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Phenotypic plasticity in *Bacillus cereus* strains isolated from various Antarctic habitats

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Abstract We studied five strains of psychrotolerant *Bacillus cereus* (*B. cereus*) isolated from Antarctic snow (BC_{sn}), ice (BC_{ic}), lake water (BC_{wr}), sediment (BC_{sd}), and soil (BC_{sl}) samples in terms of their growth, biochemical properties, and heat shock responses. Analyses of growth kinetics at 4℃ showed that BC_{sn} had the fastest generation time (16.1 h), whereas BC_{wr} had the slowest (30.8 h). Strain BC_{sd} formed the largest zone of lipid hydrolysis (18 mm) whereas BC_{sn} formed the smallest zone (3 mm).Only BC_{sd} produced gelatinase. These physiological differences illustrate adaptations of *B. cereus* isolates to different niches. Strains BC_{sl} and BC_{wr} were resistant to all 12 of the antibiotics tested. Strains BC_{sn}, BC_{ic}, and BC_{sd} were resistant to cell wall synthesis inhibitors (penicillin and ampicillin) and susceptible to protein synthesis inhibitors (tetracycline and streptomycin). A carbon-substrate utilization assay revealed that BC_{sn}, BC_{ic}, and BC_{wr} could specifically utilize D-glucose-6-phosphate, salicin, and 2'-deoxyadenosine, respectively, indicating a degree of metabolic diversity among these Antarctic *B. cereus* strains. An analysis of heat shock proteins (HSPs) produced in response to a 60℃ heat treatment revealed significant variations in the amounts of HSP33 ($p = 0.01$, $df = 4$), HSP44 ($p = 0.003$, $df = 4$), and HSP60 ($p = 0.04$, $df = 4$) among the strains. This emphasizes the importance of HSPs in bacterial taxonomy. These results show that there are considerable adaptive variations among *B. cereus* strains from extremophilic environments. This could be significant in evaluating the taxonomy and evolution of this species.

Keywords ***Bacillus cereus*, Antarctica, phenotype, heat shock protein, carbon utilization**

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1 Introduction

Microorganisms are fundamental to the functioning of Antarctic ecosystems. Bacterial diversity, community structure, abundance, and functional gene density have all been reported to be affected to different degrees by environmental conditions, and usually by interactions within each specific habitat[1-3]. Consequently, these Antarctic habitats provide models to study environmental effects on the evolution of phenotypically diverse microbial groups[4]. Microbial diversity and abundance in terrestrial and aquatic Antarctic ecosystems have been reviewed recently by Margesin and Miteva[5]. They stressed the significance of the functional activity, adaptation, and biogeography of psychrophilic microorganisms from different Antarctic habitats.

Microorganisms are dominant in most of Antarctic ecosystems. Because of their uniqueness, they are often identified as atypical strains of known species[6-7] or new species[8-9] in taxonomic studies. Strains within a single bacterial species usually have identical 16S rRNA sequences[10]. However, recent comparative studies on Antarctic and non-Antarctic bacterial strains of the same species suggest that there can be considerable variability in phenotypic and metabolic capabilities among strains[11]. The evolutionary relationships among microorganisms are often determined by comparing rRNA gene sequences. However, it is difficult to differentiate rRNA sequences among bacterial groups that diverged at nearly the same time, such as Gram-positive bacteria[10,12-13]. Other highly conserved molecules like heat shock proteins (HSPs) have been used for phylogenetic analyses because of their ubiquity and

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their high degree of sequence conservation[14-15]. This conserved group of proteins is essential for effective adaptation to changes in the environment, as well as to changes in the physiological state of the bacterial culture itself[16]. Recently, it was reported that the heatshock responses of Antarctic and non-Antarctic strains of *Cellulosimicrobium cellulans* varied markedly under similar stress conditions, and could therefore have significant ecological implications[16].

Strains of *Bacillus cereus* (*B. cereus*) adapt to various habitats from cold to hot thermal environments, and their habitat range is from alpine to temperate soils[17]. Guinebretière et al.[18] speculated that the global evolution of *B. cereus* may be strongly determined by ecological adaptation. Historically, the strains would have diverged as a result of variations in ecological tolerance limits. In the present study, we attempted to characterize the functional diversity of *B. cereus* strains isolated from Antarctica by comparing their physiological and biochemical characteristics, and their heat shock responses. We hypothesized that there is a high degree of phenotypic plasticity among *B. cereus* strains isolated from Antarctica.

2 Materials and methods

2.1 Sample collection, growth media, and colony isolation

Snow (3 samples), ice (1 sample), sediment (3 samples), soil (3 samples), and lake water (4 samples) were collected from the Larsemann Hills and Schirmarcher Oasis regions of East Antarctica during the XXVI Indian Scientific Expedition to Antarctica (March, 2007). Heterotrophic bacteria were cultured by plating the samples on 25%NA (100% NA consists of 28 g nutrient agar in 1 000 mL milliQ water) and incubating the plates for 8−10 d at 4℃ in an incubator (Sanyo, MIR 553, Wood Dale, Illinois, USA). Single colonies were selected and sub-cultured twice by streaking to ensure purity under similar growth conditions. The purity of the isolates was checked by Gram staining and a representative of each morphotype was identified by16S rRNA sequencing.

2.2 Identification by 16S rRNA sequence analysis and selection of test strains

The cells used for DNA extraction were obtained by growing each culture in Luria-Bertani medium. Chromosomal DNA was isolated and purified as described by Ausubel et al.[19]. The 16S rRNA gene was amplified using the universal bacterial 16S primers 5'-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3' (forward) and 5'-CCCGGGATCAAGCTTACGGCTACCTTGTTACGACTT-3' (reverse). Amplifications were carried out in 50-μL reaction mixtures containing 20 pmol each primer, 1.5 mM $MgCl_2$, 200 μM dNTPs, and 2.5 U *Taq* DNA polymerase. Amplifications were carried out in a thermocycler (BioRad CFX 96, Hercules, California, USA) with the following program: Initial denaturation at 95℃ for 2 min followed by 29 cycles of 30 s at 95℃, 30 s at 45℃, and 2 min at 72℃, with final extension for 10 min at 72℃. Amplification was confirmed by 1% agarose gel electrophoresis. The amplified products were eluted and purified using a Genei Pure gel extraction kit (GeNei, Bangalore, India). The eluted fragments were sequenced using an automated DNA sequencing system (Applied Biosystems, Carlsbad, California, USA). The 16S rDNA sequence of each isolate (~1 200 nt) was compared with those of type strains belonging to the same phylogenetic group obtained from a BLAST search at the National Centre for Biotechnology Information (NCBI) database.

The phenotypic characteristics the five strains of *B. cereus* isolated from snow (BC_{sn}), ice (BC_{ic}), sediment (BC_{sd}), soil (BC_{sl}), and water (BC_{wr}) were further studied. The 16S rRNA gene sequences of BC_{sn}, BC_{ic}, BC_{sd}, BC_{sl} and BC_{wr} strains were submitted to EMBL Nucleotide Sequence Database under the accession numbers HE660034-HE660038.

2.3 Growth profile of *B. cereus* strains

To evaluate the growth kinetics of each strain, 10 μL of an overnight-grown culture was seeded into 140 μL 25% Nutrient Broth (NB) in a sterile microplate (96-well flat bottom plate, BRAND, Wertheim, Germany). The culture was incubated at 4℃ on a rotary shaker (Lab companion IS-971R, Seoul, Korea) at 60 $r \cdot min^{-1}$ for 10 d. Growth was monitored at every 12 h during the 10-day period bymicroscopic cell counts after filtering 100 μL bacterial culture onto a 0.2 μm black track-etch polycarbonate membrane filter paper (Whatman, Maidstone, UK) and staining with 4',6-diamidino-2-phenylindole (DAPI)[20]. The fluorescent cells were counted under an epifluorescence microscope (Olympus BX 53, Tokyo, Japan) fitted with a U-MWU2 filter (excitation 330–385 and emission 420) at 1 000× magnification. The growth rate constant was computed for each 12-hour interval. Incubations and measurements were carried out in triplicate.

2.4 Biochemical screening and antibiotic sensitivity assay

Screening for the presence of DNase was performed on toluidine blue DNA agar (HiMedia, Mumbai, India) according to manufacturer's instructions. In brief, cultures were spot-inoculated onto the DNase plate and incubated at 4℃ for 4 d. DNase activity was identified as a color change in the medium from pink to red in a zone surrounding the area of bacterial growth. The isolates were also tested for gelatin liquefaction[21], lipase activity[22] and starch hydrolysis. Screening for starch hydrolysis was carried out by spot-inoculation onto dilute nutrient agar plates supplemented with 1% soluble starch (HiMedia). After 4 d, enzyme production was checked by flooding the plate with Gram's Iodine (1 g KI and 2 g I per 100 mL) and the zone of hydrolysis was measured. All incubations for enzyme

activity were carried out at 4℃.

The antibiotic sensitivity test was performed using the disc diffusion method. Fresh cultures were prepared in 0.9% saline by suspending 2–3 loopsful of an overnight-grown culture in 200 μL 0.9% saline, and then vortexing the mixture to distribute cells evenly. The bacterial suspension was spread onto 25% NA using sterile cotton swabs. Then, antibiotic-impregnated discs (HiMedia) of tetracyclin (30 μg·disc^{-1}), polymyxin B (300 units·disc^{-1}), neomycin (30 μg·disc^{-1}), novobiocin (30 μg·disc^{-1}), kanamycin (30 μg·disc^{-1}), streptomycin (300 μg·disc^{-1}), penicillin G (10 units·disc^{-1}), ampicillin (10 μg·disc^{-1}), chloramphenicol (30 μg·disc^{-1}), gentamycin (10 μg·disc^{-1}), vancomycin (30 μg·disc^{-1}) and erythromycin (15 μg·disc^{-1}) were placed on the seeded plates and incubated at 4℃. The zones of inhibition were measured after 4 d of incubation.

2.5 Single carbon-substrate utilization assay

We used Biolog GP2 MicroPlates (Biolog Inc., Hayward, California, USA) to analyze the ability of the strains to use 95 different organic compounds as sole carbon sources. The plates for Gram positive organisms contain 95 types of carbon sources including carbohydrates (42), carboxylic acids (16), amino acids (8), amines/amides (4), polymers (7) and miscellaneous (18). These substrates were size-fractioned into four classes (<100 Da, 100–500 Da, 500–1 000 Da, and >1 000 Da). If the bacterium is able to use the carbon source, there is a respiration-dependent reduction of a dye included with the carbon source, resulting in the formation of purple color that can be quantified and monitored over time. Negative wells remain colorless as does the reference well with no carbon source. The cultures were harvested after 4 d of incubation (at 4℃) from 25% NA plates with sterile physiological saline (0.9%). Each cell suspension was washed 5 to 6 times by centrifuging at 2 600 g for 10 min at 4℃ with subsequent vortexing each time. This step removed any nutrients from the agar plates remaining on the bacterial cells, which could otherwise result in a false-positive test. The five isolates were adjusted to a turbidity of 0.8±0.05 at OD_{600} and then each well in the plate was inoculated with 150 μL cell suspension. The plates were incubated at 4℃ and absorbance was measured at 595 nm at 0 h in a microplate reader (Biotek synergy 2, Winooski, Vermont, USA) and subsequently every 12 h for a period of 10 d to allow the development of the purple color. The percentage of substrates in each class utilized by each strain was determined.

2.6 Heat shock protein profile

Cultures were grown in dilute nutrient broth at 4℃ until they reached maximum growth (about 8 d). Then, they were incubated at 60℃ for 4 h and the total cell protein was extracted after 0, 1 h, and 4 h from a 20-mL culture sample. The control was maintained at 4℃ and total cell proteins were extracted simultaneously.

2.6.1 Protein extraction and quantification

Total proteins were extracted from 20-mL samples of the cultures. In brief, each culture was centrifuged at 6 000 g for 5 min at 4℃. Then, 0.1 mL protein extraction buffer (50 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), 0.1% Triton-X 100, 0.01% lysozyme, and 1 mM PMSF) was added to the pellet. The bacterial cells were homogenized in the extraction buffer with a sterilized pestle for 10 min on ice and then cells were disrupted in a sonicator bath (Transsonic Digital S [Elma], Singen, Germany) at 100% ultrasound power for 15 min, or in an ultrasonic homogenizer (Model 3000 Biologics, Inc., Manassas, Virginia, USA) at 30% power output for 30 s. The disrupted cell mixtures were then centrifuged at 6 000 g for 15 min at 4℃. The supernatants were collected in sterilized microfuge tubes and protein was quantified using the Bradford microassay method[23]. Absorbance was measured in a Microplate reader (Synergy 2, BioTek).

2.6.2 SDS-PAGE analysis and in-gel quantification of proteins

SDS-PAGE analysis was carried out as described by Laemmli[24]. Proteins were separated on a 12% acrylamide gel. In brief, the protein samples were dissolved in sample buffer (0.5 M Tris [pH 6.8], 2% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.01% bromophenol blue) and denatured by boiling at 100℃ for 5 min. Samples with equivalent protein contents were separated on the gel. Electrophoresis was performed at 50 V for 15 min and then at 100 V until completion. Gels were stained with 0.15% Coomassie Brilliant Blue. After destaining, gels were photographed with an Alliance 4.7 Gel doc system (Uvitec, Cambridge, UK). The molecular weights of proteins were determined by comparison with low-range SDS-PAGE standards (Bio-rad). The quantity of protein in each band was estimated by comparison with a known concentration of serum albumin (66 200 Da) as the reference band. Serum albumin was selected as the reference band because it showed the closest quantification value to the known value in an analysis using UVI Band software (Version 12.14) with the settings recommended by the manufacturer. The amount of protein in each band was determined by comparing the density of the band with that of the reference band, and the relative amounts of each protein in other lanes were calculated by the processing software. These data were used to quantify variations in the expression patterns of HSPs among the five *B. cereus* strains. The data were subjected to analysis of variance (ANOVA) using STATISTICA 9 software.

3 Results

3.1 Colony isolation, purification, and 16S rRNA sequence analysis

The plating of Antarctic snow, ice, sediment, soil and lake

water samples onto 25% NA media yielded several heterotrophic bacteria. The concentration of colony forming units (CFU) in snow, ice and sediment samples was 10^5–10^7 CFU·L^{-1}, 10^4 CFU·L^{-1}, and 10^5–10^8 CFU·L^{-1}, respectively, while soil and lake water samples contained 10^4–10^5 CFU·L^{-1}. The 16S rRNA sequence analysis using NCBI-BLAST alignments of ⩾1 200 nt of the distinct morphotypes indicated that the population was dominated by *Bacillus* spp. In total, 17 *B. cereus* strains were identified (Table 1), out of which five strains were selected to study their phenotypic characteristics.

Table 1 Details of the *B. cereus* strains obtained from Antarctic snow, ice, sediment, soil and water samples, and EMBL Nucleotide Sequence Database accession numbers of their 16S rRNA genes. Five strains selected for the study (BC_{sn}, BC_{ic}, BC_{sd}, BC_{sl} and BC_{wr}) are shown in bold

Source of isolate	*B. cereus* strains	Accession number
Snow (Larsemann Hills)	**BC_{sn}**	HE660034
	AntCr5	HF570059
	AntCr8	HF570061
	AntCr44	HF570080
	AntCr45	HF570081
Ice (Shirmarcher Oasis)	**BC_{ic}**	HE660035
Sediment (Larsemann Hills)	**BC_{sd}**	HE660036
	AntCr7	HF570060
	AntCr31	HF570073
	AntCr92	HF570100
Soil (Shirmarcher Oasis)	**BC_{sl}**	HE660037
	AntCr24	HF570071
	AntCr65	HF570089
	AntCr97	HF570102
Lake water (Larsemann Hills)	**BC_{wr}**	HE660038
	AntCr49	HF570083
	AntCr96	HF570101

3.2 Growth profiles of *B. cereus* isolates

The growth profiles of the five *B. cereus* strains at 4℃ are shown in Figure 1. The BC_{sn} strain (from snow) had the fastest generation time (16.1 h), and the BC_{wr} strain (from water) had the slowest (30.8 h). The generation times for BC_{sd}, BC_{ic}, and BC_{sl} strains were 16.2, 22.8, and 25.7 h, respectively (Table 2). The BC_{sn} and BC_{sd} strains showed similar generation times and growth patterns, with a maximum growth rate of about 0.06 h^{-1} recorded for both the strains at the end of day 2. The stationary phase for these two strains was demarcated by a reduced and almost constant growth rate commencing after 4 d of incubation. The BC_{sl} and BC_{wr} strains showed similar growth rates; both showed exponential growth until day 3 of the culture period. The BC_{ic} strain showed its maximum growth rate (0.085 h^{-1}) at the end of day 2. The BC_{ic}, BC_{sl}, and BC_{wr} strains showed a sharp decrease in cell number after 6 d of incubation.

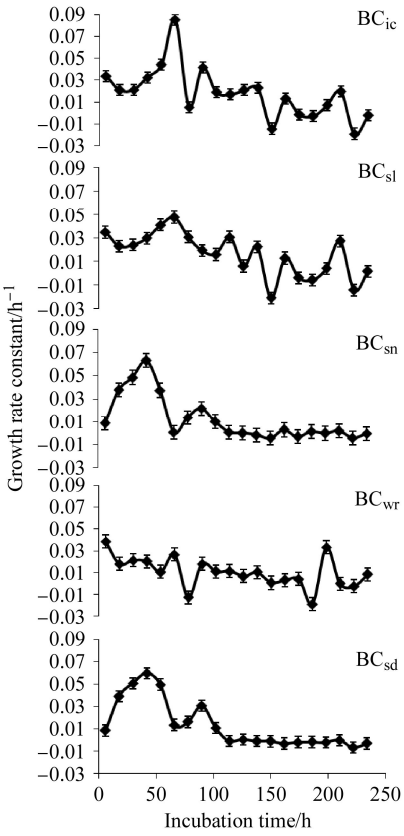

Figure 1 Growth rate constant at 4℃ measured for 10 d for five *Bacillus cereus* strains: BC_{ic}, BC_{sl}, BC_{sn}, BC_{wr} and BC_{sd}, isolated from Antarctic ice, soil, snow, water, and sediment, respectively. Error bars (±) represent standard error of the mean (n = 3).

Table 2 Physiological and biochemical properties of *B. cereus* strains BC_{sn}, BC_{ic}, BC_{sd}, BC_{sl} and BC_{wr} isolated from Antarctic snow, ice, sediment, soil, and water, respectively. "*" Values indicate diameter (mm) of zone of hydrolysis. R, resistant; S, sensitive

Characteristics	BC_{sn}	BC_{ic}	BC_{sd}	BC_{sl}	BC_{wr}
Generation time/h	16.1	22.8	16.2	25.7	30.8
Enzyme Profile*					
Amylase	—	—	—	—	—
Gelatinase	—	—	3	—	—
Lipase	3	6	18	10	12
DNase	—	—	—	—	—
Antibiotic Sensitivity					
Penicillin	R	R	R	R	R
Ampicillin	R	R	R	R	R
Chloramphenicol	R	R	R	R	R
Gentamycin	S	S	S	R	R
Kanamycin	S	S	S	R	R
Novobiocin	R	R	R	R	R
Tetracycline	S	S	S	R	R
Streptomycin	S	S	S	R	R
Vancomycin	R	R	R	R	R
Erythromycin	R	R	R	R	R
Neomycin	S	S	S	R	R
Polymyxin B	S	S	S	R	R

3.3 Biochemical screening and antibiotic sensitivity assay

All five *B. cereus* strains showed lipase activity, although the strength of lipase activity varied among the strains. Strain BC_{sd} had the largest zone of hydrolysis (18 mm) and strain BC_{sn} had the smallest (3 mm). The zones of hydrolysis for strains BC_{wr}, BC_{sl} and BC_{ic} were 12 mm, 10 mm, and 6 mm, respectively. Only one isolate (BC_{sd}) produced gelatinase, and none of the isolates produced amylase or DNase (Table 2). We tested the sensitivity of the strains to 12 different antibiotics, and found considerable variations in sensitivity among the five isolates (Table 2). Strains BC_{sl} and BC_{wr} were resistant to all 12 antibiotics tested. Strains BC_{sn}, BC_{ic}, and BC_{sd} were resistant to penicillin, ampicillin, chloramphenicol, novobiocin, vancomycin and erythromycin, and sensitive to gentamycin, kanamycin, tetracycline, streptomycin, neomycin, and polymyxin-B.

3.4 Single carbon-substrate utilization assay

The carbon-substrate utilization experiments tested the ability of the strains to use 95 different carbon sources, including carbohydrates, amides, polymers, amino acids, carboxylic acids and miscellaneous compounds (phosphorylated hydrocarbons, esters, sugar alcohols, nucleosides, etc.). The results revealed different utilization patterns among the five isolates. Strains BC_{sn}, BC_{ic}, BC_{sd}, BC_{sl}, and BC_{wr} oxidized 41, 54, 34, 49 and 54 carbon substrates, respectively, out of the 95 substrates tested. These substrates were also categorized into four different groups according to their molecular mass (<100 Da, 100–500 Da, 500–1 000 Da, and >1 000 Da). The differentially utilized substrates are shown in Table 3. Although the isolates oxidized all classes of substrates tested, they showed higher oxidation activity towards substrates of <100 Da, 100–500 Da, and >1 000 Da than towards substrates of 500–1 000 Da. All strains oxidized approximately 43% of substrates in the >1 000 Da group. For the other substrates tested, BC_{sn} oxidized 63% of substrates in the <100 Da group, 43% of the substrates in the 100–500 Da group, but none of the substrates in the 500–1000 Da group. This strain also specifically utilized D-glucose-6-phosphate, whereas none of the other strains were able to utilize this substrate. Isolates BC_{ic} and BC_{wr} showed similar trends in terms of their ability to utilize the different groups of substrates. BC_{ic} could oxidize 20% of the substrates in the 500–1 000 Da group, but BC_{wr} could not oxidize substrates in this group. Moreover, BC_{wr} differed from other strains in that it could utilize 3-methyl glucose, L-rhammnose, and thymidine substrates. Similarly, BC_{wr} was able to uptake osmoprotective disaccharides (palatinose and gentiobiose) and 2'-deoxyadenosine, but the other strains could not. BC_{sl} was able to oxidize 80% of the low molecular weight substrates (<100 Da), and specifically oxidized maltose, whereas the other strains could not. However, it was similar to the BC_{ic} strain in terms of its ability to oxidize substrates in the 500–1 000 Da group, and in its ability to utilize maltotriose. Strain BC_{sd} oxidized 63% of the substrates in the <100 Da group and 33% of substrates in the 100–500 Da group, but none of the substrates in the 500–1 000 kDa group. Unlike the other strains, BC_{sd} was unable to oxidize D-xylose, α-hydroxybutyric acid, and D-lactic acid methyl ester. Thus, the strains generally showed a greater ability to use low molecular weight substrates than high molecular weight substrates, and each strain showed a specific utilization pattern for the available carbon substrates.

Table 3 BIOLOG-GP2 substrate utilization of *B. cereus* strains BC_{sn}, BC_{ic}, BC_{sd}, BC_{sl} and BC_{wr} isolated from Antarctic snow, ice, sediment, soil, and water, respectively. C, carbohydrate; M, miscellaneous compound; A, amino acid; CA, carboxylic acid; AA, amine/amide; P, polymer. Only those substrates for which differential utilization was observed among the five strains are shown. "*" Percent oxidation indicates proportion of carbon substrates utilized out of total substrates in each size class

Substrate utilization	BC_{sn}	BC_{ic}	BC_{sd}	BC_{sl}	BC_{wr}
<100 Da					
Pyruvic acid (CA)	+	+	—	+	—
Acetic acid (CA)	—	—	+	+	+
Propionic acid (CA)	+	—	—	+	—
D-Alanine (AA)	—	+	—	—	+
L-Alanine (AA)	—	+	+	+	+
% oxidized*	63.6	72.7	63.6	81.8	72.7
100–500 Da					
Salicin (M)	—	+	—	—	—
Palatinose (C)	—	—	—	—	+
3-Methyl glucose (C)	—	+	—	—	—
D-Ribose (C)	+	+	—	—	+
D-Xylose (C)	-	+	-	+	+
D-Arabitol (C)	+	+	+	—	+
D-Galactose (C)	—	+	—	+	+
Gentiobiose (C)	—	—	—	—	+
Maltose (C)	—	—	—	+	—
D-Mannitol (C)	+	+	—	+	+
D-Mannose (C)	—	+	—	+	+
L-Rhamnose (C)	—	+	—	—	—
L-Arabinose (C)	+	+	+	—	+
Turanose (C)	—	+	—	+	+
Xylitol (C)	—	+	—	+	+
D-Psicose (C)	—	+	—	+	+
Sucrose (C)	-	-	+	+	+
D-Galacturonic acid (CA)	+	+	—	—	—
α-Hydroxybutyric acid (CA)	+	+	—	+	+
γ-Hydroxybutyric acid (CA)	—	—	—	+	+
α-Ketovaleric acid (CA)	—	+	—	+	+

(To be continued on the next page)

(Continued)

D-Lactic acid methyl ester (M)	+	+	—	+	+
Adenosine (M)	—	+	+	+	+
2'-Deoxyadenosine (M)	—	—	—	—	+
Pyruvic acid methyl ester (M)	+	+	+	+	—
Thymidine (M)	—	+	—	—	—
Uridine (M)	+	+	—	—	+
D-Glucose-6-phosphate (M)	+	—	—	—	—
D-L-α-Glycerophosphate (M)	+	—	—	+	+
L-Alanyl-glycine (AA)	—	+	—	+	+
Glycyl-L-glutamic acid (AA)	+	—	+	+	+
L-Pyroglutamic acid (AA)	—	+	—	—	+
L-Serine (AA)	+	+	—	—	+
% oxidized	43.1	58.3	33.3	50.0	59.7
500–1 000 Da					
Maltotriose (C)	—	+	—	+	—
% oxidized	0.0	20.0	0.0	20.0	0.0

3.5 Heat shock protein profiles

Heat shock proteins form a core group of conserved proteins. Therefore, we analyzed their expression profiles to evaluate differences among the five strains of *B. cereus*. A heat-shock treatment of 60℃ resulted in the upregulation of some heat shock proteins. The SDS-PAGE profiles of BC_{sn}, BC_{ic}, BC_{sd} and BC_{wr} revealed three major heat shock proteins (HSP33, HSP44, and HSP60) that were produced in large quantities (Figure 2a). However, in BC_{sl}, these proteins were downregulated under the heat shock treatment (Figure 2a). Statistical analysis showed that there was considerable variation (p = 0.01, df = 4) in the expression of HSP33 among the five *B. cereus* strains (Figure 2b). In BC_{sn}, the amount of HSP33 was increased by 139% after 1 h of heat shock, and increased by 190% after 4 h of heat shock. BC_{ic} and BC_{sd} showed an initial decrease in the amount of HSP33, but after 4 h of heat shock, the amount of HSP33 was increased by 20% and 5%, respectively. The maximum increase in the amount of HSP33 (674%) was in BC_{wr} after 4 h of heat shock. A statistical analysis showed that among the three heat shock proteins, HSP44 showed most significant variation (p = 0.003, df = 4) in expression among the strains. In all of the strains except for BC_{wr}, the amount of HSP44 decreased after 1 h of heat-shock, and then showed a marginal increase after 4 h of heat shock. In BC_{wr}, the amount of HSP44 was increased by 48% to 70% over the 4-hour heat-shock treatment (Figure 2c). The strains BC_{sn}, BC_{ic}, and BC_{wr} produced larger amounts of HSP60 than BC_{sd} and BC_{sl} did (Figure 2d). In BC_{sn}, the amount of HSP60 was increased by 159% at 1 h, and then decreased to 129% by 4 h of the heat-shock treatment (4 h). By contrast, in BC_{wr}, expression of HSP60 was upregulated (~30%) from 1 h to 4 h of the heat-shock protein. The maximum increase (173%) in the amount of HSP60 was in

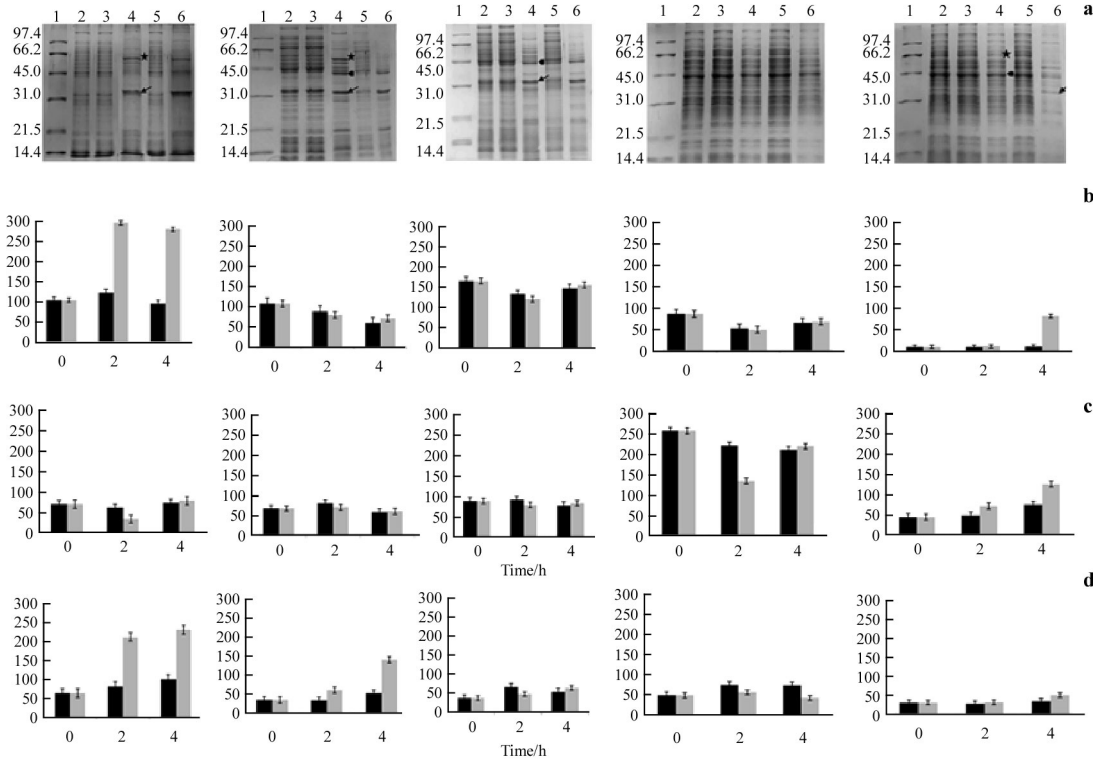

Figure 2 L-R SDS-PAGE protein profile of *B. cereus* strains BC_{sn}, BC_{ic}, BC_{sd}, BC_{sl} and BC_{wr} (**a**). Lanes: 1–molecular weight reference, 2–control at 4℃ for 0 h, 3–incubation at 4℃ for 1 h, 4–incubation at 60℃ for 1 h, 5–incubation at 4℃ for 4 h, 6–incubation at 60℃ for 4 h. Protein bands for three heat shock proteins 33 kDa (←), 44 kDa (■) and 60 kDa (★) are marked **b**, **c** and **d**, Concentration of HSP33, HSP44, and HSP60 proteins, respectively, at 4℃ (▬) and after 4 h of heat shock treatment at 60℃ (□) in BC_{sn}, BC_{ic}, BC_{sd}, BC_{sl} and BC_{wr} (from left to right). Error bars (±) represent standard error of the mean (n = 3).

BC_{ic} after 4 h of the heat-shock treatment. The expression pattern of HSP60 differed significantly (p = 0.04, df = 4) among the five Antarctic *B. cereus* strains.

4 Discussion and conclusion

We studied the phenotypic properties of five *B. cereus* strains isolated from snow, ice, sediment, soil and lake water samples from Antarctica. We analyzed the growth kinetics, enzyme production, carbon-source utilization patterns, antibiotic sensitivity, and heat shock protein expression patterns of each strain. The key phenotypic differences among these strains are summarized in Figure 3. In terms of growth kinetics, BC_{sn} had the fastest generation time (16.1 h) and BC_{wr} had the slowest (30.8 h). These differences in growth rates could reflect genotypic adaptations to different niches and temperatures, and represent the sum of many metabolic adaptations in different microorganisms[25-27]. The experiments of Harder and Veldkamp[28] showed that bacteria adapted to different niches and habitats show specific growth patterns and growth rates depending on their metabolic flexibility. Such metabolic adaptations include increasing substrate affinities in oligotrophic environments so that competition becomes dependent on the concentration of the growth-rate-limiting substrate[29].

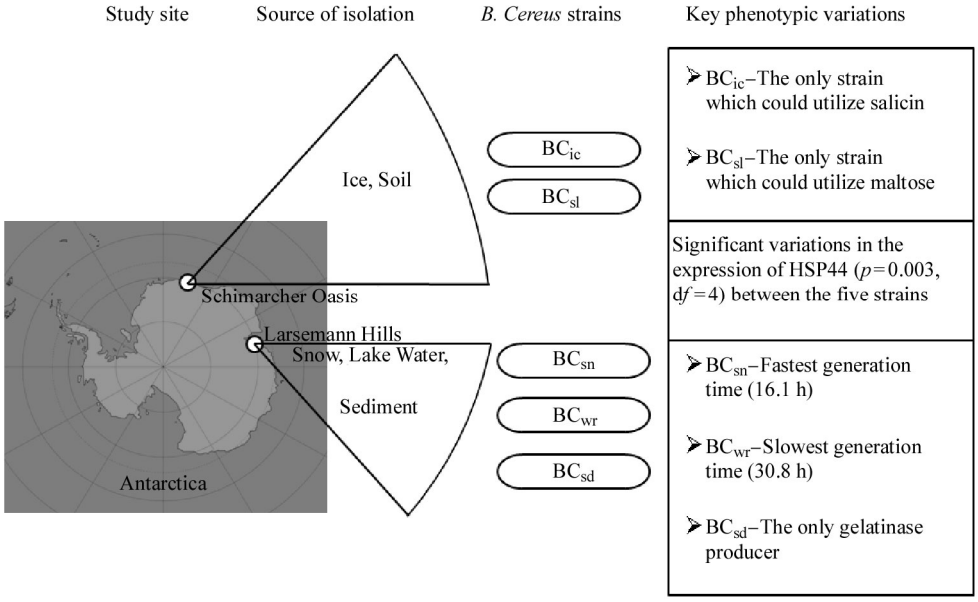

Figure 3 Schematic representation summarizes significance of the study. Study site, source of isolates, and key phenotypic variations among the five *B. cereus* strains are shown.

In these experiments, the only strain with gelatinase activity was BC_{sd}, even though the mesophilic type strain (ATCC 14579) of this species can liquefy gelatin[30]. Gelatinase might play a key role in degrading the complex organic compounds deposited in the sediment. Shivaji et al.[31] reported that many bacteria isolated from sediment of Antarctic lakes showed proteolytic activity. We detected lipase activity in all five isolates, but the activity varied markedly among the isolates; the highest activity was in BC_{wr} and the lowest was in BC_{sn}. These different patterns of enzyme production reflect the metabolic diversity within the native environment. This observation is consistent with earlier studies in which different Antarctic *Bacillus* spp. showed various abilities to catalyze hydrolysis of lipid substrates, depending on the source of the isolate[32]. We did not detect amylase activity in any of the five isolates, suggesting that they are unable to degrade complex polysaccharides. This was consistent with the results of the single carbon-substrate utilization assay. By contrast, the type strain of this species is known to produce amylase[30]. In a previous study, it was shown that the differences in bacterial enzymatic profiles are greater in more eutrophic environments than in less eutrophic environments[33].

Because *B. cereus* is widely reported as a food-borne pathogen[34-35], we analyzed the antiobiotic sensitivity of the five strains. We detected several antibiotic-resistant strains of *B. cereus* in the Antarctic soil and water samples, suggesting that the factors that maintain and perhaps promote the dispersal of resistance in natural environments should be considered carefully. BC_{sl} and BC_{wr} were resistant to all 12 of the tested antibiotics. Their resistance could be partly due their cell wall composition[36], as these isolates required intense sonication to disrupt the cellwall during the protein extraction process. An analysis of the cell wall composition of these *B. cereus* strains may increase our understanding of this mechanism of antibiotic resistance. The three other isolates, BC_{sn}, BC_{ic} and BC_{sd}, showed similar responses to the 12 antibiotics tested. These isolates were resistant to cell wall synthesis inhibitors (penicillin and ampicillin) but susceptible to common protein synthesis inhibitors (tetracycline and streptomycin). Previous studies on microbes from several different geographical locations in Antarctica

have reported the ubiquitous prevalence of antibiotic-resistant bacteria[37]. The presence of antibiotic-resistant *B. cereus* strains in the pristine Antarctic environment could also reflect anthropogenic disturbances or genetic transfer of antibiotic resistance and/or virulence determinants to bacteria in this region[38].

We conducted single carbon-substrate utilization studies on GP2 BIOLOG plates, and found that the isolates were able to use a broad spectrum of substrates including amino acids, amines, amides, carboxylic acids, carbohydrates, complex polymers, and other miscellaneous carbons. The ability of a bacterium to use a compound relies on the presence of a transport system for that particular compound and environmental conditions favorable for the activation of that transport system[39]. For instance, the strain BC_{sn} specifically used D-glucose-6-phosphate, whereas the other strains could not use this substrate. In a previous report, *Bacillus* sp. isolated from Antarctic snow were unable to oxidize this substrate[40]. Similarly, the specific utilization of salicin by strain BC_{ic} suggests that there could be metabolic interactions between this isolate from ice and algae in its natural environment; that is, algal exudates and various plant extracts can contain aryl β-glucosides, including salicin, which have been shown to play a role in signaling between bacteria and the substrate-producing organism[41]. Previous studies indicated that that the abilities of bacteria to utilize aryl β-glucosides as carbon sources could differ depending on their niches and metabolic selection pressures[42]. Substrates such as nucleosides, which the oretically are not readily utilized, were found to be the most favorable substrates for development of aquatic bacterial populations[43]. Specific utilization of 2'-deoxyadenosine by BC_{wr} is consistent with such observations. In addition, the ability of BC_{wr} to uptake osmoprotective disaccharidessuch as palatinose and gentiobiosemay reflect an adaptation to oxidative stresses in the Antarctic aquatic environment[44-45]. Similarly, BC_{sl} could specifically utilizemaltose, and all the isolates except for BC_{sd} were able to use D-xylose, α-hydroxybutyric acid, and D-lactic acid methyl ester. Thus, our observations indicate that bacteria tend to exploit the available resources as a result of differential gene expression to express specific metabolic capabilities. Previously, it was reported that substrates such as D-cellobiose, D-malic acid, adenosine 5'-monophosphate, thymidine 5'-monophosphate, uridine 5'-monophosphate, α-D-glucose 1-phosphate, glycogen, and α-cyclodextrin were utilized by a non-Antarctic mesophilic strain of *B. cereus*[46] whereas Antarctic *B. cereus* isolates were unable to oxidize these substrates. A similar observation was reported by Antony et al.[11], in that an Antarctic strain of Cellulosimicrobium cellulans showed a different metabolic profile from that of its mesophilic counterpart. These observations suggest that differences in metabolic capabilities among *B. cereus* isolates could be a result of physiological adaptations that allow them to survive in their native environment.

Analysis of highly conserved groups of proteins, such as heat shock proteins, have revealed a number of important differences with respect to rRNA-based phylogenies[47-50]. In the present study, the major HSPs were HSP33,HSP44, and HSP60. Apart from BC_{sl}, all the isolates showed increased amounts of these HSPs under heat shock. The inability of the Antarctic soil isolate BC_{sl} to upregulate heat shock proteins, while at the same time being tolerant to a 60℃ heat-shock treatment, is a subject for further investigation. The HSP33 protein showed the greatest increase (674%) in BC_{wr}. HSP33 was first characterized by Jakob et al.[51] as a redox-regulated protein in *Escherichia coli*. Heat shock induces the expression of HSP33 while oxidative stress activates its chaperone function. The prominent expression of HSP33 in BC_{wr} could be because this strain was exposed to oxidative stresses in its native environment[44]. Upregulation of HSP44 was observed in BC_{sn}, BC_{ic}, BC_{sd} and BC_{wr} strains, with the maximum increase (70%) in BC_{wr}. A likely candidate for this protein is HflK, which has an apparent molecular mass of 45 kDa. HflK together with HflC (a 38-kDa protein) are subunits of a multimeric protease which specifically degrades the λ phage protein CII in *E. coli*[52]. Many phage shock proteins can be induced by heat, ethanol, osmotic shock, and other environmental stresses[53]. In the present study, the HSP44 protein showed widest variation in quantities among strains (p = 0.003, df = 4). The ubiquitous heat shock protein HSP60 was expressed with significant variations among the five strains (p = 0.04, df = 4). The maximum upregulation of HSP60 (173%) was in BC_{ic}. Thus, our study indicates that there could be considerable differences in the expression patterns of this conserved housekeeping protein among *B. cereus* strains isolated from different habitats in the Antarctic environment. Previous studies also indicate that HSP60 plays a significant role in the heat shock response of Antarctic bacterial isolates[16]. Further studies could give valuable insights into the ecological significance of HSPs and their roles during the evolution of phenotypically diverse bacterial strains. In conclusion, our results show that there is a high degree of phenotypic dissimilarity among strains of *B. cereus* isolated from different Antarctic niches.

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