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Exploiting the natural poly(3-hydroxyalkanoates) production capacity of Antarctic *Pseudomonas* strains: from unique phenotypes to novel biopolymers

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Abstract

Extreme environments are a unique source of microorganisms encoding metabolic capacities that remain largely unexplored. In this work, we isolated two Antarctic bacterial strains able to produce poly(3-hydroxyalkanoates) (PHAs), which were classified after 16S rRNA analysis as *Pseudomonas* sp. MPC5 and MPC6. The MPC6 strain presented nearly the same specific growth rate whether subjected to a temperature of 4 °C 0.18 (1/h) or 30 °C 0.2 (1/h) on glycerol. Both *Pseudomonas* strains produced high levels of PHAs and exopolysaccharides from glycerol at 4 °C and 30 °C in batch cultures, an attribute that has not been previously described for bacteria of this genus. The MPC5 strain produced the distinctive medium-chain-length-PHA whereas *Pseudomonas* sp. MPC6 synthesized a novel polyoxoester composed of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate-*co*-3-hydroxyoctanoate-*co*-3-hydroxydecanoate-*co*-3-hydroxydodecanoate). Batch bioreactor production of PHAs in MPC6 resulted in a titer of 2.6 (g/L) and 1.3 (g/L), accumulating 47.3% and 34.5% of the cell dry mass as PHA, at 30 and 4 °C, respectively. This study paves the way for using Antarctic *Pseudomonas* strains for biosynthesizing novel PHAs from low-cost substrates such as glycerol and the possibility to carry out the bioconversion process for biopolymer synthesis without the need for temperature control.

Keywords Antarctic *Pseudomonas* · Poly(3-hydroxyalkanoates) · Low temperature · Glycerol · Exopolysaccharide · Psychrophiles

Introduction

Much of the surface of planet Earth has a temperature below 5 °C [48], thus driving the evolution of microorganisms that inhabit these harsh environments in a very particular fashion [13, 14]. Most of the bacteria isolated from Polar Regions

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fall into the category of *psycrophiles*, where they have been arbitrarily classified into two groups: psychrophilic sensu stricto and psychrotolerant [9]. The first group has the ability to grow to a maximum temperature of 20 °C whereas the latter can propagate at 0 °C, but with an optimal growth temperature above 20–25 °C [31]. Recent research has shown that psychrophiles are more lavish than psychrotolerant microbes in seawater [60]. On the other hand, psychrotolerant bacteria have been found in high abundance in soil, where they are constantly facing cyclical patterns of extreme temperature variation [30]. This environment has endowed psychrophilic bacteria with several molecular mechanisms to cope with cold and ice formation. In particular, low temperatures trigger various metabolic responses including changes in membrane composition of the cell [5, 53], synthesis of exopolysaccharides (EPS) [33], carotenoids [19], polyphosphates [50, 58], antifreeze and cold-shock proteins, and production of compatible osmotic solutes [14]. Accumulation of biopolymers in the cytosolic space of the cell has proven to be another surviving mechanism of cold-adapted bacteria to extreme temperatures [4, 55]. The search for novel biopolymers, in particular ones which can be produced using low-cost carbon sources, has drawn a lot of scientific and industrial attention. In this context, several Antarctic bacteria have been isolated and evaluated for their capacity to produce poly(3-hydroxyalkanoates) (PHAs). Given its high metabolic versatility and resistance to toxic compounds and harsh conditions, *Pseudomonas* is one of the most relevant bacterial genera for bioremediation [34] as well as for industrial production of PHAs [42, 45]. *Pseudomonas* and *Janthinobacterium* have been found to be the major PHA-producing microbes isolated from Antarctic soil [3, 12, 21, 49]. Several studies have demonstrated that PHAs granules are key stress resistance factors against freezing, elevated pressure, and toxic compounds [36]. Besides its protective function and its role as an important reserve of carbon and energy during famine, these polyoxoesters display similar mechanical and physical properties to petroleum-based plastics [28]. During the past three decades, PHAs have been industrially produced via bacterial fermentation and used as raw material for manufacturing containers, fibers, films, and more recently drug delivery systems [11, 62]. PHAs are classified into two families, namely short- (*scl*) and medium-chain length (*mcl*), according to the number of carbon atoms of the monomeric chain [28]. All of them display different thermal features that are necessary to meet the requirements for various thermoforming processes. *Pseudomonas* strains accumulate *mcl*-PHAs from a wide variety of carbon sources normally accompanied by limitation of inorganic compounds e.g. N, O_2, P, where the monomer composition of the synthesized PHA can be modulated via culturing conditions, metabolic pathways engineering, and mixing different carbon substrates [22, 39]. Antarctic *Pseudomonas* strains have been described as producing both poly(3-hydroxybutyrate) (PHB) and *mcl*-PHAs [12, 21], but when cultured at low temperature (4–10 °C) their specific growth rate, biomass and product yields show a drastic reduction by more than tenfold [3, 21]. In addition, the use of glycerol as carbon substrate for the microbial synthesis of chemicals has drawn considerable attention given its low cost and high surplus e.g. the major waste byproduct from the biodiesel industry, where bacteria of the *Pseudomonas* genus are appropriate cell factories to efficiently produce a myriad of value-added products [43]. In this work, we isolated and characterized two Antarctic *Pseudomonas* strains, from the Deception Island in the South Shetland archipelago, able to synthesize PHAs at different growth temperatures using the inexpensive substrate glycerol as the sole carbon source. For the first time, we showed that cold-adapted *Pseudomonas* bacteria could grow and accumulate PHAs to nearly equal levels at 30 °C and 4 °C. Additionally, we characterized the monomer composition of the biosynthesized PHA via GC/MS and NMR, along with their thermal properties. We also monitored the EPS and PHA formation during glycerol consumption in well-controlled batch bioreactors, where the *Pseudomonas* sp. MPC6 strain displayed very unique phenotypes and different properties of the biosynthesized PHA in response to temperature downshift.

Materials and methods

Chemicals

All chemicals were purchased from Sigma-Aldrich unless otherwise stated in the text.

Isolation of Antarctic bacterial strains and phylogenetic analysis

For the isolation of bacteria, the soil samples from Deception Island (Fig. S1, 53th Chilean Antarctic Scientific Expedition) were mixed with sterile PBS, vortexed, plated in different culture media (marine agar and Luria–Bertani agar), and incubated at 10 °C for 1–3 weeks. The recovered isolates were subjected to Gram staining and microscopic analysis as a first step for its classification and characterization. To construct the tree showing the phylogenetical relationships among *Pseudomonas* spp. MPC5, MPC6 and other *Pseudomonas* strains, we PCR-amplified and sequenced the full gene coding for 16S rRNA using the universal primers Fd1 (5′ AGA GTT TGA TCC TGG CTC AG) and Rp2 (5′ ACG GCT ACC TTG TTA CGA CTT). The 16S rRNA gene sequences were deposited in NCBI GenBank with accession numbers MK335919 and MK335925 for *Pseudomonas* MPC5 and MPC6 strain, respectively. The determined

sequence was compared among the set of *Pseudomonas* strains, defining a region of 1490 bp present in the available sequence of all the strains, and then aligned using MUSCLE [16]. The Maximum likelihood tree was inferred from the 16S rDNA alignment by running RAxML v7.7.2 [52] five times, using the generalized time-reversible (GTR) model and a Gamma distribution, selecting the final tree with the highest likelihood. The bootstrap support value for each node was calculated by RAxML from 100 iterations.

Growth conditions in shaking flask experiments

Pseudomonas putida KT2440 (DSM 6125), *Pseudomonas antarctica* (DSM 15318), and the isolated Antarctic strains *Pseudomonas* sp. MPC5 and MPC6 were kept as frozen stocks in 25% glycerol at − 80 °C. To obtain single colonies, they were plated onto Luria–Bertani agar plates after 1 day incubation at 30 °C. Inoculum was prepared by picking up a single colony from the plate and inoculating it into a 50 mL shake flask containing 10 mL of the defined minimal medium (M9) consisting of (per liter): 12.8 g $Na_2HPO_4{\cdot}7H_2O$, 3 g KH_2PO4, 1 g NH_4Cl, 0.5 g NaCl. This medium was autoclaved and subsequently supplemented with 0.12 g of $MgSO_4{\cdot}7H_2O$, trace elements (6.0 $FeSO_4{\cdot}7H_2O$, 2.7 $CaCO_3$, 2.0 $ZnSO_4{\cdot}H2O$, 1.16 $MnSO_4{\cdot}H_2O$, 0.37 $CoSO_4{\cdot}7H_2O$, 0.33 $CuSO_4{\cdot}5H_2O$, 0.08 H_3BO_3 (mg/L) (filter-sterilized), and 4 g/L of glycerol as the unique carbon source in the *Pseudomonas* strains. They were grown under aerobic conditions at 30 °C in an Ecotron incubator shaker (INFORS HT, Switzerland) set at 160 rpm. Growth curves of *Pseudomonas* strains and PHA synthesis were performed at 4 °C using a cabin refrigerator (Guangzhou Appliance, China), where the shaking flasks were placed on a rotary shaker (DLab, model SK-L180-E, China) set at 160 rpm.

Bench-scale fermentation

Single colonies from agar plates, incubated for 24 h at 30 °C, were used to propagate the cells in liquid M9 medium supplemented with 4 (g/L) glycerol for the first pre-inoculum. The cells were harvested in the middle of the exponential phase and used to inoculate the final inoculum, which was prepared in 250 mL flasks with 50 mL of M9 salt medium and 4 (g/L) glycerol and incubated for 12 h at 30 °C in a rotary shaker (INFORS HT, Switzerland) set at 160 rpm. Taking a portion of the culture, all experiments performed in bioreactors started with an initial OD_{600} of 0.1. *Pseudomonas* sp. MPC6 was grown in M9 medium, magnesium sulfate, and trace elements, supplemented with 30 (g/L) glycerol to promote PHA accumulation. Bioreactor batch fermentations were carried out in a 1 L top-bench Labfor5 bioreactor (INFORS HT, Switzerland) with a working volume of 0.9 L, at 30 °C. For fermentations carried out at 4 °C, a coolant fluid was passed through the jaket of the vessel of the bioreactor using a shiller (Julabo F500, Germany). The aeration rate was set to 0.5 vvm using a mass flow controller. The dissolved oxygen level was kept above 20% air saturation by control of the agitation speed up to a maximum of 800 rpm. The pH was maintained at 7.0 by automatic pH controlled addition of 0.5 M H_2SO_4 or 1 M of KOH.

Quantification of biomass and substrates

Cell growth was recorded as optical density (OD) at 600 nm (Ultraspec 2000, Hitachi, Japan). The cell dry mass was determined gravimetrically after collection of 10 mL culture broth for 10 min at 4 °C and 7000 *g* (Eppendorf 5810 R, Hamburg, Germany) in pre-weighed tubes, including two washing steps with a NaCl solution (0.9%). Then the final pellet was dried at 100 °C in an oven (Labtech, Korea) until constant weight. Cell-free supernatants obtained from the centrifugation step for biomass quantification were filtered (0.22 µm) and used to monitor the concentrations of glycerol in the culture broth of the bioreactor via HPLC as previously described by Ref. [40] and ammonium ions using the Spectroquant® ammonium test following the manufacture instructions (Merck, Germany). The detection limit of the test is 2.6 (mg/L).

EPS extraction, quantification, and characterization

Through the fermentation period, 50 mL of broth culture was centrifuged at 15,000*g* for 2 h at 4 °C and the supernatant was pressure-filtered (0.22 µm). Next, EPS was precipitated from the filtered supernatant using three volume of pure cold ethanol (150 mL), where the mixture was incubated at − 20 °C for 24 h, and the precipitate washed two times with concentrated ethanol. The resulting precipitate was separated by centrifugation, re-suspended in milli-Q water and purified by dialysis against distilled water at 4 °C. Excess water was removed under vacuum and further lyophilized (frozen samples) for 12 h. The obtained exopolysaccharide was quantified by weight after freeze-drying (total EPS). The total neutral carbohydrates were measured using a phenol–sulfuric acid method [24], where 100 µg of purified EPS was mixed with 125 µL of concentrated sulfuric acid. Then, 25 µL of phenol (10%) was added to the mixture and vortexed for 1 min and further incubated at 95 °C for 5 min. After cooling, the mixture was placed into 96-well plate and the absorbance (490 nm) recorded with a spectrophotometer (Synergy HT, BioTek, USA). Concentrations were obtained with glucose standards. Protein content was determined by Bradford method with bovine serum albumin as standard. DNA content in the EPS was obtained by fluorometry using the fluorescent dye 4,6-diamidino-2-phenylindole.

PHA quantification and characterization by GC–MS

Monomeric composition of PHA was determined by gas chromatography–mass spectrometry (GC–MS). For this purpose, methanolysis was carried out by re-suspending 5–10 mg of lyophilized cell dry mass in 2 mL chloroform and 2 mL methanol, containing 15% (v/v) sulfuric acid and 0.5 (mg/mL) 3-methylbenzoic acid as internal standard, respectively, followed by incubation at 100 °C for 4 h in a thermoblocker. After cooling to room temperature, 1 mL of demineralized water was added and the organic phase, containing the resulting methyl esters of the PHA monomers. Analysis was performed in a gas chromatographer coupled with a mass spectrometer model YL6900 (Young Instruments, Korea). An aliquot (2 µL) of the organic phase was injected into the gas chromatograph at a split ratio of 1:10. Separation of the methyl esters of 3-hydroxyacid compounds was achieved by a FactorFour VF-5 ms capillary column (30 m × 0.25 mm i.d. × 0.25 mm film thickness, Varian Inc., Agilent Technologies). Helium was used as carrier gas at a flow rate of 0.9 (mL/min). Injector and transfer line temperatures were 275 °C and 300 °C, respectively. The oven temperature program was: initial temperature 40 °C for 2 min, then from 40 °C up to 150 °C at a rate of 5 °C min and finally up to 280 °C at a rate of 10 °C min. Positive ions were obtained using electron impact ionization at 70 eV and mass spectra were generated by scanning ions from *m*/*z* 50 to *m*/*z* 650. The PHA concentration was determined by the method described by Lageveen et al. [25]. Briefly, quantification of PHA was carried out using calibration curve generated from 0.5 to 2 mg of a purified poly(3-hydroxyhexanoate-*co*-3-hydroxyoctanoate-*co*-3-hydroxydecanoate), obtained from a previous study [38] and polyhydroxybutyrate P(3HB) from Sigma-Aldrich to interpolate sample data. The PHA content (wt%) was defined as the percentage of the CDM represented by the PHA.

^{1}H and ^{13}C NMR analysis

For nuclear magnetic resonance (NMR) analysis, the biosynthesized PHA was extracted and purified using the method described by [6]. Briefly. PHA was extracted from lyophilized biomass with pure chloroform (1:10 w/v) by heating it for 4 h using sealing tubes placed in a thermoblock set at 80 °C. After cooling at room temperature, the chloroform was then filtered to remove cell debris and concentrated by rotary evaporation. PHA was precipitated from the chloroform solution through dropwise addition of cold methanol. The methanol-chloroform mixture was decanted and the pure biopolymer was washed twice with fresh iced ethanol. NMR spectra were obtained on a BRUKER ADVANCE III HD-400 (400 MHz for ^{1}H and 100 MHz for ^{13}C) at 25 °C. All samples were dissolved in deuterated chloroform ($CDCl_3$) as a solvent. Chemical shifts were reported in ppm relative to tetramethylsilane (TMS, Me_4Si) for ^{1}H-NMR and $CDCl_3$ for ^{13}C-NMR spectra. MestReNova version 6.0.2 software was used to analyze the spectra information.

FTIR analysis

The Fourier transform-infrared (FT-IR) spectra were recorded on a BRUKER VECTOR 22 in the 4000–600 cm^{-1} region. Pellets for infrared analysis were obtained by grinding a mixture of 3 mg of purified EPS with 200 mg of dry KBr.

Differential scanning calorimetry (DSC)

20 mg of purified PHA were loaded into aluminium pans of 40 µL and subjected to thermal scans using a DSC-1 (Mettler-Toledo, Switzerland). Prior to performing the measurements, the DSC was calibrated using indium (melting temperature of 156.6 ± 1.56 °C and melting enthalpy of $\Delta H = 28.6 \pm 1$ J/g). The reference used during the analysis was an empty pan. All experiments were performed in triplicate using the following thermal profile: cooling down from 25 to − 40 °C at 40 °C/min, holding at − 40 °C for 5 min, heating up from −40 °C 120 °C at a heating rate of 10 °C/min, holding at 120 °C for 5 min, cooling down to − 40 °C at 40 °C/min, holding at − 40 °C for 5 min and heating up again up to 120 °C at 10 °C/min. The transition temperature (T_m) related to melting of biopolymer was determined as the onset of the endothermic peak observed in the second heat scan. The energy associated with helix to coil transition (melting energy) defined as the change in enthalpy (ΔH) was calculated from the area under their corresponding endothermic peak and expressed as a function of dry PHA mass. The glass transition temperature (T_g) value was obtained from the second heating scan and defined as the midpoint of the change in heat capacity.

Gel permeation chromatography

Molecular weight distributions were determined by gel permeation (HPLC 6000 Waters with RI detector 1260 Agilent) with a column PLgel 5 µm MiniMIX-C 250 × 4.6 mm with a pre-column PLgel 5 µm MiniMIX-C 50 × 4.6 mm. Chloroform was used as eluent at a flow rate of 0.3 mL/min with a sample concentration of 5 mg/mL and an injection volume of 10 µL. The calibration curve was obtained using a polystyrene standards kit (Agilent) in the M_w range of 500–3,000,000 Da.

Scanning electron microscopy

Samples were fixed by chilling the cultures to 4 °C and addition of glutaraldehyde (2%) and formaldehyde (5%), washed with cacodylate buffer and then washed with TE-buffer (20 mmol, 1 mmol TRIS, 1 mmol EDTA, pH 6.9). Washed bacteria were applied to poly-L-lysine precoated cover slips (12 mm in diameter), which were left for 5 min, washed in TE-buffer, incubated with 2% glutaraldehyde in TE-buffer for 15 min and washed again with TE-buffer. Dehydration was carried out with a graded series of acetone (10, 30, 50, 70, 90, 100%) on ice for 15 min for each step, followed by 100% acetone at room temperature and critical-point drying with liquid CO_2 (CPD 30; Bal-Tec, Balzers, Liechtenstein). Samples were then gold shadowed by sputter coating (SCD 500; Bal-Tec) and examined with a field emission scanning electron microscope Zeiss DSM 982 Gemini (Carl Zeiss, Oberkochen, Germany), using the Everhart–Thornley SE detector and the inlens detector in a 50:50 ratio at an acceleration voltage of 5 kV.

Statistical analysis and data treatment

All experiments were repeated and the average values were shown with deviation of the repetition as error bars. Using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA), we compared the resulting values of the groups via one-way analysis of variance (ANOVA). Differences between groups were considered to be significant at a P value of <0.05.

Results

We evaluated the PHA production capacity of 25 bacterial strains isolated from Deception Island (Antarctica) with glycerol as the only carbon source using a Nile red staining method. *P. putida* KT2440 and *P. antarctica* were used as positive and negative controls for PHA synthesis, respectively (Fig. 1a). The highest fluorescence intensities, indicative of PHA accumulation, were found for bacterial isolates MPC5 and MPC6. Regarding their general microbiological properties, both isolates typically formed creamy and loose colonies when grown on LB agar plates at 15 °C, colored white in the case of MPC5 and light brown in case of MCP6. Microscopic analysis showed rod-shaped cells negative for Gram staining in both cases. To investigate the phylogenetic identity of MPC5 and MPC6, we PCR-amplified and sequenced the full-length 16S rRNA gene of each isolate and analyzed it using the SILVA ribosomal RNA gene database.

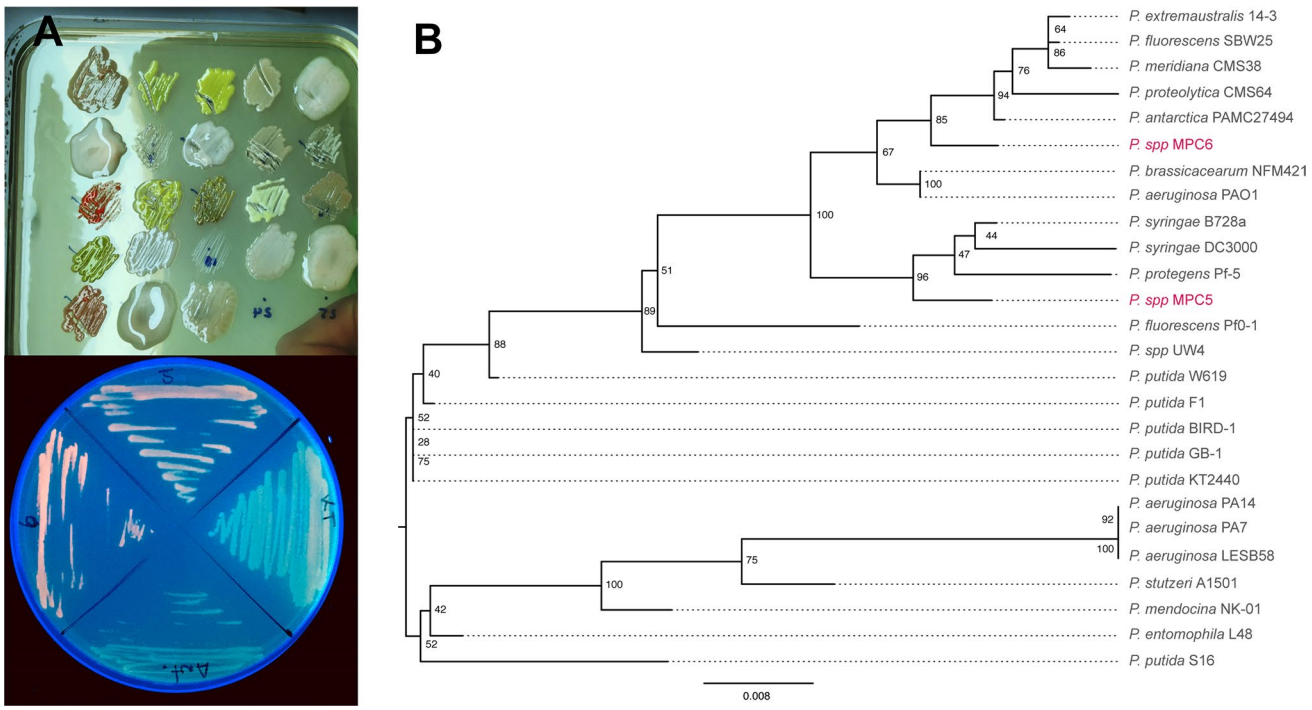

Fig. 1 **a** A collection of 25 Antarctic bacterial isolates was tested for PHA production using Nile Red staining. From them, MPC5 and MPC6 showed the highest production, which was compared to *Pseudomonas putida* KT2440 and *Pseudomonas antarctica*, as positive and negative controls, respectively. **b** Maximum likelihood phylogenetic tree inferred from the multiple alignments of the gene coding for the 16S rRNA of different strains belonging to the *Pseudomonas* genus. The value showed for each node indicates the bootstrap support (%)

As a result, MPC5 and MPC6 strains were classified as part of the genus *Pseudomonas*. Next, we investigated the phylogenetic relationship between MPC5, MPC6 and other *Pseudomonas* strains. For this, we extracted the 16S rRNA gene sequence of 24 strains of this genus from the NCBI database and used them to generate a multiple alignment and infer a maximum likelihood phylogenetic tree using MUSCLE and RAxML tools (Fig. 1b). *Pseudomonas* sp. MPC5 and MPC6 formed two separated phylogroups (bootstrap: 100%), indicating that although part of the same genus and isolated from the same soil sample, they are not clones, possibly being members of different species. MPC5 grouped close to *P. syringae* B728a, *P. syringae* DC3000, and *P. protegens* Pf-5, while MPC6 formed a single branch whose closer relatives were *P. antarctica* and a group formed by *P. proteolytica* CMS64, *P. extremaustralis* 14-3, *Pseudomonas fluorescens* SBW25, and *P. meridiana* CMS38. Further approaches using more sequence information, such as MLST or core genome MLST, are needed to classify these strains at the species level.

Growth pattern on glycerol of MPC5, MPC6 and other *Pseudomonas* strains at 4 °C and 30 °C

We first monitored the growth profile of *Pseudomonas* sp. MPC5, MPC6, *P. putida* KT2440, and *P. antarctica* on glycerol (4 g/L) at two different temperatures (30 °C and 4 °C) in shaking flask experiments. *P. putida* KT2440, a mesophilic bacterium, had a lag phase of 25 h when grown at 4 °C but reached the same biomass yield as cultures grown at 30 °C, after 90 h of cultivation (Fig. 2a). There was a sharp decline in specific growth rate of KT2440 when compared to cultures subjected at 30 °C against 4 °C [0.2 vs. 0.048 (1/h)]. *Pseudomonas* sp. MPC5 showed a specific growth rate of 0.21 (1/h), lacking the lag phase when growing at 30 °C. A different scenario was observed when the MPC5 strain was grown at 4 °C, taking more than 8 h to start propagating and seeing a reduction of the specific growth rate to 0.11 (1/h) (Fig. 2b). We grew *P. antarctica* to evaluate a niche specific bacterium adapted to grow at low temperature, where the strain surprisingly showed a very extended lag phase of 60 h and the yield of biomass was halved compared to the yield displayed at 30 °C. Moreover, *Pseudomonas* sp. MPC6 started growing immediately after inoculation (30 °C), whereas at 4 °C, it presented a short lag stage of 4 h where growth rate decreased from 0.20 to

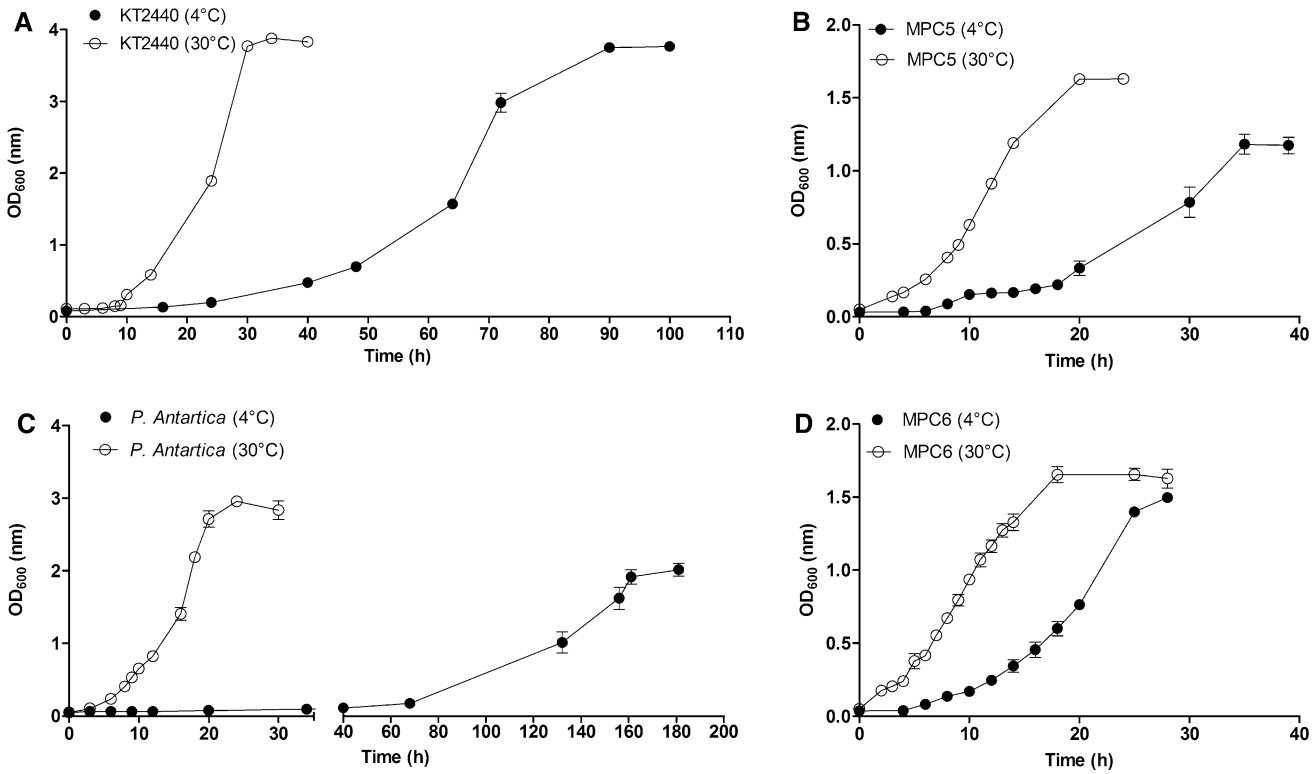

Fig. 2 Growth profile of *P. putida* KT2440, *P. antarctica*, *Pseudomonas* sp. MPC5, and MPC6 on glycerol at 4 °C and 30 °C. Each experiment was performed in triplicate

0.18 (1/h) (Fig. 2d). These features have not been previously found where the physiological parameters of bacterial cells are usually arrested when subjected to grow at cold temperatures.

PHAs and exopolysaccharides synthesis are promoted at 4 °C and 30 °C in batch cultures

To promote PHA accumulation in *Pseudomonas* sp. MP5 and MPC6, the glycerol concentration was increased from 4 to 30 (g/L) in M9 medium. We quantified and characterized the biosynthesized PHAs and EPS of both strains after 72 h of cultivation (Table 1). As previously reported for several Antarctic *Pseudomonas* bacteria [21], the PHA synthesized by the MPC5 strain exhibited a medium-chain length composition, being the major monomers of the polymeric chain 3-hydroxydecanoate (C10), 3-hydroxyoctanoate (C8), and 3-hydroxydodecanoate (C12) at 30 °C. A total PHA content of 0.58 (g/L) was found for cultures grown at 30 °C, which was nearly equal to the concentration of the produced PHA at cold temperature (Table 1). Nevertheless, growing the MPC5 strain at 4 °C was found to have large impact on monomer composition with 31.7% of 3-hydroxy-5-*cis*-dodecanoate (C12:1) being synthesized by the cell. Furthermore, *Pseudomonas* sp. MPC6 reached a total cell dry mass (CDM) of 3.76 and 3.54 (g/L) and amassed a PHA content of 33.4% and 26.6% of the CDM, at 30 and 4 °C, respectively. These values are similar to PHA titers reported for various *P. putida* strains when producing *mcl*-PHA from glycerol at 30 °C [8, 40]. The biopolymer produced by the MPC6 strain was composed of more than 80% of 3-hydroxybutyrate (C4) with the rest distributed as small quantities of monomers of medium-chain length (C6–C12) (Table 1). No unsaturated monomers were identified in the PHA synthesized by MPC6 cells at 30 °C, whilst at 4 °C, 3.7% of the side chains were (C12:1). It is clear that both Antarctic *Pseudomonas* bacteria investigated in this study have the ability to synthesize high levels of biomass and PHA at both imposed temperatures, incorporating unsaturated monomers in the polymeric chain of the resulting PHA in response to temperature downshift. As psychrophilic bacteria are good producer of EPS when exposed to low temperatures [33, 35], we also quantified the synthesized EPS at the end of the fermentation period (72 h). The MPC5 strain secreted higher levels of EPS (4.6 g/L) when cultured at 4 °C as compared to concentrations obtained at 30 °C (2.4 g/L), while the MPC6 strain showed the opposite results (Fig. 3a). We further quantified the neutral sugars, proteins, and DNA content from the purified EPS (Fig. 3b). High levels of proteins were found within the EPS (more than 20% of the total EPS) synthesized by *Pseudomonas* sp. MPC6 at 4 °C, but not at 30 °C. Neutral sugars represented more than 80% of the constituents of the synthesized EPS for MPC5 strain (Fig. 3). The DNA content was also similar for all tested conditions (~2%).

The functional groups of the purified EPS were also analyzed using a FTIR approach. Figure 4 depicts the FTIR spectrum of the secreted EPS by *Pseudomonas* sp. MPC5 (a, b) and MPC6 (c, d). We assigned numbers to the transmittance peaks that represent functional groups of the constituents of the biosynthesized EPS. The peak 1 corresponded to the sulfur and phosphorus groups whereas peaks 2 were associated with the C–O–C and O–H stretching in the polysaccharides. The band at 1382 (1/cm) was attributable to C–H stretching and peak 4 was associated with C=O stretching (Amide I). The P–O bonds were identified in the region stretching at 2450–2550 (1/cm). Finally, peaks 6 and 7 were assigned to C–H and O–H stretching, most likely of tyrosine-like substances. At low temperature the MPC5 strain presented lower intensities for the functional groups associated with O–H and polysaccharides than that found at 30 °C, confirming that lower levels of EPS are synthesized at higher temperatures (Fig. 4a, b). The FTIR analysis of EPS secreted by MPC6 showed nearly the same functional group patterns at both growth temperatures (Fig. 4c, d). In addition, we inspected by 1H-NMR analysis whether the bacterial EPS contains acetyl and succinyl groups, which are important agents for binding to other cations as well as exerting a polyanionic feature to the biosynthesized EPS for creating

Table 1 Biomass and PHA titer as well as monomer composition of the biosynthesized PHA in *Pseudomonas* sp. MPC5 and MPC6 grown on glycerol (30 g/L) in shaking flask experiments after 72 h of cultivation

Strain	Temp. (°C)	CDM (g/L)	PHA[b] (%)	PHA (g/L)	Monomer composition (%)[c]						
					C4	C6	C8	C10	C12	C12:1	C14
MPC5	4	2.75 ± 0.1	12.5	0.54 ± 0.0	N.D	3.1	11.5	37.7	13.1	31.7	2.8
	30	3.03 ± 0.1	19.2	0.58 ± 0.1	N.D	4.2	23.1	42.7	20.9	8.6	N.D
MPC6	4	3.54 ± 0.0	26.6	0.94 ± 0.1	82.1	2.1	2.7	9.7	3.5	3.7	N.D
	30	3.76 ± 0.1	33.4	1.28 ± 0.1	89.5	1.8	3.3	4.4	1.1	N.D	N.D

[a]The data reflect mean values and deviation from three replicates

[b]PHA content relative to cell dry mass (CDM)

[c]C4: 3-hydroxybutyrate, C6: 3-hydroxyhexanoate, C8: 3-hydroxyoctanoate, C10: 3-hydroxydecanoate, C12: 3-hydroxydodecanoate, C12:1: 3-hydroxy-5-*cis*-dodecanoate, C14: 3-hydroxytetradecanoate

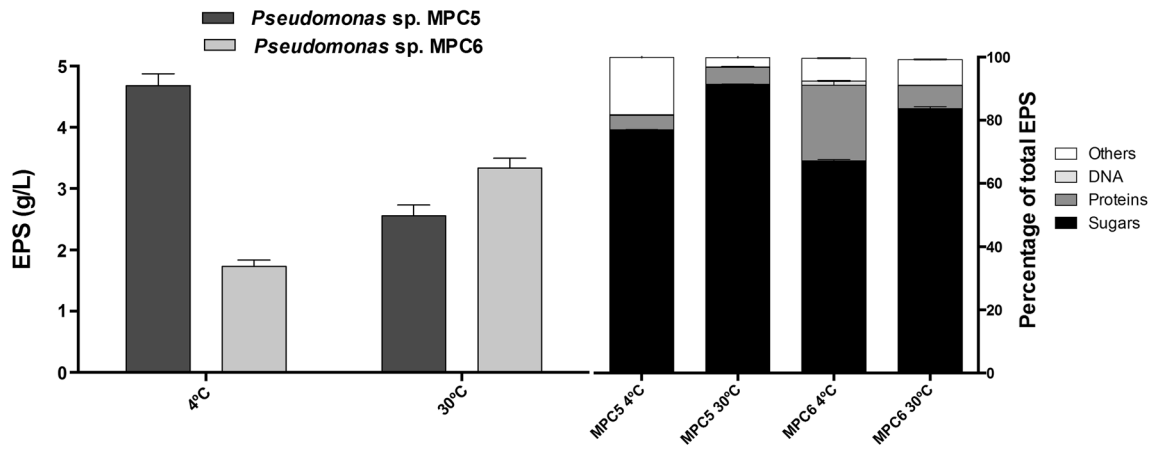

Fig. 3 EPS synthesis and characterization in batch culture by *Pseudomonas* sp. MPC5, and MPC6 on glycerol (30 g/L) after 72 h cultivation at different temperatures. Each experiment was performed in triplicate

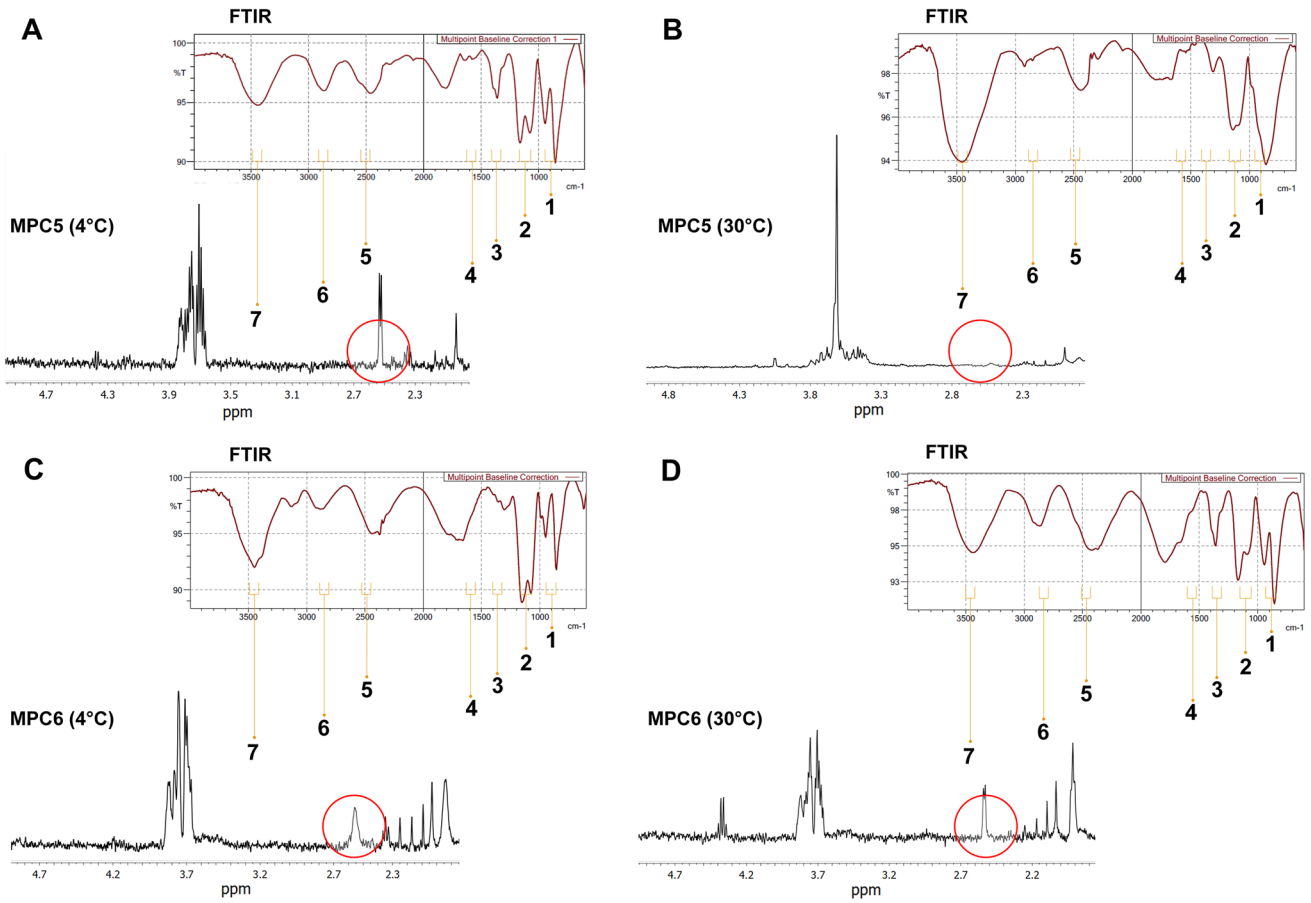

Fig. 4 ^{1}H-NMR and FTIR analysis of EPS produced by **a**, **b** *Pseudomonas* sp. MPC5, and **c**, **d** MPC6 on glycerol (30 g/L) after 72 h cultivation

a sticky layer to protect the cells [24, 33]. The succinyl group was identified at δ 2.55 ppm (highlighted in circle) and the acetyl group at δ 2.05 (Fig. 4). The succinyl group was linked to the EPS synthesized by *Pseudomonas* sp. MPC6 cells propagating at 4 and 30 °C. It was not the case for the EPS produced at 30 °C by the MPC5 strain, where no signal was found at δ 2.55 ppm (Fig. 4b), but identified within the EPS synthesized at 4 °C, demonstrating that

some Antarctic bacteria in response to low temperatures have the ability to activate this protective mechanism.

Batch PHA synthesis in bioreactors and characterization of novel *scl-co-mcl*-PHAs and their thermal properties

Given the high biomass and biopolymer production as well as the particular monomer composition of the synthesized PHA in *Pseudomonas* sp. MPC6 from glycerol in shaking flask, we next performed top-bench batch bioreactors to monitor the time course of substrate consumption and synthesis of biomass, EPS, and PHA at 4 and 30 °C. In Fig. 5a we depicted the growth behavior of the MPC6 strain at 30 °C, where the maximum biomass formation of 5.5 (g/L) was attained after 48 h of cultivation. This value is 2 (g/L) higher than the PHA concentration found in shaking flask experiments (Table 1). It is worth noting that nitrogen limitation was not required for EPS production, which began to be secreted by the cell from the start of the fermentation process, achieving a maximal overall concentration of 5 (g/L). The PHA accumulation at the end of the fermentation period (72 h) was also higher than in flask cultures, reaching a titer of 2.6 (g/L) and representing 47.3% of the CDM (Fig. 5a). When the Antarctic biocatalyst MPC6 strain was subjected to produce PHA on glycerol at 4 °C, indeed an arrested uptake of the substrate and biomass formation was observed (Fig. 5c). Nitrogen extinction occurred at 25 h of cultivation, which is needed to promote PHA synthesis in *Pseudomonas* strains when using glycerol as C source [40]. The secretion of EPS was associated with biomass synthesis but at a lower rate as compared to cultures carried out at 30 °C. Additionally, about a 54% reduction in the PHA formation was observed at low temperature relative to elevated temperatures. Although we could detect remaining glycerol in the fermentation broth, PHA accumulation in the cell ceased at 55 h of fermentation, accounting for 36% of the CDM (Fig. 5c). Overall, the monomer composition of the synthesized PHA was stable throughout the fermentation process (Fig. 5a, c).

We also evaluated possible changes in cell morphology in response to different temperatures during PHA producing conditions by visualizing the cells obtained at the end of the fermentation period using scanning electron microscopy (SEM) (Fig. 5b, d). *Pseudomonas* sp. MPC6 cells cultured at 30 °C showed an average length of about 8 µm, being several times longer than cells (2 µm) grown at 4 °C. This could explain the superior accumulation of PHA by MPC6 observed at 30 °C. In addition, the cells constructed nanowires, sticking to each other and to surfaces and forming lines and films (Fig. 5d).

Next, we purified the PHAs produced in the bioreactor at both temperatures and characterized their monomer composition through ^{1}H- and ^{13}C-NMR, as well as their thermal properties using differential scanning calorimetry (DSC). Based on the NMR data, we determined that *Pseudomonas*

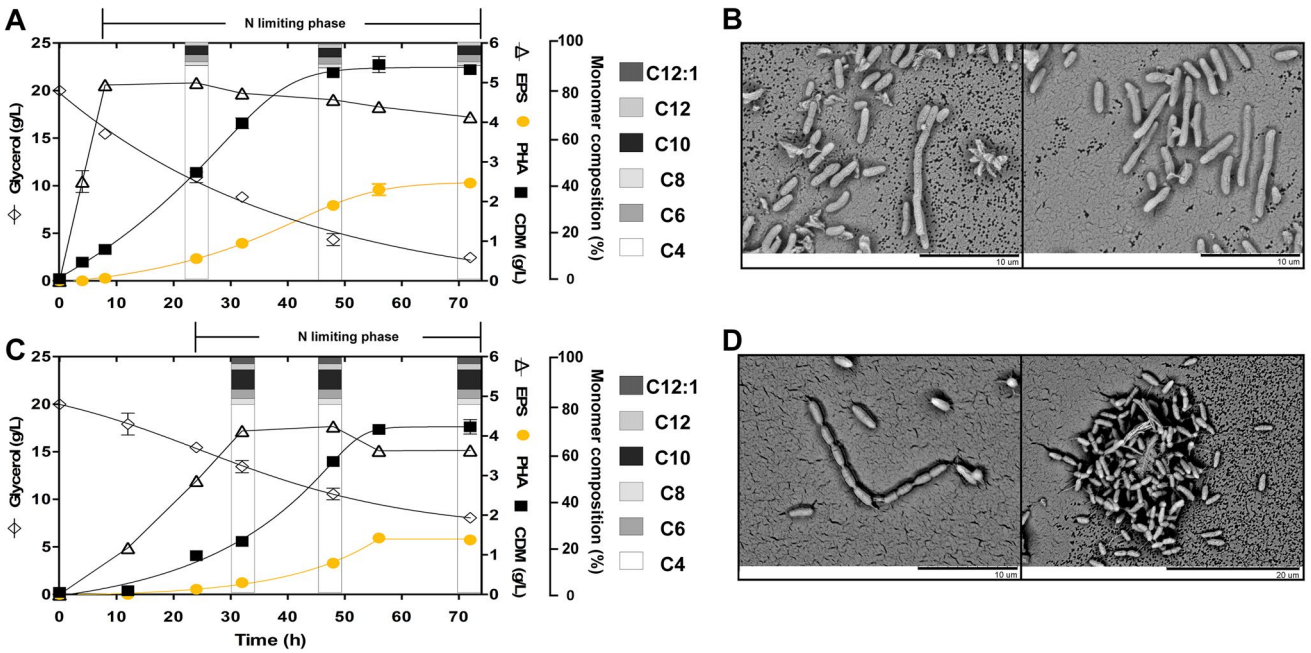

Fig. 5 Time course of glycerol consumption and biomass, PHA, and EPS synthesis by *Pseudomonas* sp. MPC6 in batch bioreactor at **a** 30 °C and **b** 4 °C. C4: 3-hydroxybutyrate, C6: 3-hydroxyhexanoate, C8: 3-hydroxyoctanoate, C10: 3-hydroxydecanoate, C12: 3-hydroxydodecanoate, C12:1: 3-hydroxy-5-*cis*-dodecanoate, C14: 3-hydroxytetradecanoate. Each batch was performed in duplicate

sp. MPC6 synthesizes a copolymer of 3-hydroxybutyrate (3HB), 3-hydroxyhexanoate (3HH), 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD), and 3-hydroxydodecanoate (3HDD). In response to temperature downshift, the MPC6 strain also produced the unsaturated 3-hydroxy-5-*cis*-dodecanoate (Fig. 6a, b). These monomer compositions are in good agreement with the results obtained by GC/MS. To discriminate whether the biosynthesized PHA is a copolymer or a blend of homopolymers, we carefully inspected the bands at 169–170 ppm of the quantitative ^{13}C NMR spectrum (magnified in Fig. 6b, d). Based on the resonance of the carbonyl groups at 169.3, 169.42, and 169.54 ppm, three independent bands were identified which are characteristic signals of a copolymer of 3HB with 3HH, or 3HO. For the thermal properties of the purified *scl-co-mcl*-PHAs, we used DSC analysis to determine the glass transition temperature (T_g), a parameter that allows to classify the PHA in terms of its homo- or heteropolymeric composition [10]. We observed a single T_g for the *scl-co-mcl*-PHAs accumulated by the MPC6 strain at both 4 and 30 °C, as well as a slight difference between the two glass transition temperatures (− 0.29 vs. 2.26) (Fig. S2, Table 2). Both obtained T_g

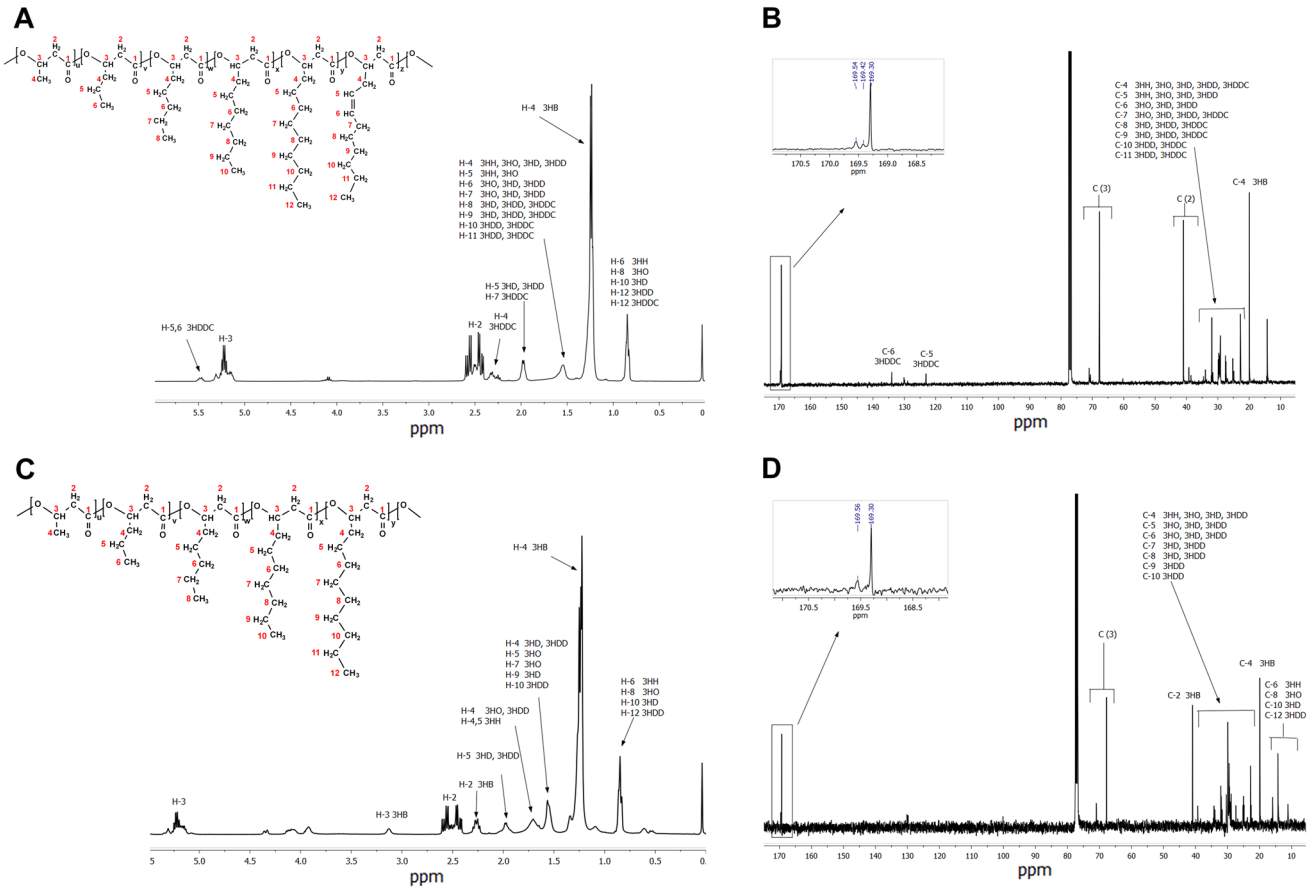

Fig. 6 ^{1}H- and ^{13}C-NMR spectrum of the biosynthesized *scl-co-mcl*-PHA by *Pseudomonas* sp. MPC6 at **a**, **b** 4 °C and **c**, **d** 30 °C after 72 h cultivation in bioreactor

Table 2 Polydispersity index and thermal properties of the synthesized *scl-co-mcl*-PHA by *Pseudomonas* sp. MPC6 at 4 °C and 30 °C after 75 h of cultivation in bioreactors

Growth temperature	T_m (°C)	T_g (°C)	T_c (°C)	M_w (kDa)	M_n (kDa)	PDI
4 °C	157.5 ± 3.9	−0.29 ± 0.1	41.3 ± 0.1	806	448	1.8
30 °C	163.5 ± 0.4	2.26 ± 0.04	45.9 ± 0.2	490	118	4.1

The Data reflect mean values and deviation from two replicates

T_m melting temperature, T_g glass transition temperature, T_c crystallization phase transition temperature, M_w molecular mass, M_n molecular mass distribution, *PDI* polydispersity index

values are distinctive in copolymers of PHAs [10]. Conversely, blocks of homopolymers of PHAs have been shown to have multiple T_g values [10, 56]. Additionally, the PHA synthesized at 30 °C showed a higher melting point than that produced at 4 °C (Table 2), where both values are relatively close to the T_m displayed by other *scl-co-mcl*-PHAS copolymers of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoates) [15, 26]. Moreover, it has been widely reported that cultivation conditions can have a large impact on the M_w and polydispersity of biosynthesized PHAs [57]. Accordingly, we next obtained the M_w and M_n through gel permeation chromatography (GPC) of the PHAs synthesized by *Pseudomonas* sp. MPC6, where lower growth temperatures result in higher molecular weights (806 compared to 490 kDa) but lower PDI values in comparison to PHAs synthesized at 30 °C (Table 2).

Discussion

Extreme environments on Earth offer a source of unique microorganisms encoding metabolic capacities that remain largely unexplored. Only a fraction of the Antarctic territory has been sampled with the aim of isolating PHA-producing bacterial strains. In this study, we have evaluated the growth behavior and PHA production performance of a mesophilic model strain *P. putida* KT2440, a cold-loving bacterium *P. antarctica*, and two isolated Antarctic *Pseudomonas* sp. strains using glycerol as C source at 4 and 30 °C. Under standard growth condition (4 g/L glycerol) at 4 °C, specific growth rates of KT2440 and *P. antarctica* strains were highly arrested as compared to cells growing at 30 °C (Fig. 2), a phenomenon commonly reported for bacteria exposed to low temperatures [20, 32], including psychrophilic microorganisms [4]. This was not the case for *Pseudomonas* sp. MPC6, where this physiological parameter remained nearly equal in response to temperature downshift (Fig. 2d). Antarctic microbes have been reported to show a more than tenfold reduction in specific growth rate including *P. extremeaustralis*, *P. antarctica*, and *Pseudoalteromonas* [18]. Low environmental temperatures trigger a series of molecular responses and metabolic shifts in the cell such as lower protein synthesis given their thermodynamic basis, production of compatible osmotic solutes, higher synthesis of osmotic shock and anti-freeze proteins, and upregulation of LPS, membrane synthesis, EPS, and membrane transport genes, to name a few [14, 48]. These metabolic reprogramming systems indeed operate at the expense of the rate of the cell propagation, which is one of the most important factors of industrial biocatalysts for process design in the synthesis of value-added chemical compounds [1, 41]. It was very surprising to observe that *Pseudomonas* sp. MPC6 strain reached nearly the same yield of biomass at both growth temperatures, indicating that the yield-rate tradeoff of this bacterial strain is very unique since rapid growth rate necessarily implies a more unbalanced catabolism/anabolism, normally resulting in energy spilling and/or overflow metabolism and, as a final consequence, lower ATP and biomass formation [27]. When the Antarctic strains were subjected to PHA production conditions, low temperatures highly affected the monomer composition of the *mcl*-PHA-produced by *Pseudomonas* sp. MPC5, where a high proportion of unsaturated 3-hydroxy-5-*cis*-dodecanoate (C12:1) was synthesized—37% of the monomers content. Cold environments stimulate the synthesis of unsaturated fatty acids in bacterial cells, which tend to make the membrane less rigid by overexpressing enzymes that belong to de novo synthesis fatty acid pathway [29], and in turn are the same compounds (e.g. 3-hydroxyacyl-ACP moieties) that fuel the PHA biosynthetic pathway in *Pseudomonas* strains [46]. This might provide an explanation for the shift in the monomer composition of the biosynthesized PHA that we observed in the MPC5 strain. For *Pseudomonas* sp. MPC6, low temperatures did not highly affect the monomer composition of PHA in comparison to the produced biopolymer at 30 °C, where unsaturation was also found within the biopolymer but to a minor extent (Table 1, Fig. 5d). This could be explained in two manners since the characterized PHA was a copolymer of *scl-co-mcl*-PHA, being the main monomer 3-hydroxybutyrate (3HB): (1) it can be attributed to the specificity of the PHA synthase that possesses the MPC6 strain, or/and (2) an alternative pathway, other than de novo synthesis fatty acid. PHAs containing the 3HB monomer usually used the first described PHB biosynthetic pathway, where acetyl-CoA is converted in various enzymatic steps into 3-hydroxybutyl-CoA. We propose that both pathways are functioning together to generate 3-hydroacyl-CoA precursors of various lengths for PHA synthesis, but this is a hypothesis that needs to be further tested. In relation to the level of biomass and PHA synthesis by both Antarctic *Pseudomonas* strains, there was no remarkable difference when cells were grown at low or elevated temperatures. It has been postulated that environmental bacteria with the capacity to accumulate PHA are more resistant to abiotic stresses [36]. PHA-negative mutant strains of *C. necator* and *P. extremaustralis* have shown lower cell viability to cold environments and freezing compared to parental strains [37, 55]. Nevertheless, one of the few reports showing the production of *mcl*-PHA at 5 °C was carried out in the Arctic bacterium *Pseudomonas* sp. PAMC28620, where the concentration of the biosynthesized biopolymer was diminished at least 20-fold compared to that produced at 25 °C [49]. Taken together, as both Antarctic *Pseudomonas* presented in this study produce high titers of the biopolymer on glycerol, these bacteria are suitable biocatalysts for the synthesis of biopolymer under uncontrolled temperature conditions in bioreactor, which can have

a positive impact on the economics of the bioproduction process. Another notable feature displayed by the MPC6 strain is the elevated concentration of EPS that was found at 30 °C, which is detrimental for PHA production due to carbon loss towards competing biosynthetic pathways [61]. Psychrotolerant bacteria are known to secrete higher levels of EPS when exposed to low temperature [33, 35], conferring a mechanism of protection to compensate for the freezing effect. Indeed, the MPC5 strain follows such postulate (Fig. 3a), but in the case of the MPC6 strain the EPS produced at 30 °C would play a different role. The constitutive functioning of the EPS biosynthetic pathways of the MPC6 strain, independently of growth temperature, is a trait that deserves further study, especially the variation in the content of proteins associated with the produced EPS observed at different temperatures (Fig. 3b). These variations might have profound implications for bacterial adaptation to cold environments that could depend on the synthesis of intracellular and secreted biopolymers.

We then focused on the most promising PHA-producing Antarctic *Pseudomonas* strain (MPC6), which was monitored over time when grown on glycerol under controlled conditions in bioreactor. When the bioreactor was set at 4 °C, this condition affected the cell morphology of *Pseudomonas* sp. MPC6 (Fig. 5b, d). Previous reports have indicated that microbes tend to reduce their cell size as a survival mechanism to changing temperatures [44, 51]. It was postulated that the cell wall determines the shape of bacterial cells, with the peptidoglycan (PG) layer and the chemical composition of the cell membrane being the main drivers of this phenomenon [59]. Another relationship between temperature and bacterial body size relates to ecology, where higher abundance is associated with smaller bacterial size (temperature-size rule, TSR) [2]. Taking the above postulates, one could consider that the reduction in size of MPC6 cells in response to temperature downshift is a strategy to maintain a high cellular division rate (protein synthesis) under such stressful condition [44], along with the development of a specific cell population (biofilm) that may sustain a microenvironment for nutrient exchange [47]. Regarding PHA synthesis, the biopolymer titer reached by the MPC6 strain in bioreactors was higher compared to concentrations found in shaking flask experiments, a trait already reported by several works [7, 41]. As the resulting PHA from *Pseudomonas* sp. MPC6 was characterized as a *scl*-co-*mcl*-PHA using GC/MS when cultured in shaking flasks, we wanted to fully confirm these findings by analyzing it via NMR (Fig. 6) and unveil their thermal properties as well as M_w and PDI (Table 2). We confirmed that the biopolymer produced by the MPC6 is a copolymer of 3-hydrobutyrate and various *mcl*-monomers (Fig. 6). These types of PHAs display superior physical and mechanical properties than that of P(3HB) and *mcl*-PHAs given the combination of the strength of P(3HB) and the elasticity of mcl-PHAs. Additionally, this is the first report showing the natural production of a copolymer of poly(3HB-*co*-3HH-*co*-3HO-*co*-3HD-3HDD) in environmental bacteria since this polyoxoester has been previously produced at low level through genetic engineering of *C. necator* [26] and *Escherichia coli* [54]. Thermal analysis of the *scl-co-mcl*-PHA copolymer synthetized at 4 and 30 °C, demonstrated the existence of singles T_g (Fig. S2), confirming the copolymer nature of the PHA [10]. The M_w of the *scl*-co-*mcl*-PHA obtained at 4 °C was 2 times higher than the biopolymer synthesized at 30 °C (Table 2), which is a desirable feature since it determines how much the biopolymer can be stretched, presenting the possibility to develop more resistant fibers and films [57]. Factors affecting the molecular weight of PHA include the level of the PHA synthase, its activity, and the occurrence of chain transfer reactions [57]. The precise molecular mechanism by which microbes produce PHAs with defined molecular weights is still elusive and based on the results presented in this study, temperature is another factor that determines the polymerization process of PHAs. PHA-producing bacteria with slow metabolism having been shown to biosynthesize PHAs with higher molecular weights [17, 23]. One possible explanation of the resulting physical properties of the produced PHAs is that, at low temperatures, the MPC6 strain polymerize PHAs at a lower rate allowing higher molecular weights but more compacts, thus resulting in lower PDI values (Table 2) as compared to PHAs produced at 30 °C. Most importantly, the psychrophilic bacterium *Pseudomonas* sp. MPC6 can be exploited as an efficient PHA-producing strain that produces *scl-co-mcl*-PHAs with varying physical properties by simply adjusting the fermentation conditions in the bioreactor.

Overall, this study presents for the first time a novel group of Antarctic *Pseudomonas* strains capable of producing high levels of biomass and PHAs at low temperatures using the inexpensive feedstock glycerol. In addition, a novel copolymer of *scl*-co-*mcl*-PHA was found and characterized, which can be synthesized at high concentrations by the psychrophilic Antarctic bacterium *Pseudomonas* sp. MPC6 at different growth temperature, opening up the possibility to further develop a PHA production process without the need of temperature control.

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Compliance with ethical standards

Conflict of interest All author participating in this study declare that they have no conflict of interest.

Human/animal rights statement This article does not contain any studies with animals performed by any of the authors.

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