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ORIGINAL PAPER

Polyhydroxyalkanoates are essential for maintenance of redox state in the Antarctic bacterium *Pseudomonas* sp. 14-3 during low temperature adaptation

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Abstract Polyhydroxyalkanoates (PHAs) are highly reduced bacterial storage compounds that increase fitness in changing environments. We have previously shown that *phaRBAC* genes from the Antarctic bacterium *Pseudomonas* sp. 14-3 are located in a genomic island containing other genes probably related with its adaptability to cold environments. In this paper, *Pseudomonas* sp. 14-3 and its PHA synthase-minus mutant (*phaC*) were used to asses the effect of PHA accumulation on the adaptability to cold conditions. The *phaC* mutant was unable to grow at 10°C and was more susceptible to freezing than its parent strain. PHA was necessary for the development of the oxidative stress response induced by cold treatment. Addition of reduced compounds cystine and gluthathione suppressed the cold sensitive phenotype of the *phaC* mutant. Cold shock produced very rapid degradation of PHA in the wild type strain. The NADH/NAD ratio and NADPH content, estimated by diamide sensitivity, decreased strongly in the mutant after cold shock while only minor changes were observed in the wild type. Accordingly, the level of lipid peroxidation in the mutant strain was 25-fold higher after temperature downshift. We propose that PHA metabolism modulates the availability of reducing equivalents, contributing to alleviate the oxidative stress produced by low temperature.

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Introduction

Many environmental conditions impact microbial life. Among them, low temperature is a critical factor in controlling cell growth and survival. Exposure to low temperature results in decreased protein synthesis, reduced membrane fluidity, protein cold-denaturation, crystal ice formation, low diffusion rates across the membrane, and increased production of toxic reactive oxygen species (D'Amico et al. 2006). Microorganisms that inhabit cold environments are frequently exposed simultaneously to several stress conditions. For example, bacteria living in deep-sea environments have to tolerate low temperature as well as high pressure (Kato et al. 1998), while Antarctic microorganisms usually endure low temperature, nutrient deprivation, and high ultraviolet radiation (Margesin et al. 2007). In such extreme conditions, microorganisms that develop resistance to multiple stresses are selected (Hirsch et al. 2004).

Polyhydroxyalkanoates (PHAs) are highly reduced carbon storage compounds that are accumulated in most bacteria during unbalanced growth conditions (Madison and Huisman 1999). Accumulation and degradation of PHAs endow bacteria with enhanced survival, competition abilities, and stress tolerance, increasing fitness in changing environments (López et al. 1995; Ruiz et al. 2001; Kadouri et al. 2003; Ruiz et al. 2004; Pham et al. 2004; Kadouri et al. 2005).

We have analyzed an Antarctic bacterium, *Pseudomonas* sp. 14-3, that is able to accumulate polyhydroxybutyrate

(PHB), the best known PHA. *Pseudomonas* sp. 14-3 synthesizes large amounts of PHB from octanoate, and shows high stress resistance compared with other related *Pseudomonas* species (Ayub et al. 2004). Genetic analysis demonstrates that *Pseudomonas* sp. 14-3 has a complete PHA biosynthesis cluster containing genes *phaR*, *phaA*, *phaB,* and *phaC* (Ayub et al. 2006). PHA biosynthesis in *Pseudomonas* sp. 14-3 proceeds from fatty acids but not from glucose or gluconate, due to a defective thiolase gene (*phaA*) (Ayub et al. 2006). It was recently found that PHA biosynthesis genes in this strain are located within a genomic island that also contains other genes probably related with its adaptability to Antarctic environments (Ayub et al. 2007). The fixation of horizontally transferred genes suggests that they confer a selective advantage on the recipient organism. However, the nature of this advantage usually remains unclear (Koonin et al. 2001).

In this paper, we investigated the role of PHA on the adaptation of *Pseudomonas* sp. 14-3 to cold conditions using a *phaC* mutant.

Materials and methods

Bacterial strains

Pseudomonas sp. 14-3, a PHB-producing strain with high stress resistance, was previously isolated in our laboratory from a temporary water pond in Antarctica (Ayub et al. 2004). Analysis of 16S rDNA (EMBL accesion # AJ583501) showed that this isolate was closely related with *Pseudomonas veronii* (Ayub et al. 2004). *Pseudomonas putida* KT2440 (Franklin et al. 1981) able to synthesize medium chain lengh PHA and *P. putida* GPp104 a PHA negative mutant (Huisman et al. 1991) were also used in this study.

Plasmids, mutant and recombinant strains construction

A 1,827 bp fragment from *Pseudomonas* sp. 14-3 *phaC* classI gene (EMBL accession # AM262984) containing *Eco*RI and *Sac*I internal restriction sites obtained by colony PCR amplification using primers 5′CGCTCGAGCGCCTT TCATCACCAGCTTCGCCCT3′ and 5′CGCTCGAGCAT TGGCGCTGGCGAACCG3′ (*Xho*I restriction sites underlined) was used to construct the mutant strain. The amplification fragment was digested with *Xho*I and subcloned into pBluescript SK (Stratagene). This plasmid was digested with *Sac*I and religated in order to eliminate the pBluescript *Eco*RI site. Next, it was digested with *Eco*RI, and ligated with a kanamycin cassette obtained from plasmid pUC4 K (Pharmacia, San Francisco, California) cut with *Eco*RI. The resulting plasmid that does not replicate in *Pseudomonas* was introduced by transformation into competent cells of 14-3 prepared according to Lee et al. (2005). Transformants were selected by plating on LB agar containing 20 μg/ml of kanamycin (Km). Km^R colonies were then screened for ampicillin (Amp) sensitivity in 0.5NE2 medium (Huisman et al. 1992) supplemented with 15 mM sodium octanoate and Amp (200 μg/ml). A single Km^R -Amp^S colony was chosen and named *Pseudomonas* sp. 14-3C. The double recombinant event in the strain 14-3C was checked by PCR analysis and its inability to synthesize PHB was confirmed by gas chromatography as previously described (Braunegg et al. 1978).

Plasmid pCTPHB, carrying the entire wild type *phaC* gene, was constructed by introducing an amplification fragment into pBBR1MCS (Kovach et al. 1995). The oligonucleotides used were 5′CGCTCGAGTGGCGACGCTG TGCATCGGC3′ and 5′CGCTCGAGCATTGGCGCTGGC GAAACCG3′ containing *Xho*I sites (underlined). The pCTPHB plasmid was used to transform competent cells of *P. putida* GPp104 and *Pseudomonas* sp. 14-3C. Transformants were selected by plating on LB agar supplemented with chloramphenicol (50 μg/ml) and Km (20 μg/ml) or only choramphenicol for 14-3C and GPp104 strains, respectively.

Growth conditions

Bacteria were grown in nutrient broth (NB) containing 15 mM sodium octanoate (NBO) or 15 mM glucose (NBG). In some experiments, NBO medium was supplemented with: cystine (20 μM), glutathione (50 μM), alanine (100 μM), arginine (100 μM), phenylalanine (100 μM) or methionine (100 μM). Cultures were performed in 125 ml Erlenmeyer flasks containing 25 ml of medium, incubated at 10 or 28°C with shaking (200 rpm). Staining with Nile Blue (Ostle and Holt 1982) was used to examine the PHA accumulation phenotype. To test growth in a cold aerobic environment, overnight cultures grown at 28°C were used to inoculate media (pre-cooled to 10°C) to an initial optical density ($OD_{580\ nm}$) of 0.05. Growth was monitored by measuring $OD_{580\ nm}$ for 72 h. In order to examine bacterial tolerance at 10°C, exponentially growing cells ($OD_{580\ nm} = 0.5$) at 28°C were downshifted to 10°C, and incubated for 2 more days. The bacterial number was measured by colony counts on nutrient agar (NA) plates.

Stress experiments

Exponentially growing cells ($OD_{580\ nm} = 0.5$) in NBO medium at 28°C were exposed to thermal and oxidative stress. To assay cell survival to freezing, 0.1 ml of the cultures were transferred to 0.9 ml of NBO medium, precooled to 4°C and exposed to −20°C. The frozen

suspension was thawed, mixed using vortex, and the number of viable cells after different times of exposure to −20°C was measured by plating aliquots on NA. Survival was expressed as a percentage of the number of colony forming units at time zero, that was taken as 100%. Sensitivity to H_2O_2 was measured by a disk inhibition assay as previously described (Ayub et al. 2004).

In order to test if cold exposure was able to induce the oxidative stress response, bacterial cells were grown in NBO medium at 28°C until $OD_{580\ nm} = 0.5$ and then transferred to 10°C. After 20 min of incubation at 10°C, 2.5 ml aliquots of the culture were inoculated into 125 ml Erlenmeyer flasks containing 22.5 ml of NBO medium supplemented with 0.05 mM H_2O_2, and growth was monitored by measuring $OD_{580\ nm}$ for 10 h. Controls were treated in the same conditions without H_2O_2.

Indicators of cellular redox state

Pseudomonas sp. 14-3 and 14-3C cells grown until $OD_{580\ nm} = 0.5$ at 28°C in NBO were transferred to an ice bath for 20 min. For the diamide sensitivity test, a 100-µl aliquot of the bacterial suspension was spread on NA plates and three sterile filter paper disks impregnated with 10 µl of 0.5 M diamide in dimethyl sulfoxide were applied over the seeded plates. After overnight incubation at 28°C, sensitivity was determined by measuring the diameter of the growth inhibition zone. Triplicate 1-ml culture samples were used for quantification of intracellular NADH/NAD ratio. Determination of these two pyridine nucleotides was performed as previously described (Leonardo et al. 1996). Absorption was measured at 570 nm, and coenzyme standards from 0.05 to 1.5 mM were used to calibrate the assay. Lipid damage was estimated through thiobarbituric acid-reactive substances (TBARS) concentration by the procedure of Semchyshyn et al. (2005). The amount of TBARS formed was expressed in picomoles of malondialdehyde (MDA) per milligram of protein, with an ε_{535} of 156 mM^{-1} cm^{-1}.

Results

Characterization of the *phaC* mutant

The *phaC* mutant of *Pseudomonas* sp. 14-3, called 14-3C, showed similar duplication time (67 ± 2 min and 64 ± 3 min, respectively) in nutrient broth suplemented with sodium octanoate (NBO) or glucose (NBG) at 28°C compared with the parent strain. Quantification of the polymer production by gas chromatography showed that strain 14-3C was unable to synthesize PHB in several accumulation media, including NBO, where PHB content was less than 0.01 wt% (Table 1). Complementation of the mutant strain with pCTPHB carrying the *phaC*, restored the PHB biosynthesis capability and also resulted in the production of an amount of PHB similar to that produced by the wild type strain (Table 1).

Growth and tolerance to low temperature

To investigate if PHA accumulation capability affects the growth at low temperatures, we performed aerobic cultures of *Pseudomonas* sp. 14-3 at 10°C in NBO and NBG. This last medium does not allow PHA acumulation in this strain. Wild type cells cultured in NBO at 10°C arrived to stationary phase in 2 days, whereas cells cultured in NBG did not grow during a 3 day period (Fig. 1a). Accordingly, strain 14-3C, defective in the synthesis of PHB (Table 1), was unable to grow in NBO medium at 10°C (Fig. 1a). In addition, on nutrient agar plates supplemented with 15 mM sodium octanoate in cold conditions (10°C), macroscopic, well developed colonies of strain 14-3 were observed after 7 days of incubation, whilst strain 14-3C exhibited no significant growth (data not shown).

To further investigate the cold sensitive phenotype of the mutant strain, we assessed its tolerance to cold shock. Exponentially growing cultures in NBO at 28°C of strains 14-3 and 14-3C were transferred to 10°C. The wild type strain showed a slight increase in cell numbers following transfer to low temperature (Fig. 1b). By contrast, the number of culturable cells of the mutant showed a strong decrease with the temperature downshift (Fig. 1b).

We also analyzed resistance of cultures of 14-3 and 14-3C grown in NBO at 28°C to freezing (−20°C), and observed that *Pseudomonas* sp. 14-3 was more resistant than its *phaC* mutant (Fig. 1c).

In all these experiments the behavior of the complemented strain, 14-3C/pCTPHB, was similar to the wild type strain (Fig. 1a, b, c).

Table 1 Production of PHB in *Pseudomonas* sp. 14-3 and recombinant strains at different incubation temperatures

Strain	PHB (wt %)	
	28°C	10°C
14-3	18 ± 2	24 ± 2
14-3C	ND	–
14-3C/pCTPHB	20 ± 3	25 ± 4

Determinations were performed during exponential growth phase in nutrient broth supplemented with 15 mM octanoate [Wild type (14-3), PHA minus mutant (14-3C), and complemented strain (14-3C/pCTPHB)]

ND not detected

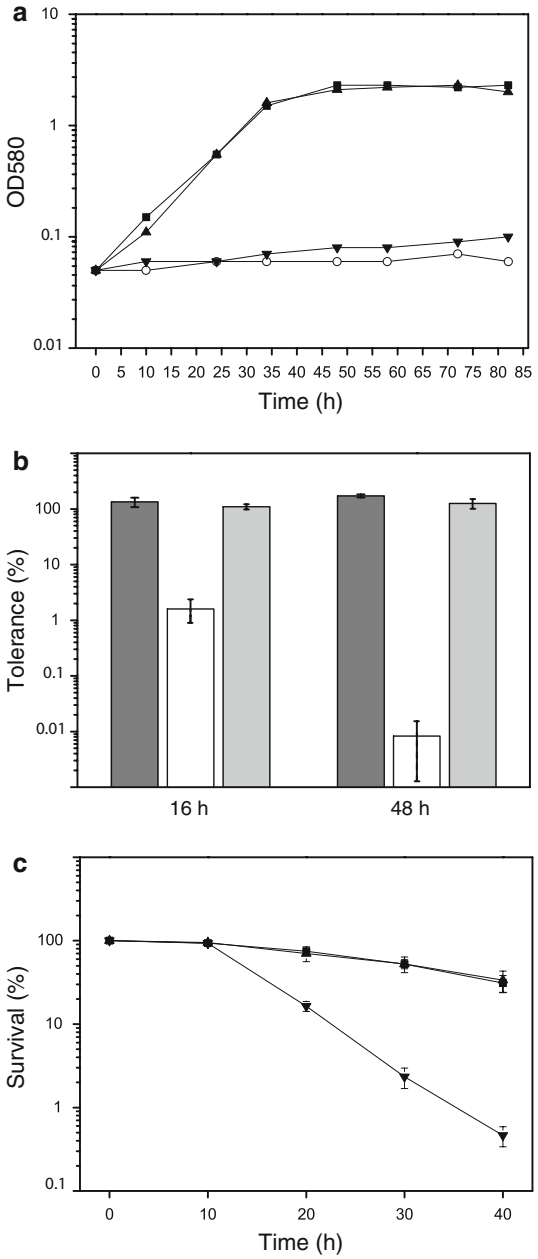

Fig. 1 Behavior of *Pseudomonas* sp. 14-3 and its PHA negative mutant under cold conditions. **a** Growth at 10°C of strains 14-3 (*rectangle*), 14-3C (*inverse triangle*), and 14-3C/pCTPHB (*triangle*) in NBO (*closed symbols*) or 14-3 in NBG (*open circle*). Each experiment was performed twice and typical results are presented. Values represent media ± SD of triplicate measurements. **b** Cold challenge. Exponentially growing cells of 14-3 (*dark grey bar*), 14-3C (*white bar*) and 14-3C/pCTPHB (*light grey bar*) strains in NBO medium were transferred from 28°C to 10°C during 16 h and 48 h. **c** Survival to freezing (−20°C) of 14-3 (*rectangle*), 14-3C (*inverse triangle*), and 14-3C/pCTPHB (*triangle*). **b**, **c** Values represent media ± SD of triplicate experiments. One hundred percent was considered to be the number of bacteria before the exposure to the stress agent

Role of PHA on the development of oxidative defenses during low temperature exposition

We examined the response of 14-3 and 14-3C to H_2O_2 after cold treatment. The cold (20 min at 10°C) and non-cold-treated cultures did not exhibit any significant changes on growth (Fig. 2a), indicating that during a short time period the cold treatment did not have important effects on growth. Addition of a low concentration of H_2O_2 to non-cold-treated cultures resulted in an increase of the lag growth phase in all strains analyzed (Fig. 2b). *Pseudomonas* sp. 14-3 showed a shorter initial lag than the mutant strain (Fig. 2b), in accordance with its higher tolerance to hydrogen peroxide (data not shown). Additionally, when cells were cold pre-treated before the addition of H_2O_2 the lag phase in the wild type strain was similar to the lag phase without H_2O_2 supplement, while no improvement in growth in the mutant strain was observed (Fig. 2b), indicating that PHA was necessary for the development of the antioxidant response induced by cold shock. The results observed for the complemented strain were comparable to those described for the wild type strain (Fig. 2b).

To investigate if the deficiency in antioxidative defenses was responsible for the cold sensitive phenotype of *phaC* mutant, we analyzed the growth of 14-3C at 10°C in NBO medium supplied with the reducing compounds glutathione (γ-Glu-Cys-Gly) or cystine (Cys-Cys). Both compounds were able to suppress this phenotype, as the mutant was able to grow in the presence of the antioxidant compounds (Table 2). No growth was observed in NBO medium supplied with different amino acids that do not have antioxidant properties (Table 2).

To test whether the increased fitness in cold environments conferred by the ability to synthesize PHA could be extended to other *Pseudomonas* species, we performed low temperature and oxidative stress experiments using *P. putida* KT2440 and their PHA negative mutant *P. putida* GPp104. The duplication time at 28°C shown by GPp104 was similar to the wild type strain (57 ± 3 min and 62 ± 2 min, respectively). However, GPp104 was unable to grow and survive at 10°C in NBO (Fig. 3a, b). Similarly to 14-3C, GPp104 showed increased sensitivity to oxidative stress when compared to the wild type strain (Fig. 3c). The introduction of *phaC* from *Pseudomonas* sp. 14-3 conferred the capability to synthesize the polymer to the mutant strain GPp104 reaching values of 56 ± 5 wt% PHB in stationary phase cultures in NBO, and also restored the wild type phenotype (Fig. 3a–c).

Effect of cold shock on PHA content and redox state

On the basis of these experiments we hypothesized that the protective role of PHA under cold conditions could be

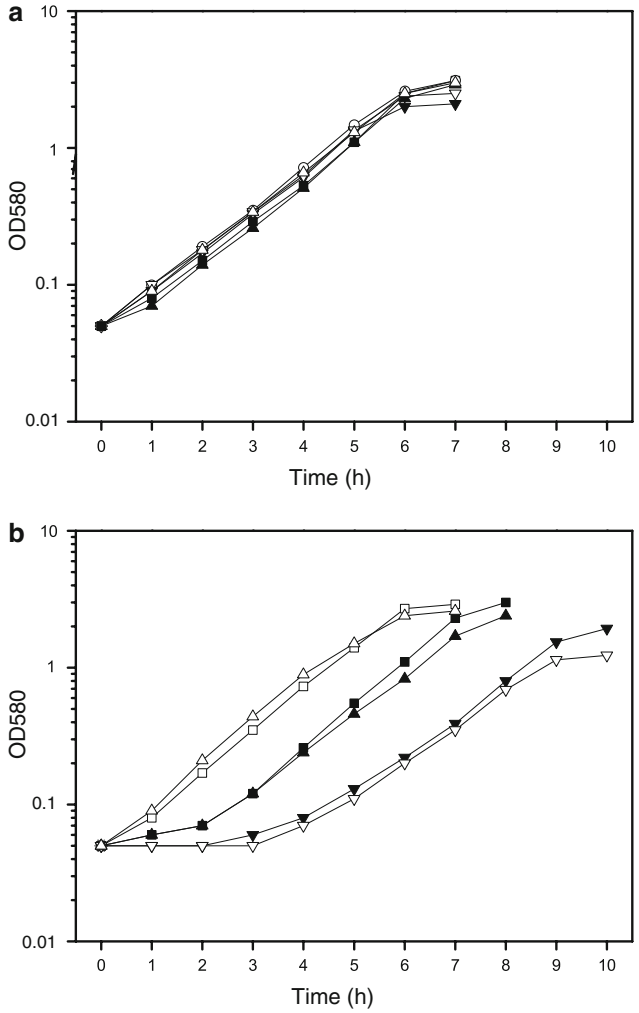

Fig. 2 Effect of *phaC* mutation on the growth in the presence of H_2O_2 after cold treatment. Cultures grown in NBO medium at 28°C until $OD_{580} = 0.5$ were shifted to 10°C for 20 min. Then, 2.5 ml aliquots of these cultures were inoculated into 22.5 ml of the same medium supplemented with 0 (**a**) or 0.05 mM (**b**) H_2O_2 and incubated at 28°C until early stationary phase. Strains: 14-3 (*rectangle*), 14-3C (*inverse triangle*), and 14-3C/pCTPHB (*triangle*). *Open and closed symbols* designate cultures cold-pretreated and non-cold- pretreated, respectively. Each experiment was performed twice and typical results are presented. Values represent media ± SD of triplicate measurements

related with its function as a modulator of the pool of reducing equivalents. To test this hypothesis, we assessed the ratio NADH/NAD as an indicator of intracellular redox state and the sensitivity to diamide as a rough estimation of NADPH availability (Butler et al. 2002) in line with PHA degradation after cold shock. In addition, the accumulation of damage products of oxidative stress resulting from cold exposure was analyzed by measuring thiobarbituric acid-reactive substances (TBARS) that indicate the amount of oxidized lipids. In all the experiments, exponentially growing cultures of *Pseudomonas* sp 14-3 and its *phaC*

Table 2 Effect of antioxidant compounds on growth at 10°C of the *phaC* mutant

Strain/medium	Growth rate (h^{-1})
14-3 NBO	0.0389
14-3C NBO	No growth
14-3C NBO cystine (20 μM)	0.0253
14-3C NBO glutathione (50 μM)	0.0242
14-3C NBO alanine (100 μM)	No growth
14-3C NBO arginine (100 μM)	No growth
14-3C NBO phenylalanine (100 μM)	No growth
14-3C NBO methionine (100 μM)	No growth

Growth rate was derived from turbidimetric measurements at 580 nm on log-phase cultures. Data are expressed as the mean from three independent experiments

mutant were cold challenged in an ice bath during 20 min, and cellular redox state indicators were measured before and after cold shock. The amount of PHB in strain 14-3 decreased drastically reaching values of 0.39 ± 0.04 wt%. The NADH/NAD ratio in the wild type strain showed a slight decrease (Fig. 4a). On the contrary, the NADH/NAD ratio in mutant cells dropped to values roughly threefold lower than those observed in non-cold shocked cells (Fig. 4a). Complementation with *phaC* gene allowed the reversion of the redox imbalance (Fig. 4a).

Despite the fact that the wild type and the mutant strain showed similar inhibition zones when exposed to diamide (12.0 ± 1.0 and 14.7 ± 0.6 mm, respectively) before ice treatment, the PHA minus strain showed higher diamide sensitivity (18.0 ± 1.0 mm) in comparison with the wild type (13.3 ± 0.6 mm) after cold shock (Student′s *t* test, $P < 0.002$). This indicated that the *phaC* mutant presented alterations in both the NADH/NAD ratio and the intracellular NADPH content under cold conditions. The amount of TBARS after cold challenge increased drastically (25-fold) in the mutant strain (from 428 ± 86 to 10,574 ± 2,229 pmol MDA/mg protein), while the increase observed for the wild type strain was significantly lower (from 107 ± 17 to 429 ± 87 pmol MDA/mg protein) (Fig. 4b). Complementation with the *phaC* decreased the level of oxidative damage in lipids (Fig. 4b).

Discussion

The *phaC* gene of *Pseudomonas* sp. 14-3 is located in a large genomic island-like element (Ayub et al. 2007). Horizontally transferred genes are believed to persist if they provide a selective benefit to the host (Lawrence 1999). In this study we have shown that the capability to synthesize PHA encoded by *phaC* was essential for the

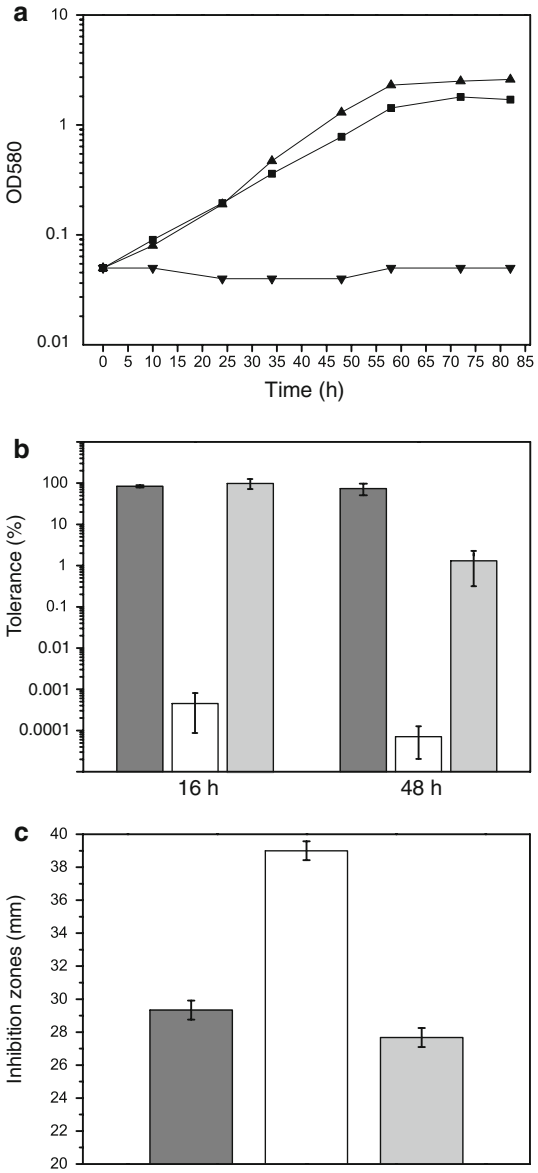

Fig. 3 Performance of *Pseudomonas putida* KT2440 and its PHA negative mutant under cold and oxidative stress. **a** Growth at 10°C of strains KT2440 (*rectangle*), GPp104 (*inverse triangle*), and GPp104/pCTPHB (*triangle*) in NBO. **b** Cold challenge. Exponentially growing cells of KT2440 (*dark grey bar*), GPp104 (*white bar*) and GPp104/pCTPHB (*light grey bar*) strains in NBO medium were transferred from 28°C to 10°C during 16 h and 48 h. One hundred percent was considered to be the number of bacteria before the temperature downshift. **c** Sensitivity to 30% of H_2O_2 of KT2440 (*dark grey bar*), GPp104 (*white bar*) and GPp104/pCTPHB (*light grey bar*). **a**, **b** and **c** Each experiment was performed twice and typical results are presented. Values represent media ± SD of triplicate measurements

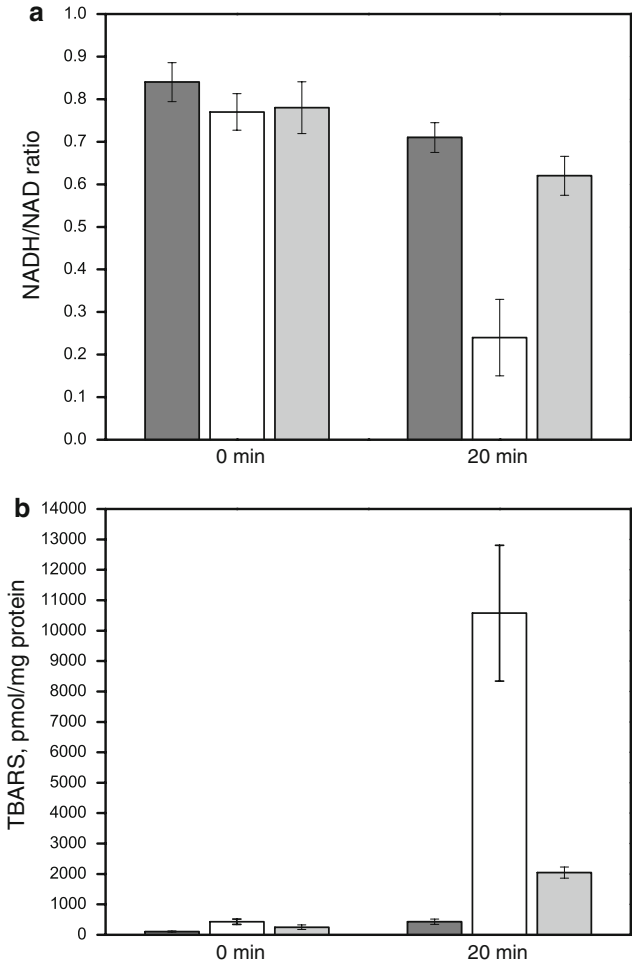

Fig. 4 Effect of *phaC* mutation on intracellular redox state and oxidative lipid damage after cold shock. NADH/NAD ratio (**a**) and TBARS (**b**) in 14-3 (*dark grey bar*), 14-3C (*white bar*) and 14-3C/pCTPHB (*light grey bar*) strains before and after cold shock (ice treatment for 20 min). Values represent media ± SD of triplicate measurements

adaptability to cold conditions in the Antarctic bacterium *Pseudomonas* sp. 14-3. Bacterial survival and stress resistance studies are important for many aspects of bioremediation, biocontrol, and plant growth promotion. *Pseudomonas* species are highly versatile microorganisms, among them *P. putida* KT2440 is one of the best characterized *Pseudomonas* used in biotechnology and agriculture applications. We found that PHA biosynthesis was also necessary for growth at 10°C and resistance to cold shock in this strain. Recently, a study based on the screening for cold sensitive Tn*5* mutants in *P. putida* KT2440 allowed the identification of 13 essential genes for life at low temperatures (Reva et al., 2006). PHA genes were not included inside this group of genes, probably due to the duplication of synthase genes (Nelson et al. 2002). Our results suggest that the increased fitness in cold environments conferred by the ability to synthesize PHA to *Pseudomonas* sp. 14-3 could be extended to other *Pseudomonas* species that were not isolated from Antarctic environments.

In order to elucidate some of the mechanisms involved in the increased fitness conferred by PHA we analyzed

several indicators of intracellular redox state. It has been reported that organisms living at low temperature either bacteria, yeasts, plants or animals, are exposed to oxidative stress generated by cold conditions (e.g. Kane et al. 1996; Salahudeen et al. 2000; Liu et al. 2002; Gocheva et al. 2006). Therefore, low temperatures induce the production of antioxidant enzymes (e.g. catalase, superoxide dismutase, and glutathione peroxidase) that utilize reduced nicotinamide dinucleotides as cofactors (Smirnova et al. 2001; Zhang et al. 2003). The *phaC* mutant of *Pseudomonas* sp. 14-3 was unable to develop the oxidative resistance after cold exposure indicating a crucial function of PHA in cold-inducible antioxidant defense. Low temperature growth experiments using the reducing compounds cystine and glutathione further suggest that the cold sensitive phenotype of the *phaC* mutant was due to an insufficient availability of reducing equivalents. PHAs are highly reduced storage materials which are involved in a dynamic process of biosynthesis and degradation (Yan et al. 2000). Because of this, polymer degradation could supply the reductive power necessary to subdue the oxidative stress under cold conditions. This hypothesis is supported by several experiments performed in this work. The wild type strain showed a very rapid PHA degradation after cold shock, associated with the maintenance of NADH/NAD ratio, total NADPH content, and low lipid damage. In addition, the occurrence of redox imbalance and lipid peroxidation in the mutant after cold shock could be related with its impaired growth at 10°C. To our knowledge, this is the first report suggesting a relationship between the intracellular redox state mediated by PHA and the adaptability to cold environments.

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