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Original Article

Isolation and cellular fatty acid composition of psychrotrophic *Halomonas* strains from Antarctic sea water

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Abstract

Microorganisms from extreme environments such as Arctic, Antarctic and Polar regions modulate their membrane fatty acids to survive in such habitats. Characterization of such microorganisms helps in understanding their physiological behavior. In view of this, the present article describes isolation, characterization and cellular fatty acid composition of three bacterial isolates from Antarctic sea water samples. All the three isolates (BRI 6, 29 and 31) were psychrotrophic Gram negative rods. Their 16S rRNA sequencing (around 1200 bp) revealed that all three of them belong to genus *Halomonas*. Each of them showed 99% sequence similarity to *Halomonas neptunia* Eplume1 (NR 027218), *H. boliviensis* LC1 (NR 029080) and *H. variabilis* DSM 3051 (NR 042068). The fatty acid analysis of our isolates indicated i) predominance of C 18:1, C 16:0 and C16:1 fatty acids and absence of trans fatty acids in all of them and ii) higher percentage of anteiso fatty acids than of iso fatty acids in BRI 6. These characteristic features may contribute to their adaptation to the Antarctic habitat.

Keywords: 16S rRNA, halotolerant, polyunsaturated fatty acids, omega- 6 fatty acids, cold adaptation

1. Introduction

Antarctic sea water is a known habitat for different types of microorganisms like bacteria, microalgae, fungi and protozoa (Vincent, 2000). Among bacteria, *Clostridium, Colwellia, Flavobacterium, Gelidibacfer, Halomonas, Pseudomonus, Psychromonas, Shewanella* etc. are the various genera reported from the Antarctic region. A plethora of species belonging to the genus *Halomonas* has been isolated from the Antarctic saline lakes, solid layer of fast ice and other locations in the Antarctic region (James *et al.*, 1990; Reddy *et al.*, 2003; Bowman *et al.*, 1997; Franzmann *et al.*, 1987) and also from different habitats all over the world (Duckworth *et al.*, 2000; Bouchotroch *et al.*, 2001). From the Antarctic region, *meridiana* (James *et al.* (1990), *subglaciescola* (Franzmann *et al.*, (1987), *glaciei* (Reddy *et al.* (2003), *alkaliantarctica* (Poli *et al.* (2007) etc. are different species belonging to the genus *Halomonas*.

It is a well known fact that decrease in growth temperature results in an increase in monounsaturated FAs and a decrease in saturated straight chain FAs in low temperature dwelling microorganisms (Freese *et al.*, 2008). Among fatty acids PUFAs play important role in their physiology of microorganisms since they help to maintain membrane fluidity for surviving in the extreme habitat (Russell and Nichols, 1999). A review of the available studies on cold adaptation indicates that cells respond and adapt to low temperatures by modulating the fluidity of their membrane which is achieved predominantly not only by altering their fatty acid composition but also by various other strategies such as altering the lipid head group, the protein content of the membrane, the type of carotenoids, the fatty acid chain

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length and the proportion of cis to trans fatty acids (Chintalapati *et al.*, 2004). Such variations were found not only between members of different phyla, but also among strains of a single genus (e.g. *Shewanella* and *Desulfovibrio*) (Freese *et al.*, 2008). Earlier research indicates that in bacteria it is virtually impossible to predict the potential changes in the membrane fatty acid composition in response to change in temperature (Freese *et al.*, 2008).

We isolated three Gram negative aerobes from Antarctic sea water samples obtained from different locations. Their 16S rRNA sequencing revealed that they belonged to the genus *Halomonas*.

Since microorganisms adapt to low temperature environment by using various strategies as mentioned above, it was subject of interest for us to observe the characteristic fatty acid composition of these strains for their adaptation to low temperature. The current paper deals with the characterization, identification and fatty acid analysis of the isolated strains.

2. Materials and Methods

2.1 Organisms

Antarctic seawater samples were collected during the Antarctic summer of 2007–2008 from different locations (Table 1). Isolation of microorganisms was carried out as described previously (Jadhav *et al.*, 2010). They were examined for salt tolerance in marine salt medium (MSM) containing varying concentrations of NaCl in the range of 8- 20% and for temperature tolerance by incubating at temperatures ranging from 15 to 45°C for 24 h.

2.2 Amplification of 16S rRNA and sequence analysis

The genomic DNA of BRI 6, 29 and 31 was isolated as described by Ausubel *et al.*, 1987. The PCR assay was performed using Applied Biosystems, model 9800 (Foster, California, USA) with 50ng of DNA extract in a total volume of 25 µl. The PCR master mixture contained 2.5 µl of 10X PCR reaction buffer (with 1.5 M $MgCl_2$), 2.5 µl of 2 mM dNTPs, 1.25 µl of 10 pm μl^{-1} of each oligonucleotide primers 8F (5' - AGAGTTTGATCCTGGCTCAG 3') and 1391R (5'- GACGGG CGGTGTGTRCA -3') (Amann *et al.*, 1992; Hauben *et al.*, 1997; Pidiyar *et al.*, 2002; Ben-Dov *et al.*, 2006), 0.2 µl of 5 U μl^{-1} Taq DNA polymerase and 15.76 µl of glass-distilled PCR water.

Initial denaturation was accomplished at 94°C for 3 min. Thirty-two cycles of amplification consisted of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1.30 min. A final extension phase at 72°C for 10 min was performed. The PCR product was purified by PEG-NaCl method (Hauben *et al.*, 1997). Briefly, the sample was mixed with 0.6 times volume of PEG-NaCl, [20% PEG (MW 6000) and 2.5 M NaCl] and incubated for 20 min at 37°C. The precipitate was collected by centrifugation at 3,800 rev min^{-1} for 20 min. The pellet was washed with 70% ethanol, air dried and dissolved in 15 il sterile distilled water.

The sample was sequenced using a 96-well Applied Biosystems sequencing plate as per the manufacturer's instructions. The thermocycling for the sequencing reactions began with an initial denaturation at 94°C for 2 min, followed by 25 cycles of PCR consisting of denaturation at 94°C for 10 s, annealing at 50°C for 10 s, and extension at 60°C for 4 min using primers 704F (5'GTAGCGGTGAAATGCGTAGA3') (Amann *et al.*, 1992; Hauben *et al.*, 1997; Pidiyar *et al.*, 2002; Ben-Dov *et al.*, 2006) and 907R (5' CCGTCAATTCMTTT GAGTTT 3') (Amann *et al.*, 1992; Ben-Dov *et al.*, 2006). The samples were purified using standard protocols described by the manufacturer (Applied Biosystems, Foster City, USA). To this, 10 µl of Hi-Di formamide was added and vortexed briefly.

The DNA was denatured by incubating at 95°C for 3 min, kept on ice for 5-10 min and was sequenced in a 3730 DNA analyzer (Applied Biosystems, Foster City, USA.) following the manufacturer's instructions.

The sequences of bacterial 16S rRNA were analysed using Sequence Scanner (Applied Biosystems) software. The 16S rRNA sequence contigs were generated using Chromas Pro and then analysed using online databases viz. NCBI-BLAST to find the closest match of the contiguous sequence. Phylogenetic analysis was carried out using MEGA software package version 5.0 (Tamura *et al.*, 2011).

2.3 Fatty acid analysis

The three strains were cultivated at 15°C for 24 h. Fatty acid extraction from BRI 6, 29 and 31 and separation of fatty acid methyl esters (FAMEs) was carried out by the Microbial Identification Inc (MIDI) standard protocol (Sasser, 1990). Fatty acid methyl esters were obtained from 40 mg cells scraped from petri dishes by saponification, methylation and extraction. Analyses of the FAMES were performed with an Agilent model 6890N gas chromatograph equipped

Table 1. Sea water samples used for isolation of Antarctic isolates

Sample no.	Latitude	Longitude	pH	Temperature (°C)	Isolates
13	S 59°40'24.6"	E 68°33'23.7"	7.8	-0.7	BRI 29
20	S 41°40'03.3"	E 42°15'53.1"	7.5	13.5	BRI 6
L-5	S 69°24'29.7"	E 76°11'57.2"	8.5	-2.1	BRI 31

with a 25 m x 0.2 mm phenyl methyl silicone fused silica capillary column and flame ionization detector. The column was temperature programmed from 170°C to 270°C at the rate of 5°C per minute and further increased to 300°C during a hold of 2 minutes. The external calibration standard used was developed and manufactured by MIDI which is a mixture of the straight chained saturated fatty acids from 9 to 20 carbons in length (9:0 to 20:0) and five hydroxy acids.

3. Results and Discussion

3.1 Isolation and characterization

Three psychrotrophic bacteria were isolated from three Antarctic sea water samples (Table 1). All of them were (1) Gram - negative rods, (2) showed neutral pH optimum for growth, (3) tolerated salt concentration up to 15%, and (4) grew well at 15-40°C (Table 2).

3.2 Phylogenetic relationship of the isolated psychrotrophs

The isolates BRI 6, 29 and 31 were identified using 16S rRNA sequencing. The sequences were deposited in EMBL+Genbank under the accession numbers HQ 600586, JX 123568 and JX 123569 for BRI 6 (1235 bp), BRI 29 (1234 bp) and BRI 31 (1233 bp) respectively. The analysis clearly revealed that all the three isolates belonged to Gammaproteobacteria and are the members of the genus *Halomonas*. Each of them indicated 99% sequence similarity with the 16S rRNA sequences of *Halomonas neptunia* Eplume1 (NR 027218), *H. boliviensis* LC1 (NR 029080) and *H. variabilis* DSM 3051 (NR 042068) in BLAST analysis. Phylogenetic analysis is shown in Figure 1 depicting the relationship of our isolates with their closest matches.

3.3 Fatty acid analysis

Fatty acid compositions of the three strains are shown in Table 3. C 18:1 was the predominant fatty acid in all the three strains. Other predominant fatty acids were C16:1 followed by C 16:0. All members of the Halomonadaceae contain the same major fatty acids (Franzmann and Tindall, 1990) and are usually rich in C18:1 and C16:0 acyl chains (Giordano *et al.*, 2007). Our observations are in line with this and also with the earlier reports on different *Halomonas* species isolated from various locations of Antarctic region (Dobson *et al.*, 1993; Reddy *et al.*, 2003; Poli *et al.*, 2007; Kim *et al.*, 2010). Noticably, we have recorded C20 fatty acids in the range of 0.02 to 0.19 % in the three strains. Omega - 6 PUFAs (around 0.12%) were also detected in two of the three strains. They were gamma linolenic acid C (18:3) in BRI 6 and BRI 31 and eicosadienoic acid C (20:2) and arachidonic acid (AA) C (20:4) in BRI 31. Similar levels of omega-6 fatty acids have also been reported in different *Halomonas* species from various habitats (Jung-Hoon *et al.*, 2002; Kaye and Baross, 2004). However, earlier work on *Halomonas glacei* revealed absence of C20 fatty acids (Reddy *et al.*, 2003). Significance of long chain fatty acids in the organisms surviving at lower temperature is very well justified. These fatty acids maintain membrane fluidity at lower temperature by spanning the width of bilayer more easily as compared to short chain fatty acids (Chintalapati *et al.*, 2004).

Table 2. Physiological characterization of BRI 6, 29 and 31 isolates

	Temperature (°C)			
Isolate	15	30	40	45
BRI 6	++	+	+	-
BRI 29	++	+	+	-
BRI 31	++	+	+	-

	pH				
Isolate	3	5	7	9	11
BRI 6	-	+	++	+	(+)
BRI 29	+	+	++	(+)	(+)
BRI 31	-	+	++	(+)	(+)

	NaCl (%)			
Isolate	8	10	15	20
BRI 6	++	++	(+)	-
BRI 29	++	++	+	-
BRI 31	++	++	+	-

++ = Good Growth, + = Moderate growth, (+) = Weak growth,
- = No growth

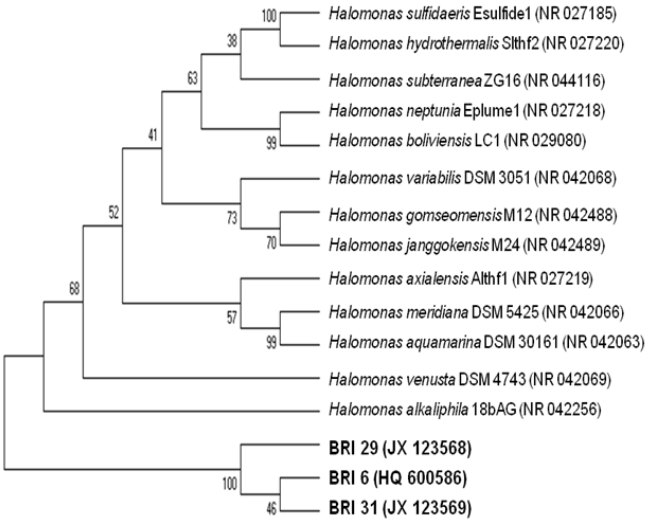

Figure 1. Phylogenetic analysis based on 16S rRNA sequences of isolates BRI 6, 29 and 31 and related *Halomonas* species. Gene Bank accession numbers are listed with species names. Bootstrap values were generated from 1000 replicates and are shown as percentages at nodes.

Table 3. Percentage fatty acid composition of Antarctic isolates

Fatty acid	Isolates		
	BRI 6	BRI 29	BRI 31
$C_{10:0}$	1.95±0.07	1.95±0.04	1.88±0.07
$C_{10:0}$ 3-OH	0.25±0.05	0.31±0.04	0.25±0.04
$C_{12:0}$	1.56±0.03	1.60±0.03	1.47±0.08
$C_{12:0}$ 3-OH	8.92±0.42	9.61±0.1	9.12±0.11
$C_{14:0}$	0.46±0.05	0.40±0.04	0.36±0.04
iso-$C_{16:0}$	0.67±0.04	0.06±0.01	0.08±0.01
$C_{16:1}$ w6c/ w7c	18.69±0.2	19.80±0.06	19.69±0.09
$C_{16:0}$	13.15±0.13	14.34±0.12	14.04±0.07
anteiso-$C_{17:0}$	0.88±0.06	0.13±0.02	0.1±0.03
$C_{17:1}$ w8c	0.14±0.02	0.16±0.02	0.14±0.02
$C_{17:0}$	0.16±0.03	0.17±0.02	0.15±0.02
$C_{17:0}$ cyclo	0.74±0.04	0.67±0.05	0.83±0.04
$C_{18:0}$	0.43±0.02	0.47±0.02	0.21±0.01
$C_{18:1}$ w7c	47.44±0.07	46.19±0.12	48.46±0.08
$C_{18:3}$ w6c (6,9,12)	0.10±0.01	N.D.	0.06±0.01
$C_{19:0}$ cyclo w8c	1.10±0.04	0.96±0.04	1.23±0.04
$C_{20:0}$	0.14±0.02	0.19±0.02	0.09±0.01
$C_{20:1}$ w7c	0.11±0.02	0.17±0.01	0.09±0.02
$C_{20:2}$ w6,9c	N.D.	N.D.	0.02±0.01
$C_{20:4}$ w6,9,12,15c	N.D.	N.D.	0.04±0.01

The analyses were performed in triplicates. The values represent Means±SD
N.D. = Not detected

Our results indicated a higher percentage of anteiso fatty acids than of iso fatty acids in BRI 6. Though both the structural isomers increase fluidity in comparison to the straight chain isomer, anteiso-form has more fluidizing effect than the iso-form (Chintalapati *et al.*, 2004). Moreover, geometrical isomers, like the cis-trans isomers also affect membrane fluidity (Morita *et al.*, 1993; Okuyama *et al.*, 1990). Increase in trans fatty acids was observed with increase in temperature in *Vibrio* sp. strain ABE-1 and *Pseudomonas* sp. E-3 strain (Okuyama *et al.*, 1990; 1991). This could effectively reduce the fluidity of the membrane (Cronan and Gelman, 1975; Kiran *et al.*, 2004; Okuyama *et al.*, 1991; Weber *et al.*, 1994). On the contrary absence of trans fatty acids and predominance cis isomers in BRI 6, 29 and 31 (this work) supports their role in adaptation of psychrotrophic microorganisms to low temperatures.

In conclusion, athough PUFA production appears as a phylogenetically linked genotypic strategy for selective pressures, their presence may not be essential for the growth of bacteria in such environments (Nichols, 2003). This is evident from the absence of long chain fatty acids in *H. variabilis* isolated from deep-sea and *Halomonas* species isolated from Antarctic region (Reddy *et al.*, 2003; Yi-Guang *et al.*, 2009; Kim *et al.*, 2010). Similarly, our isolates BRI 6, 29, 31 adapted to low temperature conditions without producing long chain fatty acids.

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