree of fatty acid unsaturation and fatty acid chain length was similar to that of the *Pseudomonas psychrophila* strain as reported by Wada et al. (1991). The synthesis of PUFA into membrane phospholipids is suggested to play an important role in adaptation in extreme conditions. Bacterial resistance was found to be enhanced against harmful substances in the presence of DHA which improves the biosynthesis of certain membrane proteins such as porins and TolC family proteins (Kannan et al., 2021). The presence of such proteins in *Acidiphilium symbioticum* H8 cells revealed by Singh et al. (2010) suggests that the cell membranes fortified with excess PUFA help to maintain membrane fluidity in adverse conditions. However, certain barophilic and psychrophilic organisms and other bacterial species are also known to produce PUFA in adverse/extreme conditions (Kannan et al., 2021). CFAs are synthesized in the bacterial membranes from monoenoic fatty acids via their further cyclization by the cyclopropane synthase, adding a methylene carbon bridge from s-adenosyl-L methionine across the double bond. Temperature-induced alteration in CFA content in the *Acidiphilium symbioticum* H8 strain is similar to that of *Limosilactobacillus fermentum* as revealed by Suutari and Laakso (1994), where the decrease in the UFA content compensated for the increase in CFA

Table 1. Fatty acid spectrum of *Acidiphilium symbioticum* H8 cells grown at different temperatures

Fatty Acids	22°C	30°C	41°C
$C_{12:0}$	01.44 ± 0.8	–	03.42 ± 1.1
$C_{14:0}$	02.06 ± 1.1	–	06.09 ± 0.9
$C_{16:0}$	10.16 ± 1.1	33.49 ± 1.2	47.02 ± 0.8
$C_{18:0}$	07.71 ± 1.4	08.46 ± 1.6	18.80 ± 1.1
Minor saturated	<0.5	<0.8	<0.7
cis-$C_{16:1\Delta9}$	15.72 ± 0.9	–	–
cis-$C_{18:1\Delta11}$	10.68 ± 0.9	05.13 ± 1.4	04.43 ± 1.2
cis-$C_{20:1\Delta11}$	15.18 ± 1.2	22.51 ± 1.6	02.27 ± 1.3
cis-$C_{22:6\ \Delta4,7,10,13,16,19}$	28.24 ± 1.3	16.32 ± 1.3	–
Minor unsaturated	<0.8	<0.5	<0.6
$\Delta C_{19cyc11}$	**08.99 ± 1.3**	**13.35 ± 0.9**	**18.02 ± 1.1**

Data represent an average of five independent experiments.

content at elevated temperature. It may be suggested that the significant amount of CFA in the cells might play a role in their acidophilicity, as acidophilic *Acidithiobacillus* species contain high levels (nearly 60%) of CFAs (Kerger et al., 1986). Dufourc et al. (1984) claimed that the inclusion of a cyclopropane ring in the membrane fatty acids increased the stability of the structural and dynamic characteristics of biological membranes. Like other gram-negative bacteria, such as *Escherichia coli* and *Pseudomonas fluorescens*, the degree of fatty acid cyclization in *Acidiphilium symbioticum* H8 cells appeared to decrease as the growth temperature decreased (Suutari and Laakso, 1994). Though Brown et al. (1997) speculated that cyclopropanation of monounsaturated fatty acids might protect the cells from chemical disturbances and that relative increase in CFAs acted as an adaptive response in *Escherichia coli* during acid shock treatment, the change in fatty acid cyclization however not be an absolute requirement for the adaptation of gram-negative bacteria to variations in environmental stresses. As the proteins have a rigidifying effect on lipid fatty acyl chains, the increase in the protein-lipid ratio in membranes at suboptimal temperatures appears to compensate for the fluidizing effect caused by the increase in UFA contents. A similar effect has been observed in *Escherichia coli* when grown at elevated temperatures (Dombek and Ingram, 1984). It may therefore be assumed that the increasing biosynthesis of double bonds by β-hydroxydecanoyl–ACP β,γ–dehydrase appears to occur over the whole decreasing growth temperature range, while the relative efficiency of elongating palmitoleic acid to *cis*-vaccenic acid by β-ketoacyl-ACP synthetase-II decreases on lowering the temperature. Thus, the fatty acid unsaturation on lowering the temperature by the increase of *cis*-vaccenic acid and DHA at the expanse of palmitic acid is apparently due to the temperature regulation of the anaerobic biosynthesis of monoenes.

Effect of temperature on the membrane protein profile. Cellular proteins of different *Acidiphilium* strains under metal stress conditions have been extensively studied by Mahapatra and Banerjee (1996), but the spectra of membrane protein profiles of *Acidiphilium symbioticum* strain H8 were not revealed. This study observed growth temperature-associated changes in membrane protein expression when *Acidiphilium symbioticum* H8 cells were grown at 22, 30, and 41°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of membrane proteins gave well-resolved polypeptide bands (Fig. 4a). Most of the bands were found in the investigated temperature range but the relative amounts of certain protein bands, as judged from staining intensities increased or decreased (to disappearing completely) due to thermal stress. At 30°C, the apparent molecular weight of the membrane proteins was determined to be 98.2, 91.0, 85.5, 66.0, 40.0, 32.2, 28.5, 26.9, 14.6, 11.4 and 2.5 kDa (lane 4), containing five significant bands that stood out in particular (bands nos. 2, 4, 5, 6, and 7 in Fig. 4a). Under the conditions employed, the total membrane fraction was resolved into approximately 15 protein bands (Fig. 4a); at suboptimal temperature (Lane 3) the band intensities at 32.2 and 11.4 kDa (band a,c) protein were decreased about 40 and 56%, respectively. The band at 'c' was absent when the cells were grown at the elevated temperature (Lane 2). Similarly, the band near 90 kDa was not found at this temperature. Moreover, the intensities of bands 'a' and 'b' decreased by about 30 and 42% at this temperature when compared with the optimal growth temperature. A spectrophotometric scanning of the gel pattern of the strain showed that the 66-kDa polypeptide band made up 18% and the 40-kDa band made up 25% of the total strain. Thus, roughly 43% of the membrane

proteins in *Acidiphilium symbioticum* strain H8 consisted of these high molecular weight proteins. When the membrane protein was fractioned into inner and outer parts by the method of Guiliani and Jerez (2000), followed by SDS-PAGE (Fig. 4b), it was found that the 40-kDa protein (Lane 1, band 'a') and 28.5 kDa (Lane 1, band 'b') were present only in the outer membrane fraction of the cell grown at the optimum temperature. A binding protein is involved in maintaining the cell shape, division and elongation. The involvement of such PBPs in maintaining the rod shape of *Escherichia coli* was reported by Spratt (1975). The PBP of 91 kDa is considered to be involved in maintaining cell shape and elongation in *Escherichia coli* and was found to be absent when the cells were grown at an elevated temperature. Since a similar phenomenon was observed in the *Acidiphilium symbioticum* H8 strain, it may be assumed that the 91-kDa peptide band is a PBP present in the cells. Acidophiles require a different kind of molecular sieve in their outer membrane to control the passage of ions in the presence of a very high concentration of protons. This may require the existence of various types of outer membrane proteins in them. Two distinct protein bands (40 and 28.5 kDa), which were found in the membrane fractions, might be transmembrane pro-

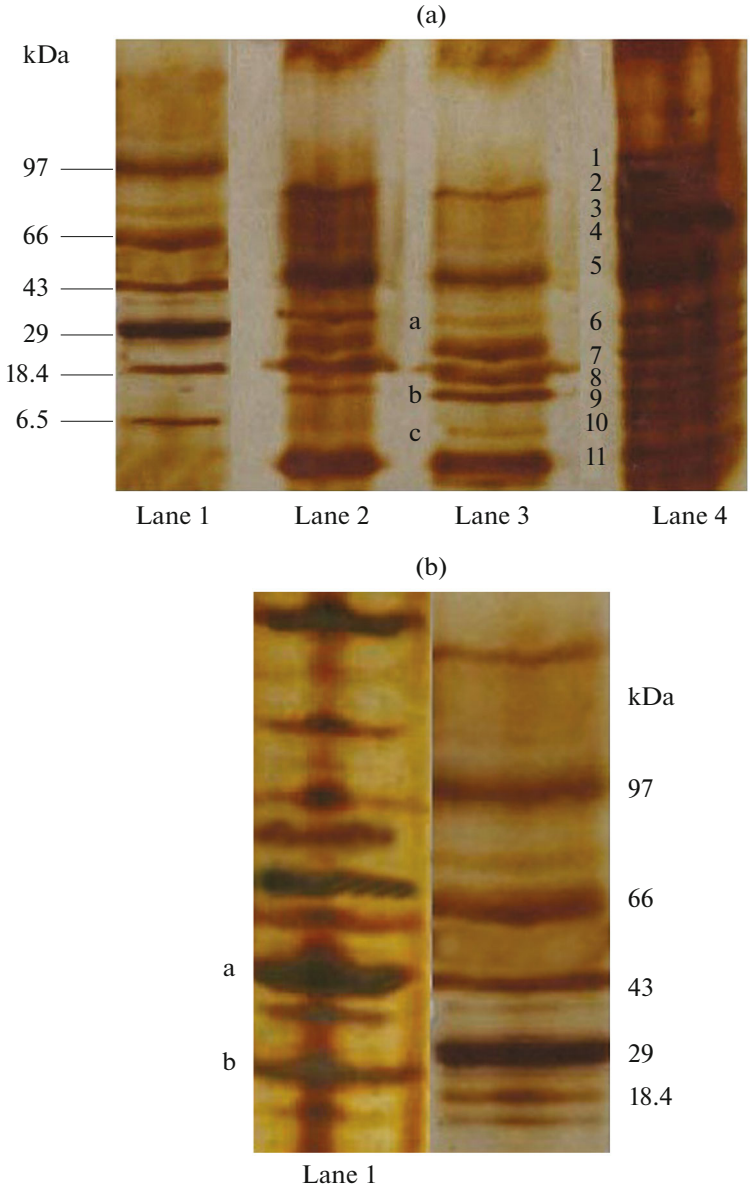

Fig. 4. (a) Silver nitrate stained SDS-PAGE of membrane protein profile of *Acidiphilium symbioticum* H8 cells. Lane 4: 30°C; Lane 3: 22°C; Lane 2: 41°C. (b) Silver nitrate stained SDS-PAGE of outer membrane protein profile of the cell grown at optimum temperature (band 'a': 40 kDa; band 'b': 28.5 kDa).

teins and possibly a metal pump. The *omp40* component from the acidophilic *Acidithiobacillus ferrooxidans* was supposed to be a porin-like protein, apparently regulated by external pH (Rodriguez et al., 1986). The relative amounts of the porins are interdependent and influenced by environmental factors such as osmolarity of the medium, temperature and external pH as shown for *Escherichia coli* and other heterotrophs (Heyde and Portalier, 1987). Thus, gram-negative bacteria adapt themselves to the environment by adjusting the number of pores on their surface, depending on the conditions they are exposed to. It should be noted that the absence or low-level synthesis of some normal membrane proteins or generation of novel proteins under thermal stress are very common bacterial phenomena and such proteins are distinguished as resistance-nodulation-cell division (RND) group proteins (Nies, 2003). Earlier, we have shown that the *Acidiphilium symbioticum* strain H8 had a strong binding capacity for certain heavy metal ions (Mahapatra and Banerjee, 1996; Chakravarty and Banerjee, 2012) and membrane proteins of this strain may play a vital role in this phenomenon. Cd^{2+} induced synthesis of 14.5-kDa proteins involved in cadmium efflux in *Staphylococcus aureus* (Nies, 2003). A similar protein band is found in the *Acidiphilium symbioticum* strain H8, which may be involved in heavy metal binding. In *Pseudomonas syringae*, the new protein bands at 12, 39, and 72 kDa were observed in the presence of Cu^{2+} (Brown et al., 1995). Co^{2+}, Zn^{2+} and Cd^{2+} induced synthesis of *czc* proteins of 24, 41.5, 62.5 and 128 kDa in *Wautersia eutropha* (Nies, 2003). Moreover, inducible Co^{2+} and Ni^{2+} resistance proteins of 10.6, 11, 11.6, 15.5, 40, 44, and 105 kDa were expressed in the same organism (Liesegang et al., 1993). In *Salmonella enterica*, transport proteins of 42, 91 and 102 kDa were synthesized in the presence of Cd^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+} (Nies, 2003). The protein bands, expressed in the presence of different heavy metal ions, resembled the bands observed in our strain. Thus, it may be assumed that these proteins are also involved in heavy metal binding in the *Acidiphilium symbioticum* cell membrane. As the cellular membrane plays a critical role in (i) thermal sensing and signaling, (ii) protecting the cell against heat damage and (iii) the chain of events in thermotolerance adaptation, the changes in the membrane protein profile due to thermal stress may have a close relationship with other morphological, biochemical and biophysical properties of the cells. However, further detailed investigation regarding the proteomic analysis of the *Acidiphilium symbioticum* H8 strain may help to understand the survival strategies in a stressful environment.

In this study, we have reported the unique adaptational strategies of the acidophilic bacterium *Acidiphilium symbioticum* strain H8 to temperature stress. The results revealed that the mechanism for maintaining the optimal physiological conditions involved morphological aberrations and membrane fluidity. Membrane homeostasis is not restricted to a constant membrane fluidity but also contributes to the dynamic range of cytoplasmic membrane behavior in the cell by altering the phospholipid-fatty acid composition. It is also revealed that the growth in thermal stress conditions typically modulates membrane phase transition points to a greater extent. The presence/absence of different protein bands also influences the survival strategies of the cells. In this context, further study is required to correlate with the membrane physiology, acidophilicity and heavy metal resistance of the strain.

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COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any studies involving animals or human participants performed by any of the authors.

The authors declare that they have no conflicts of interest.

SUPPLEMENTARY INFORMATION

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