



## Diversity and phylogenetic analysis of pseudomonas strains isolated from Mingyong glacier, China

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### Abstract

On the planet genus of *Pseudomonas* encompasses debatably the most varied and ecologically important group of bacteria. In all of the main natural environments (Fresh-water, marine and terrestrial) members of *Pseudomonas* family are found in great numbers and these members also establish intimate relations with animals and plants. This universal spread and distribution represents a notable degree of genetic and physiological adaptability. Within the genus physiological traits do not limit diversity. The variety of phenotypes is also revealed at the genetic level, and evidence is increasing to advocate that the diversity of genome architecture of both the chromosomes and accessory genetic elements is of certain importance. In the present study different *Pseudomonas* strains were isolated from mingyong glacier. Phylogenetic analysis of 16S rRNA was done and 16S rRNA structures were applied to examine the diversity of *Pseudomonas* strains. Phylogenetical analysis showed that four branches are formed on the phylogenetic tree. These branches included *Pseudomonas* strains MY1402, MY1403, MY1408, MY1410, MY0503, MY1412, MY1416, MY1420 and MY1405 which were 98~99.9% close to 11 16S rRNA gene sequences of *Pseudomonas* sp (NCBI GenBank). These four groups can be easily distinguished by comparing their special characteristics in 16S rRNA secondary structures and sequences. The comparison of the 16S rRNA secondary structures of the strains showed that the *Pseudomonas* strains from mingyong glacier have diversity than other glaciers around the world.

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## Introduction

In order to determine if life can exist elsewhere in our solar system more importance is being given to the survival of microorganism in environments that are cold. After the evidence of ice on Europa and Mars, the study and isolation of psychrophiles has become principally important in order to determine the types of organisms that can exist in frozen environments and also to develop improved procedures for their cultivation. In this concern, sheets of glacier ice represent most suitable alternative for extraterrestrial cold habitats. These habitats are also vital as chronological and long term sources of microorganisms. Despite of their relative importance studies of viability, physiology and diversity of the organisms in frozen habitats are in initial stages (Miteva *et al.*, 2004). The genus of *Pseudomonas* is one of the most versatile and ecologically important group of bacteria in our planet earth (Spiers *et al.*, 2000) and it plays a very significant role in the nitrogen and carbon cycles (Cladera *et al.*, 2004). It is easy to isolate *Pseudomonas* bacteria from animal tissue, plants, soil and fresh water. The members in the genus *Pseudomonas* play a vital role in the bacterial structures present in the environment (Kwon *et al.*, 2003).

*Pseudomonas* spp. belongs taxonomically to the subclass gamma of Proteobacteria i.e rRNA group I (Kerstens *et al.*, 1996). The species which are in this group have been differentiated on the bases of cataloging 16S rRNA (Woese, 1987). In the bacteria, 16S rRNA sequence represents the most important present target of study in both bacterial evolution and its ecology, in which the determination of phylogenetic relationships among taxa, the quantification of the relative abundance of taxa of various ranks and the exploration of bacterial diversity in the environment are included (Hugenholtz *et al.*, 1998).

It is well known that the actual culturable bacteria in the environment are quite low in comparison with total amount present. However, a combination of culture-dependent and culture-independent methodologies should result in a more complete characterization of microbial diversity.

In this paper, the diversity of *Pseudomonas* strains isolated from mingyong glacier was studied based on the phylogenetic and secondary structure analysis of their 16S rRNA genes sequences. This is the first ever report on the diversity of *Pseudomonas* strains isolated from mingyong glacier in the narrated above condition.

## Materials and methods

### Culture Media

The Tryptic soy medium containing tryptone 15g, soytone 5g and sodium chloride 5g per 1,000 ml of water (pH 7.5). For a solid medium, 20 gram of agar was added before autoclaving.

### Isolation of bacterial strains

Mixtures of ice sheet and soil dust were collected at the ice tongue of glacier. Nearly 0.1 gram of sample was spread on the agar of the plate uniformly and plates were sealed with parafilm. All the plates were kept at 4°C to 8°C before transformation to lab and finally incubated at 8°C for a week. Cultures were purified by sub culturing in laboratory and were preserved at -80 °C in medium containing 15% glycerol.

### 16S rRNA gene sequencing and phylogenetic analysis

To amplify the 16S rRNA gene, two primers, forward primer (5'-GCGGATCCGCGGCCGCTGCAGAGTTT-GATCCTGGCTCAG-3') and reverse primer (5'-GGCTCGAGCGGCCCGCCGGTTACCTTGTTACGAC TT-3') were designed according to the method used by Robb *et al.*, (1995).

The 16S rRNA genes were amplified in the rmocycler (iCYCLE) using the La Taq PCR kit (TaKaRa). The temperature profile was set as following: 5 min 95°C, followed by 30s at 94°C, 30s at 50°C and 1 min at 68°C for 30 cycles while, finally extended at 68°C for 15 min. All PCR fragments were sub cloned into pCR4TOPO vector (Invitrogen) and the nucleotide sequences were determined.

The full length sequences were assembled using software Vector NTI-v9 (Infor Max, Inc). NCBI BLASTn (BLASTn 2.2.14) was used for sequence alignment with the 16S rDNA sequences

obtained in this study and representative sequences of related *Pseudomonas* species from the Gen bank. A phylogenetic dendrogram was generated by using program AlignX (Infor Max, Inc) and the neighbor-joining methods by use of Tree View v1.5 package (Page, 1996).

*16 S rDNA comparison and 16S rRNA secondary structure prediction*

Three 16S rDNA gene fragments ranged from 70 to 100 bp, 139 to 167 bp and 454 to 479 bp (referred to the nucleotide positions in *E. coli* 16S rDNA), respectively, were selected for 16S rDNA and 16S rRNA secondary structure prediction with program RNA structure 3.71 (Mathews *et al.*, 1999) and program RNA Viz (De Rijk *et al.*, 1997) was used to display the model structures.

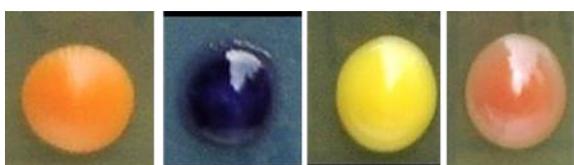
*Nucleotide sequence accession numbers*

The accession numbers and strain designations of the 16S rDNA/RNA reference sequences used in the phylogenetic and secondary structure analyses are *P. Antarctica*, AJ 537601; *P. fluorescens*, AY 512614; *P. gessardii*, AF 074384; *P. jessenii*, AF 068259; *P. libaniensis*, AF 057645; *P. meridiana*, AJ 537602; *P. migulae*, AF 074383; *P. orientalis*, AF 064457; *P. putida*, AY 958233; *P. synxantha*, AF 267911; *P. syringae*, AJ 576247; *P. trivialis*, AJ 492831.

**Results**

*Isolation of strains, morphological characteristics*

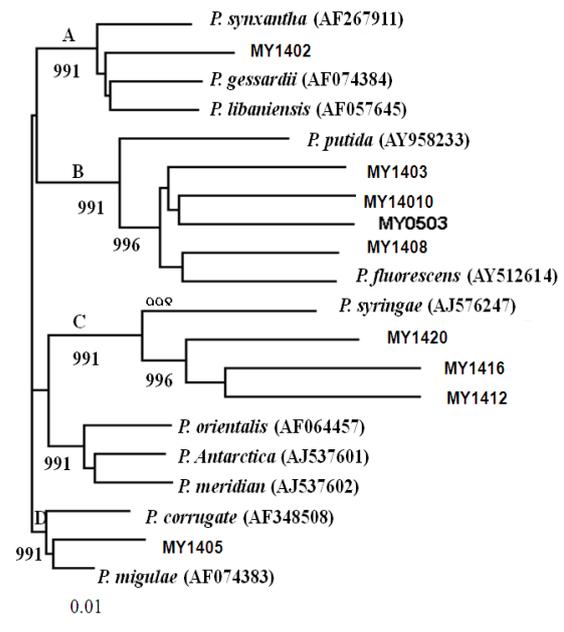
A total of 9 strains with different morphology and clone colors were purified from 100 samples. These strains can produce intracellular yellow, orange, red or purple pigments as shown in Fig. 1. All the isolated can grow at 4°C but cannot grow at 37°C. The optimum grown temperature of most strains was about 13°C.



**Fig. 1.** Morphological characteristic of 9 strains.

*16 S rRNA genes determination and phylogenetic analysis*

16S rRNA gene sequences of the nine *Pseudomonas* strains were determined. Which were further deposited into NCBI Genbank database under the accession numbers MY1402, EF062802; MY1403, EF062803; MY1408, EF062804; MY1410, EF062805; MY1416, EF062806; MY1420, EF062807; MY0503, EF062808; MY1405, EF062809; MY1412, EF079867, respectively. BLASTn 2.2.14 program were used to align the sequences against other sequences from the public databases. Eleven 16S rRNA gene sequences of *Pseudomonas* sp with higher similarity values (98~99.9%) against the sequences from mingyong glacier were chosen for further phylogenetic analysis. The result showed that there are four branches formed on the phylogenetic tree as shown in Fig 2.



**Fig. 2.** 16S rRNA phylogenetic tree of nine strains isolated from Mingyong glacier.

Branch A included strains MY1402 that had 99% similarity to *P. synxantha* and *P. libaniensis*. Branch B included strains MY1403, MY1408, MY1410 and MY0503, which clustered with *P. fluorescens* and *P. putida* (99% similarity). Branch C which included strains MY1412, MY1416 and MY1420 appeared to form the monophyletic branch with *P. syringae* (99% similarity).

Branch D included strain MY1405 which was very close to *P. corrugata* and *P. migulae* (similarity 99%).

These four monophyletic branches, A, B, C and D were supported with bootstrap values of 994, 993, 991 and 999 respectively.

The comparison of 16S rDNA sequences in branches A, B, C and D were shown in table 1. Branch A and D shared the same nucleic acid sequence in helix 6, and branch B and C had their specific sequences, respectively. But in helix 8, branch A and C had the same sequences while branch B and D were distinguished by their special sequences.

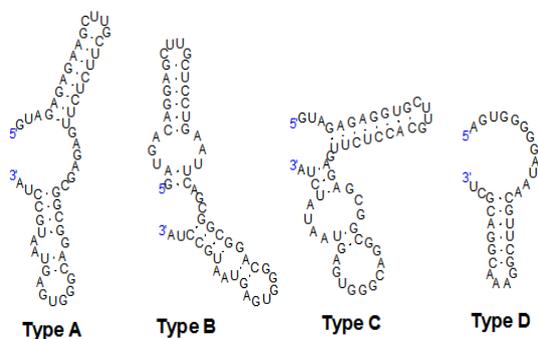
**Table 1.** Comparison of 16S rDNA sequences and their 16S rRNA structure in helix 6, helix 8 and helix 17 of stains isolated from Mingyong glacier and some species of genus *Pseudomonas*.

Branch Species	Helix 6*		Helix 8		Helix 17			
	Nucleic acid sequence		rRNA type	Nucleic acid sequence		rRNA type	Nucleic acid sequence	
A <i>P. synxantha</i>	GTAGAGAGAAGCTTGCTTCTCTGAGAGC		A	AGTGGGGGATAACGTTTCGAAAACGGAC		D	GGTTGTAGATTAATACTCTGCAAT TTT	
A <i>P. gessaidii</i>	GTAGAGAGAAGCTTGCTTCTCTGAGAGC		A	AGTGGGGGATAACGTTTCGAAAACGGAC		D	GGTTGTAGATTAATACTCTGCAAT TTT	
A <i>P. libanensis</i>	GTAGAGAGAAGCTTGCTTCTCTGAGAGC		A	AGTGGGGGATAACGTTTCGAAAACGGAC		D	GGTTGTAGATTAATACTCTGCAAT TTT	
A MY1402	GTAGAGAGAAGCTTGCTTCTCTGAGAGC		A	AGTGGGGGATAACGTTTCGAAAACGGAC		D	GGTTGTAGATTAATACTCTGCAAT TTT	
B <i>P. putida</i>	GATGAAAGGAGCTTGCTCCTGGATTGAGC		B	AGTGGGGGACAACGTTTCGAAAAGGAAC				
B <i>P. fluorescens</i>	GATGACAGGAGCTTGCTCCTGAATTCAGC		B	AGTGGGGGACAACGTTTCGAAAAGGAAC				
B MY1403	GATGACAGGAGCTTGCTCCTGAATTCAGC		B	AGTGGGGGACAACGTTTCGAAAAGGAAC				
B MY1408	GATGACAGGAGCTTGCTCCTGAATTCAGC		B	AGTGGGGGACAACGTTTCGAAAAGGAAC				
B MY1410	GATGACAGGAGCTTGCTCCTGAATTCAGC		B	AGTGGGGGACAACGTTTCGAAAAGGAAC				
B MY0503	GATGACAGGAGCTTGCTCCTGAATTCAGC		B	AGTGGGGGACAACGTTTCGAAAAGGAAC				
C <i>P. syringae</i>	GTAGAGAGGTGCTTGCACCTCTGAGAGC		C	AGTGGGGGATAACGTTTCGAAAACGGAC		D	GGCATTAACTAATACGTTAGTGC TTT	
C MY1412	GTAGAGAGGTGCTTGCACCTCTGAGAGC		C	AGTGGGGGATAACGTTTCGAAAACGGAC		D	GGCAGTTACTTAATACGTTGATTGT CTT	
C MY1416	GTAGAGAGGTGCTTGCACCTCTGAGAGC		C	AGTGGGGGATAACGTTTCGAAAACGGAC		D	GGCAGTTACTTAATACGTTGATTGT CTT	
C MY1420	GTAGAGAGGTGCTTGCACCTCTGAGAGC		C	AGTGGGGGATAACGTTTCGAAAACGGAC				
D MY1405	GTAGAGAGAAGCTTGCTTCTCTGAGAGC		A	AGTGGGGGATAACGTTTCGAAAACGGAC				
D <i>P. corrugata</i>	GTAGAGAGAAGCTTGCTTCTCTGAGAGC		A	AGTGGGGGATAACGTTTCGAAAACGGAC				
D <i>P. migulae</i>	GTAGAGAGAAGCTTGCTTCTCTGAGAGC		A	AGTGGGGGATAACGTTTCGAAAACGGAC				

\* Positions from 70 to 100, from 140 to 168 and from 455 to 481 in *E. coli* 16S rRNA were helix 6, helix 8 and helix 17, respectively.

**Secondary structure comparison**

Comparison of the 16S rRNA secondary structures of the strains showed that there were three different types in helix 6 (type A, B and C) and helix 8 type D. In helix 6 two branches (A and D) shared the type A, branch B shared types B and branch C shared type C. In helix 8, branch B and D had their specific types as shown in Fig. 3.



**Fig. 3.** Appearance of 16S rRNA secondary structures from Mingyong Glacier.

**Discussion**

Many kinds of microorganisms, including snow algae and bacteria, have been found on glacier surfaces in various parts of the world. These microorganisms make up an ecosystem adapted to snow and ice, which includes cold-tolerant animals such as insects and copepods in the Himalayas and Patagonia. It is well known that as compared to lower productivity environment diversity would often be greater in more productive environments. Mingyong glacier stretches are present in the valley and valleys flourish forests and the mingyong glacier also stretch across valley so it is easy for nutrients and bacteria to transfer from forests to the glacier. As a matter of fact this transfer can have an effect on the distribution of *Pseudomonas* sp on the surface and as well as in the glacier interior (Rees *et al.*, 2004).

Nine strains of *Pseudomonas* from mingyong glacier fell into the branch of A, B, C and D on the phylogenetic tree, respectively and these *Pseudomonas* strains were shown diversity as Abyzov *et al.*, (2001) reported that deep glacier microorganisms have high diversity. The phylogenetic diversity was also demonstrated by the comparison of 16S rDNA sequences and 16S rRNA secondary structure of helices 6, 8 and 17. This research is the first attempt to reveal the diversity of *Pseudomonas* strains that are contributed in the mingyong glacier.

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