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ORIGINAL PAPER

Poly(3-hydroxybutyrate) influences biofilm formation and motility in the novel Antarctic species *Pseudomonas extremaustralis* under cold conditions

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Abstract Polyhydroxyalkanoates (PHAs) are highly reduced bacterial storage compounds that increase fitness in changing environments. It has previously shown that polyhydroxybutyrate (PHB) accumulation is essential during the growth under cold conditions. In this work, the relationship between PHB accumulation and biofilm development at low temperature was investigated. *P. extremaustralis*, an Antarctic strain able to accumulate PHB, and its *phaC* mutant, impaired in the synthesis of this polymer, were used to analyze microaerobic growth, biofilm development, EPS content and motility. PHB accumulation increased motility and survival of planktonic cells in the biofilms developed by *P. extremaustralis* under cold conditions. Microaerobic conditions rescued the cold growth defect of the mutant strain. The PHB accumulation capability could constitute an adaptative advantage for the colonization of new ecological niches in stressful environments.

Keywords Biofilms · Polyhydroxybutyrate · *Pseudomonas* · Cold · Motility

Introduction

Bacterial biofilms are known as complex bacterial communities embedded in a polysaccharide matrix and attached to a surface (Costerton et al. 1995). It is becoming increasingly clear that biofilms have an enormous impact on bacterial survival as they are the preferred lifestyle in natural and artificial environments. Biofilm formation represents a protected lifestyle that allows cells to survive in hostile environments, to cope with the stress, and to disperse to colonize new niches (Decho 2000). In medicine, biofilms represent one of the major problems due to the higher resistance of biofilm-associated bacteria to antimicrobial compounds giving rise to serious nosocomial infections (Mah and O'Toole 2001). In comparison to planktonic cells, biofilms show increased resistance to several stress factors such as reactive oxygen species (ROS) derived from antibiotic action or from H_2O_2 exposure, high osmotic pressure and extreme pH (Lapaglia and Hartzell 1997; Wai et al. 1998; Cochran et al. 2001, Albesa et al. 2004) and is also believed to protect bacterial cells from protozoa predation (Matz and Kjelleberg 2005).

The extent of attachment of microbial cells is a complex process that is influenced by several factors including cell surface hydrophobicity, presence of fimbriae and flagella, production of exopolysaccharides (EPS), metabolism of carbon polymers and microaerobic respiration (Pratt and Kolter 1998; Donlan 2002; Van Alst et al. 2007). Motility is a key factor in biofilm development. It has been demonstrated that flagella motility is required for biofilm formation by *P. aeruginosa* PA14, and that type IV pili is important for microcolony formation (O'Toole and Kolter 1998). *P. aeruginosa* can develop three types of movement: swarming, swimming, and twitching motility (Klausen et al. 2003). Swarming is important for movement across semisolid surfaces (Kohler et al. 2000) and is influenced by type IV pili and flagella (Shrout et al. 2006). Swimming is a movement in liquid medium by means of flagella; and twitching motility, powered by extension and

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retraction of type IV pili, is involved in microcolony formation which drives cell aggregation (Klausen et al. 2003).

Pseudomonas extremaustralis is a highly stress-resistant bacterial strain isolated from an Antarctic environment (López et al. 2009) that is able to synthesize polyhydroxyalkanoates (PHA), carbon and energy reserve polymers that are produced under imbalanced growth conditions. PHAs are interesting since their involvement in survival under different stress conditions and their plastic and elastomeric characteristics similar to petroleum derivative plastics (Hazer and Steinbüchel 2007). *P. extremaustralis* is able to synthesize polyhydroxybutyrate (PHB), the most studied PHA, using octanoate and other fatty acids, but not glucose, as carbon sources (Ayub et al. 2006). In this species, *pha* genes are organized in a cluster, containing genes *phaR*, *phaB*, *phaA* (a defective β-ketothiolase), and *phaC*, and are located within a genomic island (Ayub et al. 2007) that also encloses other genes probably related with the adaptability to stress conditions. In *P. extremaustralis,* PHB is essential for bacterial growth under low temperature and to tolerate the oxidative stress derived from cold (Ayub et al. 2009). Studies from our laboratory demonstrated that *P. extremaustralis* is able to grow under low oxygen conditions (Tribelli et al. 2010), similar to those found it in biofilms (Xu et al. 1998).

Cold environments constitute stressful habitats that limit the bacterial survival and the colonization of new ecological niches due to the effect of unfavorable conditions impacting on physical and biochemical cellular process. Exposure to these conditions provokes changes in solubility, reaction kinetics, membrane fluidity, degradation, protein stability, conformation, and gene expression (D'Amico et al. 2006; Strocchi et al. 2006). As a result, bacteria that live in such conditions must have some physiological adaptations. The aim of this work was to investigate the relationship between PHB and biofilm development during growth under cold conditions. We also analyzed EPS content and motility as relevant characteristics of biofilm formation at low temperature.

Materials and methods

Bacterial strains and culture conditions

Pseudomonas extremaustralis DSM 17835^{T} (López et al. 2009), a *phaC* mutant and a complemented strain containing the plasmid pBBR1MSC carrying the *phaC* gene (Ayub et al. 2009) were grown in nutrient broth (NB) supplemented with 15 mM sodium octanoate, incubated overnight at 28°C and used as initial inocula. In order to analyze the effect of different oxygen levels, cultures were grown under three different aeration conditions, defined as follows. Standard aerobiosis was achieved using a 1:5 medium volume/Erlenmeyer flask volume ratio with vigorous rotatory agitation (200 rpm). For microaerobiosis, 100 ml bottles were filled with 25 ml (slight microaerobiosis) or 50 ml of medium (moderate microaerobiosis) and slow shaking (100 rpm) in order to avoid cellular aggregation. This experimental design was similar to that described in Lüthi et al. (1986) and Pessi and Haas (2000). Initial optical density was 0.05 (OD_{600nm}). All cultures were incubated at 10°C during 30 h and growth was monitored by measuring OD_{600nm}. Results were expressed as growth rate (ln OD/h). PHB accumulation was qualitatively monitored at OD = 0.5 by staining with Nile Blue (Ostle and Holt 1982).

Biofilms experiments

Static biofilms were developed in polystyrene microplates during 72 h. Precultures used as inocula were grown in NB with sodium octanoate (PHB accumulation condition) or 15 mM glucose (PHB non-accumulation condition). Initial optical density was 0.025 (OD_{600nm}). Planktonic cells were collected and OD_{600} (A_{PL}) was measured. The attached biofilm was stained with crystal violet (CV) as described (O'Toole and Kolter 1998) and the total attached biomass (CV_{550nm}) was determined.

Total cell counts (planktonic and surface attached cells) were determined using eight wells as experimental unit. Bacterial number was determined by colony counts on nutrient agar (NA) plates, incubated at 28°C. Planktonic cells were obtained by extracting with a pipette the free cells in the culture medium of each well. Surface attached cells were removed with sterile physiological solution and the resulting suspension was disrupted by vortexing during 20 min. The ratio ($C_{A/P}$) = colony forming units of attached cells (CFUA)/colony forming units of planktonic cells (CFUP) was calculated.

Exopolysaccharides determination

Exopolysaccharides formation was measured using anthrone reagent (Quelas et al. 2006). Briefly, 1 ml of culture, obtained from 8 wells as an experimental unit, was centrifuged for 40 min at 9,500 rpm at 4°C. The supernatant was transferred to an assay tube and 3 volumes of absolute ethanol were added. The mix was maintained overnight at −20°C and after this, centrifuged for 40 min at 4°C. The supernatant was discarded and the pellet was resuspended in 0.5 M NaCl. Different aliquots of each preparation were used to determine reducing monosaccharides with 0.2% w/v of anthrone in 95% H_2SO_4 by measuring absorbance at 620 nm (Trevelyan and Harrison 1952). Glucose was used as a standard. The EPS content

was normalized to the cell growth by measuring the total OD_{600} of the experimental unit and a ratio EPS/OD_{600} was determined.

Motility assays

One colony of *P. extremaustralis* grew in PHB accumulating (with octanoate as carbon source) or non-accumulating conditions (with glucose as carbon source) was used as inocula for motility experiments using a sterile toothpick. The culture media used for the different mobility assays were:

Swimming: tryptone broth (10 g/l tryptone and 5 g/l NaCl) containing 0.3% (wt/vol) agarose. Swarming: NB (8 g/l) solidified with 0.5% (wt/vol) agar–agar. Twitching: LB broth (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl) solidified with 1% (wt/vol) agar–agar. All plates were supplemented with sodium octanoate and incubated at 10 or 28°C for 3 and 7 days. The diameter of growth of each strain was measured.

Statistical analysis

To determine the significance of each treatment, a Student's *t* test with two tails and 95% of confidence was used. The *p* value is indicated in the significant experiments.

Results

Effect of microaerobiosis on the growth of *P. extremaustralis* under cold conditions

P. extremaustralis is able to survive and grow under cold conditions due its capability to synthesize PHB; we previously showed that a *phaC* mutant strain was impaired for growth under this condition (Ayub et al. 2009). To further investigate the effect of oxygen availability on growth under cold conditions, the wild type and the *phaC* mutant strain were grown at 10 and 28°C under aerobic and microaerobic conditions. At 28°C, both strains showed a similar growth rate ($p > 0.05$) that decreased under low oxygen availability (Table 1). By contrast, at 10°C, only the wild-type strain was capable of growing at all the oxygen levels, while the mutant strain only was able to grow under microaerobic conditions reaching growth rates significantly lower than those of the wild-type strain (Table 1, $p = 0.008$ for slight microaerobiosis and $p = 0.027$ for moderate microaerobiosis). These growth rates represented an increase in the doubling time (g) of the mutant at 10°C in comparison to those found at 28°C. The values reached 6.6 ± 1.9 h at 10°C and 1.6 ± 0.06 h at 28°C for slight microaerobiosis, and 8.3 ± 1.2 and 2.2 ± 0.1 h at 10 and 28°C, respectively, for moderate microaerobiosis. Even though this, microaerobic conditions rescued the growth defect of the mutant strain under low temperature**.** The presence of PHB was detected qualitatively in both temperatures in the wild-type strain in all oxygen conditions.

These results showed that a low oxygen environment allowed for growth under cold conditions without PHB accumulation.

Biofilm formation at cold conditions

The biofilm is a structured heterogeneous community with oxygen and nutrient gradients in which microaerobic respiration seems to be important (Xu et al. 1998). On the basis of the results obtained in cold microaerobic conditions, we hypothesized that PHB would not be essential for survival when cells are grown in a biofilm. We studied static biofilm formation in *P. extremaustralis* and *phaC* mutant. We found that the total number of viable cells in the biofilm was similar in all strains ($p > 0.05$) when the culture was incubated at 28°C (Fig. 1a), while at 10°C a significant decrease in cell number was observed in the mutant compared with the wild-type strain ($p = 0.048$, Fig. 1b). The wild-type strain cultured without octanoate showed similar results to the mutant strain (Fig. 1b). The survival of planktonic cells, free cells in the culture medium in biofilms assays, was similar at 28°C with no significant differences between the strains. While at 10°C planktonic survival of the mutant strain decreased significantly ($p = 0.030$) in comparison with that observed at

Table 1 Growth rate at 10 and 28°C under different aeration conditions

T (°C)	Condition	Growth rate (ln DO/h)	
		Wild type	*phaC*
10	Aerobic	0.054 ± 0.006	NG
	Slight microaerobic	0.056 ± 0.001	0.031 ± 0.003
	Moderate microaerobic	0.055 ± 0.004	0.034 ± 0.003
28	Aerobic	0.33 ± 0.010	0.33 ± 0.020
	Slight microaerobic	0.19 ± 0.020	0.16 ± 0.020
	Moderate microaerobic	0.15 ± 0.020	0.17 ± 0.020

NG not growth

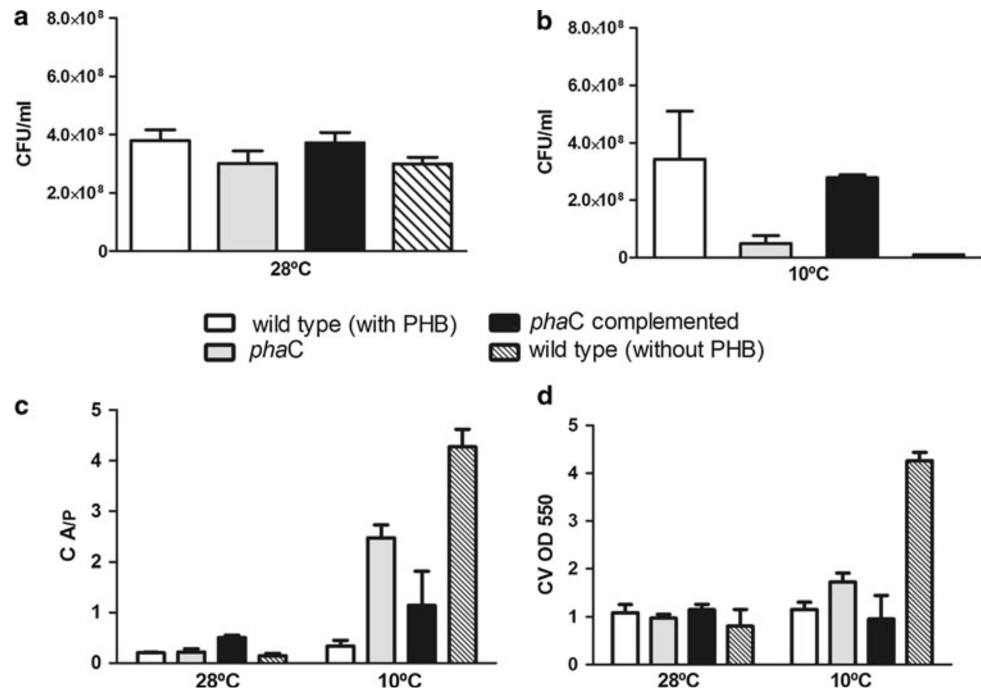

Fig. 1 **a** Bacterial total counts from biofilms experiments at 28°C. **b** Bacterial total counts from biofilms experiments at 10°C. **c** $C_{A/P}$ index (ratio between attached cells and planktonic cells from the biofilms) at 28 and 10°C. **d** Crystal violet assay in biofilm cultures at 10 and 28°C, the OD was measured at 550 nm. All cultures were supplemented with octanoate to allow PHB accumulation and the wild-type strain was also grown in non-accumulation conditions by adding glucose to the culture medium. Each experiment was performed three times and typical results are presented. Values represent media ± SD of triplicate measurements

28°C ($1.2 \times 10^6 \pm 5.2 \times 10^5$ and $1.9 \times 10^8 \pm 7.3 \times 10^7$ cfu/ml, respectively), the wild-type strain showed similar values at both temperatures ($p > 0.05$, $4.4 \times 10^8 \pm 9.9 \times 10^7$ and $2.6 \times 10^8 \pm 8.9 \times 10^7$ cfu/ml for 28 and 10°C, respectively).

A significant decrease ($p = 0.001$) was also observed in the wild-type strain under non-PHB accumulating conditions (using glucose as the carbon source) showing values of $1.3 \times 10^8 \pm 2.1 \times 10^7$ cfu/ml at 28°C and $8.7 \times 10^5 \pm 9.8 \times 10^4$ cfu/ml at 10°C.

The complementation with the *phaC* gene significantly increased the planktonic survival at 10°C, reaching to a value of $1.9 \times 10^7 \pm 6.2 \times 10^5$ cfu/ml ($p = 0.038$).

According to that, the $C_{A/P}$ ratio at 28°C was similar in all strains (Fig. 1c). The wild-type strain, growing in conditions leading to PHB accumulation, showed a similar $C_{A/P}$ value at both temperatures. However, in cold conditions the *phaC* mutant presented a significant increase in this ratio in comparison to the wild-type strain (Fig. 1c, $p = 0.001$). These results indicate that the mutant strain has a higher number of viable cells in the attached form in comparison with the planktonic state (Fig. 1c). The complementation of the mutant with the *phaC* gene, allowed for PHB accumulation and restored the wild-type phenotype (Fig. 1c). In addition, cultures of the wild-type strain performed in PHB non-accumulating conditions showed similar values to the mutant strain (Fig. 1c). The biofilm total biomass was also calculated by staining with crystal violet (CV). At 28°C, the value was similar for all strains with no significant differences. However, at 10°C, the *phaC* mutant showed a significant increase in the CV staining in comparison to that of the wild-type strain and the mutant strain at 28°C, $p = 0.002$ and $p = 0.038$, respectively (Fig. 1d). These results are in accordance with the index calculated by viable counts.

EPS content of static biofilms at low temperature

Biofilm formation depends on several factors, including carbon compounds, therefore, we investigated the exopolysaccharide content (EPS) in both strains. In all strains, the exopolysaccharide content (EPS) was lower at 28°C in comparison with the EPS content at 10°C (Fig. 2). The EPS production observed for the *phaC* mutant strain in cold conditions (Fig. 2) was significantly higher than that observed at 28°C ($p = 0.002$) and it was also higher in comparison to the EPS content of the wild-type strain at 10°C ($p = 0.001$). A similar trend was observed in the wild-type strain under PHB non-accumulating conditions that showed significant differences with the EPS content at 28 and 10°C ($p = 0.001$) (Fig. 2). The results of EPS content are in line with the results observed in biofilm experiments.

Effect of cold and PHB accumulation on *P. extremaustralis* motility

Flagella and pili type IV motility is known to be important in biofilm formation. Motility assays were carried out in *P. extremaustralis* wild-type strain using inocula that previously had or had not accumulated PHB. We studied swarming, swimming and twitching movements. After

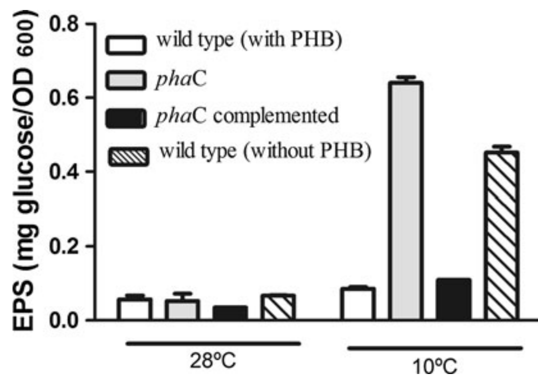

Fig. 2 EPS content at 28 and 10°C. All cultures were supplemented with octanoate to allow PHB accumulation and the wild-type strain was also cultured in PHB non-accumulation conditions by supplemented the media with glucose instead with octanoate. Each experiment was performed three times and typical results are presented. Values represent media ± SD of triplicate measurements

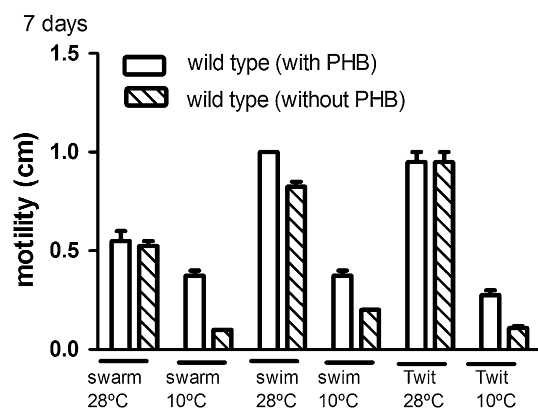

Fig. 3 Motility of *P. extremaustralis* at 28 and 10°C. All motility plates were supplemented with octanoate as carbon source to allow PHB accumulation. The inocula were prepared using octanoate (with PHB) or glucose (without PHB) as carbon source. Values represent media ± SD of triplicate measurements. *Swar* swarming, *Swim* swimming, *Twit* twitching

7 days, the wild-type strain in both growth conditions presented similar capability of movement at 28°C, while at 10°C the higher motility was observed when the polymer had been previously accumulated showing significant differences in comparison with the non-accumulation condition (Fig. 3). Interestingly, after 3 days of culture the strain was able to grow and to move at 10°C only when it had previously accumulated PHB (data not shown).

Discussion

Bacteria have developed several strategies to cope with stressful environments, among them accumulation of carbon polymers and biofilm formation have been widely reported (e.g. López et al. 1995; Mah and O'Toole 2001; Pham et al. 2004; Kadouri et al. 2005). We have previously showed that PHB is essential for growth under cold conditions alleviating the oxidative stress produced at low temperatures (Ayub et al. 2009). In addition, *P. extremaustralis* is able to grow and accumulate PHB under low oxygen levels (Tribelli et al. 2010). In this study, we showed that PHB accumulation condition allowed the formation of biofilms with a higher number of living planktonic cells in comparison with non polymer production condition, in which cells presented significantly higher total adherent biomass, as was observed by CV staining, and the dependence of microaerobiosis to thrive in the cold. The cold sensitive phenotype of the *phaC* mutant was due to an insufficient availability of reducing equivalents (Ayub et al. 2009) necessary for the functioning of NADH/NADPH dependent antioxidant enzymes to cope with the oxidative stress derived from low temperature. The growth defect of the mutant strain could be partially alleviated by decreasing oxygen levels in both biofilm and microaerobic cultures.

Biofilms are organized structures that offer a protective lifestyle due to the extracellular matrix in which the cells are embedded (Costerton et al. 1995). Extracellular polysaccharides and proteins have been shown to be key components of the matrix in addition to extracellular DNA (Branda et al. 2005). The EPS content increased in all strains under cold conditions, with the highest exhibited by the mutant strain in concordance with its higher attached biomass. Low temperature produces ROS and an increase in the activity of key antioxidant enzymes (Chattopadhyay et al. 2011). Our results that showed a lower planktonic cell survival, higher EPS content and also a higher attached biomass at low temperatures agree with previous reports which suggest that EPS could scavenge ROS and other toxic compounds present in the environment (Simpson et al. 1989; Bylund et al. 2006; Wang et al. 2007; Chang et al. 2009). In addition, EPS and PHB production appear to be related as both metabolic pathways consume carbon molecules (Martinez et al. 1997). The defect in PHB production observed in the mutant strain may result in an increase in the availability carbon molecules availability that could redirect PHB production to other metabolic processes such as EPS synthesis. The wild-type strain also showed increased EPS production at low temperature, although the differences were not significant, this trend suggests that EPS could contribute to enhanced survival under these conditions.

A key factor in biofilm development is the capability of motility. In *E. coli* the role of motility appears to be dual by promoting initial cell-to-surface contact and contributing to the spread of a growing biofilm along an abiotic surface (Pratt and Kolter 1998). In addition, in *P. aeruginosa* and *P. putida* it was observed that motility is important for the

dispersion stage in biofilms (Sauer et al. 2002) and also it was shown that hypermotile mutants of *P. aeruginosa* have decreased biofilm formation capability in comparison to the wild type (Caiazza et al. 2007). We found that *P. extremaustralis* at cold conditions exhibited a higher motility when the inoculum had accumulated PHB. These results together with biofilm formation capability at low temperatures could be important to understand the lifestyle of bacteria in the environment.

Natural environments are generally poor in nutrients with transient carbon pulses (Chesson et al. 2004) and PHA accumulation has been detected in these environments (Kadouri et al. 2005). Our results suggest that the PHB accumulation could constitute an advantage for the colonization of new niches by promoting the motility and the viability of planktonic cells. PHA metabolism is a dynamic process since polymerases and depolymerases are concomitantly active, resulting in parallel polymer synthesis and degradation. It has been suggested that the overall PHA flux is governed by intracellular ratios of NADH/NAD and acetyl-CoA/CoA (Ren et al. 2010). The depolymerization process is a source of carbon and reducing equivalents. At cold conditions the PHB depolymerization contribute to maintain the redox state avoiding the oxidative damage of lipids molecules (Ayub et al. 2009).

In this study, we demonstrated that PHB accumulation increased motility and survival of planktonic cells in the biofilms developed by *P. extremaustralis* under cold conditions. The PHB accumulation capability could constitute an adaptative advantage for the dispersion in stressful environments.

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