

Multilocus sequence based systematics of *Pseudomonas* sp. from supraglacial site of Sikkim Himalaya and their adaptational strategies

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Abstract

Background Being one of the most complex and diverse genera, deciphering the correct taxonomy of *Pseudomonas* species has always been challenging. This study investigates and resolves the taxonomic ambiguity of 11 strains of *Pseudomonas* obtained from the supraglacial site of East Rathong glacier. Since the supraglacial region represents an extreme, stressful environment, the inhabitant microorganisms must have evolved multiple adaptive traits that define their origin. Hence, for adaptation study, we examined the survivability of the 11 strains in physical conditions of freezing and ultraviolet radiation, and their ability to produce extracellular cold-active enzymes.

Results Multilocus sequence analysis (MLSA) using five housekeeping genes (1140 polymorphic sites) supported the taxonomic assignment of these strains to *Pseudomonas antarctica*, further supported by their lesser mean genetic distances with *P. antarctica* (0.73%) as compared to *P. fluorescens* (3.65 %). The studied strains displayed significant tolerance to freezing for 96 hours as compared to the mesophilic control strain, while except 4 strains, all strains exhibited substantial tolerance to UV-C radiations, and all strains produced cold active enzymes as well.

Conclusion MLSA successfully resolved the taxonomy of these significant group of bacteria from physical extremes of temperature and radiation. The isolates ERGC3:01 and ERGC3:05, owing to their polyadaptational attributes, may be considered promising for exploitation in various industries.

Background

The members of the genus *Pseudomonas* are known for their metabolically versatile nature and occurrence in diverse ecological niches [1]. The taxonomy of this genus has been revised from time to time and is now classified into two major lineages: *P. aeruginosa* group that includes all the clinical isolates and *P. fluorescens* complex containing the environmental isolates which are further subdivided into nine subgroups [2]. The *P. fluorescens* complex forms a relatively diverse group whose vast genetic and phenotypic heterogeneity poses complexity in their taxonomic assessment. It has been proposed that *P. antarctica* forms a separate subgroup within the *P. fluorescens* complex, together with the Antarctic species *P. antarctica* PAMC 27494 and *P. extremaustralis* 14-3 [1]. These species were previously placed under the *P. fluorescens* subgroup. So, there lies a narrow line between the subgroups *P. antarctica* and *P. fluorescens*.

At our institute we have been classifying the psychrotrophic bacteria to maintain its repository of genomic data from East Rathong glacier of Sikkim Himalaya [3-9]. While exploring the supraglacial site, we observed taxonomic ambiguity among a dominant group of *P. fluorescens* species. We obtained 11 unique morphotypes with distinct physiological characteristics, showing the closest 16S rRNA gene sequence similarity to *P. fluorescens* DSM 50090^T (Supplementary Table S1). Further evaluation of its taxonomic position with phylogenetic clustering (maximum-likelihood) based on the 16S rRNA gene sequence (Fig. 1) revealed taxonomic ambiguity.

Although 16S rRNA gene analysis is the best method with the largest database for bacterial taxonomic resolution, it is insufficient for efficient discrimination of bacterial taxa at the species level [5, 10]. Due to the extremely slow rate of evolution of the 16S rRNA gene, it often does not lead to the proper resolution of closely related species. On the contrary, the evolution of protein-encoding house-keeping genes is faster and hence provides a better solution for the discrimination of closely related *Pseudomonas* species (Mulet et al. 2009). Multilocus sequence analysis (MLSA) based on the combination of multiple housekeeping genes has been a reliable and preferred method for establishing the taxonomy at the species level [10].

Glacier ice has recently gained recognition as a biome driven exclusively by microorganisms [12, 13]. Encompassing the topmost layer of ice, the supraglacial zone receives ample sunlight and subject to deposition of microbial cells plus nutrients by the wind. Moreover, the liquid water produced during the surface melting of ice provides a medium for growth that supports a rich microbial life on the glacial surfaces [14, 15]. Microorganisms inhabiting the supraglacial site are bestowed with special adaptive features as they are constantly subjected to subfreezing temperatures, high hydrostatic pressure, low nutrient availability, and high exposure to ultraviolet radiations [16]. The mechanisms of their survival include the production of useful biomolecules in the form of cold-active enzymes and ice-binding proteins for maintaining the molecular central dogma and membrane fluidity at freezing conditions. Cold-active proteases from microorganisms isolated from cold habitats represent a major group of enzymes essential for metabolic and physiological functioning of an organism [17]. Another cellular protective response involves the production of photo protective compounds that respond to osmotic and oxidative stress resulting from high radiation exposure [18, 19].

Considering the significance of such bacteria from physical extremes of temperature and radiation, we conducted MLSA of eleven strains of *Pseudomonas* genus obtained from the supraglacial site of East Rathong glacier to resolve its taxonomy. To establish the fact that these unique group of bacteria are the inhabitants of the pristine supraglacial environment and not the result of any anthropogenic disturbance, we investigated their survivability in physical conditions of freezing and UV radiation, and examined their ability to produce extracellular cold-active enzymes.

Results

Sampling source and bacterial taxonomic affiliation

Ice meltwater samples were collected from three points on the supraglacial site [glacial surface of the ablation zone, (GPS: 27°33.149' N; 88°07.406' E)] of East Rathong glacier (Fig. 2). The pH of the collected ice melt water ranged from 6 to 6.7 at the collection site while the temperature was noted 8°C at the laboratory before further processing of the samples (Supplementary Table S3). The cultivated bacteria with unique morphotypes were selected. The eleven strains reported in the current study showed white to creamish pigmentation and produced fluorescent pigment on King's B medium [20] (Supplementary Fig. S1). The physiological characteristics of these eleven strains were evaluated (Supplementary Table S1).

The strains exhibited growth in the temperature range of 4-28°C, indicating their psychrotolerant nature. Five of the strains could grow till 37°C, the temperature optima for all being 20°C. Among eleven, ten strains were able to grow over a pH range of 4-12 while one strain showed growth in the pH range of 5-12. The majority of the strains showed tolerance to NaCl concentration ranging from 1-4% (w/v), while ERCE:11 could only tolerate up to 3% NaCl, and ERGC9:06 was tolerant to 5% NaCl.

Molecular characterization based on the 16S rRNA gene sequence (accession numbers provided in Supplementary Table S1) showed the closest sequence similarity (top-hit) of all eleven strains with *Pseudomonas fluorescens* DSM 50090^T. However, the sequences showed a similarity of above 99% (threshold) with other *Pseudomonas* strains as well.

The phylogenetic tree was constructed with all the neighbouring sequences showing >99% similarity to determine the affiliations of these eleven strains. In the phylogenetic tree, however, the strains showed close clustering with both *P. fluorescens* DSM 50090^T and *P. antarctica* PAMC 27494 (Fig. 1), which creates a taxonomic discrepancy. To clarify the phylogenetic affiliation of the strains, we partially sequenced the housekeeping genes *gyrB*, *ileS*, *nuoD*, *recA*, and *rpoD*, and concatenated the sequences to form a single sequence of 2526 bp. We analyzed the maximum likelihood trees of each housekeeping gene (Supplementary Fig. S2).

In the case of *gyrB* gene, the eleven *Pseudomonas* strains were grouped in a single cluster with *P. antarctica* and *P. fluorescens* strains, of which nine of the strains showed closer relatedness to *P. antarctica* strain based on evolutionary distances. With *ileS*, among eleven strains two of the strains ERCE:11 and ERGC7:16 showed clear affiliation to *P. antarctica* PAMC 27494, while *P. fluorescens* and *P. azotoformans* were the next closest neighbors. All of the eleven strains formed a separate cluster with *P. antarctica* and *P. veronii* in the *nuo D* gene based tree, with higher bootstrap value in case of *P. antarctica*. Similarly, with *recA*, the eleven strains formed one separate clade with *P. antarctica* strain, while ERCE:11 exhibited clear associations to *P. antarctica*. Phylogeny with *rpoD* revealed clustering of the eleven strains with *P. antarctica*, *P. fluorescens* and *P. orientalis* in a single clade, where ERGC7:16, ERGC8:03, and ERCE:11 formed a distinct clustering with *P. antarctica*. Thus, the single-gene trees based on housekeeping gene sequences gave a higher resolution than the 16S rRNA gene tree alone. The ML tree with MLSA showed clustering of the eleven strains with *P. antarctica* PAMC 27494 with a bootstrap value of 99, while *P. fluorescens* formed a sister taxon with a lower bootstrap value (Fig. 3). The nucleotide polymorphism statistics of 11 strains for each gene and five concatenated genes are displayed in Table 1. Neutrality test, i.e. Tajima's D was performed to test whether any of the tested genes have undergone recent selective events in each locus. There were no significant Tajima's D values for any housekeeping genes, which indicated a neutral evolution pattern for these genes. A significant negative Tajima's D value for the 16S rRNA gene depicts an excess of rare alleles resulting from a recent selective sweep. The *gyrB* gene showed the most parsimony informative sites with 318, followed by *nuoD* with 291, *rpoD* with 258, *recA* with 257, and *ileS* with 88. These values were higher than that of the 16S rRNA gene, hence providing more genetic information. The mean G+C content of the genes varied between 53.4 to 61.9%. The neutrality of sequence polymorphism was also checked by the ratio of non-synonymous (d_N) to

synonymous (d_S) nucleotide substitution values. Variation in the d_N/d_S ratio was observed for all the 5 genes. The $d_N/d_S < 1$ for the housekeeping genes *recA* and *rpoD* could be predictive that these genes were under purifying selection, while the remaining genes had values higher than 1, indicating their diversifying selection [21]. With MLSA, the concatenated sequences of 2526 bp showed a parsimony informative sites of 1140 and a G+C content of 56.1 mol%.

Table 1 Gene characteristics of 11 *Pseudomonas* strains

Locus	Length (bp)	S	P	Mean G+C content (%)	K2P distance (range)	π	Θ_W	Tajima's D	d_N/d_S
16S rRNA	1326	207	33	53.4	0-0.00455	0.01709	0.04067	-2.25705	0.97691
<i>gyrB</i>	504	379	318	53.4	0-0.0144	0.23835	0.20022	0.74543	1.34062
<i>ileS</i>	548	242	88	55.3	0-0.00933	0.06923	0.11407	-1.53265	2.33873
<i>nuoD</i>	526	351	291	58.5	0-0.00389	0.13681	0.17769	-0.89988	1.33249
<i>recA</i>	444	308	257	61.9	0-0.00693	0.18444	0.18541	-0.02047	0.46937
<i>rpoD</i>	494	295	258	61.1	0-0.00208	0.17852	0.16259	0.38264	0.92327
MLSA*	2526	1604	1140	56.1	0-0.00695	0.15881	0.17089	-0.27819	1.07161

S, number of segregating sites; P, parsimony informative sites; π , nucleotide diversity; Θ_W , theta (per site) from *Eta*; d_N/d_S , the ratio of non-synonymous to synonymous changes; *, *gyrB-ileS-nuoD-recA-rpoD* concatenated gene sequences.

The mean genetic distance calculated using Kimura 2-Parameter distance model was minimum with *P. antarctica* PAMC 27494 (0.73%), while with *P. fluorescens* DSM 50090^T the value was higher (3.65%). The distance values further increased with other close neighbours (Supplementary Table S4).

Based on the amplified DNA bands of ERIC-PCR products, clustering analysis revealed three main groups (Supplementary Fig. S3). Cluster 1 contained ERGC9:04 and ERGC5:06; cluster 2 comprised of two sub-clusters, one with two of the strains ERGC3:01 and ERGC3:05 and the other with six strains; while cluster 3 contained a single strain ERGC8:04. The results thus suggest genetic heterogeneity among the investigated strains.

Adaptational characteristics

Most of the strains showed survivability >65% after preliminary screening at 150 Jm⁻² UV-C dose. Further testing at 300 Jm⁻² screened for the strains that could efficiently survive the given dose of UV-C radiation. Among all, ERGC3:01 and ERGC3:05 showed 100% survival on UV-C exposure of 300 Jm⁻². ERGC2:04, ERGC8:03 and ERGC8:04 showed survivability above 60%, while ERGC5:06 and ERCE:11 showed comparatively lesser tolerance of <50% (45% and 33% respectively) to the tested UV-C dosage. On subsequent exposure to 450 Jm⁻² UV-C dosage, two strains i.e. ERGC3:01 and ERGC3:05 showed remarkable survival percentage of 58% and 69% respectively. This was closely followed by ERGC8:04,

ERGC8:03, and ERGC5:06. ERGC5:06 and ERCE:11 diminished to 15% and 13% respectively. Four of the strains i.e. ERGC7:07, ERGC7:16, ERGC9:04, and ERGC9:06, and the mesophilic control strain *Pseudomonas aeruginosa* MTCC 2453 showed zero survivability after all the given UV-C treatments (Fig. 4A). UV-C resistance of bacteria was determined using fractionated fluences in an earlier study [22].

The colony counts of most of the test strains increased after subsequent freezing conditions, except ERGC5:06 and ERCE:11 which showed decreased survival than the unfrozen control during the 96 h freezing experiment. Among all, ERGC2:04 maintained its percentage survival till 96h. ERGC3:01 showed decreased survivability for 48 h, after which an increased survival was observed, while ERGC3:05 displayed increasing colony counts for 48 h followed by a steep decrease in survival. ERGC7:07 maintained its CFUs with slight variations till 72 h, decreasing thereafter. Some strains like ERGC7:16 and ERGC8:03 showed unusual responses by exhibiting a rise and a sharp decline in percent survival only at one point over the time course respectively. ERGC8:04 exhibited increased survivability right after an initial decrease at 24 h of freezing. It was observed that ERGC9:04 showed decreasing survivability with a slight increase at 96 h, and ERGC9:06 also showed decreasing survival after a maximum survival at 48 h, while the survivability of ERGC5:06 and ERCE:11 decreased linearly during the entire experiment. Overall, the negative effect of freezing was most pronounced in ERGC5:06, ERGC7:07, ERGC7:16, and ERGC9:06 that reduced to <50% survival percentage by 96 h. The mesophilic control strain *P. aeruginosa* exhibited diminished survivability of 4% by 96 h of freezing (Fig. 4B).

Production of extracellular cold-active enzymes

Qualitative screening for extracellular hydrolytic enzymes in eleven strains of *Pseudomonas* showed the presence of one or more enzymatic activity at 10°C. Two of the strains ERGC5:06 and ERGC8:03 were positive for all of the four enzymes tested. Lipolytic and proteolytic activities were predominantly exhibited by all eleven strains, followed by five strains with cellulolytic and four with amylolytic activity respectively.

We conducted the quantitative estimation of protease, where the extracellular enzyme of eleven bacterial strains was first harvested after 52 h of production at 20°C. The activities were assayed using casein as a substrate. Eight of the strains, namely ERGC2:04, ERGC3:01, ERGC3:05, ERGC5:06, ERGC7:07, ERGC8:03, ERGC8:04, and ERCE:11, showed higher specific activities at 5°C as compared to 15°C. Among all the strains, the maximum specific activity of 8.46 U/mg was exhibited by *Pseudomonas* sp. ERGC2:04 at 5°C. No activity was observed at 5°C in case of the strains ERGC9:04 and ERGC9:06, but both showed a considerable activity at 15°C. ERGC3:01 displayed a highest activity of 1.36 U/mg at 15°C (Fig. 5).

Discussion

The presence of bacterial communities in supraglacial habitats has been documented before and many reports are available on the occurrence of *Pseudomonas* in the supraglacial ecosystems [23, 27]. It has been suggested that microbes in supraglacial environments are involved in the cycling of carbon and nutrients [15]. Being a common inhabitant of a cold environment, *Pseudomonas* is considered to be of

scientific and industrial significance as it is a metabolically versatile organism and an excellent producer of extracellular enzymes [28].

Our present study focussed on exploring, for the first time, the bacterial diversity from a supra-glacial ecosystem of the East Rathong glacier in Sikkim Himalaya with possible bioprospection and adaptational studies. In the course, many unique morphotypes of bacteria were obtained by culturing, and 32 of them were identified up to species level. Among 32 identified bacteria, 21 of them belonged to *Pseudomonas*, of which 11 strains notably showed the closest similarity to *P. fluorescens*.

In a previous study, MLSA has been proven to be a useful tool for the identification and determination of *Pseudomonas* sp. from Antarctica, where they studied a concatenate of six genes i.e. 16S rRNA, *aroE*, *glnS*, *gyrB*, *ileS* and *rpoD* [1]. Mulet et al. [29] used a concatenated sequence of 16S rRNA, *gyrB* and *rpoD* genes to establish the phylogeny and taxonomy of 33 strains of *Pseudomonas*. In another study by Mulet et al. [30], MLSA of four concatenated genes (16S rRNA, *gyrB*, *rpoB*, and *rpoD*) was used for the taxonomic affiliation of a *Pseudomonas* strain. From the multilocus sequence typing of the concatenated genes, which has been a more reliable method for taxonomic resolution than the traditional 16S rRNA gene, we inferred that our strains can be assigned to *P. antarctica*, based on the branching and bootstrap values. Although they show very close association to *P. fluorescens*, they formed a distinct cluster with *P. antarctica*, which we are referring to as *P. antarctica* cluster. Individual phylogenies of the genes were also analyzed, which provided a clearer picture. Especially *recA* gene tree placed all the strains in one cluster with *P. antarctica*, while other single genes did not reveal clear demarcation among species. The assigning of the 11 strains to *P. antarctica* was also supported by the genetic distance calculated by the Kimura-2-parameter model, which showed closer relatedness of these strains to *P. antarctica* (0.00734) than with *P. fluorescens* (0.0365). To estimate the genetic distance, evolutionary distances between two sequences are calculated on the basis of the number of nucleotide base substitutions [31]. The type strain *P. fluorescens* NCTC 10038 is a mesophilic strain with optimal growth temperature of 30°C (NCBI database), while our strains are all psychrotrophic with optimal growth temperature of 20 °C and probably shows more relatedness to the Antarctic strain *P. antarctica* PAMC 27494.

Highly similar 16S rRNA gene sequences between individuals do not essentially correspond to the high similarity of the genomes of different strains [32]. In our case, although the strains showed very similar 16S rDNA patterns, they differed by their morphological and physiological characteristics. To differentiate between the closely related strains of bacteria, repetitive element-based PCR (Rep-PCR) is reliable and widely used. Among Rep-PCR, Enterobacterial Repetitive Intergenic Consensus (ERIC) elements are commonly used for the molecular typing of Gram-negative bacterial genera [33]. ERIC-PCR results depicted genetic variability among the 11 strains.

The bacterial strains that we obtained from the glacial surface are psychrotrophic and are not strict psychrophiles, as they grew best at 20°C rather than 4°C, and the maximum limit of growth was 28°C for most and 37°C for a few. Frequent exposure of bacterial cells in glacier ice to repeated freeze-thawing

cycles and high ultraviolet radiations is common, and microorganisms isolated from such environments are expected to resist multiple harsh conditions.

Physiological assays were performed to evaluate the characteristics expected from bacteria isolated from a unique, cold niche. Since UV-C radiation is the most effective bactericidal agent, this provides an easy means for assessing highly radiation-resistant microorganisms [34, 35]. Bacteria isolated from high-altitude glacier ecosystems are likely to be UV-resistant owing to their constant exposure to UV-radiations with an increased reflection by ice [36]. Seven strains were more tolerant than the mesophilic *Pseudomonas aeruginosa* MTCC 2453 in the tested UV-C doses. Two of the strains ERGC3:01 and ERGC3:05 efficiently survived the dosage of 300 Jm^{-2} , equivalent to *Deinococcus radiodurans* MTCC 4465 ($p < 0.0001$). In fact, the given strains showed efficient survivability even at 450 Jm^{-2} . Five strains could tolerate the UV-C exposures with decreased survival percentage, while four strains could not survive any of the tested dosages. This depicts a variation in traits even among the same bacterial species obtained from the same niche. DNA repair mechanisms, including mismatch repair and recombination repair, have been suggested to be the common strategies for surviving the DNA damage caused by high UV radiations [34].

All strains exhibited notable tolerance to freezing conditions until the entire experiment period of 96 h, which is likely as freeze-thaw is a regular process in a high-altitude environment. The tolerance of eleven glacier strains to freezing was significantly higher than the mesophilic *P. aeruginosa* ($p < 0.05$). Such a physiological feature probably is important to support the survival of the *Pseudomonas* strains in the glacier environment where freeze-thaw is common physiological stress. It is known that the susceptibility of bacterial cells to freeze-thaw varies with strains, their physiological state, growth conditions, and other factors [37]. Each bacterial strain within the group demonstrated a unique response to the continuous freezing and thawing treatment. A subsequent increase in colony count was displayed by certain strains after different days of continuous freezing and thawing. Such an enhanced survival after freezing is common and has been documented in an earlier freeze-thaw study of ice core bacteria [38].

Microorganisms in frozen biomes have been reported to be metabolically active even under frozen conditions, through specific adaptive mechanisms. Upregulation of cell wall and membrane maintenance genes, cold-shock proteins, particularly RNA helicase protein CsdA as a key protein, and alterations in cellular energy metabolism have been proposed to be relevant for survival in icy conditions [39-41]. The structural and molecular adaptations in enzymes for maintaining a low temperature catalytic activity are vital for the survival and functioning of an organism in extreme cold niches [42]. All of the strains showed some or other hydrolytic enzyme activities at low temperatures. This particular trait shows the ability of these organisms to utilize complex organic matters, present in a limiting environment, as a source of their nutrients. Production of enzymes, as indicated by clear halos around the colonies, was observed at both 5°C and 20°C . The growth of the colony and zone of clearance developed much slower in the case of incubation at 5°C . Among hydrolytic enzymes, cold-active proteases catalyze the hydrolysis of peptide bonds in proteins and peptides and constitute an important class of enzymes with huge demand in the enzyme industries [43]. In our findings, most of the strains showed better enzymatic

activity at 5°C, which thus demonstrate that our strains exhibit the characteristics of cold-active enzymes. Owing to their higher activities at lower temperatures, these proteases may find potential industrial applications with economic and ecological benefits.

Conclusion

The approach of MLSA with five housekeeping genes was applied to establish the taxonomy of 11 strains of *Pseudomonas* isolated from the supraglacial site of Sikkim Himalaya. 16S rRNA gene analysis was helpful in identifying the strains at the genus level but was not significant in species identification. Based on the MLSA results supported by mean genetic distance values, the strains were identified as *P. antarctica*. Studies on adaptation demonstrated some of the strains i.e. ERGC2:04, ERGC3:01, ERGC3:05 and ERCE:11, to be the finest producers of protease enzyme at 5°C among others. Interestingly, the strains ERGC3:01 and ERGC3:05 exhibited superior tolerance to UV radiation up to 450 Jm⁻² and comparatively good survival on 96 hours of freezing that suggest the polyadaptational attributes of these particular strains that could be exploited for bioprospection potential in varied industry like detergents, cosmetics and pharmaceuticals.

Methods

Sampling site

Samples were collected from the supraglacial site of the East Rathong glacier, located between latitudes 27°33'36 'N and 27°36'40 'N, longitudes 88°06'03 'E and 88°07'38 'E [44]. It is a south-east facing, debris-free and summer-nourished glacier in the West district of Sikkim that forms the dominant glacier in Eastern Himalayas. The area experiences a cool and wet climatic condition, and snowfall is not unusual even during the monsoon season [45]. The sample collection was done in the month of May. Ice meltwater samples were collected in sterile 250 ml amber wide mouth bottles (Tarson, India), and transported in ice buckets with ice packs. Samples were stored at 4°C until analysis.

Isolation and identification of bacteria

The ice melt-water and the water samples of the glacier surface were enriched in sterile distilled water in shaking conditions for 2 h at 10°C. Subsequently, tenfold serial dilutions of each samples were made to plate in triplicate on R2A agar medium (pH 7.0; Himedia, India) and Antarctic Bacterial Medium (ABM) plates [peptone (0.5%, w/v), yeast extract (0.2%, w/v) and agar (2%, w/v) and incubated at 10° C for 10-15 days. Viable bacteria obtained in the agar plates were counted as colony forming units (CFU). Unique morphotypes from each plate were purified and maintained on ABM plates. The pure cultures obtained were preserved using 20 % glycerol at -80° C for further studies.

Growth parameters were checked at different temperatures (4, 20, 28, 37, 50°C), pH (1, 3, 5, 7, 9, 11, 13) and varying salt concentrations (1-6%) in ABM agar plates.

Molecular characterization

16S rRNA gene sequencing and phylogenetic analysis

Genomic DNA was extracted from each of the isolates using the CTAB method, as given by Chen and Kuo [46]. The 16S rRNA gene was amplified by PCR using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR was performed with 20 µl reaction mixtures containing approximately 50 ng of template DNA, 2 µM forward primer, 2 µM reverse primer, and 1X GoTaq Green PCR Master Mix (Promega, US). DNA amplification was carried out in G-Storm Thermocycler (Somerset, United Kingdom) with an initial denaturation step of 94° C for 5 minutes, followed by 30 cycles of denaturation at 94° C for 1 min, annealing at 55° C for 1 min, and extension at 72° C for 2 min and then a final extension step of 72° C for 5 min. The PCR products were purified prior to sequencing by treatment of ExoSAP-IT solution as per manufacturer's instructions (Affymetrix, US).

Purified PCR products were subjected to cycle sequencing using the forward, reverse and internal primers with Big Dye Terminator cycle sequencing kit v.3.1 (Applied Biosystems, US) protocol as described earlier [47]. The sequencing reaction was performed with 5 µL reaction mixture containing approximately 50 ng template DNA and 1 pmol of sequencing primers. Post reaction cleanup was performed using Montage Sequencing Reaction clean up kit (Millipore, US) using a Vacuum Pump Assembly (Millipore, US). Cleaned samples after cycle sequencing were bi-directionally sequenced using an automated Genetic Analyzer ABI 3130XL (Applied Biosystems, US).

The generated sequences were used to perform Basic Local Alignment Search Tool (BLAST) [48] analysis to determine the nearest phylogenetic neighbors against the database of type strains. For phylogenetic analysis, 16S rRNA gene sequences of the neighbors were obtained from the GenBank database (NCBI) and Molecular Evolutionary Genetics Analysis software (MEGA version X) was used for phylogenetic analyses [18]. The sequences of identified phylogenetic neighbors were aligned using Clustal W inbuilt with MEGA X. *E. coli* K12 MG1655 was used as the outgroup organism. Maximum likelihood method using Kimura 2-parameter model was employed to construct the Phylogenetic trees with 1000 bootstrap replications to assess nodal support in the tree.

Genotyping by ERIC-PCR

The isolates that showed similar phenotypes were compared through enterobacterial repetitive intragenic consensus (ERIC)-PCR. Briefly, genomic DNA was extracted and subjected to PCR amplification using the primers ERIC1 (5'-ATGTAAGCTCCTGGGGATTAC-3') and ERIC2 (5'-AAGTAAGTACTGGGGTGAGCG-3'), as given by Khosravi et al. [49]. The thermocycling conditions consisted of first denaturation cycle at 95 °C for 7 min followed by 30 cycles, including denaturation at 94 °C for 1 min, annealing for 1 min at 52 °C for ERIC-PCR, extension at 65 °C for 8 min, one final extension cycle at 65 °C for 16 min, and hold at 4 °C. The amplified products were subjected to electrophoresis on 1.5% agarose gel, stained with 0.5 µg/µl ethidium bromide (Qiagen, Germany) and analyzed under UV light in a gel documentation system

(Syngene G-BOX transilluminator, US). The banding profiles were observed. The visible bands were converted into a binary matrix and used to construct a dendrogram using the Jaccard similarity index and the unweighted pair group method (UPGMA) with the aid of software Past, version 3.25.

Multilocus Sequence Analysis (MLSA)

The eleven strains belonging to the species of *Pseudomonas* were studied for their phylogenetic relationship by multilocus sequence analysis of five housekeeping genes. The genes selected were *gyrB*, *ileS*, *nuoD*, *recA*, and *rpoD*, based on the criteria that they are present as single copies in the genome, and are homologous and ubiquitous in the studied taxa [10]. The primers used for PCR amplification of the housekeeping genes were based on previous studies [50] (Supplementary Table S2). The PCR condition for all genes consisted of: an initial denaturation step of 2 min at 94°C, followed by 35 cycles of 30s at 94°C, 20s at 54°C, and 2 min at 72°C, and a final extension of 7 min at 72°C. After Sanger sequencing, the partial sequences obtained for these five genes were submitted in GenBank (NCBI). Based on 16S rRNA gene similarity, seventeen closely related *Pseudomonas* species were taken and the partial sequences of these five housekeeping genes were retrieved from the complete genomes of corresponding type strains of *Pseudomonas* from the GenBank database. Multiple sequence alignment of the nucleotides was performed with CLUSTAL W and the sequences were trimmed manually for subsequent phylogenetic analyses. Sequences were translated to amino acid and the open reading frame was determined in MEGA X. For phylogenetic analysis, the partial sequences of the five protein-coding genes were concatenated into a single alignment using MEGA X in the order: *gyrB-ileS-nuoD-recA-rpoD*. Partitionfinder v2.1.1 [51] was used to determine the best-fit partitioning schemes and substitution models of molecular evolution. Maximum likelihood (ML) trees were constructed with the same partitioning schemes and model using RAxMLGUI v1.5 [52] through the CIPRES Science Gateway for individual and concatenated datasets [53].

Kimura 2-Parameter (K2P) genetic distances [31] were calculated between the eleven *Pseudomonas* strains and their nearest neighbours taken from the NCBI database using MEGA, Version X.

Nucleotide polymorphism

Gene parameters, such as GC content, number of polymorphic sites, parsimony-informative sites, synonymous and non-synonymous sites, percentages of mean sequence similarities, number of nucleotide differences per site (θ), nucleotide diversity per site (π) and Tajima's D statistic for individual gene sequences and the concatenated sequence were computed using MEGA X. The confidence of the branches of the ML tree was based on 1000 bootstrap replicates. *E. coli* K12 MG1655 was used as outgroup.

Physiological characterization

Tolerance to UV-C

For the radiation resistance test, the colony count method corresponding to UV-C irradiated aliquots and non-irradiated control was performed as described previously [54], with minor modifications. The

bacterial strains were grown in ABM broth till cell O.D. 600 of ≈ 1.0 was attained. After centrifugation at 8,000 rpm for 5 minutes, the pellet was washed and re-suspended in normal saline. UV-C exposures of 150, 300 and 450 Jm⁻² were given to the cell suspensions. Serial dilutions of UV irradiated as well as non-irradiated bacterial cultures were made and spread plated. After incubation for 3-5 days at 20°C, the number of colony-forming units was determined and the survival percentage was calculated. For comparison, a mesophilic type strain *Pseudomonas aeruginosa* MTCC 2453 and a radioresistant type strain *Deinococcus radiodurans* MTCC 4465 were subjected to the same conditions of UV-C exposure.

Tolerance to freezing

Tolerance to freezing was checked for 96 hrs. Cultures were grown till the stationary phase in ABM broth and culture tubes in triplicates were placed in -20°C freezer. After every 24 h of freezing, tubes were removed, thawed for 1 h at 20°C and 100 µL of the thawed culture was serially diluted in normal saline. The diluted culture was spread on ABM agar and incubated at 20°C for 2-4 days. The average of the triplicate colony counts was used for determining the survival percentage. Plates of unfrozen culture served as 0-time point control and a mesophilic type strain *Pseudomonas aeruginosa* MTCC 2453 was subjected to the same condition of freezing.

Production of extracellular cold-active enzymes

Screening of the isolates for extracellular hydrolytic activities

The isolates were screened for the production of hydrolytic enzymes such as amylase, cellulase, lipase, and protease at 10°C by spotting the cultures on their selective media containing specific substrate. Protease activity was assayed in Skim-milk agar. A clear zone around the colony indicated the production of proteases [55]. Lipase activity was checked on the enrichment medium Tributyrin agar. The colonies with clear hydrolysis zones were marked as lipase producers [56]. Carboxymethyl cellulose (CMC) agar was used for the detection of cellulase activity. On flooding the plates with Gram's iodine, the zone of clearance around the bacterial colonies represented positive cellulase producers [57]. Amylase activity was checked on starch agar plates. Amylolytic isolates were selected by flooding the starch agar plates with Gram's iodine solution. Isolates with distinct clear zone around the colonies were identified as amylase producers [58].

Quantitative estimation of extracellular Protease Activity

Extracellular protease activities of eleven bacterial isolates were determined by a previously described method [59]. In brief, eleven bacterial isolates were grown in Antarctic Bacterial Medium (0.2% yeast extract, 0.5% peptone) broth for 12h at 20°C. The bacterial growth was checked at 600 nm. 1% (v/v) bacterial culture was seeded into the protease production medium (K₂HPO₄ 0.1%, KH₂PO₄ 0.05%, CaCl₂ 0.02%, MgSO₄.7H₂O 0.05%, Glucose 1%) with 1% skim milk as substrate and incubated at 20°C, 120 rpm. After 48h of incubation, the cultures were centrifuged at 10,000 rpm for 15 mins at 4°C. The cell-free supernatant was used as a source of enzyme. For the enzymatic assay, the reaction mixture containing

100 µl of the enzyme and 400 µl of 1 % casein solution in 50 mM Tris buffer (pH 8) was incubated for 10 min at 5 and 15°C. The reaction was terminated by adding 0.5 ml of trichloroacetic acid (1.2 M) and centrifuged for 10 min at 6000xg. 500 µl of the filtrate was mixed with 1 ml of 400 mM Na₂CO₃ solution and 50 µl Folin-Ciocalteu's reagent. The amount of tyrosine released was determined spectrophotometrically at 660nm against the enzyme blank. The control was treated in the same way, except TCA was added before enzyme addition. One unit of protease activity was equivalent to the amount of enzyme that required releasing 1 µg of tyrosine/ml/min under standard assay conditions.

Statistical Analysis

All experiments were conducted in triplicate, and the results were presented as the mean ± standard deviation. Statistical analysis for significant differences was performed by one-way ANOVA followed by Dunnett's multiple comparison test (at $p < 0.05$ statistical significant differences) using the software GraphPad Prism7 (GraphPad Software, Inc., San Diego, USA).

Nucleotide sequence accessions numbers

The accession numbers obtained from NCBI GenBank for the 16S rRNA gene sequences against each strain are provided in Supplementary Table S1, and those for partial housekeeping gene sequences lie from MN238515-MN238591.

List Of Abbreviations

(w/v): weight by volume

d_N/d_S : non-synonymous to synonymous ratio

Jm⁻²: joule per square metre

Declarations

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Authors' contributions

SM collected the samples, performed initial screening and sequencing, wrote the manuscript; SM and AK performed the enzyme assays and statistical analyses; SM and PK performed the phylogenetic analyses; RK conceived and designed the research study, wrote and finalized the manuscript; SK provided overall supervision.

Competing interests

The authors declare no competing interests.

Availability of data and material

All the partial 16S rRNA and housekeeping gene sequences generated and analysed in the study are submitted in NCBI GenBank database and included in the article and its supplementary file (accession numbers are provided in Supplementary Table S1 and results section).

Consent for publication

Full authorization is provided by the people appearing in Figure 2(D) to BMC Springer Nature, granting full permission for the images to be published in an online open-access publication.

Ethics approval and consent to participate

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

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Figures

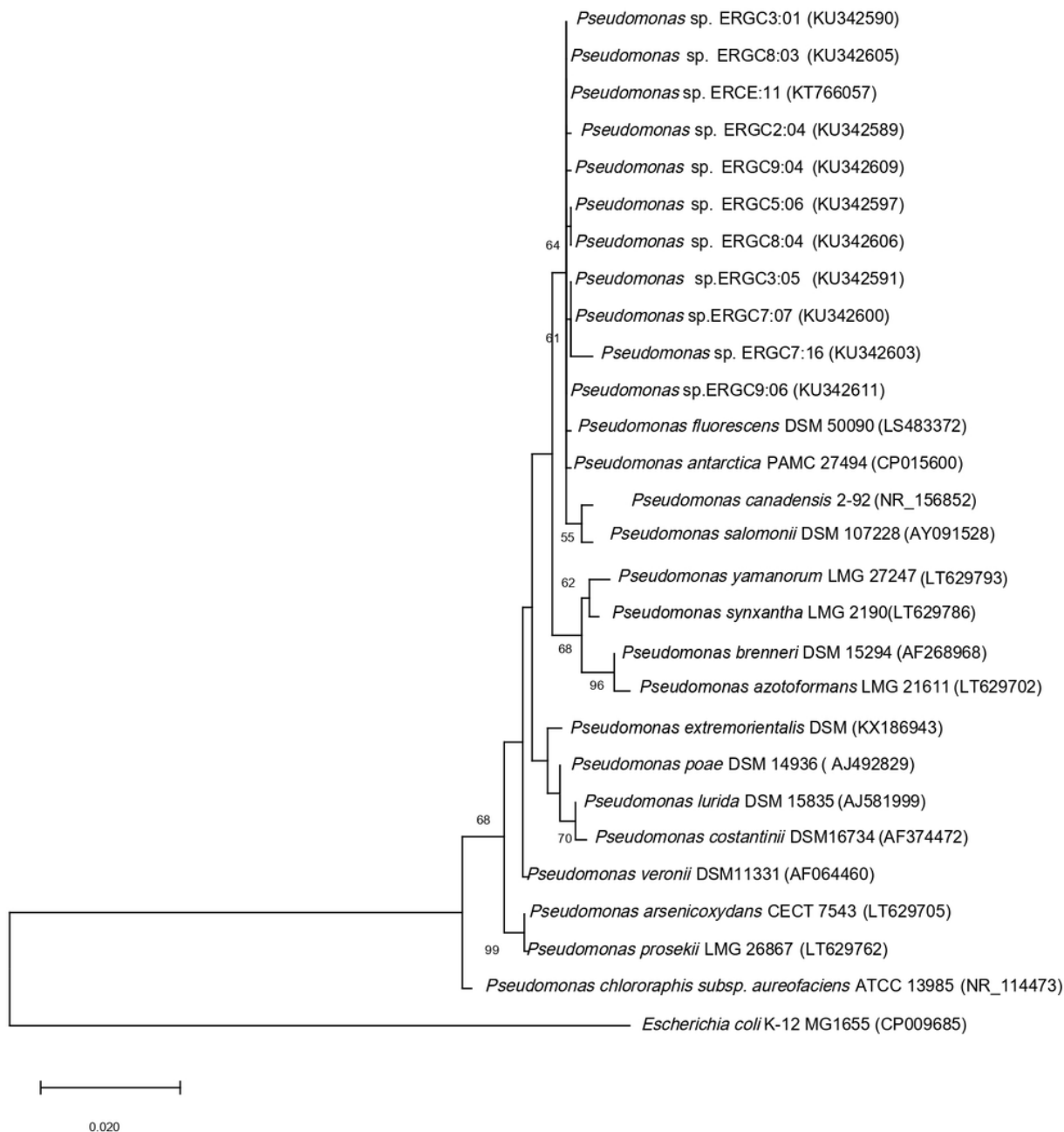


Figure 1

Maximum likelihood tree based on the partial 16S rRNA gene sequences of the 11 *Pseudomonas* strains and closely related validly published strains of the *Pseudomonas* genus obtained from NCBI database. The 11 *Pseudomonas* strains show clustering with both *P. fluorescens* and *P. antarctica*. *E. coli* K12 MG1655 was used as the outgroup organism. The tree was constructed using MEGA X software based on the Kimura 2 parameter model with 1000 bootstrap replications and the scale bar corresponds to the average number of nucleotide substitutions per site. Accession numbers are given in parenthesis.

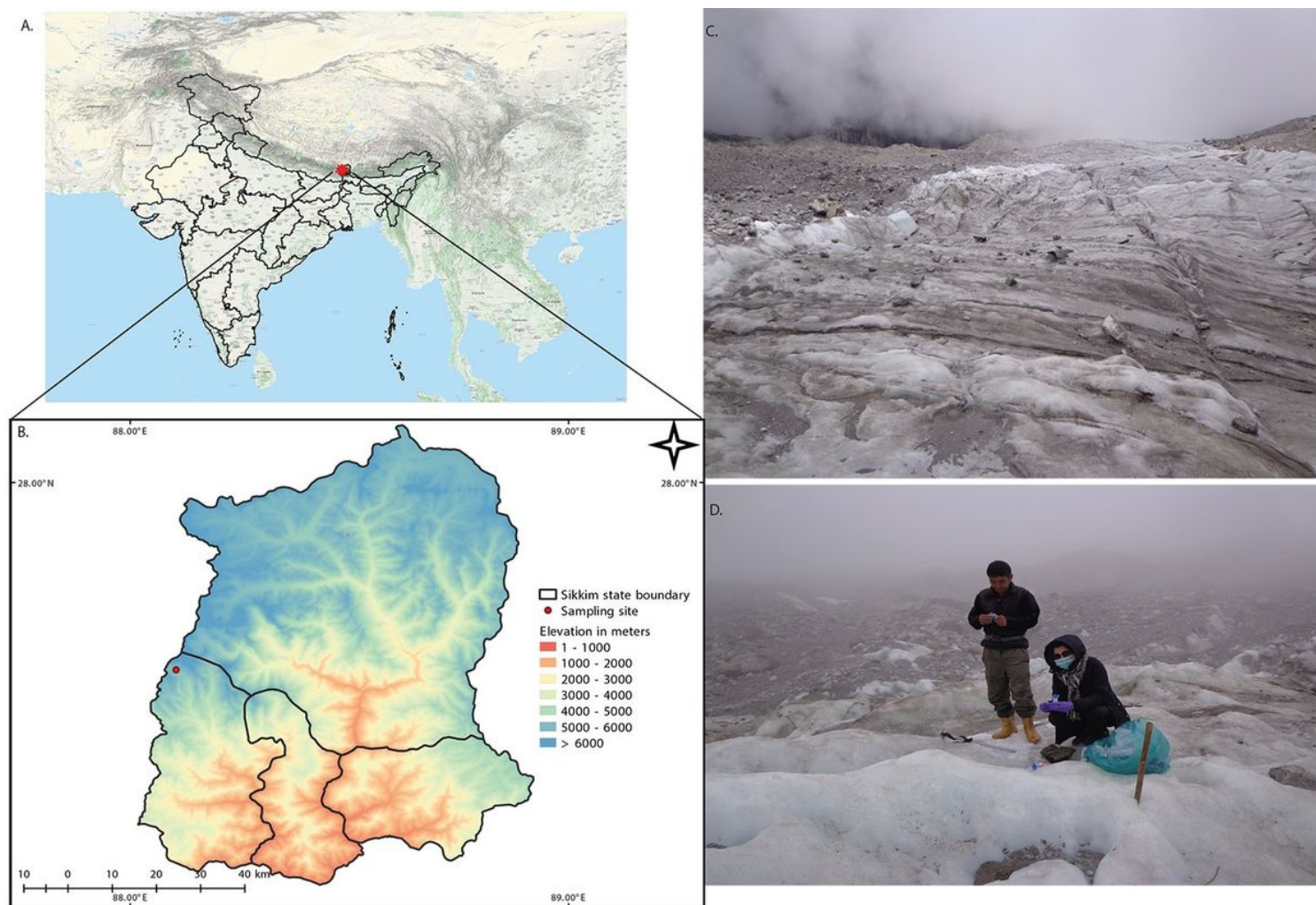


Figure 2

Map illustrating (A-B) geographical location of the sample collection site in Sikkim Himalaya, India. The red circle depicts the specific spot of the collected ice melt water sample; (C-D) sampling site comprising the supraglacial site in the ablation zone of the East Rathong glacier. The map shows sampled locations plotted on near 30 m resolution elevation data (source: Shuttle Radar Topography Mission, SRTM). The map was made in QGIS version 3.0.0-Girona (URL: <http://qgis.org>). Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

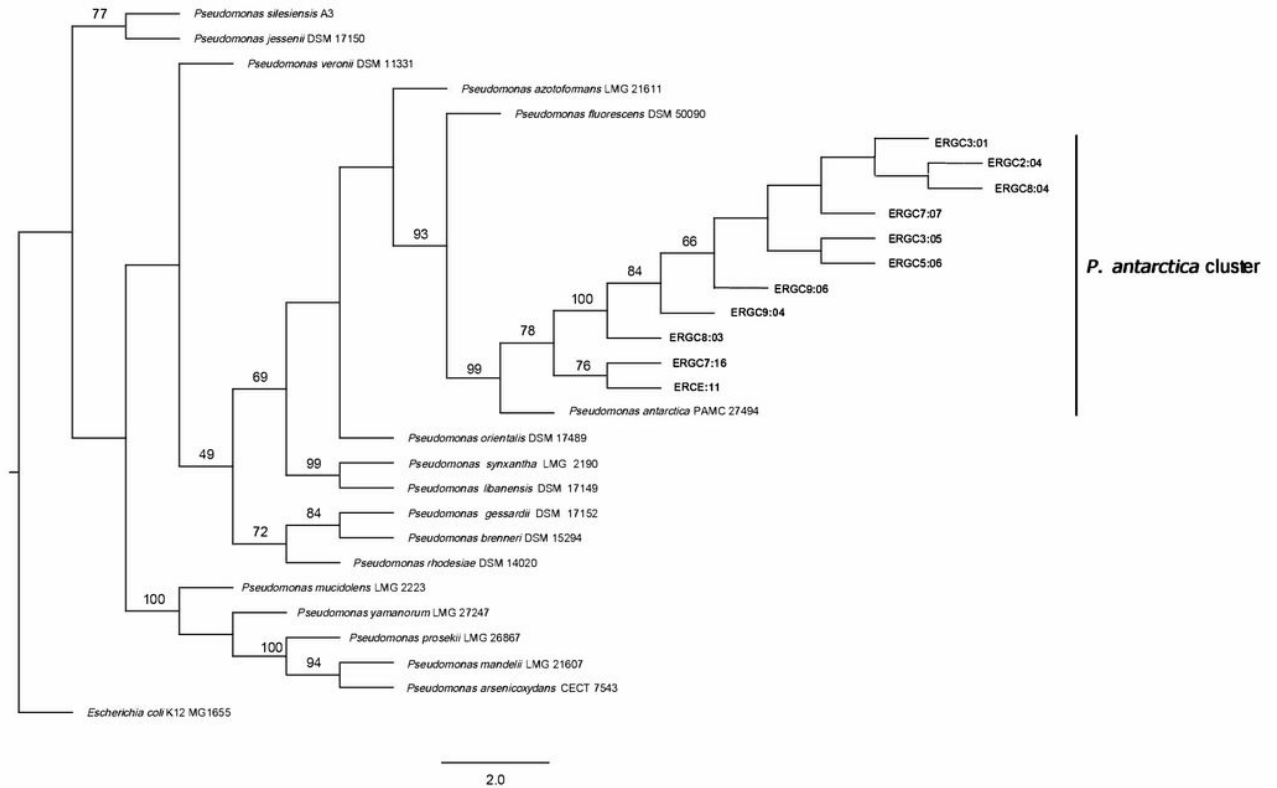
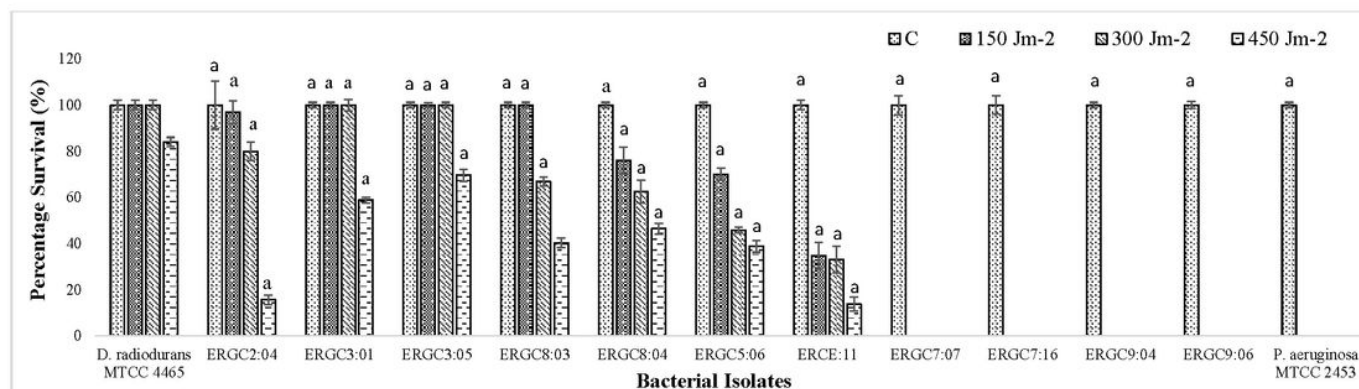


Figure 3

Phylogenetic tree of 11 *Pseudomonas* study strains based on the concatenated sequences of protein-coding genes *gyrB-ileS-nuoD-recA-rpoD* using RAxML. Support values are calculated from 1000 rapid bootstrap replicates (BT). BT values of 50 or more are indicated at branching points. The scale bar corresponds to the average number of nucleotide substitutions per site. Our test strains are marked in bold, that form a separate cluster with *P. antarctica* PAMC 27494. *E. coli* K12 MG1655 was used as the outgroup organism. Partitionfinder v2.1.1 was used to determine the best-fit partitioning schemes and substitution models of molecular evolution.

(A)



(B)

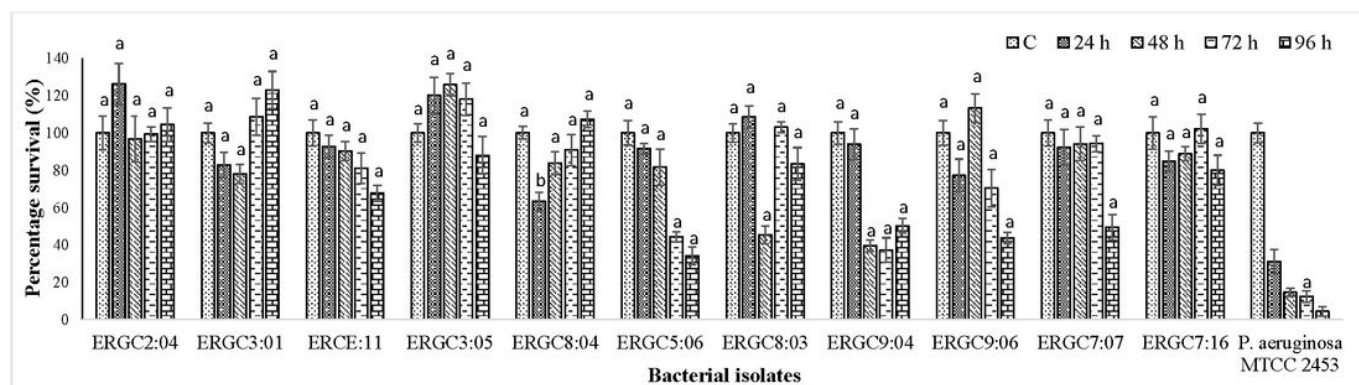


Figure 4

Tolerance of the 11 *Pseudomonas* strains to (A) UV-C irradiation at 150, 300 and 450 Jm⁻², (B) freezing for 96 h, whereby percentage survival was calculated relative to the colony count of non-irradiated and unfrozen control as 100% respectively. Mean of triplicate colony count was used to calculate percentage survival. Error bars denote standard deviation of mean of the three biological replicates (n = 3). Letters a, b denotes significant differences between the (A) positive control strain (*Deinococcus radiodurans* MTCC 4465) and test strains and, (B) negative control strain (*Pseudomonas aeruginosa* MTCC 2453) and test strains; at p<0.0001 and p<0.0002 respectively, using one-way ANOVA by Dunnett's multiple comparison test.

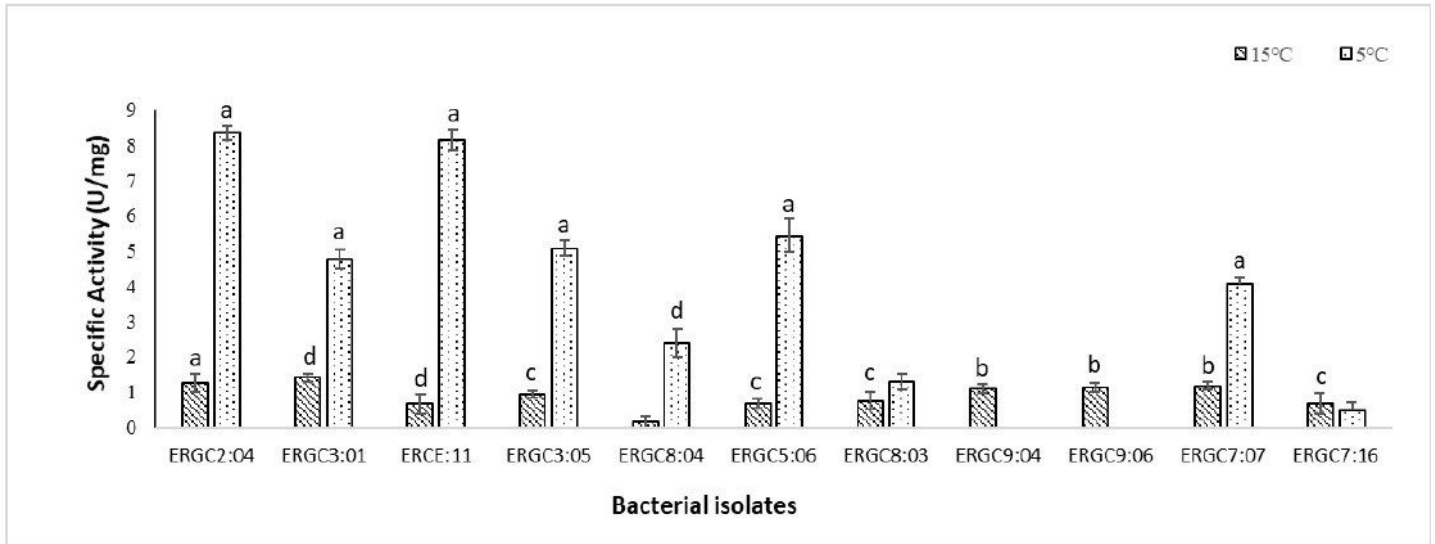


Figure 5

Protease activities of 11 *Pseudomonas* strains at 5°C and 15°C, with casein as substrate. Error bars denote standard deviation of mean of the three biological replicates (n = 3). Letters a, b, c, d denote significant differences between the reaction control and reaction test at $p < 0.0001$, $p < 0.0002$, $p < 0.0003$, $p < 0.0004$ respectively, using one-way ANOVA by Dunnett's multiple comparison test (No letter = non-significant value).

Supplementary Files

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