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Alginate overproduction and biofilm formation by psychrotolerant *Pseudomonas mandelii* depend on temperature in Antarctic marine sediments

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ABSTRACT

Background: In recent years, Antarctica has become a key source of biotechnological resources. Native microorganisms have developed a wide range of survival strategies to adapt to the harsh Antarctic environment, including the formation of biofilms. Alginate is the principal component of the exopolysaccharide matrix in biofilms produced by *Pseudomonas*, and this component is highly demanded for the production of a wide variety of commercial products. There is a constant search for efficient alginate-producing organisms.
Results: In this study, a novel strain of *Pseudomonas mandelii* isolated from Antarctica was characterized and found to overproduce alginate compared with other good alginate producers such as *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*. Alginate production and expression levels of the alginate operon were highest at 4°C. It is probable that this alginate-overproducing phenotype was the result of downregulated MucA, an anti-sigma factor of AlgU.
Conclusion: Because biofilm formation is an efficient bacterial strategy to overcome stressful conditions, alginate overproduction might represent the best solution for the successful adaptation of *P. mandelii* to the extreme temperatures of the Antarctic. Through additional research, it is possible that this novel *P. mandelii* strain could become an additional source for biotechnological alginate production.

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1. Introduction

Alginates, linear polysaccharides composed of β-D-mannuronic and α-L-guluronic acid, are produced by brown algae and bacteria such as *Azotobacter vinelandii* and many *Pseudomonas* species [1]. Alginates have been applied as thickening agents, stabilizers, and hydrogels in food, cosmetics, pharmaceutical, textile, and paper industries [2]. Especially interesting are those bacteria that can be engineered to produce high-quality alginates, which are even suitable for medical applications [3]. One of the principal features of the high-quality alginates is that they form strong gels and form thick aqueous solutions [4,5]. Some of the medical applications of alginates are as follows:

i) Pharmaceutical applications: Alginates are used as carriers for delivering small chemical drugs and proteins [6,7];
ii) Wound dressing applications: Alginate dressings in dry form absorb wound fluid to re-gel, and gels can moisturize dry wound, thereby maintaining a physiologically moist microenvironment and minimizing bacterial infection at the wound site [8];
iii) Cell culture applications: Alginate gels are being used as scaffolds for 2-D and 3-D cell culture systems [9]; and
iv) Tissue regeneration applications: In this aspect, alginates are considered excellent materials for cell adhesion, regenerative properties, and good slow carriers of proteins, DNA, and antibodies [10].

Alginates are important components of extracellular polysaccharides, or exopolysaccharides, in all pseudomonads [11]. In turn,

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exopolysaccharides form the extracellular matrix structure of biofilms [12].

The genes directly involved in pseudomonads alginate synthesis are organized in an operon controlled by a single promoter [1,13,14,15], the regulation of which is highly complex and influenced by the quorum sensing system [16,17]. In *Pseudomonas* species, this operon is formed by 12 genes (*algD-alg8-alg44-algK-algE-algG-algX-algL-algI-algJ-algF-algA*), and operon expression is controlled by *algU*, a sigma factor and activator of the alginate operon [18]. Operon expression is also regulated by the MucA protein coded by *mucA*, which binds to AlgU to prevent the transcription of the alginate operon [19]. In *Pseudomonas aeruginosa,* the model species of the genus, more than 80% of alginate overproducer strains present a mutated *mucA* that inhibits AlgU binding, thus resulting in the uncontrolled production of alginate [20].

Biofilms originate from the assembly of individual sessile cells into a complex and organized “multicellular” system by a highly regulated developmental process that involves a coordinated sequence of events, including primary surface attachment, micro-colony formation, maturation, expansion, and, finally, dissemination [21]. This transition from a single cell state to the biofilm architecture is dependent on the production of adhesins and on the formation of a polysaccharide-rich extracellular matrix responsible for intertwining individual cells to provide fundamental support for the newly formed bacterial community. In addition to the aforementioned polysaccharides, the matrix is also composed of proteins and extracellular DNA in proportions carefully regulated by a complex cellular system [16,22]. Biofilm formation by many environmental bacteria appears to be an efficient strategy for overcoming stressful conditions [23,24].

Antarctica is the coldest, driest, and windiest continent in the world where the minimum and maximum winter temperatures reach -25°C and barely over -2°C, respectively. During summer, temperatures range between -8 and 3°C [25]. The most adapted bacteria to these harsh conditions are the psychrophilic bacteria because they can grow at temperatures as low as -15°C, while optimum growth temperatures never reach above 10°C [26]. In recent years, however, many psychrotolerant bacteria have been isolated from the Antarctic and Arctic regions [27,28,29,30,31]. Although psychrotolerant bacteria display optimum growth at temperatures >20°C, they can grow at 0°C [26,32]. Considering the wide and abrupt seasonal temperature variations that psychrotolerant Antarctic bacteria must endure for survival [33,34], it can be expected that these bacteria have efficient and highly regulated strategies for tolerating low temperatures.

Alginates, which are important components of exopolysaccharides in pseudomonads, could play an important role in bacterial adaptation to low temperatures. Therefore, the main objective of this study was to determine whether alginate synthesis increases at low temperatures as an adaptation strategy by a novel Antarctic isolate of *Pseudomonas mandelii* (6A1). To achieve this objective, the following assessments were performed: 1) the 6A1 isolate was phylogenetically characterized to determine its possible species, 2) biofilm formation capacity at low temperatures was assessed, 3) the expression of genes forming the alginate operon at low temperatures was evaluated, and, finally, 4) the amount of alginate produced at different temperatures was measured. The Antarctic 6A1 isolate was classified as a new strain of *P mandelii*. This strain produces high amounts of alginate, thus providing a possible adaptation strategy at low temperatures.

2. Materials and methods

2.1. Bacterial strains and growth media

The bacterial strain 6A1 was isolated in 2008 from a marine sediment sample collected at Fildes Bay (62°13′28.8″S, 58°58′42.7W) of King George Island, Antarctica. The strain was maintained under standard conditions in lysogeny broth (LB) agar and routinely grown in the LB medium at 25°C. The doubling time was determined by OD_{600} monitoring at different temperatures (4, 15, 25, 30, and 37°C) in 25 ml of LB medium in 100 ml flasks and with agitation at 240 rpm. All experiments were performed on culture duplicates on three different days (six cultures in total). *Pseudomonas fluorescens* (ATCC® 31948™) and *P. aeruginosa* PAO1-V [35] were used as controls of alginate production.

2.2. Amplification of the 16S rRNA gene and phylogenetic analysis

To identify and phylogenetically characterize the 6A1 isolate, DNA was isolated using the AxyPrep™ Bacterial Genomic DNA Miniprep Kit (Axygen® Biosciences, Corning) following the manufacturer's instructions. The 16S rRNA gene was amplified by PCR using the primers EubB/27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and EubA/1522R (5′-AAGGAGGTGATCCANCCRCA-3′). The generated PCR product (≈1530 base pairs) was purified using the AxyPrep™ PCR Clean-up Kit (Axygen® Biosciences, Corning) and sequenced using Macrogen (Korea). The obtained sequences were assessed by BLAST analysis against the NCBI GenBank database. Phylogenetic analysis was performed using the Phylogeny.fr software [36,37] by using the maximum likelihood method and a GTR approach.

2.3. Multilocus sequence analysis

Analysis of the 16S rRNA gene sequence demonstrated that the 6A1 isolate belonged to the fluorescens group of the genus *Pseudomonas.* The primers of housekeeping genes (*glnS*, *gyrB*, *ileS,* and *rpoD*; Table 1) previously used in a multilocus sequence typing analysis of the *P. fluorescens* group were used in the present study [38]. Additionally, the *aroB* gene was used in the multilocus sequence analysis (MLSA); the primers for this gene were designed on the basis of the alignment with the homologous gene of *P. mandelii* (complete genome NZ_CP005960.1), *P. fluorescens* SBW25, and *P. fluorescens* A506 [39]. PCR amplifications were performed with an initial denaturation at 95°C for 10 min; then 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min. The PCR products were cloned into the pCR2.1-TOPO® TA Cloning System (Invitrogen) and sequenced using Macrogen (Korea). The obtained sequences were alphabetically organized, phylogenetically analyzed, and compared with a concatenated structure with the orthologous genes of 30 different *Pseudomonas* species from the sampled site www.pseudomonas.com [39]. Similar to that done for 16S rRNA, the

Table 1
Primers used in this study.

Primer	Sequence	Annealing T°
aroE F	5′-ACCGTGCCGTTCAAGGAAGA-3′	55°C
areE R	5′-TACATCATGTCGTAGCA-3′	55°C
glnS F	5′-ACCAACCCGGCCAAGGAAGACCAGG-3′	55°C
glnS R	5′-TGCTTGAGCTTGCGCTTG-3′	55°C
gyrB F	5′-GGTGGTCGAYAAYTCCATCG-3′	55°C
gyrB R	5′-CGYTGWGGAATGTTGTTGGT-3′	55°C
ileS F	5′-TTCCCAATGAAGGCCGGCCTGCC-3′	55°C
ileS R	5′-GGGGTRGTGGTCCAGATCACG-3′	55°C
rpoD F	5′-CTGATCCAGGAAGGCAACATYGG-3′	55°C
rpoD R	5′-ACTCGTCGAGGAAGGAGCG-3′	55°C
16S 27 F	5′-AGAGTTTGATCMTGGCTCAG-3′	55°C
16S 1492 R	5′-AAGGAGGTGATCCANCCRCA-3′	55°C
*algU*F	5′-AGCACAAAATTCTCGGGTTG-3′	55°C
*algU*R	5′-CCACGTGTAAAACGCACTGT-3′	55°C
*mucA*F	5′-CGTGACACCTGGTCTCGTTA-3′	55°C
*mucA*R	5′-CCATGGACCACGAGTAGCTT-3′	55°C
algA F	5′-TCAAGCACATCTCGGTCAAG-3′	55°C
algA R	5′-ATCGGGATGTAGGTCGACTG-3′	55°C
*rpsL*F	5′-GCAAGCGCATGGTCGACAAGA-3′	55°C
*rpsl*R	5′-CGCTGTGCTCTTGCAGGTTGTGA-3′	55°C

sequences were analyzed using the Phylogeny.fr software [36,37] by using the maximum likelihood method.

2.4. Real-time reverse transcription PCR (RT-PCR) assays

RNA was isolated as described previously [40]. cDNA was synthetized using the M-MLV Reverse Transcriptase System (Promega). Real-time RT-PCR was performed in triplicate using the CFX96 Touch™ Real-Time PCR Detection System (BioRad) and the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent) with 100 mM of each primer and 50 ng of RNA. The *rpsL* gene was used as a housekeeping gene to normalize the real-time RT-PCR results, and the amount of each RNA was calculated following the $2^{-\Delta\Delta Ct}$ method [41]. The oligonucleotides used in the real-time RT-PCR experiments are listed in Table 1.

2.5. Differential expression of the alginate operon genes

Primers to measure the expressions of *algU*, *mucA* (regulators: sigma and anti-sigma factors, respectively), and *algA* (enzyme: mannose-6-phosphate isomerase) were designed using the complete genome of the *P. mandelii* JR-1 (NZ_CP005960.1) and are listed in Table 1. An overnight culture grown at 25°C in the LB medium was used to seed 6-well plates at an initial concentration of $OD_{600} = 0.1$. The plates were then incubated at 4, 15, and 25°C. RNA was isolated after 24, 48, 72, and 48 h of incubation, and gene expression was measured by real-time RT-PCR according to the protocol detailed above.

2.6. Crystal violet biofilm assays

Biofilm formation was estimated using the crystal violet procedure [42]. An initial inoculum was grown overnight at 4, 15, and 25°C in a 100-ml flask containing the LB medium. Subsequently, 96-well microtiter plates were seeded with the LB medium at an initial concentration of $OD_{600} = 0.1$. The plates were incubated for 24, 48, 72, and 96 h. After incubation, the cells were removed by turning the plates upside down and draining the liquid. Next, 125 μl of 0.1% crystal violet was added to each dish, and the plates were incubated for 20 min. The plates were then washed four times with water, shaken, and blotted on a stack of paper towels to remove excess dye. The plates were dried overnight at room temperature. To quantify the amount of biofilm formed, 125 μl of 30% acetic acid was added to each well and incubated for 15 min at room temperature. Finally, the solution was transferred to a new flat-bottomed microtiter dish, and its absorbance was read at 550 nm using 30% acetic acid in water as the blank.

2.7. Confocal scanning microscopy

Biofilms were formed at 4, 15, and 25°C in six-well plates with coverslips incubated for 72 and 96 h. After incubation, the coverslips were washed three times with PBS buffer (NaCl 137 mM; KCl 2.7 mM; Na_2HPO_4 10 mM; KH_2PO_4 2 mM). The samples were fixed and permeabilized with absolute methanol (Merck) for 15 min at room temperature. Cell aggregation was identified by staining the samples with 20 μM of SYTO® 9 (Thermo-Fisher) for 5 min. The stained samples were then washed five times with PBS buffer. Exopolysaccharides were stained using 100 μg/ml of the Concanavalin A, Alexa Fluor® 594 Conjugate (Molecular Probes®, Thermo-Fisher) and incubated for 2 h at room temperature. Finally, all samples were mounted for confocal scanning microscopy on a slide with the Dako Mounting Medium (Agilent Technologies). Samples were analyzed in a Leica TCS SP5 II spectral confocal microscope (Leica Microsystems Inc.), and the images were obtained using a Leica 40X/1.25 Oil HCX PL APO CS lens (Leica Microsystems Inc.). The Java-based image analysis program Image J (http://rsb.info.nih.gov/ij/) was used for image analyses. The images were assembled to generate a single image based on the sum of pixel brightness values through the image stack (ImageJ: z-project). Fluorescence intensity was measured for each channel, and three fields were analyzed in all samples. The experiments were performed in triplicate.

2.8. Alginate measuring assay

To measure the amount of alginate produced, all bacterial strains used in this study were grown in 50 ml of LB broth at 4, 15, and 25°C until the culture reached an OD_{600} of 2.0. The bacterial cells were then collected by centrifugation at 7000 × g for 20 min and suspended in 10 ml of PBS buffer. Simultaneously, another culture was used to correlate OD_{600} 2.0 with the dry cell weight. To remove any contaminants such as RNA and DNA from the alginate, the samples were treated with RNAse A (Promega) and DNAse I (Sigma). The samples were then incubated at 37°C for 1 h. To remove the cells, the mixture was vortexed and centrifuged at 8000 × g for 20 min. The alginate remaining in the supernatant was precipitated with 25 ml of 95% ethanol. The alginate precipitates were collected by centrifugation at 10000 × g for 30 min and suspended in 2 ml of 0.85% NaCl. The uronic acid concentration was determined by a standard colorimetric assay [43].

2.9. Statistical analysis

Student's t-test was performed to determine the statistical significance of the qRT-PCR results.

3. Results

3.1. 16S and MLSA analyses reveal that 6A1 is a new strain of P. mandelii

To identify at least the genus of the 6A1 Antarctic isolate, the 16S rRNA sequence was assessed (GenBank accession no. KT377040). The best homology was obtained with *P. mandelii* (99%), a member of the *P. fluorescens* group in the genus *Pseudomonas*. To corroborate this result, an MLSA analysis was performed. The amplified genes were first individually evaluated by BLAST analysis and then alphabetically concatenated and compared with 30 concatenated sequences from 30 different *Pseudomonas* species. From this, the *aroE* gene (GenBank accession no. KT820723) was observed to have a 98% identity with *P. mandelii*, followed by 87% identity with *P. fluorescens* SBW25 strain. Similarly, *glnS* showed 97% identity with the homologous *P. mandelii* gene, while *gyrB* (GenBank accession no. KT820725) presented 98% identity with *P. mandelii*. Similar results were obtained for *ileS* and *rpoD* (GenBank accession nos. KT820726 and KT820727), which had 98% and 99% identity, respectively, with orthologous *P. mandelii* genes (Table 2). Finally, phylogenetic analyses of the concatenated genes (Fig. 1) strongly demonstrated the 6A1 isolate to be a novel strain of *P. mandelii*.

Table 2
Percentage identities of individual genes used in MLSA analyses indicating that 6A1 is a novel strain of *Pseudomonas mandelii.*

	% identity	Species
aroE	98%	*Pseudomonas mandelii* JR-1
	87%	*Pseudomonas fluorescens* NCBIMB
glnS	97%	*Pseudomonas mandelii* JR-1
	95%	*Pseudomonas fluorescens* NCBIMB
gyrB	98%	*Pseudomonas mandelii* JR-1
	93%	*Pseudomonas fluorescens* NCBIMB
ileS	98%	*Pseudomonas mandelii* JR-1
	93%	*Pseudomonas fluorescens* FW300
rpoD	99%	*Pseudomonas mandelii* JR-1
	97%	*Pseudomonas fluorescens* FW300

Fig. 1. MLSA phylogenetic analysis revealed that the 6A1 isolate is a novel strain of *Pseudomonas mandelii*. Maximum-likelihood phylogenetic tree based on concatenated partial *aroE*, *glnS*, *gyrB*, *ileS*, and *rpoD* of strain 6A1 and the type strains of closely related species of the genus *Pseudomonas*. Numbers indicate the branch length.

3.2. The growth rate showed the psychrotolerant nature of the 6A1 strain

To investigate the effect of temperature on the growth rate of the bacterial isolate, 6A1 was grown in the LB medium at different temperatures (4, 15, 25, 30, and 37°C). The optimal temperature to grow, the best doubling time, was observed at 25°C (Table 3), and there was no detectable growth at 37°C. These results support the psychrotolerant nature of this strain, a behavior that is common among all known *P. mandelii* strains.

Table 3
Doubling time of 6A1 at different temperatures. The optimum growth of *P. mandelii* 6A1 was at 25°C.

	Doubling time (min) ± SD				
	4°C	15°C	25°C	30°C	37°C
6A1	381 ± 26	158 ± 18	49 ± 9	78 ± 8	N/G[a]

[a] N/G = no growth

3.3. Biofilm formation and exopolysaccharide production increase at lower temperatures

To determine whether there was a correlation between low temperatures and the increase in biofilm formation, biofilm formation was assessed with growth at 4, 15, and 25°C by using a microtiter dish assay. After 72 h, 6A1 showed at least six-fold and three-fold more biofilm formation at 4 and 15°C, respectively, than that at 25°C (Fig. 2). After 96 h, biofilm formation at 4 and 15°C increased at least ten-fold and five-fold, respectively, compared with that at 25°C. To explain the increase in biofilm formation at lower temperatures, the amount of exopolysacharides produced was measured. One of the principal components of the bacterial biofilm is the exopolysaccharide matrix [16,17]. This matrix is secreted and formed by the bacterial population [44]. The principal component of pseudomonads

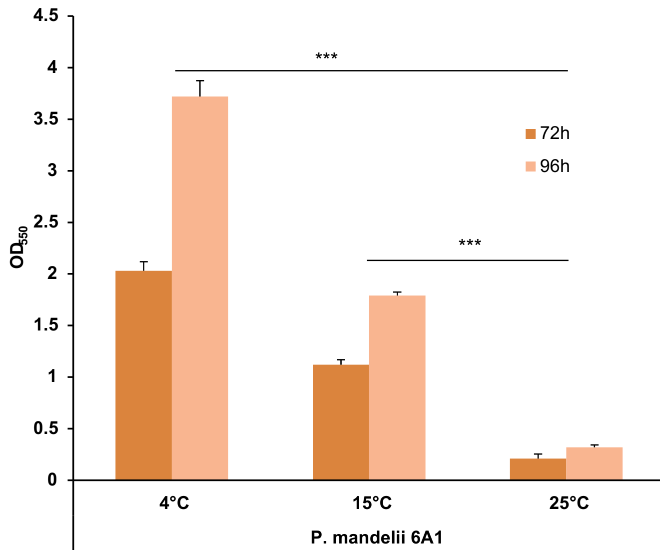

Fig. 2. Crystal violet assays revealed that biofilm formation increases at 4 and 15°C. Overnight cultures of *P. mandelii* 6A1 were subcultured at an OD_{600} of 0.1 in 96-well plates using 150 μl of LB medium and incubated at 4, 15, and 25°C for 72 h (black bars) and 96 h (grey bars). At 4 and 15°C, biofilm production increased at least 10-fold and 4-fold, respectively, compared with those at 15 or 25°C, thus demonstrating the effect of temperature on biofilm formation (*** = $P > 0.05$).

exopolysaccharide is alginate, and the production of this molecule is highly regulated [45,46,47]. Confocal laser microscopy was used to investigate the production of exopolysaccharides during biofilm formation in *P. mandelii* 6A1 (Fig. 3). After 72 h of incubation at 4°C, a very low number of bacterial cells were observed, while a high amount of polysaccharides was detected (Fig. 3a). This was significantly different from that observed in biofilm formed after incubation at 15 and 25°C, where the number of bacterial cells was consistent with the amount of extracellular matrix generated (Fig. 3b, c). After 96 h of incubation, the number of bacterial cells increased depending on temperature, whereas exopolysaccharide production did not appear to depend exclusively on the number of cells, but temperature may play a relevant role here.

3.4. Alginate production significantly increases at low temperatures due to downregulation of mucA

As mentioned above, alginate is the principal component of the extracellular matrix in pseudomonads biofilms [21,48,49]. This compound is a negatively charged copolymer formed by O-acetylated D-mannuronic and L-glucoronic acids [50]. Alginate production is controlled by the alginate operon in all pseudomonads described to date [51]. To determine the correlations between low growth temperature, biofilm formation, and alginate production, the expression levels of *algU* and *mucA*, two relevant regulatory genes involved in alginate synthesis, and *algA*, a component of the alginate synthesis operon, were measured. The expression of these genes was assessed at three different temperatures (4, 15, and 25°C) after 24, 48, 72, and 96 h of growth. No changes were observed in *algU* expression at any of the temperatures or growth times. However, *mucA* expression decreased at 4 and 15°C after 24 and 48 h of incubation (Fig. 4), and this low expression at 4°C was maintained up to 72 h post incubation. In contrast, *algA* was overexpressed at 4 and 15°C, reaching at least six-fold higher by 24, 48, and 72 h of incubation than the culture growing at 25°C (Fig. 4). After 96 h, the expression levels of *algA* at 4 and 15°C were similar to those of the culture grown at 25°C (Data not shown). This evidence supports the temperature dependence of alginate operon overexpression in this novel strain of *P. mandelii*. To validate the results obtained by transcriptional analysis, the amount of alginate produced was also measured at 4, 15, and 25°C when the cultures reached an OD_{600} value of 2.0 and was compared with those produced by two good alginate producers *P. aeruginosa* and *P. fluorescens*. The amount of alginate produced was fully dependent on temperature and increased as the temperature decreased (Table 4) in all tested strains. Specifically, the amount of alginate produced in 6A1 at 4°C was at least 20-fold higher than that produced by a similar number of cells at 25°C. Similarly, the amount of alginate produced in 6A1 at 15°C was 4.5-fold higher than that produced by cultures grown

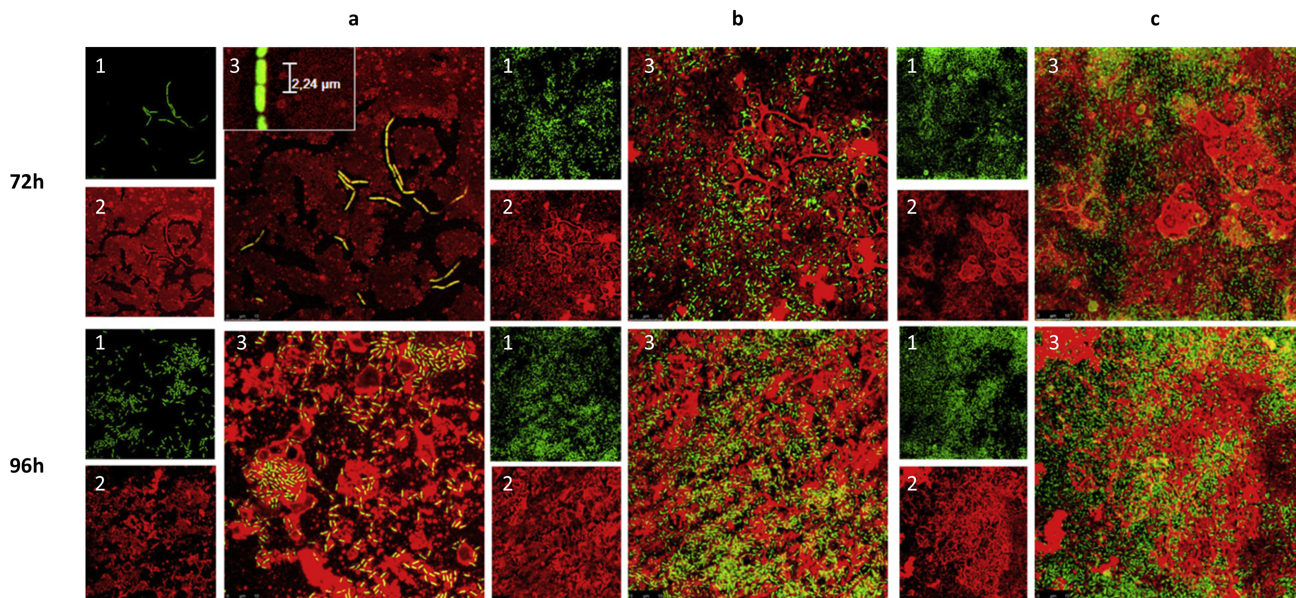

Fig. 3. Exopolysaccharide production increases at lower temperatures independent of cell density. Confocal scanning microscopic images of concanavalin A detection. Bacterial DNA was stained with SYTO® 9 (Green), and the exopolysaccharide matrix was detected with ConA-Alexa592 (red). The EPS matrix increased at (a) 4°C compared with that in cultures at (b) 15°C and(c) 25°C. Box 1: bacterial DNA stained with SYTO® 9; Box 2: EPS detected with ConA-Alexa592; and Box 3: Merge.

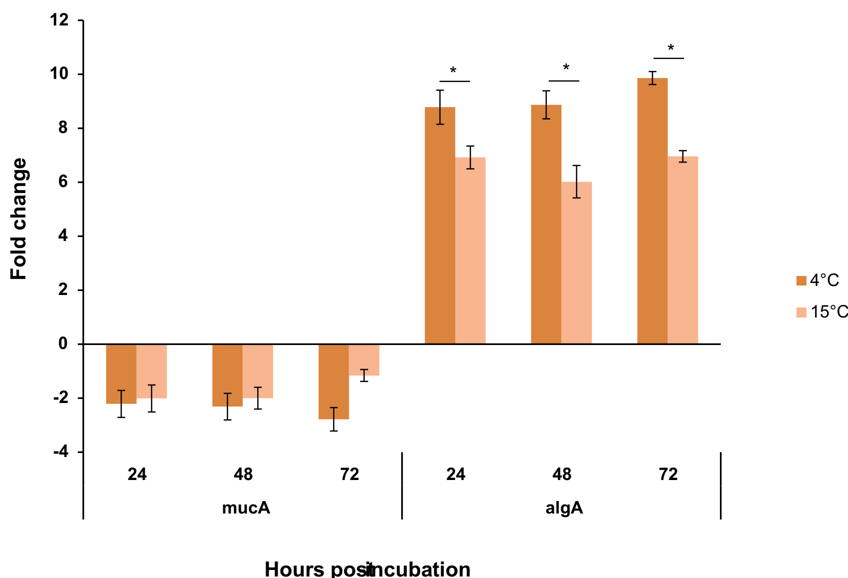

Fig. 4. *mucA* was downregulated at lower temperatures, while *algA* expression increased. The expression of *mucA*, the anti-sigma factor of the alginate operon, decreased at 15 and 4°C after 24 h of incubation, and these levels were maintained until 72 h of incubation. In contrast, the expression of *algA*, the first gene in the alginate operon, increased at 15 and 4°C at least six-fold compared with that at 25°C (* = $P > 0.1$).

at 25°C. The decreased expression of *mucA*, which is perhaps mediated by acclimatization to low temperatures, may be responsible for the overproduction of alginate.

4. Discussion

The present study analyzed the effect of temperature on biofilm formation and alginate production in a bacterial strain isolated from the marine sediments of Fildes Bay, King George Island, Antarctica. The growth rate of this isolate was estimated at three different temperatures. The behavior of this novel *P. mandelii* strain was more similar to that of typical environmental *Pseudomonas* growing in less stressful environments, where optimal growth temperatures range between 25 and 30°C. This indicates that the 6A1 strain is different from psychrophilic bacteria, which are fully adapted to conditions of extreme cold [52].

P. mandelii was first isolated from mineral waters [53] but has since then been isolated from agricultural fields [54,55], with both isolation environments (i.e., water and fields) located in temperate climates. However, this species is characterized as a cold-adapted bacterium [56] that shows non-halophilic features and that flourishes at low temperatures by producing extracellular enzymes [57]. Nevertheless, the optimum growth temperature of *P. mandelii* is from 25 to 30°C [58], although this bacterium can grow at 4°C but not at 37°C [57]. Therefore, *P. mandelii* is an excellent example of a psychrotolerant bacterium.

As stated, this bacterium has been isolated from mineral waters in France [53] and Korea [56] and from agricultural fields in China [55]; the climates in these countries are vastly different from that in Antarctica. With the exceptions of the psychrophilic *Pseudomonas antarctica, Pseudomonas meridiana*, and *Pseudomonas proteolytica* [59], all members of the *Pseudomonas* genus are described as psychrotolerant microorganisms [33].

The current study obtained evidence for the capacity of the novel *P. mandelii* 6A1 strain to improve biofilm formation at low temperatures (4°C), which was mediated by alginate overproduction. The alginate operon is responsible for the synthesis of all enzymes participating in the formation of exopolysaccharide extracellular matrix [19]. To demonstrate the effect of temperature on biofilm formation, the expression was measured for two genes involved in alginate synthesis: *algU* and *mucA*. In *P. aeruginosa*, MucA controls AlgU binding, the transcriptional activator of the alginate operon, to prevent the expression of the operon acting as an anti-sigma factor [19]. In the present study, no changes were found in the expression of *algU* at different temperatures; however, *mucA* was downregulated at lower temperatures (4 and 15°C). This downregulation would explain the increased alginate production recorded at lower temperatures.

Many strategies for overcoming extremely low temperatures have been reported in bacteria. One of them is the production of biofilm to persist in stressful environmental conditions [60,61,62]. Biofilm formation depends on the capacity of bacteria to sense environmental signals such as nutrient availability, pH, temperature, osmolality, fluctuations in oxygen concentration, and the presence of antibiotics [63,64,65,66]. At 23°C ("low temperature" for *Escherichia coli*), the biofilm formation capacity of *E. coli K-12* considerably increases [67]. The following three mechanisms have been proposed for classifying biofilms as resistance structures against environmental stress: i) the barrier properties of the slime matrix and exopolysaccharide hydrogel

Table 4
Alginate production of the *Pseudomonas* sp. 6A1 strain. Alginate concentrations were indirectly determined using colorimetric reactions that measured the concentration of uronic acid. Production by the 6A1 strain was compared against the strains *P. aeruginosa* PAO1-V and *P. fluorescens* (ATCC® 31948™) at 4, 15, 25, 30, and 37°C.

	Alginate production in µg/ml of uronic acid per gram of dry weight culture				
	4°C	15°C	25°C	30°C	37°C
Pseudomonas sp. 6A1	26.18 ± 4.63	6.32 ± 0.46	1.28 ± 0.31	1.02 ± 0.18	N/G
P. fluorescens (ATCC® 31948™)	1.94 ± 0.41	1.00 ± 0.27	1.02 ± 0.37	0.78 ± 0.24	0.62 ± 0.12
P. aeruginosa PAO1-V	N/G[a]	1.02 ± 0.30	0.83 ± 0.12	0.82 ± 0.12	2.22 ± 0.51

[a] N/G = no growth.

that protect bacteria against ultraviolet light and dehydration [68]; ii) the creation of starved, stationary, and dormant zones inside biofilms, which is a characteristic that allows bacteria to generate a reservoir population that can withstand adverse conditions and begin a new colony when conditions are more favorable [69,70,71]; and iii) the presence of subpopulations better adapted to the particular stressor, termed persisters [69]. These three features make biofilms powerful structures that could fundamentally contribute to the survival of bacteria in Antarctica.

In conclusion, the present study described that the novel strain 6A1 strain of *P. mandelii* isolated from Antarctica increases biofilm formation at lower temperatures due to alginate overproduction. This behavior might be caused by the downregulation of MucA, which is the repressor of the alginate operon. Biofilm over-production in this Antarctic isolate might represent a useful strategy for adaptation to low temperatures. These data contribute toward a better understanding of environmental adaptations in psychrotolerant Pseudomonads bacteria to low temperatures. Future studies are needed to elucidate the genetic and metabolic bases for alginate overproduction in the 6A1 strain. Ultimately, this knowledge could be used for the biotechnological production of this component.

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Conflict of Interest

No conflict of interest is declared.

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