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Antimicrobial properties of the bacterial associates of the Arctic lichen *Stereocaulon* sp.

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Four bacterial associates were isolated from the Arctic lichen *Stereocaulon* sp. and identified based on their complete 16S rRNA gene sequences. Cell cultures were extracted using different solvents and tested for their antimicrobial properties using paper disk diffusion assay and MIC test against six test bacteria. Results show that the ethyl acetate of bacterial isolate is a most effective solvent to extract metabolites from the bacterial associates. *Bosea vestrissi* 34635^T (KOPRI 26642) of four bacterial associates showed highest activity as shown as inhibition zone was 7 to 14, and MIC value was 52.7 to >1000 against six test bacteria.

Key words: Lichen, *Stereocaulon sp*., bacterial associate, antimicrobial activity, MIC test.

INTRODUCTION

Various infectious diseases are caused by bacterial pathogens such as *Streptococcus* and *Pseudomonas* species. Although, several strong antibiotics have been developed, many bacterial pathogens are becoming resistant to available antibiotics. Isolation of bioactive compounds from natural products is thus a popular approach to develop new drugs because they often play an important role in treatment of many diseases, especially infectious ones. The polar area serves as an attractive source for microorganisms with potentially bioactive compounds. Lichen is an association of a fungus with its photosynthetic partner, either an alga or a cyanobacterium. They can grow in extreme environments ranging from the desert to the polar area. The photosynthetic partner provides nutrient to the fungus while the fungus provide the structure that protects the photosynthetic partner from drying (REF). Lichens produce various bioactive metabolites that were used as medicines, cosmetics, dyes, foods and decorations (Boustie and Grube, 2005; Dayan and Romagni, 2001; Oksanen, 2006). About 800 lichen metabolites have been found with usnic acid as one of the most common and investigated lichen compounds (Hager et al., 2008). Their antiviral, anti-proliferative, anti-inflammatory, anti-tumor, anti-mycobacterial and analgesic activities have been previously reported (Ingolfsdottir et al., 1998; Lauterwein et al., 1995; Lawrey, 1989; Molnár and Farkas, 2010; Muller, 2001). Several lichen compounds also showed antibacterial activities against pathogenic microorganisms (Paudel et al., 2008).

Recent studies also showed that the bacterial symbionts isolated from lichens contributes to the lichens structurally and ecologically (Bates et al., 2011; Cardinale et al., 2006, 2008; Gonzalez et al., 2005; Grube et al., 2009). However, the biological activities of the bacterial symbionts of this lichen are unknown in detail in contrast to the many reported studies on the biological activities of the host lichens. In particular, the bacterial communities of the lichen species *Stereocaulon* are not well-known, although, the antimicrobial activities of *Stereocaulon*

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alpinum, *Stereocaulon arcticum*, *Stereocaulon vanoyei* and *Stereocaulon vesuvianum* were already reported (Ingolfsdottir et al., 1985). In this study, we isolated four bacterial associates of the Arctic lichen *Stereocaulon* sp. Cell cultures were extracted with different solvents and assessed for their antimicrobial activities.

MATERIALS AND METHODS

Isolation of lichen-associated microorganisms

The lichen *Stereocaulon* sp. was collected in Ny-Ålesund, Svalbard, Arctic (78, 54.738' S/011, 57.278' E) on July 13, 2010. It was transported at room temperature and stored at -20°C until further use. The specimen was identified by morphological characteristics with unaided eye and there is no herbarium. A segment from a lichen thallus was separated by scissors or knife. Sterilized 0.85% NaCl solution was added followed by vortexing for 10 min. The solution was discarded and the above steps were repeated twice. The tissue was subsequently broken with mortar in sterilized 0.85% NaCl solution. After spreading the tissue on selection media, it was incubated at 28°C for 15 to 21 days. Four selective media used included Humic acid vitamin agar (Humic acid 10 g, Na_2HPO_4 0.5 g, KCl 1.71 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, $CaCO_3$ 0.02 g, vitamin 1.0 ml, distilled water 1.0 L, agar 16.0 g), Bennett's vitamin agar (D-glucose 10.0 g, yeast extract 1.0 g, peptone 2.0 g, beef extract 1.0 g, vitamin 1.0 ml, distilled water 1.0 L, agar 16.0 g), ISP4 (Difco soluble starch 10.0 g, K_2HPO_4 anhydrous 1.0 g, $MgSO_4 \cdot 7H_2O$ 1.0 g, NaCl 1.0 g, $(NH_4)_2SO_4$ 2.0 g, $CaCO_3$ 2.0 g, ISP trace salt solution 1.0 ml, distilled water 1.0 L, agar 16.0 g) and water agar (distilled water 1.0 L, agar 16.0 g). The subculturing was repeated three times, the pure culture of bacterial isolate was preserved at -80°C in 20% glycerol. The obtained bacterial associates were identified by sequence similarity and phylogenetic analysis of their 16S rRNA gene sequences. The 16S rRNA gene was amplified from a single colony of pure cultures with two universal primers, 27F; 5'-AGA GTT TGA TCM TGG CTC AG-3' and 1492R; 5'-GGT TAC CTT GTT ACG ACT T-3', as described by Lane (1991). PCR was carried out with 25 ul reaction mixtures containing 1X PCR reaction buffer, 200 uM of dNTPs, 0.2 uM of each primer, a single colony as a template and 1 unit of *Taq* DNA polymerase (In-Sung Science, Suwon, Korea). The PCR procedure included an initial denaturing step at 95°C for 5 min and 30 cycles of amplification (95°C for 30 s, 56°C for 30 s, and 72°C for 30 s) and a final extension step at 72°C for 5 min. PCR products were purified using the AccuPrep PCR Purification Kit (Bioneer, Korea) and sequenced with the same primers used for PCR amplification. The sequence of the 16S rRNA gene was compared with that of type strains available in the database to find closely related species.

In addition, for cellular fatty acid analysis, the isolated bacterial associates were grown on R2A at 28°C for three days. Cellular fatty acid was methylated and analyzed using gas chromatography followed by microbial identification Inc. (MIDI) standard protocol. Identification of fatty acid was confirmed using a standard protocol of Hewlett-Packard Co. microbial identification system (HP MIS).

Culture and extraction of bacterial associates

The four bacterial associates were cultured in 50 ml of Bennett's vitamin liquid media and ISP4 liquid media at 15°C for 10 to 15 days. The culture broth was added to double volume of various organic solvents: acetone, chloroform, diethyl ether, ethanol, ethyl acetate, methanol and petroleum ether. Extraction was performed individually to each solvent at room temperature and then the layer of culture media was discarded after 2 h. Solvent layer was concentrated using rotary evaporator and the obtained dried crude extract was dissolved in 500 ul of same extracting solvent.

Assay for antimicrobial activities

Test bacteria

Gram-positive pathogenic bacteria *Staphylococcus aureus* (KCTC1928), *Bacillus bacillus* (KCTC1918), *Micrococcus luteus* (KCTC1915), and Gram-negative pathogenic bacteria *Escherichia coli* (KCTC2441), *Pseudomonas aeruginosa* (KCTC1637) and *Enterobacter cloacae* (KCTC1685) used in the study were purchased from the Korean Collection for Type Cultures (KCTC), Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejon, Korea. All bacterial strains were kept on Luria-Bertani (LB; trypton 10.0 g, yeast extract 5.0 g, NaCl 10.0 g, distilled water 1.0 L, pH 7.2) at 4°C.

Paper disk diffusion assay

We carried out the paper disk diffusion test according to Bauer et al. (1966) with some modifications. Bacterial cells were standardized to 0.5 McFarland and then mixed with soft agar (0.04 g/ml), and 9 ml of this mixture was inoculated onto LB agar plate. Then, each extract was loaded into paper disks (6 mm in diameter, ADVANTEC, Japan) and transferred onto the plates inoculated with the bacterial strains. Disks loaded with the solvents were used as a control. All inoculated culture plates were incubated at 37°C, and the inhibition zones of bacterial growth were measured after 12 to 18 h. All experiments were done twice and compared with the control.

Determination of minimum inhibitory concentration (MIC)

MIC was determined using the broth dilution method (Swenson et al., 1982) with some modifications. Dilutions of culture extract ranging from 0 to 1000 µg/ml in 5 ml of Mueller-Hinton broth was prepared in duplicate in 15 ml sterile falcon tubes. Final concentration of test organisms was adjusted to 10^6 CFU/ml. The inoculated tubes were incubated in a shaker (250 rpm) at 37°C for 24 h. MIC was measured by UV-Vis spectrophotometer at 530 nm to determine the growth.

RESULTS AND DISCUSSION

Identification of bacterial associates

Four bacterial associates were isolated from the Arctic lichen *Stereocaulon* sp. Their closest strains are summarized using 16S rRNA sequence analysis (Table 1). They grew well on the Bennett's vitamin agar media at 28°C. Their morphological, physiological biochemical characteristic results are similar with other species identified from resource as shown in Table 2. All of these strains were isolated from lichen for the first time in our study. Thus, when compared with other species that isolated from plant root, soil and food, they have similar physiological and biochemical characteristics.

Antibacterial activity of bacterial associates

The various solvent extracts of all bacterial associates

Table 1. Bacterial isolated from *Stereocaulon* sp. and taxonomical identification.

Bacterial associate No.	16S rRNA sequence analysis (closest strain)	Similarity (%)
KOPRI 26639	*Pseudomonas graminis* DSM 11363^T	99.6
KOPRI 26640	*Mucilaginibacter rigui* WPCB133^T	96.3
KOPRI 26641	*Pseudomonas graminis* DSM 11363^T	99.6
KOPRI 26642	*Bosea vestrisii* 34635^T	98.2

Table 2. Physiological and biochemical test results.

Characteristics	26639	26640	26641	26642
Pigment	**Yellow**	**Orange**	**Yellow**	**Beige**
Growth range				
Temparature (°C)	15-28	15-28	15-28	15-28
pH	6-8	6-8	6-8	6-8
Oxidase activity	+	+	+	+
Nitrate reduction	-	-	-	-
H_2S production	-	-	-	-
Acid production from :				
Arabinose	+	+	+	-
Glucose	+	+	+	-
Assimilation of :				
N-Acetyl-D-glucosamine	-	-	-	-
D-Arabinose	-	+	-	+
L-Arabinose	+	-	+	+
Fructose	+	-	+	+
D-Mannose	+	-	+	+
Xylose	+	+	+	+
Major Quinone(s)	UQ-9	MK-7	UQ-9	UQ-10
DNA G+C contents (mol%)	60.0	43.4	60.0	65.0

Bacterial isolates: 26639, *Pseudomonas graminis* DSM11363^T; 26640, *Mucilaginibacter rigui* WPCB133^T; 26641, *Pseudomonas graminis* DSM11363^T; 26642, *Bosea vestrisii* 34635^T.

resulted in variable inhibition diameter ranging from 7 to 14 mm (Table 3). None of the aqueous extracts of 4 microorganisms showed any activity against six target strains suggesting that active compounds are not dissolved in aqueous. Extracts of 26639 has activity against all target strain when compared with 6 mm diameter of paper disk. Among the various solvent extracts, extracts with ethyl acetate (EtoAC) showed activity, especially ethyl acetate extract of 26639 showed best activities (9 mm) against *E. coli*. Similarly, 26640 extracts showed activity. It was not sensitive against *S. aureus* and *E. cloacae*. Acetone, chloroform and ethyl acetate extracts of 26640 showed activity and among them, chloroform extract and acetone extract showed better activity than other extracts of 26640. 26641 have activity against all test microorganisms. Chloroform and ethyl acetate extracts have only activity and other solvents were not effective. Ethyl acetate extract showed better activity (9 mm) than other extract of 26641 against *E. coli*. 26642 have a good activity in comparison to other bacterial associates, especially against Gram-negative pathogenic bacteria. Although, acetone, chloroform and diethyl ether extracts have activity, ethyl acetate extraction was more effective.

Ethyl acetate extract of 26642 showed 14 mm of inhibition zone diameter against *E. coli* and followed by 13 mm against *P. aeruginosa*, 10 mm against *B. subtilis* and *M. luteus.* Until now, many cases have reported that extract of crude lichens, not bacterial associates of lichens, has good antibacterial activity. Although, these strains were isolated from various natural sources, this is the first study to show antibacterial activity of bacterial associates of lichens. MIC test results of extracts were similar to those of the disk diffusion test, as shown in Table 4. Ampicillin used as a positive control showed range 0.28 to 0.59 µg/ml MICs against six kinds of target stain. Four tested samples exhibited very high MIC values against all six target bacteria. It means that our bacterial associates inhibit the six pathogenic bacteria at high concentration, in other words they have weak activity

Table 3. Antibacterial activities of bacterial associates isolated from Arctic lichen *Stereocaulon* sp. The activity was expressed as inhibition zone diameter in mm and NA means no activity against target bacteria.

Target bacteria		Solvent*	Polar microorganisms**				
			26639	26640	26641	26642	Standard***
Gram positive	*S. aureus*	Aq	NA	NA	NA	NA	NA
		Ace	NA	7	NA	7	NA
		Chl	NA	NA	NA	8	NA
		Di	NA	NA	NA	NA	NA
		Et	NA	NA	NA	NA	NA
		EtoAC	7	NA	7	9	NA
		Met	NA	NA	NA	NA	NA
		Pe	NA	NA	NA	NA	NA
	B. subtilis	Aq	NA	NA	NA	NA	NA
		Ace	NA	NA	NA	NA	NA
		Chl	NA	7	NA	NA	7
		Di	NA	NA	NA	8	7
		Et	NA	NA	NA	NA	NA
		EtoAC	8	NA	8	10	NA
		Met	NA	NA	NA	NA	NA
		Pe	NA	NA	NA	NA	NA
	M. luteus	Aq	NA	NA	NA	NA	NA
		Ace	NA	NA	NA	NA	NA
		Chl	NA	7	NA	9	7
		Di	NA	NA	NA	9	7
		Et	NA	NA	NA	NA	NA
		EtoAC	7	7	7	10	NA
		Met	NA	NA	NA	NA	NA
		Pe	NA	NA	NA	NA	NA
	E. cloacae	Aq	NA	NA	NA	NA	NA
		Ace	NA	NA	NA	NA	NA
		Chl	7	NA	7	8	NA
		Di	NA	NA	NA	NA	7
		Et	NA	NA	NA	NA	NA
		EtoAC	NA	NA	NA	12	NA
		Met	NA	NA	NA	NA	NA
		Pe	NA	NA	NA	NA	7
Gram negative	*P. aeruginosa*	Aq	NA	NA	NA	NA	NA
		Ace	NA	8	NA	NA	NA
		Chl	7	7	7	8	NA
		Di	NA	NA	NA	NA	7
		Et	NA	NA	NA	NA	NA
		EtoAC	7	NA	NA	13	NA
		Met	NA	NA	NA	NA	NA
		Pe	NA	NA	NA	NA	7
	E. coli	Aq	NA	NA	NA	NA	NA
		Ace	NA	7	NA	NA	NA
		Chl	7	8	7	8	NA
		Di	NA	NA	NA	NA	7

Table 3. Contd.

	Et	NA	NA	NA	NA	NA
	EtoAC	9	7	9	14	NA
	Met	NA	NA	NA	NA	NA
	Pe	NA	NA	NA	NA	7

*Aq, aqueous; Ace, acetone; Chl, chloroform; Di, diethyl ether; Et, ethanol; EtoAC, ethyl acetate; Met, methanol; Pe, petroleum ether; **The number was given from KOPRI; ***standard: only solvents without extract.

Table 4. Minimum inhibitory concentration (MIC) of bacterial associates of the Arctic lichen *Stereocaulon* sp. against the test organisms. Values given as µg/ml for lichen associated with microorganism extracts and as µg/ml for antibiotics. NA means no activity.

Target bacteria		Solvent*	Polar microorganism**				
			26639	26640	26641	26642	Standard***
Gram positive	*S. aureus*	Aq	NA	NA	NA	NA	0.59
		Ace	>1000	>1000	>1000	>1000	
		Chl	>1000	>1000	896.3	748.5	
		Di	NA	NA	NA	NA	
		Et	>1000	>1000	>1000	>1000	
		EtoAC	942.1	799.6	543.3	332.6	
		Met	NA	NA	NA	NA	
		Pe	NA	NA	NA	>1000	
	B. subtilis	Aq	NA	NA	NA	NA	0.43
		Ace	>1000	>1000	>1000	>1000	
		Chl	889.1	752.9	602.1	598.2	
		Di	>1000	>1000	>1000	>1000	
		Et	NA	NA	NA	NA	
		EtoAC	737.2	594.6	660.2	142.6	
		Met	NA	NA	NA	NA	
		Pe	NA	NA	NA	NA	
	M. luteus	Aq	NA	NA	NA	NA	0.41
		Ace	NA	NA	NA	NA	
		Chl	612.5	>1000	495.8	330.3	
		Di	>1000	>1000	>1000	>1000	
		Et	>1000	>1000	>1000	>1000	
		EtoAC	>1000	>1000	752.6	123.4	
		Met	NA	NA	NA	NA	
		Pe	NA	NA	NA	>1000	
Gram negative	*E. cloacae*	Aq	NA	NA	NA	NA	0.32
		Ace	NA	NA	NA	NA	
		Chl	>1000	>1000	>1000	>1000	
		Di	>1000	>1000	>1000	>1000	
		Et	NA	NA	NA	NA	
		EtoAC	170.6	655.3	189.6	98.1	
		Met	NA	NA	NA	NA	
		Pe	NA	NA	NA	NA	
	P. aeruginosa	Aq	NA	NA	NA	NA	0.39
		Ace	>1000	NA	NA	>1000	
		Chl	NA	NA	NA	>1000	

Table 4. Contd.

	Di	>1000	>1000	>1000	>1000	
	Et	NA	NA	NA	NA	
	EtoAC	530.6	565.6	147.6	77.8	
	Met	NA	NA	NA	NA	
	Pe	>1000	>1000	>1000	>1000	
	Aq	NA	NA	NA	NA	
	Ace	>1000	>1000	>1000	>1000	
	Chl	NA	NA	NA	>1000	
E. coli	Di	NA	NA	NA	NA	0.28
	Et	NA	NA	NA	NA	
	EtoAC	416.2	203.2	150.3	52.7	
	Met	NA	NA	NA	NA	
	Pe	>1000	>1000	>1000	>1000	

*Aq, aqueous; Ace, acetone; Chl, chloroform; Di, diethyl ether; Et, ethanol; EtoAC, ethyl acetate; Met, methanol; Pe, petroleum ether; **The number was given from KOPRI; ***standard: only solvents without extract and ampicillin is used as control.

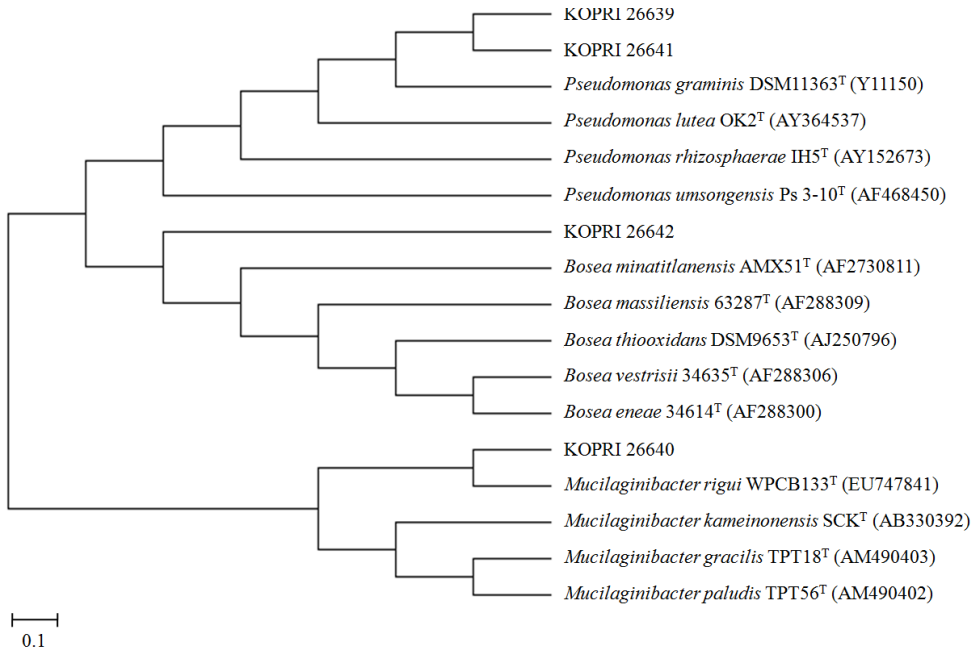

Figure 1. Phylogenetic tree of the bacterial associates based on 16S rRNA gene sequences. The tree was constructed by the neighbor-joining method. Bar 0.02 changes per nucleotide. KOPRI 26639, *Pseudomonas graminis* DSM11363^T; 26640, *Mucilaginibacter rigui* WPCB133^T; 26641, *Pseudomonas graminis* DSM11363^T; 26642, *Bosea vestrisii* 34635^T.

activity when compared with antibiotics. Aqueous extract has no activity against six target bacterial strains. It is probably that active compounds do not dissolved in water. Thus, ethanol and methanol extracts showed almost no activity. In case of solvents, activity of ethyl acetate was the highest followed by chloroform, acetone and petroleum ether, respectively. In case of bacterial associates, 26642 have a good activity (147.6 µg/ml of ethyl acetate extract against *P. aeruginosa*) compared with other bacterial extractions.

To summarize, four bacterial associates were isolated from the lichen *Stereocaulon* sp. In both paper disk diffusion test and MIC test, 26642 showed good activities in all solvents. According to 16S rRNA gene sequence analysis, this strain is close to *Bosea vestrisii* 34635^T (Figure 1). Furthermore, gas chromatography results of fatty acid analysis indicate that this bacterial associate has similar fatty acid profile with the genus *Bosea.* Fatty

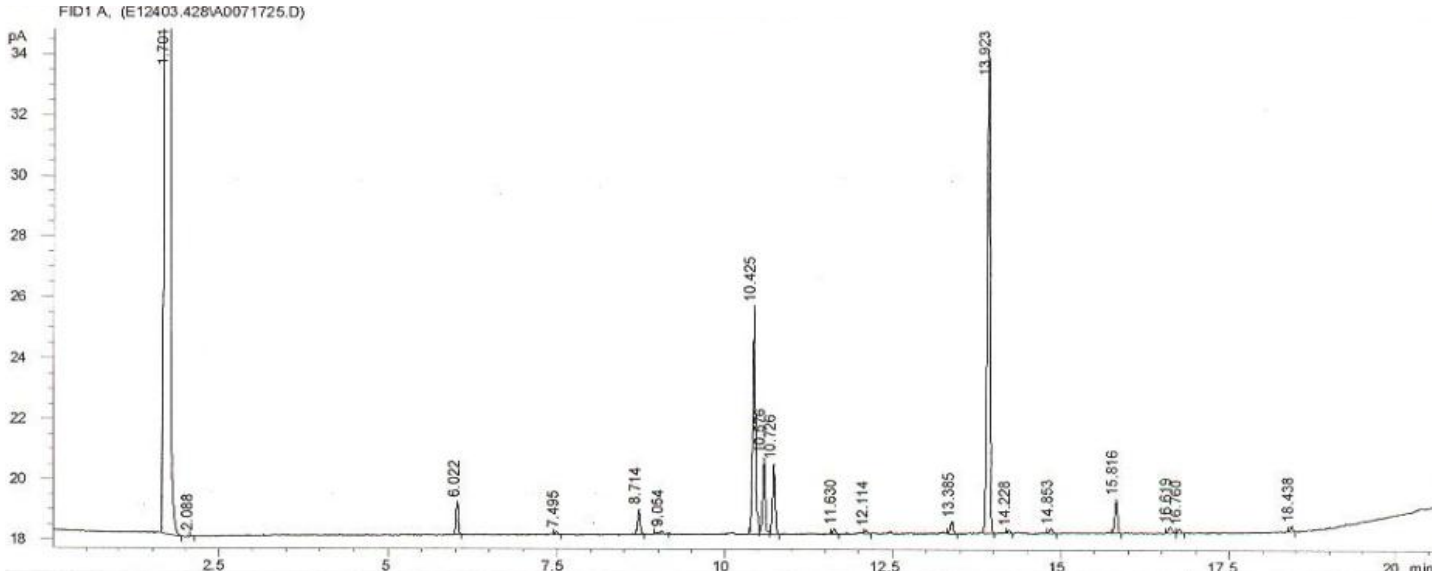

Figure 2. Fatty acid profile. Numerical data in figure indicated retention time of cellular fatty acids and expressed in follow form: fatty acid (retention time). Solvent peak (1.701); $C_{13:1}$ (6.022); $C_{14:0}$ (7.495); $C_{15:0}$ (9.054), summed in $C_{16:1}$ w7c/$C_{16:1}$ w6c (10.425); $C_{16:1}$ w5c (10.576); $C_{16:0}$ (10.726); $C_{17:1}$ anteiso w9c (11.630); $C_{17:1}$ w8c (12.114); $C_{16:0}$ 3OH (13.385); summed in $C_{18:1}$ w7c/$C_{18:1}$ w6c (13.923); $C_{18:0}$ (14.228); $C_{19:0}$ cyclo w8c (15.816); $C_{19:0}$ 10-methyl (16.619).

acid profile is summarized in Figure 2. There is no report about active compounds from *B. vestrisii* 34635^T. In our results, however, this strain might have antimicrobial compound. To our knowledge, this is first study investigating the bacterial associates from the lichen *Stereocaulon* sp. and their antibacterial activities. Considering various active compounds, it has been identified from fungal symbionts of lichen but not from bacterial symbionts, it is a novel approach to search for active antibacterial compounds. Therefore, it is worthwhile to continue searching and isolating meaningful microorganisms of lichen associate to investigate their bioactivities further detail.

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