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ORIGINAL ARTICLE

Response of cellular fatty acids to environmental stresses in endophytic *Micrococcus* spp.

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Abstract The response of cellular fatty acids to various environmental stresses was studied using two endophytic species of *Micrococcus*. A total of 18 samples with three biological replicates from low, moderate and high stress conditions of salt (0.5, 5 and 10 % NaCl), pH (5, 7 and 10) and temperatures (15, 25 and 41 °C) were analysed. Branched chain fatty acids dominated in both the organisms, while saturated and unsaturated fatty acids were detected less frequently. The mole percentage of isoforms of branched chain fatty acids gradually increased with increasing salinity and showed more than a twofold increase at higher concentration of salt (10 %). Unlike *Micrococcus yunnanensis* DSM 21948^T, *Micrococcus aloeverae* MCC 2184^T showed more agreement with previous findings related to stress tolerance in other bacteria. Data indicate that iso fatty acids are responsible for the growth of *Micrococcus* at high salt concentration. In addition, instead of individual fatty acids, the ratio of the total content of iso/anteiso forms modulates membrane fluidity and functions during environmental stress in *Micrococcus*. For a comparative study of salinity stress in Gram-positive and Gram-negative bacteria, the strain of *Halomonas* was alsoincluded.

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Introduction

The genus *Micrococcus* was established by Cohn (1872), with *Micrococcus luteus* as the type species. Stackebrandt et al. (1995) dissected members of the genus *Micrococcus* into four different genera, including *Kocuria*, *Nesterenkonia*, *Kytococcus*, and *Dermacoccus*. Later, Wieser et al. (2002) re-evaluated the traits of *Micrococcus* and published its emended description. *Micrococcus* is widely distributed and has been isolated from a variety of habitats (Cohn 1872; Wieser et al. 2002; Liu et al. 2007; Rieser et al. 2013). The emended description of the genus *Micrococcus* revealed that growth at high pH (pH 10) is a characteristic feature of members of the genus *Micrococcus* (Wieser et al. 2002). Despite growth at high pH, most of the type species of the genus *Micrococcus* have been isolated from extreme habitats and are known to grow in a wide range of salt concentrations, pHs and temperatures. Due to growth in a wide range of salt concentrations, pHs and temperatures, *Micrococcus* seems an ideal candidate for the study of physiological adaptations to harsh conditions of salt, pH and temperature.

During stress, bacteria modulate their membrane lipid and fatty acid content to maintain membrane fluidity, stability and transport. The stress tolerance capacity of the bacteria is related to their high content of odd-numbered iso and anteiso branched-chain fatty acids, and their capacity to modulate the amount of saturated and unsaturated fatty acids (Julaka et al. 1989; Giotis et al. 2007). Although adaptation studies, in terms of cellular and membrane fatty acids, have been conducted with other groups, including yeast, *Listeria monocytogenes*, *Bacillus subtilis*, *Lactobacillus*, and *Pseudomonas*, data on the genus *Micrococcus* are lacking.

In the present article, we studied the effect of various levels of different environmental stresses on membrane adaptability and changes in fatty acids content in two different endophytic species of *Micrococcus*. Our results show that *M. yunnanensis* DSM 21948^T and *M. aloeverae* MCC 2184^T demonstrated totally opposite patterns of adaptation for temperature and pH; however, the response for salt tolerance was more or less similar in both organisms. Almost all isoforms of branched-chain fatty acids (BCFA) increased in content with salt in both strains of *Micrococcus*, while no drastic change was reported in *Halomonas* sp. strain HBL-15 (taken as model organism for Gram-negative bacteria). Our findings indicate that isoforms of BCFA play an important role in the adaption of *Micrococcus* to high salt content.

Materials and methods

Chemicals and bacterial strains Media and chemicals were purchased from HiMedia (Mumbai, India) and Sigma-Aldrich, USA, respectively. *Micrococcus yunnanensis* DSM 21948^T was isolated by Zhao et al. (2009) from the roots of *Polyspora axillaris*, procured from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Germany. *Micrococcus aloeverae* MCC 2184^T was isolated in our laboratory from the leaf tissues of *Aloe barbadensis* (Aloe Vera), as described by Prakash et al. (2014). *Halomonas* sp. strain HBL-15 was also isolated in our laboratory, from sediment samples of an alkaline and saline lake. Identity of the strains was confirmed using the polyphasic approach of bacterial taxonomy (Prakash et al. 2012; Prakash et al. 2014). Strains were maintained with 15 % glycerol in liquid nitrogen (Prakash et al. 2013) and deposited in the Microbial Culture Collection, Pune, India with the accession no. MCC 2184. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain M. aloeverae is KF524364.

Culture conditions and biological replicates Growth data for *M. yunnanensis* DSM 21948^T were taken from Zhao et al. (2009) and re-confirmed in our laboratory. Growth data for *M. aloeverae* MCC 2184^T and *Halomonas* sp. strain HBL-15 were generated during this study. Tryptic soy broth agarised (TSBA) was selected to grow the organisms for fatty acid analysis, due to the availability of the extensive FAME database of Microbial IDentification Inc (MIDI) Newark, Delaware, USA, on this medium. At low pH (pH 5), TSBA did not support growth, and hence strains were cultivated on nutrient agar for the pH study. To adapt the cells to selected stress conditions before the extraction of fatty acids, cultures were sub-cultured up to three consecutive generations on the same media and under similar stress conditions. To create authentic biological replicates of each sample, the same bacterium was streaked on three different plates (three replicates) of same the medium and incubated in similar conditions. Using the above-mentioned approach, biological replicates of both the organisms were created for different levels of stress by salt (0.5, 5 and 10 % NaCl), pH (5, 7, 10) and temperatures (15, 25, 40 °C). To achieve a pH of 5.0, 7.0 or 10.0 during the pH experiment, medium was buffered with 10 mM homopiperazine-N,N'-bis-2-(ethane sulfonic acid) (HOMOPIPES, Fisher Scientific), HEPES (Fisher Scientific) and sodium carbonate, respectively. After 72 h of growth, 40 mg cells were scraped from each of the biological replicates and used for fatty acid extraction.

Fatty acid extraction and analysis

Prior to running the actual samples, extraction efficiency of the protocol and authenticity of fatty acid peaks were verified using the type strain of *Stenotrophomonas maltophilia* ATCC 13637^T (with known fatty acid profile) as a positive control. Accuracy of the instrument and analytical procedure was confirmed using six analytical replicates from the same sample. All biological replicates of the same sample were extracted and injected separately.

The extraction and analysis of FAME was conducted as described by Buyer (2008). In brief, at first, bacterial cells were saponified with 15 % NaOH prepared in 50 % methanol and water. The saponified solution obtained above was methylated for 10 min at 80 °C with 3 N HCl in 45 % methanol and extracted using the solvent hexane-methyl tert butyl ether (1:1). Extracted FAME solution (1 ml) was gently washed with 3 ml of 0.3 N NaOH, pipetted off in pre-cleaned vials with teflon coated septum, and used for injection and analysis.

Methyl esters were analysed on a gas chromatograph (Agilent Technologies, CA, USA, Model 7890A), equipped with a flame ionization detector (FID), auto-sampler and capillary column (HP-5 column, 25 m x 0.2 mm x0.33 μm). Hydrogen was used as the carrier gas at a flow rate of 1.3 ml min^{-1}. The column oven temperature was in gradient mode; the initial temperature was set at 170 °C and it increased to 310 °C at a rate of 28 °C min^{-1}. The injector and the detector temperature was set at 250 and 300 °C, respectively. Samples (2 μl) were injected in split mode with a split ratio of 40:1. Before starting the sample analysis, the gas chromatography system was calibrated twice with a calibration standard no. 1300AA (MIDI, USA). After that; calibration was automatically rechecked after every 11th sample. Peaks were identified based on retention time of standard run in a similar set of conditions, using the software and database (RTSBA6) of MIS, MIDI Inc.

In order to conduct a comparative study on "membrane response for salinity stress in Gram-positive as well as in Gram-negative bacteria" along with *Micrococcus* (Gram-

positive), a halotolerant strain of *Halomonas* sp. strain HBL-15 (Gram-negative) was also included as a model system for this study. Biological replicates of *Micrococcus* and *Halomonas* were grown simultaneously at three different concentrations of salt. Extraction and analysis of fatty acids was performed using the similar set of conditions discussed earlier.

Statistical analysis of data

Data for individual peaks (mol %) from all the biological replicates were tabulated in excel. The means of the replicates, and standard deviations and standard errors of the means were calculated in excel. Furthermore, analysis of the variance of all the means (ANOVA) for all three treatments for every factor was calculated separately, using the free statistical software (ezANOVA) available at http://www.mccauslandcenter.sc.edu/mricro/ezanova.

Polar lipid analysis

To support the findings of FAME data, polar lipids were also extracted and analysed from cells grown under different conditions of stress. Fatty acid analysis data indicated that the major variation in FAME occurred in the case of salinity and temperature; therefore, we analysed the change in polar lipids from variable conditions of salinity and temperature only. Extraction and analysis of polar lipids was conducted using a protocol similar to that described in Prakash et al. (2014). In brief, cells were grown in different conditions of salinity and temperature, harvested by centrifugation, and lyophilised. Polar lipids were extracted from an equal biomass of lyophilised cells using chloroform:methanol (2:1) as solvent. After extraction, the solvent was evaporated to dryness, and residual lipids were dissolved in chloroform:methanol (1:1) and spotted on silica gel 60 F_{254} plate (Merck, Germany) using glass capillary. One dimensional TLC was run using chloroform:methanol:water (65:25:4) as the solvent system, and developed with 5 % molybdophosphoric acid in ethanol. Spots were visualized by heating the plate at 110 °C for 10–15 min using a hot air gun, and identified using previously published data as a standard reference (Albuquerque et al. 2008; Zhao et al. 2009; Prakash et al. 2014).

Results

Physiological data indicate that *M. yunnanensis* DSM 21948^T and *M. aloeverae* MCC 2184^T grew in a wide range of salt concentrations (up to 12 % NaCl), pHs (5–12) and temperatures (15–42 °C). Qualitative (number of peaks) as well as quantitative (mol % of peaks) data of all the six analytical replicates were almost the same, with lower values of standard deviation (nSD±0.01), standard error (nSE±0.01) and variance (nVar 0.01). This indicates that analytical accuracy of the instrument was good and the protocol was optimized. Furthermore, small values of nSD and nSE (less than 1.0 and 0.5) in the case of most of the biological replicates (Tables 1, 2 and 3), respectively, indicated that values of each individual sample were less deviated from the mean and the sampling width was appropriate. In addition, lower p values ($p<0.05$) of all means, which is desired to prove the null hypothesis in most of the cases, indicated that our hypothesis (different levels of various factors effect membrane fatty acid contents) was right, and showed a higher confidence interval (CI, more than 95 %). Experiments were repeated twice. Data from both the experiments were almost similar and complemented each other.

Temperature

In both the organisms, saturated fatty acids increased with temperature. The amount of $C_{14:0}$ and $C_{16:0}$ increased almost eightfold and 15-fold, respectively, from 15 to 41 °C in *M. aloeverae* MCC 2184^T. Unsaturated fatty acids were only detected at the lower temperature in the case of *M. yunnanensis* DSM 21948^T, but they were absent in *M. aloeverae* MCC 2184^T. Among the dominant BCFAs, the contents of iso-$C_{14:0}$ increased with increasing temperature in *M. yunnanensis* DSM 21948^T but was almost the same in *M. aloeverae* MCC 2184^T. In contrast, iso-$C_{15:0}$ iso showed the opposite trend, and it increased in *M. aloeverae* MCC 2184^T, but decreased in *M. yunnanensis* DSM 21948^T. The quantity of iso-$C_{16:0}$ initiallyincreased from 15 to 25 °C, but decreased later at higher temperatures in both the organisms. The contents of $C_{13:0}$ and anteiso-$C_{15:0}$ decreased in *M. aloeverae* MCC 2184^T, but increased in *M. yunnanensis* DSM 21948^T with increasing temperature. The amount of anteiso-$C_{17:0}$ initiallyincreased from 15 to 25 °C, and then decreased in both the organisms (Table 1 and Fig. 1). Figure 1 indicates that the total content of iso and saturated fatty acids gradually increased with temperature, but the contents of anteiso decreased in *M. aloeverae* MCC 2184^T. We also found similar trends in *M. yunnanensis* DSM 21948^T from 15 to 25 °C. Unlike *M. aloeverae* MCC 2184^T, at higher temperature, isoforms decreased and anteiso increased from 25 to 40 °C in *M. yunnanensis* DSM 21948^T.

Salt

Saturation decreased with salinity in *M. aloeverae* MCC 2184^T, but increased in *M. yunnanensis* DSM 21948^T. Unsaturated fatty acids were only detected at high salt in *M. aloeverae* MCC 2184^T, but were not detected in *M. yunnanensis* DSM 21948^T. The contents of iso-$C_{13:0}$, iso-$C_{14:0}$ and iso-$C_{15:0}$ increased with salinity in both the organisms. Also, the content of iso-$C_{16:0}$ initiallyincreased from

Table 1 Cellular fatty acid composition of *M. aloeverae* MCC 2184^T and *M. yunnanensis* DSM 21948^T in variable conditions of temperature

FAME	*Micrococcus aloeverae* MCC 2184^T				*Micrococcus yunnanensis* DSM 21948^T			
	15 °C	25 °C	40 °C	PV	15 °C	25 °C	40 °C	PV
Saturated								
14:0	0.54±0.15	0.82±0.27	4.15±0.87	0.00	0.24±0.03	0.38±0.05	0.53±0.05	0.00
16:0	0.85±0.19	3.64±1.06	12.66±1.59	0.00	0.42±0.05	0.85±0.08	1.46±0.15	0.00
17:0	nd	nd	0.29±0.05	0.00	nd	nd	nd	nd
18:0	0.21±0.01	nd	0.13±0.02	0.00	nd	nd	nd	nd
Iso-branched chain								
13:0 iso	0.41±0.11	0.11±0.01	0.35±0.07	0.00	0.23±0.01	0.29±0.005	0.32±0.01	0.00
14:0 iso	**3.56±0.34**	**3.26±0.26**	**4.06±0.10**	**0.02**	**4.64±0.06**	**3.89±0.06**	**8.9±0.03**	**0.00**
15:0 iso	**8.01±0.27**	**8.23±0.50**	**18.01±2.16**	**0.00**	**12.49±0.11**	**14.07±0.27**	**9.17±0.30**	**0.00**
16:0 iso	**4.15±0.40**	**12.24±1.92**	**7.51±1.12**	**0.00**	**5.86±0.20**	**13.33±0.36**	**7.5±0.28**	**0.00**
17:0 iso	nd	0.35±0.01	0.8±0.07	0.00	0.15±0.005	0.46±0.02	0.13±0.05	0.00
Anteiso-branched chain								
11:0 ante	0.45±0.07	nd	nd	*	0.18±0.02	nd	nd	*
13:0 ante	1.69±0.22	0.52±0.03	0.74±0.05	0.00	0.53±0.02	0.46±0.01	1.01±0.03	0.00
15:0 ante	**75.54±0.93**	**65.17±0.49**	**47.52±2.35**	**0.00**	**65.47±1.54**	**60.96±0.29**	**68.33±0.47**	**0.00**
17:0 ante	**3.44±0.28**	**5.49±0.25**	**3.26±0.75**	**0.00**	**1.67±0.05**	**4.74±0.10**	**2.09±0.06**	**0.00**
Unsaturated								
20:1 w7c	nd	nd	0.08±0.01	*	nd	nd	0.22±0.03	*
18:1 w9c	nd	nd	0.11±0.005	*	nd	nd	nd	nd
16:1 w5c	Nd	nd	nd	*	0.55±0.01	nd	nd	
15:1 w5c	nd	nd	nd	*	1.46±0.02	nd	nd	*
15:1 w8c	nd	nd	nd	*	0.2±0.005	nd	nd	*
15:1 w6c	nd	nd	nd	*	0.26±0.06	nd	nd	*
14:1 w5c	nd	nd	nd	*	0.81±0.01	nd	nd	*
16:1 iso H	nd	nd	nd	*	2.91±0.04	0.25±0.02	nd	0.00
16:1 w9c	nd	nd	nd	*	0.3±0.01	nd	nd	*
17:1 ante w9c	nd	nd	nd	*	1.34±0.02	0.12±0.02	nd	0.00
Hydroxy								
15:0 iso 3OH	nd	nd	0.14±0.01	0.00	nd	nd	nd	nd
16:0 iso 3OH	0.16±0.02	nd	0.09±0.02	0.00	0.18±0.05	0.12±0.01	0.15±0.01	0.00
17:0 2OH	0.18±0.03	nd	nd	0.00	nd	nd	nd	*
16:0 3OH	nd	nd	0.08±0.01		0.2±0.01	nd	nd	*
15:0 2OH	1.03±0.23	0.24±0.10	0.27±0.03	0.00	0.29±0.01	0.16±0.01	0.32±0.01	0.00

Data are means of three biological replicates and are mole % of total fatty acids contents; values after (±) are standard deviations

In most of the cases, values of standard errors are almost half the values of standard deviations (data not shown)

Values in bold represent quantitatively dominant fatty acids

nd not detected; *PV p* value of ANOVA; * *p* values are not calculated due to single mean

25 °C was treated as a control for the comparison of the effects of lower and higher temperatures

0.5 to 5 % in *M. aloeverae* DSM 21948^T and then decreased at 10 %, while it was almost same in *M. yunnanensis* DSM 21948^T. Anteiso-$C_{15:0}$ initiallyincreased from 0.5 to 5 % and then decreased at 10 % salt in both organisms, while 17:0 anteiso decreased continuously in both (Table 2 and Fig. 2). Thus, it was found that the total content of the isoforms of fatty acids increased with salinity in both organisms, but the change in anteiso forms was variable (Fig. 2).

pH

Unlike other stresses (salt and temperature), change was not very prominent at different levels of pH. Except for $C_{16:0}$ in the case of *M. aloeverae* DSM 21948^T, change in other saturated fatty acids was not noticeable in either organism. All forms of iso fatty acids increased with alkalinity in *M. yunnanensis* DSM 21948^T, but were almost the same in

Table 2 Cellular fatty acids composition of *M. aloeverae* MCC 2184^T and *M. yunnanensis* DSM 21948^T in variable conditions of salt

FAME	*Micrococcus aloeverae* MCC 2184^T				*Micrococcus yunnanensis* DSM 21948^T			
	0.5 %	5 %	10 %	PV	0.5 %	5 %	10 %	PV
Saturated								
12:0	0.17±0.05	nd	nd	*	nd	nd	nd	*
14:0	1.59±0.27	0.43±0.01	0.52±0.06	0.00	0.52±0.09	0.31±0.06	0.74±0.04	0.00
16:0	8.74±0.37	1.08±0.15	1.02±0.12	0.00	1.16±0.12	0.81±0.07	2.21±0.27	0.00
17:0	2.01±0.66	nd	nd	*	nd	nd	nd	*
Iso-branched chain								
13:0 iso	0.19±0.05	0.27±0.04	0.97±0.15	0.00	0.27±0.03	0.35±0.01	0.52±0.03	0.00
14:0 iso	**1.85±0.33**	**5.3±0.35**	**8.74±0.92**	**0.00**	**2.07±0.15**	**2.93±0.45**	**4.54±0.39**	**0.00**
15:0 iso	**15.3±1.96**	**12.67±1.29**	**20.53±1.73**	**0.00**	**10.04±0.91**	**12.74±0.64**	**16.72±1.04**	**0.00**
16:0 iso	**7.39±0.60**	**9.97±0.99**	**5.48±0.65**	**0.00**	**11.51±0.42**	**9.56±0.18**	**11.47±1.48**	**0.06**
17:0 iso	0.89±0.05	0.29±0.03	0.24±0.03	0.00	0.52±0.02	0.48±0.02	0.66±0.11	0.04
Anteiso-branched chain								
11:0 ante	nd	nd	0.18±0.05	*	nd	nd	nd	*
13:0 ante	0.23±0.01	0.81±0.07	1.62±0.23	0.00	0.41±0.01	0.57±0.04	0.71±0.09	0.00
15:0 ante	**54.08±1.54**	**64.72±0.16**	**55.38±2.76**	**0.00**	**63.1±0.48**	**66.07±1.37**	**57.31±3.12**	**0.00**
17:0 ante	**7.68±1.38**	**3.48±0.56**	**1.52±0.12**	**0.00**	**10.2±0.62**	**6.1±0.41**	**5.12±0.05**	**0.00**
Unsaturated								
16:1 w5c	nd	nd	1.36±0.05	*	nd	nd	nd	*
14:1 w5c	nd	nd	2.83±0.12	0.00	nd	nd	nd	*
15:1 w5c	nd	nd	5.33±0.57	0.00	nd	nd	nd	*
16:1 w5c	nd	nd	1.36±0.01	0.00	nd	nd	nd	*
16:1 iso H	nd	nd	nd	*	0.28±0.01	nd	nd	0.00
17:1 ante w9c	nd	nd	nd	*	0.32±0.05	nd	nd	0.00
Hydroxy								
15:0 2OH	0.15±0.01	0.2±0.02	0.24±0.06	0.09	nd	0.17±0.01	nd	*
16:0 iso 3OH	nd	0.11±0.01	nd	*	nd	0.21±0.01	nd	*
17:0 2OH	nd	nd	nd	*	nd	0.16±0.01	nd	*

Data are means of three biological replicates and are mole % of total fatty acids contents; values after (±) are standard deviations

In most of the cases, values of standard errors are almost half the values of standard deviations (data not shown)

Values in bold represent quantitatively dominant fatty acids

nd not detected; *PV p* value of ANOVA; * *p* values are not calculated due to single mean

0.5 % salt was treated as a control for comparison of the increasing effects of salinity

the case of *M. aloeverae* MCC 2184^T. In contrast, all forms of anteiso fatty acids increased with alkalinity in *M. aloeverae* MCC 2184^T but the contents of anteiso forms were almost the same in *M. yunnanensis* DSM 21948^T (Table 3 and Fig. 3). Iso-$C_{14:0}$, iso-$C_{15:0}$, iso-$C_{16:0}$ and anteiso-$C_{15:0}$ and $C_{17:0}$ were detected as dominant fatty acids at all the pH levels.

Polar lipid analysis

Spots of phosphatidylinosito (PI), phosphatidylglycerol (PG), and diphosphatidylglycerol (DPG) were detected in the chromatogram, along with minor quantities of glycolipid and an unidentified phospolipid (Fig. 4). Only phospholipids constitute a major part of the total lipid profile of *Micrococcus*, while the contributions of other lipids (amino, glyco sphingo, etc.) are negligible. Diphosphatidylglycerol (DPG) is detected as a prominent phospholipid in both organisms, as reported in previous findings (Zhao et al. 2009; Prakash et al. 2014). Qualitative as well as quantitatve variability in the polar lipid profile is visible in the different conditions of salinty and temperature, and more prominent in the case of salinity stress (Fig. 4).

Discussion

In comparison to the saturated and unsaturated fatty acids, iso and anteiso forms dominated the profiles of both the organisms (Figs. 1, 2 and 3). Among the branched-chain fatty acids,

Table 3 Cellular fatty acids composition of *M. aloeverae* MCC 2184^T and *M. yunnanensis* DSM 21948^T in variable conditions of pH

FAME	*Micrococcus aloeveare* MCC 2184^T				*Micrococcus yunnanensis* DSM 21948^T			
pH	5	7	10	PV	5	7	10	PV
Saturated								
12:0	0.22±0.02	0.17±0 .01	nd	0.00	nd	nd	nd	*
14:0	**2.37±0.11**	**1.59±0.27**	**2.13±0.86**	**0.25**	**0.48±0.02**	**0.52±0.08**	**0.4±0.05**	**0.15**
16:0	**18.64±0.83**	**8.74±0.37**	**6.9±2.08**	**0.00**	**1.27±0.04**	**1.16±0.11**	**1.21±0.10**	**0.41**
17:0	0.33±0.02	2.01±0.66	0.84±0.03	0.00	nd	nd	nd	*
Iso-branched chain								
13:0 iso	0.13±0.01	0.19±0.01	nd	0.00	0.27±0.03	nd	0.3±0.02	0.00
14:0 iso	1.63±0.08	1.85±0.33	2.03±0.40	0.35	1.86±0.02	2.07±0.15	2.67±0.17	0.00
15:0 iso	**16.2±0.31**	**15.3±1.96**	**16.43±1.85**	**0.69**	**10.36±0.13**	**10.04±0.91**	**14.81±4.87**	**0.29**
16:0 iso	**8.32±0.26**	**7.39±0.60**	**6.91±0.36**	**0.02**	**11.18±0.40**	**11.51±0.42**	**13.42±0.36**	**0.00**
17:0 iso	1.05±0.06	0.89±0.05	0.82±0.07	0.01	0.57±0.02	0.52±0.02	0.64±0.03	0.00
Anteiso-branched chain								
13:0 ante	0.13±0.05	0.23±0.01	0.24±0.03	0.00	0.36±0.03	0.41±0.01	0.41±0.02	0.08
15:0 ante	44.55±0.60	54.08±1.54	55.9±2.75	0.00	62.16±0.07	63.1±0.48	62.3±3.96	0.87
17:0 ante	6.37±0.26	7.68±1.38	7.63±2.11	0.29	10.78±0.46	10.2±0.62	10.09±1.44	0.50
Unsaturated								
16:1 iso H	nd	nd	nd	*	0.35±0.11	0.28±0.01	0.31±0.05	0.58
17:1 ante w9c	nd	nd	nd	*	0.62±0.02	0.32±0.05	0.35±0.01	0.00

Data are means of three biological replicates and are mole % of total fatty acids contents; values after (±) are standard deviations

In most of the cases values of standard errors are almost half the values of standard deviations (data not shown)

Values in bold represent quantitatively dominant fatty acids

nd not detected; *PV p* value of ANOVA; * *p* values are not calculated due to single mean

pH 7.0 was treated as a control for comparison of the effects of alkalinity and acidity

odd-numbered (iso-$C_{15:0}$ and anteiso-$C_{15:0}$) dominated in both the organisms, followed by even-chain iso-($C_{14:0}$ and $C_{16:0}$) and odd-chain anteiso-($C_{17:0}$ and $C_{13:0}$) forms (Tables 1, 2 and 3). The pattern of change in fatty acid contents varied with different kinds of stresses in both the organisms. High

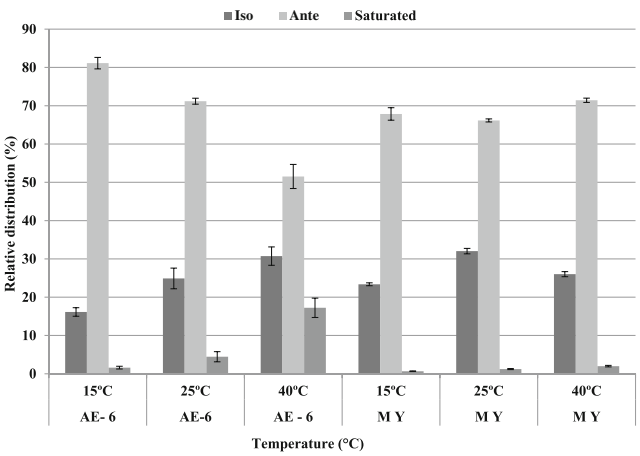

Fig. 1 Relative distributions of different classes of cellular fatty acids of *M. aloeverae* MCC-2184^T and *M. yunnanensis* DSM 21948^T in variable conditions of temperature. Unsaturated fatty acids were not detected. Data are sums of the means of different fatty acids belonging to the same group and obtained from three biological replicates. Error bars indicate standard deviations in the data. The abbreviations AY and MY represent *Micrococcus aloeverae* MCC 2184^T and *Micrococcus yunnanensis* DSM 21948^T, respectively

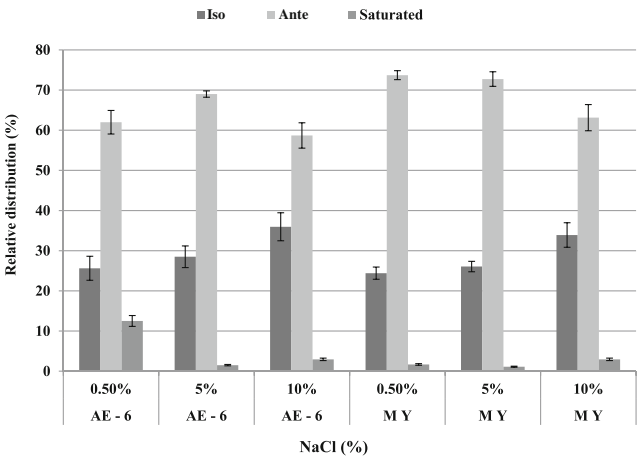

Fig. 2 Relative distributions of different classes of cellular fatty acids of *M. aloeverae* MCC 2184^T and *M. yunnanensis* DSM 21948^T in variable conditions of salinity (NaCl). Unsaturated fatty acids were not detected. The data are sums of the means of different fatty acids belonging to the same group and obtained from three biological replicates. Error bars indicate standard deviations in the data. The abbreviations AY and MY represent *Micrococcus aloeverae* MCC 2184^T and *Micrococcus yunnanensis* DSM 21948^T, respectively

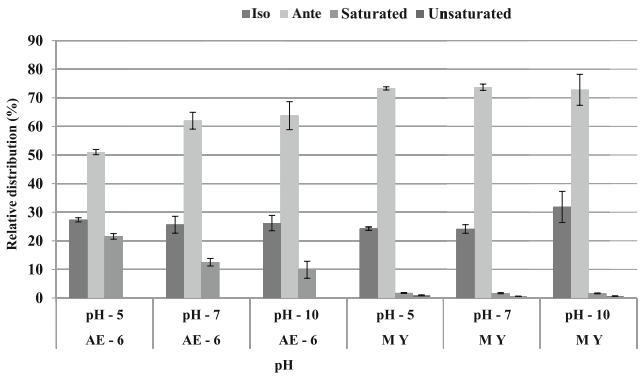

Fig. 3 Relative distributions of different classes of cellular fatty acids of *M. aloeverae* MCC 2184^T and *M. yunnanensis* DSM 21948^T in variable conditions of pH. Unsaturated fatty acids were detected in minor quantity. The data are sums of the means of different fatty acids belonging to the same group and obtained from three biological replicates. Error bars indicate standard deviations in the data. The abbreviations AY and MY represent *Micrococcus aloeverae* MCC 2184^T and *Micrococcus yunnanensis* DSM 21948^T, respectively

contents of iso-$C_{15:0}$ and anteiso fatty acids are characteristic of Gram positive bacteria, and dominance of odd numbered branched chain fatty acids in *Micrococcus* shows agreement with previous findings (Kaur et al. 2005)

Similar to our findings, accumulation of saturated fatty acids and a declined level of unsaturated fatty acid content with increasing temperature has been reported in several other groups of organisms (Denich et al. 2003; Canion et al. 2013). It has been found that temperature not only affects the activity of fatty acid desaturates, but also affects the formation and availability of primers required for biosynthesis of fatty acids, and consequently affects the membrane fluidity and stability (Paulucci et al. 2013). Increased saturation and chain length with increasing temperatures and accumulation of short-chain, unsaturated and branched-chain fatty acids at lower temperatures are in fact essential to maintain the homeoviscous adaptation of the membrane during temperature stress (Sinensky 1974; Chihib et al. 2003; Mrozik et al. 2004). Similar to *M. aloeverae* MCC 2184^T, an increase in anteiso-$C_{15:0}$ content with decreasing temperature was also reported in several different strains of *Lysteria monocytogenes* (Juneja and Davidson 1993; Mazzota and Montville 1997; Chihib et al. 2003). It has been reported that anteiso fatty acids have a lower melting point than iso and saturated fatty acids (Russel and Fukunga 1990; Kaneda 1991). Increased contents of saturated and isoforms of BCFA at higher temperatures in *M. aloeverae* MCC 2184^T is in agreement with previous findings, and substantiates the fact that bacteria need these kinds of fatty acids to modulate membrane fluidity and function at higher temperatures. In contrast, increased contents of anteiso forms at higher temperature in *M. yunnanensis* DSM 21948^T were unexpected and need further investigation to predict their probable role in membrane adaptation at higher temperatures.

Unlike in our observation, increased anteiso-$C_{15:0}$ content with salinity was previously reported in *Planococcus*, *Staphylococcus epidermidis* and *S. aureus* (Kanemasa et al. 1972;

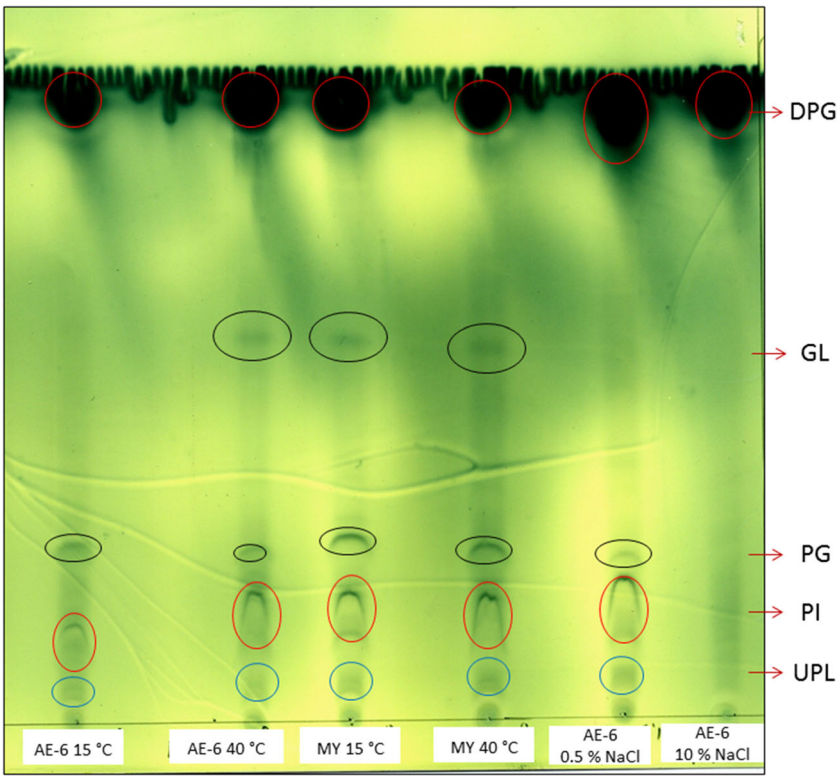

Fig. 4 Polar lipid profile of *M. aloeverae* (AE-6) and *M. yunnanensis* DSM 21948^T (*MY*), as depicted in one-dimensional thin layer chromatography (*TLC*). Unidentified Phospholipid (*UPL*), glycolipid (*GL*), phosphatidylinositol (*PI*), phosphatidylglycerol (*PG*) diphosphatidylglycerol (*DPG*). Spots were identified based on the Retentation Factors (R_f) of specific lipids and using the published polar lipids profile of *Micrococcus yunnanensis* DSM 21948^T and *Micrococcus aloeverae* MCC 2184^T (Zhao et al. 2009, Prakash et al. 2014) as a standard reference. Abbreviations AY and MY represent *Micrococcus aloeverae* MCC 2184^T and *Micrococcus yunnanensis* DSM 21948^T, respectively

Miller 1985). Machado et al. (2004) reported increased saturated and cyclic forms of fatty acids in *Lactobacillus casei* at high osmolality. A similar type of observation was also reported in the case of *L. monocytogenes* strain CNL and *Bacillus subtilis* (Chihib et al. 2003; Lopez et al. 2000; Zhu et al. 2005). Unlike previous findings, our data show that iso fatty acid content increased with osmolality, while the content of anteiso forms decreased in both the organisms (Fig. 2). A similar observation was also reported with temperatures of 15–40 °C in the case of *M. aloeverae* MCC 2184^T, but it was found to be true only in the 15 to 25 °C range in the case of *M. yunnanensis* DSM 21948^T (Fig. 1).

Both organisms showed an almost similar pattern of adaptation (increased iso and decreased anteiso) for temperature and salt stress, but the pattern of adaptation varied with the range of stresses. It indicates that similar intensities of environmental stresses affect different organisms differently. This behaviour traces its antecedents to one of the basic themes of diversity amongst the life forms. It is interesting to note that though the organisms are both endophytes, their reactions towards the same stress varied. Utilization of a more or less similar nature of adaptation at high temperature and salinity might be due to the similar nature of effects (loss of cytoplasmic water, shrinkage of cells and membrane impermeability for Na^+ and protons) exerted by both kinds of stresses (Andreishcheva et al. 1999; Tymczyszyn et al. 2005). Increased contents of total iso fatty acids with increasing salinity in both strains of *Micrococcus* indicated that the isoforms of branched-chain fatty acids are essential components of the cell membrane of *Micrococcus* in coping with conditions of high salt.

Unlike *Micrococcus*, the result of fatty acid analysis in *Halomonas* sp. strain HBL-15 revealed that the bacterium did not show any response to increasing salt concentration, and the cellular fatty acid composition was almost similar at all three different concentrations of salt (Table 4). It indicated that, due to structural differences in the cellular envelope of Gram-positive and Gram-negative bacteria (Zhang et al. 2010); *Micrococcus* and *Halomonas* showed different responses for salt stress. As discussed earlier, iso and anteiso branched-chain fatty acids are characteristic of the Gram-positive cell wall, but this kind of fatty acid is lacking in the cell wall of Gram-negative bacteria. It is hypothesised here that the presence of outer membrane with outer membrane protein and lipopolysaccharide in Gram-negative bacteria might provide more of a protective barrier for stress exerted by high osmotic medium and resistance to change in membrane structure.

As TSBA did not support the growth of *Micrococcus* at pH 5, both the organisms grew on nutrient agar. Narendranath et al. (2001) and Thomas et al. (1994) observed that despite providing nutritional support, yeast extract also protects the bacterium at low pH by providing buffering strength to the medium. Due to the presence of yeast extract in nutrient broth, it supported the growth of *M. yunnanensis* DSM 21948^T and *M. aloeverae* MCC 2184^T. Although both organisms showed different patterns of membrane adaptation in terms of change of fatty acids, the overall data indicated that branched-chain fatty acids (iso-$C_{15:0}$-, iso-$C_{16:0}$- and anteiso-$C_{15:0}$ - and anteiso-$C_{17:0}$ -) play an important role in acid and alkali adaptation mechanisms of bacteria. Our data also suggests that increased isoforms and anteiso forms of fatty acids are required for growth of *M. yunnanensis* DSM 21948^T and *M. aloeverae* MCC 2184^T, respectively, at high pH. Our findings substantiate the observations of Giotis et al. (2007) with *L. monocytogenes*, and proved that branched-chain fatty acids, particularly anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, anteiso-$C_{13:0}$ and iso-$C_{15:0}$, iso-$C_{14:0}$, and iso-$C_{16:0}$ play an important role in the stress tolerance mechanism of bacteria.

The change in cellular fatty acids in *M. aloeverae* MCC 2184^T showed some agreement with previous reports published

Table 4 Cellular fatty acids composition of *Halomonas* sp. HBL-15 in variable conditions of salt

FAME	*Halomonas* sp. strain HBL-15			
	0.5 %	5 %	10 %	PV
Saturated				
10:0	**3.18±0.86**	**2.56±0.20**	**2.41±0.45**	**0.289**
12:0	**4.17±1.15**	**3.39±0.36**	**3.20±0.56**	**0.325**
14:0	0.22±0.02	0.21±0.01	0.33±0.20	0.41
16:0	**18.56±0.61**	**17.65±0.21**	**19.17±0.30**	**0.01**
17:0	0.56±0.1	0.34±0.04	0.49±0.05	0.00
18:0	0.35±0.05	0.42±0.03	0.59±0.05	0.00
Unsaturated				
17:1 w8c	0.22±0.02	0.15±0.02	0.18±0.01	0.00
17:1 w6c	0.17±0.01	nd	0.17±0.01	0.00
18:1 w5c	0.15±0.02	0.32±0.02	0.57±0.05	0.00
18:1 w7c 11-methyl	**0.20**±0.01	0.22±0.02	nd	**0.00**
20:2w6,9c	nd	0.16±0.02	nd	0.00
Hydroxy/Cyclo				
10:0 3OH	nd	0.17±0.02	0.16±0.02	0.00
12:0 3OH	5.27±0.99	4.98±0.70	4.45±0.12	0.41
19:0 Cyclo w8c	4.78±2.01	7±1.58	4.10±0.91	0.13
17:0 Cyclo	**0.35**±0.10	0.46±0.02	0.16±0.02	**0.00**
Summed feature 3*	**5.28±0.63**	**4.89±0.5**	**5.48±0.08**	**0.35**
Summed feature 8**	**55.90±3.67**	**57.45±0.22**	**57.71±3.38**	**0.72**

*16:1w7c/16:1w6c, **; 18:1w7c/ 18:1w6c

The data are means of three biological replicates and are mole % of total fatty acids contents; values after (±) are standard deviations

In most of the cases, values of standard errors are almost half the values of standard deviations (data not shown)

Values in bold represent quantitatively dominant fatty acids

nd not detected, *PV p* value of ANOVA

0.5 % salt was treated as a control for the comparison of increasing effects of salinity

on stress response with other organisms, while the strain *M. yunnanensis* DSM 21948^T showed almost the opposite pattern of physiological adaptation. We also observed that when the contents of the anteiso form decreased, the content of the isoform or saturated form increased in the same ratio. Therefore, not only the content of individual fatty acids, but also the total ratio of different species of fatty acids matters to modulate the membrane fluidity and stability in the condition of any given stress. Unlike the previous studies on other groups of organisms, through this work, we propose that isoforms of branched chain fatty acids are crucial for the high salt (NaCl) tolerance potential of *Micrococcus*. Furthermore, this study also indicates that saturation is only required to modulate the membrane fluidity at higher temperatures, but isnot required for the growth and survival of *Micrococcus* at high salinity or in conditions of acidity or alkalinity. Our polar lipids data indicate that lipids of both of the micrococci mainly contain phospholipid. Phospholipids are the main constituent of bacterial cell membranes. Thus, any structural as well as quantitative variations in the fatty acid profiles in both strains of *Micrococcus* are directly related to variations in membrane phospholipid structure, membrane fluidity and stress response.

It is believed that bacteria accumulate lowmelting–point (unsaturated, branched-chain and short chain) and high-melting–point (long-chain and saturated) fatty acids in their membrane to maintain the membrane fluidity, stability and function at low and high temperatures, respectively. Although most organisms support the above statement, contradictions also exist. Variability in the mode of membrane adaptation, as reported for *M. yunnanensis* DSM 21948^T and *M. aloeverae* MCC 2184^T, has indicated that even members of two different species of the same genus isolated from the same habitat show variability in terms of adaptation for the same kind of stress. Unlike temperature, lesser data are available on salt and pH adaptations, and the findings are based on only a limited group of organisms. Therefore, we need more data using different groups of organisms and a wide range of stress conditions to understand the exact role of different fatty acids in membrane adaptability for different kinds of environmental stresses.

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Conflict of interest The authors declare that they do not have any conflict of interest regarding this manuscript.

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