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Hydroxybutyrate prevents protein aggregation in the halotolerant bacterium *Pseudomonas* sp. CT13 under abiotic stress

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Abstract Polyhydroxybutyrate (PHB), a typical carbon and energy storage compound, is widely found in Bacteria and Archae domains. This polymer is produced in response to conditions of physiological stress. PHB is composed of repeating units of β-hydroxybutyrate (R-3HB). It has been previously shown that R-3HB functions as an osmolyte in extremophile strains. In this study, *Pseudomonas* sp. CT13, a halotolerant bacterium, and its PHB synthase-minus mutant (*phaC*) were used to analyze the chaperone role of R-3HB. The production of this compound was found to be essential to salt stress resistance and positively correlated with salt concentration, suggesting that PHB monomer acts as a compatible solute in *Pseudomonas* sp. CT13. R-3HB accumulation was also associated with the prevention of protein aggregation under combined salt and thermal stresses in *Pseudomonas* sp. CT13. Physiological concentrations of R-3HB efficiently reduced citrate synthase (CS) aggregation and stabilized the enzymatic activities of CS during thermal stress. Docking analysis of the CS/R-3HB interaction predicted the stability of this complex under physiological concentrations of R-3HB. Thus, in vivo, in vitro and in silico analyses suggest that R-3HB can act as a chemical chaperone.

Keywords Chemical chaperone · Hydroxybutyrate · Stress · Protein aggregation

Introduction

Polyhydroxybutyrate (PHB) is a carbon and energy storage compound that is accumulated by many bacterial species under abiotic stress (Kadouri et al. 2005). The degradation of PHB releases the β-hydroxybutyrate monomer, which has an R stereo-configuration (R-3HB) (Fig. 1). Recently, accumulation and complete degradation of this polymer has been associated with the control of the redox status during low-temperature adaptation in the Antarctic bacterium *Pseudomonas* sp. 14-3 (Ayub et al. 2009). Thus, PHB degradation could supply the reductive power necessary to subdue the oxidative stress induced by cold conditions (Ayub et al. 2009).

The content of R-3HB and R-3HB oligomers is also positively correlated with the levels of NaCl in *Methylarcula marina*, *Methylarcula terricola*, *Photobacterium profundum* strain SS9, *Sinorhizobium meliloti* JB1 and *Sinorhizobium meliloti* MTCC 3402 (Doronina et al. 2000; Martin et al. 2002; Arora et al. 2006), suggesting that the R-3HB molecule is an osmolyte in halotolerant and halophilic bacteria. In addition, *Photobacterium profundum* strain SS9 preferentially accumulates R-3HB and R-3HB oligomers as a response to changes in both the hydrostatic and the osmotic

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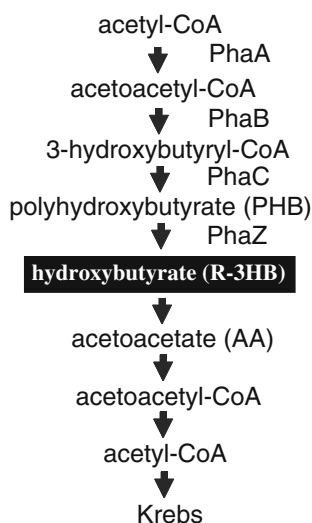University of Camerino, 62032 Camerino, MC, Italy

acetyl-CoA
↓ PhaA
acetoacetyl-CoA
↓ PhaB
3-hydroxybutyryl-CoA
↓ PhaC
polyhydroxybutyrate (PHB)
↓ PhaZ
hydroxybutyrate (R-3HB)
↓
acetoacetate (AA)
↓
acetoacetyl-CoA
↓
acetyl-CoA
↓
Krebs

Fig. 1 Biosynthesis and degradation of polyhydroxybutyrate (PHB). Thiolase II (PhaA), reductase (PhaB) and PHB polymerase (PhaC) and PHB polymerase (PhaZ) involved in β-hydroxybutyrate (R-3HB) production are shown

pressure (Martin et al. 2002). Thus, the R-3HB molecules are an atypical class of osmolytes from halotolerant bacteria, termed piezolytes (Roberts 2005). However, many molecular aspects of R-3HB function remain unclear.

Compatible solutes such as betaine, proline, trehalose, choline and glycerol can act as "chemical chaperones" by increasing the stability of native proteins or assisting refolding of unfolded polypeptides in *Escherichia coli* during high-temperature adaptation (Caldas et al. 1999; Diamant et al. 2001). Moreover, low physiological concentrations of proline, glycerol and betaine can activate molecular chaperones under combined salt and heat stress (Diamant et al. 2001). The presence of molecular mechanisms to mitigate multiple forms of stress is not unexpected considering that microorganisms are exposed to various stresses at the same time.

In this study, we investigated the role of R-3HB in the prevention of protein aggregation of the halotolerant bacterium *Pseudomonas* sp. CT13 using a *phaC* mutant. This recombinant strain is defective in the production of R-3HB. The function of R-3HB as chemical chaperone was also evaluated both in vitro, by studying thermoprotection of citrate synthase (CS) activity and thermal aggregation of this enzyme, and in silico, by the docking analysis of the citrate synthase/R-3HB complex.

Experimental procedures

Sample collection

Samples were collected from El Bebedero salt mine, an area of scientific interest located at $33°30'00''$S and $66°37'00''$W, San Luis, Argentina (Sgroy et al. 2009). In this area, the average maximum temperature ranges between 42 and 45 °C in summer. Samples were screened for PHB-producing *Pseudomonas* strains. Screening to obtain these strains was made using PIPI medium (10 g K_2HPO_4, 3.5 g $NaHNH_4PO_4 \cdot 4H_2O$, 10 g glucose, 2 g citric acid, 2 g $MgSO_4 \cdot 7H_2O$, 5 mg $FeSO_4 \cdot 7H_2O$, 4 mg $MnCl_2 \cdot 4H_2O$, 6 mg $CoSO_4 \cdot 7H_2O$, 3 mg $CaCl_2 \cdot 2H_2O$, 0.4 mg $CuCl_2 \cdot 2H_2O$, 0.6 mg $ZnSO_4 \cdot 7H_2O$, 1L, pH = 7.5) supplemented with 100 g/L of NaCl (PIPI$_{10\%}$ medium) and then plating them in PIPI$_{10\%}$ agar. The resulting bacterial colonies were tested for PHB accumulation by staining with Nile Blue and gas chromatography (Braunegg et al. 1978; Ostle and Holt 1982). *Pseudomonas* sp. CT13 was isolated under these specific conditions.

PCR amplification of 16S rRNA and *phaC* genes from *Pseudomonas* sp. CT13

The 1460-bp fragment of the 16S ribosomal RNA gene from *Pseudomonas* sp. CT13 (JQ027335) was obtained by colony PCR amplification using fD1 and rP2 primers according to Weisburg et al. 1991. The 1576-bp fragment from the *Pseudomonas* sp. CT13 *phaC* gene (JQ269639) was obtained by colony PCR amplification using primers 5′CGCCTTTCATCACCAGCTTCGCCCT3′ and 5′GTTCTAGGGGAGGGTATTCGGCATTGCC3′. The sequencing reactions were performed by Macrogen.

Phylogenetic analysis

Molecular evolutionary analysis was conducted using MEGA version 4.0 (Tamura et al. 2007). The phylogenetic tree was constructed using the neighbor-joining (NJ) method with genetic distances computed using Poisson correction model. This analysis was developed by setting the following parameters: substitutions to include = all, gaps/missing data = pair wise deletion, phylogeny test = 500 bootstrap replicates and root on midpoint. The NJ tree was shown with bootstrap values. The tree topologies obtained using the NJ method, minimum evolution and maximum parsimony methods were identical.

Bacterial strains, cell growth, R-3HB and PHB production and chemical chaperone assay

The *phaC* mutant (PC) and complementary (PC/pCTPHB) strains derived from *Pseudomonas* sp. CT13 (PS) were constructed as previously (Ayub et al. 2009). Cultures were performed in 125 ml Erlenmeyer flasks containing 25 ml of PIPI medium and incubated at 28 °C with shaking (250 rpm). To test the growth in saline conditions, overnight cultures grown in PIPI medium were used

to inoculate PIPI medium with or without NaCl (100–400 mM) to an initial optical density (OD 580 nm) of 0.05. After 48 h, growth, and PHB and RS-3HB contents were analyzed by colony forming units (CFU), NMR spectroscopy and gas chromatography, respectively (Brauneg et al. 1978; Martin et al. 2002). Sensitivity to thermal stress was measured as previously described (Ayub et al. 2004). Protein aggregates in *Pseudomonas* sp. CT13 cells were evaluated according to Diamant et al. (2001) with very slight modifications. Frozen bacterial pellets from 1 ml aliquots were lysed in 0.5 mg/ml lysozyme on ice for 5 min and then sonicated. Aggregated proteins were separated from the insoluble cell membranes and debris by two washes with 200 μl of 2 % (v/v) Nonidet P-40. Nonidet P-40-insoluble pellets were resuspended in 50 μl of guanidine-HCl (6 M). Thermal aggregation and thermal inactivation of citrate synthase were measured as described in Richarme and Caldas (1997). The native enzyme (100 μM) was diluted 100-fold in 40 mM HEPES, 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM KCH_3CO_2, pH 8 at 43 °C in the presence or absence of R-3HB. Citrate synthase aggregation was monitored by measuring the OD at 650 nm. For the citrate synthase inactivation assay, citrate synthase was diluted to a final concentration of 80 nM at 42 °C in the presence or absence of R-3HB. Citrate synthase activity was determined as described by Jakob et al. (1993). Protein content was determined by the Bradford method (Bradford 1976) using bovine serum albumin as standard.

Bioinformatic analysis

To identify the most probable conformation of the R-3HB/ citrate synthase complex, molecular docking analysis was performed on an Intel Core2Duo/Linux Red Hat-based platform using Autodock 4.0, a software program performing a Lamarckian genetic algorithm to explore the binding possibilities of a ligand in a binding pocket (Morris et al. 2009). The X-ray crystal structure of mammalian citrate synthase [pdb entry: 1CTS (Remington et al. 1982)] was retrieved from the Protein Data Bank (Berman et al. 2000), while the tree-dimensional structure of R-3HB was constructed and optimized using HyperChem (release 8.0.6; Hypercube Inc.). Hydrogen atoms were added to the protein prior to any analysis. The Autodock genetic algorithm was performed with a grid of 64, 50, and 60 points (in the x, y, and z directions) and a grid spacing of 0.375 Å around the citrate synthase active site, and with a grid of 100, 100, and 100 points and a grid spacing of 0.719 Å around the whole enzyme. A root-mean-square (rms) tolerance of 0.8 Å, a maximum of 2500000 energy evaluations and other parameters set to default values were used (Cuccioloni et al. 2011).

Binding affinity was expressed as predicted equilibrium dissociation constants (K_d) derived from free energy of binding (ΔG_{bind}), according to the standard relation: $K_d = e^{\Delta G_{bind} 1000/RT}$. The output from Autodock, and all modeling studies and images were rendered with PyMOL (Python Molecular Graphics—2010; DeLano Scientific LLC, San Carlos, CA). PyMOL was also used to calculate the length of theoretical hydrogen bonds, measured between the hydrogen and its putative binding partner.

Results

Phylogenetic and phenotypic characterization of *Pseudomonas* sp. CT13

Pseudomonas sp. CT13 was isolated from a saline soil sample that was collected in San Luis Province, Argentina. The complete sequencing of 16S ribosomal DNA showed that this isolate belongs to the *Pseudomonas syringae* group (Fig. 2a). In accordance with these data, this isolate was included in the genus *Pseudomonas* and named *Pseudomonas* sp. CT13. Phenotypic characterization showed that this isolate was a Gram-negative straight, mobile rod and grew on 0.5 NE2 medium, a semi-synthetic medium of *Pseudomonas* (Huisman et al. 1992). *Pseudomonas* sp. CT13 grew at temperatures ranging from 4 to 35 °C, with an optimum at 28 °C at pH 7.5, and in the presence of NaCl concentrations ranging from 0 to 3.8 %, with an optimum 0.15 % NaCl at pH 7.5 and 28 °C. This result shows that *Pseudomonas* sp. CT13 is a halotolerant bacterium.

To study the role of the R-3HB molecules, a 1576-bp DNA fragment from *Pseudomonas* sp. CT13 was cloned using primers designed from PHB polymerase (*phaC* gene) conserved motifs. The PCR product was cloned into a pGEM-t easy vector and sequenced. This DNA fragment from *Pseudomonas* sp. CT13 (JQ269639) shares 99 % identity with *phaC* genes from *Pseudomonas* sp. 14-3 (CAK18904) and *Pseudomonas stutzeri* A1501 (ABP78389).

Characterization of the *phaC* mutant

The *phaC* mutant of *Pseudomonas* sp. CT13 showed a duplication time in nutrient broth at 28 °C similar to that of the parent strain, suggesting that *phaC* is not a housekeeping gene. Quantification of R-3HB and PHB production confirmed that the mutant was unable to synthesize these compounds in several accumulation media, including PIPI medium, where the production of the polymer and monomer was <0.05 and 0.09 ± 0.03 wt%, respectively. Complementation of the mutant with pCTPHB carrying the *phaC* from *Pseudomonas* sp. CT13 restored the PHB
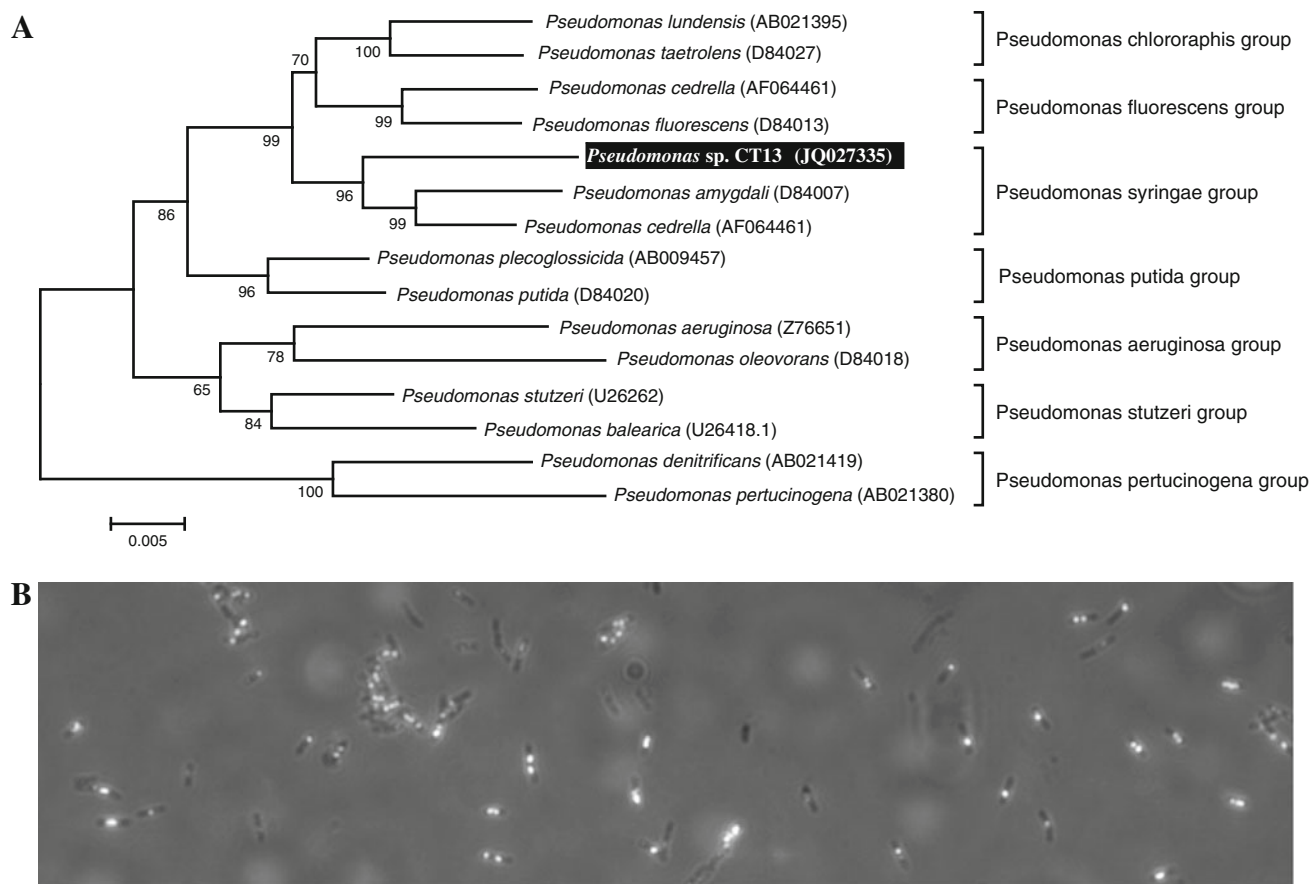
A

B

Fig. 2 **a** Phylogenetic relationships between representative species of the genus *Pseudomonas* based on neighbor-joining (NJ) analysis of the 16S rDNA gene. *Pseudomonas* sensu stricto species are divided in seven groups (Anzai et al. 2000). These subclasses are shown. Bootstrap percentages are indicated at the branch points. **b** Phase-contrast microscopy of *Pseudomonas* sp. CT13 strain showing PHB polymer granules

biosynthesis ability and also resulted in the production of an amount of PHB similar to that produced by the wild-type strain in PIP medium (17 ± 5.4 and 13.5 ± 3.2 wt%, respectively) (Fig. 2b).

Role of the R-3HB molecules in the salt and heat stress response

To investigate if PHB and R-3HB production affects *Pseudomonas* sp. CT13 growth in high salinity, we performed aerobic cultures of the wild-type strain (PS), the *phaC* mutant (PC) and the complemented strain (PC/pCTPHB) derived from *Pseudomonas* sp. CT13 in PIPI medium at 28 °C. The growth of PC in PIPI medium without NaCl supplementation was similar to that of the parent strain (Fig. 3). While PS was able to tolerate diverse NaCl concentrations and heat shock, PC was sensitive to moderate concentrations of NaCl (Fig. 3) and high
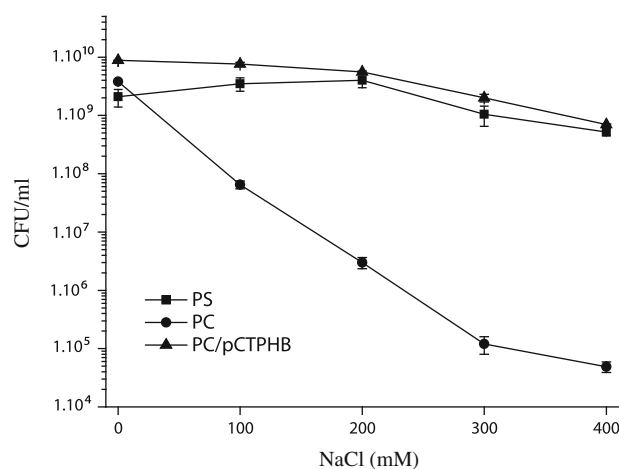

Fig. 3 Effect of different salt concentrations on the growth of *Pseudomonas* sp. CT13. Growth at 28 °C of strains in PIPI medium supplied with NaCl to an initial optical density (580 nm) of 0.05. Values represent mean $\pm$ SD of triplicate experiments
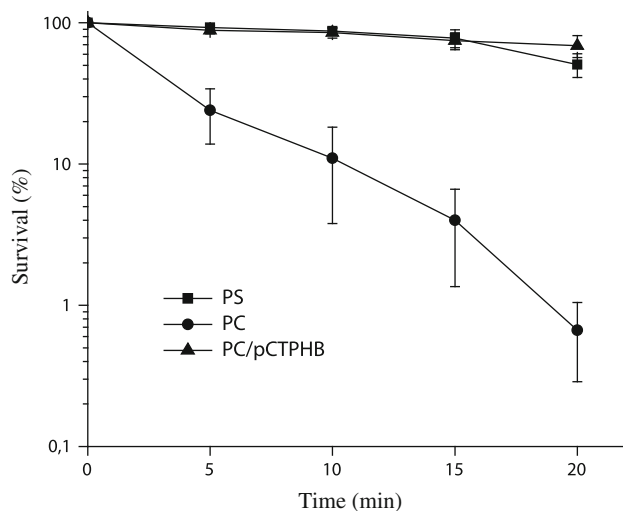
Fig. 4 Behavior of *Pseudomonas* sp. CT13 and its R-3HB negative mutant under thermal stress. Exponentially growing cells in PIPI$_{1.7}$ % medium were transferred from 28 to 47 °C for 20 min. Values represent mean ± SD of triplicate experiments. One hundred percent was considered to be the number of bacteria before the exposure to the stress agent
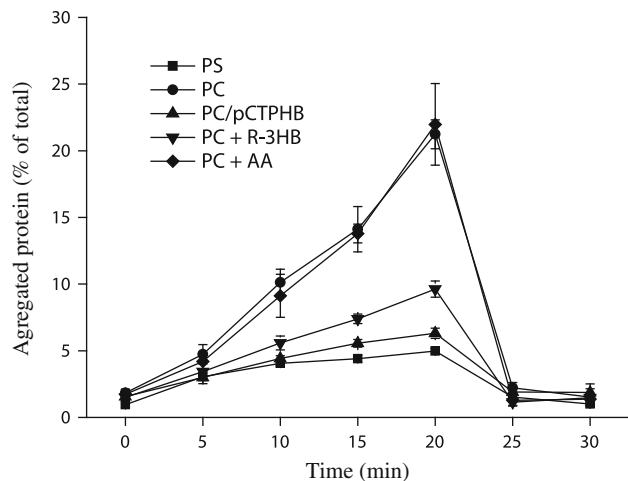

Fig. 5 Protein aggregation in wild-type and recombinant strains under high temperature. Time-dependent protein aggregation at 47 °C (0–20 min) and disaggregation at 30 °C (20–30 min) in cells in PIPI medium supplemented with 300 mM NaCl (PIPI$_{1.7}$ %). In some experiments, PIPI$_{1.7}$ % was supplemented with 100 mM of R-3HB or acetoacetate (AA)

Table 1 Biosynthesis of PHB monomer and PHB polymer by *Pseudomonas* sp. CT13 under water stress

NaCl (mM)	Monomer content (wt%)	Polymer content (wt%)	Monomer/ polymer (%)
0	ND	13.5 ± 3.2	0
100	3.4 ± 0.3	11.9 ± 0.8	28
200	5.6 ± 0.7	10.0 ± 0.9	56
300	7.1 ± 0.6	8.8 ± 1.2	80
400	10.5 ± 0.6	11.2 ± 1.5	94

Monomer and polymer content is expressed as a percentage of total cell dry weight. Values represent media ± SD of triplicate experiments

temperature (Fig. 4). Then PC/pCTPHB was also able to tolerate salinity conditions and heat shock, suggesting that *phaC* is involved in stress tolerance.

PS exhibited no significant R-3HB production and low accumulation of the PHB polymer in PIPI medium without NaCl (Table 1), suggesting that all monomer molecules are in the polymer form. However, when we increased NaCl concentration in PIPI medium from 0 to 400 mM NaCl, PS increased the R-3HB content and maintained PHB production (Table 1). In addition, PS accumulated the maximum R-3HB at 400 mM NaCl where R-3HB represents 94 % of the PHB production (Table 1). These results suggest that R-3HB is an essential osmolyte in *Pseudomonas* sp. CT13.

Effect of R-3HB on protein aggregation in *Pseudomonas* sp. CT13 under abiotic stress

We next analyzed the effect of the availability of R-3HB on protein aggregation in wild-type and recombinant

strains both during and after heat stress. Less than 2 % of the total proteins were found to be insoluble aggregates in cells grown in PIPI medium at 28 °C supplemented with 300 mM NaCl (PIPI$_{300}$) in PS, PC and PC/pCTPHB (Fig. 5). This aggregated fraction was severely increased to 21 % in PC during a 20-min treatment at 47 °C (Fig. 5). In contrast, PS and PC/pCTPHB showed a slight increase in aggregated protein (2 to 2.5-fold increased) at this time. Interestingly, PC showed a decrease in the heat-induced protein aggregation when the R-3HB was added to PIPI$_{300}$ medium (Fig. 5). However, the addition of an alternative source of energy (acetoacetate) to PIPI$_{300}$ medium did not reverse the sensitive phenotype of heat-induced protein aggregation in PC (Fig. 5), suggesting that the R-3HB protection is not due to an energetic effect (Fig. 1).

Effect of R-3HB on thermal protection of citrate synthase

To address the thermoprotective nature of R-3HB, the protein aggregation and thermal inactivation of citrate synthase was analyzed in vitro under heat stress conditions. The physiological concentrations of R-3HB examined (50 and 100 mM) significantly stabilized the native CS at 42 °C for 30 min (CS activities of 34 and 47 %, respectively), whereas without R-3HB, native CS showed a severe inactivation (CS activity of 3 %) under these conditions (Fig. 6). We also observed that 50 and 100 mM R-3HB reduced CS aggregation in vitro under high temperature (42 °C). For example, treatment with 100 mM R-3HB showed 5-fold lower aggregation of CS with
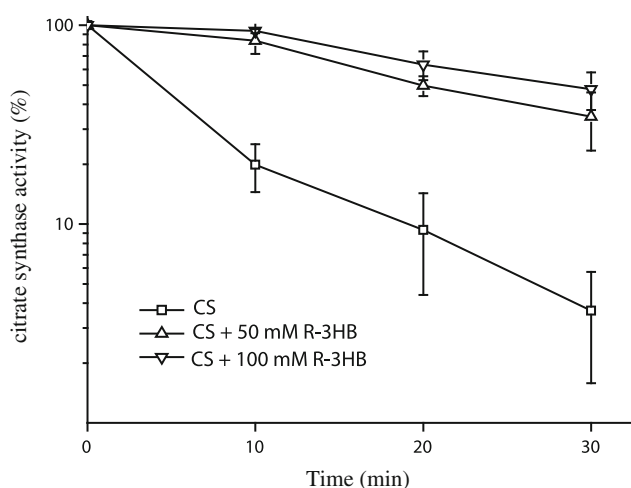
Fig. 6 Thermal protection of citrate synthase activity. Citrate synthase (70 nM) was incubated at 42 °C in the presence or absence of 50 and 100 mM R-3HB. Samples were removed at intervals, cooled, and enzyme activity was subsequently determined at 25 °C, as described in "Experimental procedures"
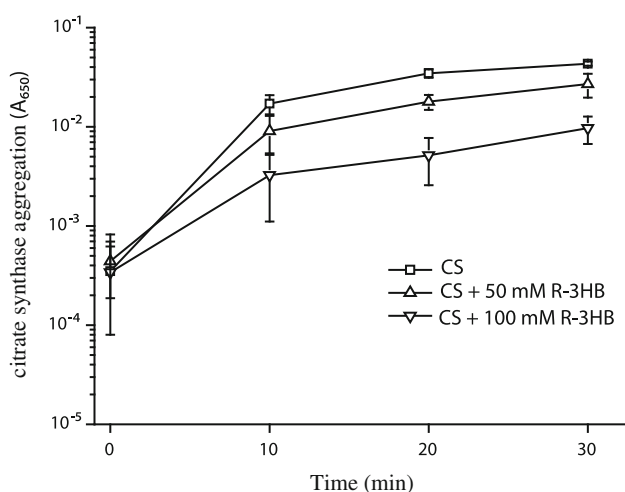

Fig. 7 Thermal aggregation of citrate synthase in the presence of R-3HB. The kinetics of citrate synthase aggregation was determined by light scattering at 650 nm. Native citrate synthase was diluted to a final concentration of 0.8 μM at 42 °C, in the presence or absence of 50 and 100 mM R-3HB

respect to CS aggregation without the monomer under high temperature for 30 min (Fig. 7). Thus, in vitro assays further suggest a chaperone role of R-3HB.

Molecular docking of the complex between R-3HB and citrate synthase

Docking analyses on both the active-site neighborhood and the whole citrate synthase resulted in 20 distinct conformational clusters with comparable binding affinities in the range between 1 and 12 mM. The best binding pose placed

R-3HB out of the active site ($\Delta G_{\text{bind}} = -3.99$ kcal/mol and $K_d = 1.19$ mM). In particular, R-3HB interacted with the amino acids Arg[164], Arg[313], Val[314], Val[315] and Lys[366] of the enzyme, establishing seven H bonds (mean length = 2.9 Å), as shown in Fig. 8.

Discussion

While several *Pseudomonas* species have been found to produce medium chain length polyhydroxyalkanoates (PHAs), the inability to produce PHB by the *Pseudomonas sensu stricto* species has been proposed as a critical taxonomic value (Kessler and Palleroni 2000). Nevertheless, PHB genes have been found in *Pseudomonas* sp. 14-3 (Ayub et al. 2004, 2006) and *Pseudomonas stutzeri* A1501 (Yan et al. 2008). In both cases, the PHB genes were found within genomic islands (Ayub et al. 2007; Yan et al. 2008). The localization within these mobile elements, phylogenetic studies and functional analysis of the PHB genes support that these genes were acquired by horizontal transfer (Ayub et al. 2007, 2009; Yan et al. 2008). Based on these results, it has been proposed that horizontal transfer of the PHB genes is a mechanism of adaptability to changing environments.

Here, we describe the isolation and characterization of a PHB-producing halotolerant bacterium *Pseudomonas* sp. CT13. The taxonomic position of this strain and the extremely high nucleotide identity of the PHB genes from *Pseudomonas* sp. CT13, *Pseudomonas* sp. 14-3 and *Pseudomonas stutzeri* A1501 suggest that the PHB genes from *Pseudomonas* sp. CT13 were also acquired by horizontal transfer. Normally, the fixation of horizontally transferred genes suggests that they present a selective benefit on the recipient organism. In this work, we explored the nature of this advantage and found that the capability to produce the R-3HB molecules was essential for the adaptability of *Pseudomonas* sp. CT13 to heat and salt conditions. Previously, the function of R-3HB as a compatible solute of the deep-sea bacterium *Photobacterium produndum* was demonstrated by the laboratory of Dr. Mary Roberts (Martin et al. 2002). In our work, we observed that R-3HB is a critical osmolyte in *Pseudomonas* sp. CT13. We also showed a chaperone role of R-3HB in vivo under abiotic stress. This function is supported by the thermal protection of citrate synthase experiments and docking analysis performed in this study. The predicted low affinity of R-3HB for citrate synthase excludes the presence of a specific interaction ($K_d \geq 1$ mM) (Mendel and Mendel 1985), suggesting an osmolyte/chemical chaperon role of the ligand that largely shields the enzyme at concentrations higher than the predicted equilibrium dissociation constant. In fact, chemical chaperones prevent protein aggregation
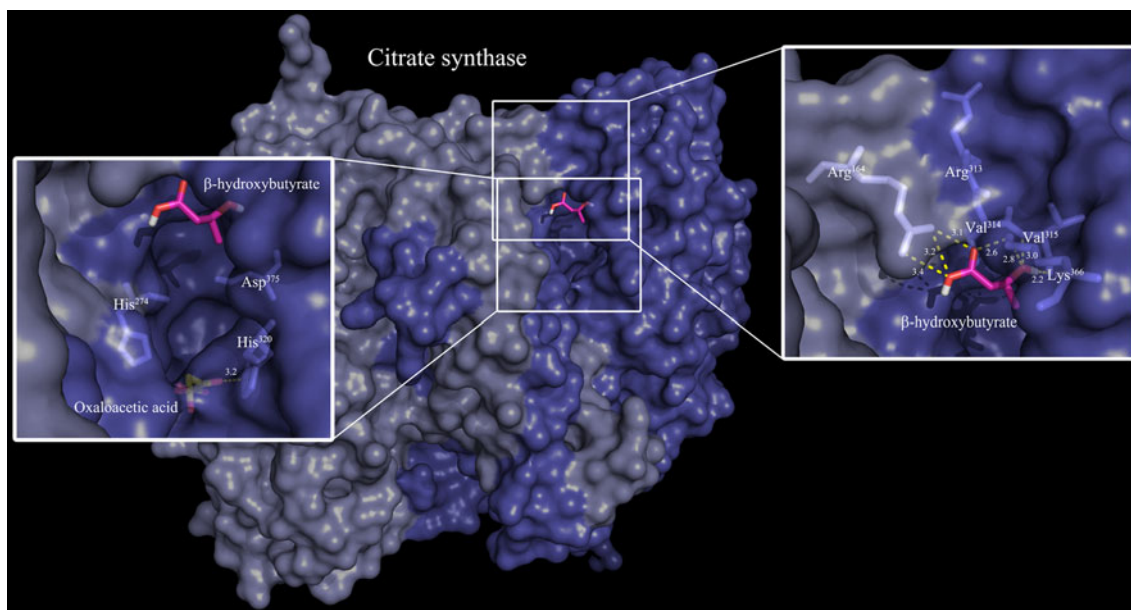
Fig. 8 Predicted R-3HB/citrate synthase complex. Catalytic amino acids complexed to oxaloacetic acid (*left box*) and amino acids involved in the interaction with R-3HB (*right box*) are shown. H bonds formed, represented as *yellow dotted lines*, and their length in Å are also shown

(confirming protein aggregation data), stabilizing protein structure due to their ability to stabilize the hydration shell around proteins (Smith et al. 1998). To our knowledge, this is the first time that R-3HB has been used by an organism to increase the stability of native proteins.

S-3HB, the enantiomer of PHB monomer (R-3HB), is well known as the main component of ketone bodies produced by hepatocytes and astrocytes in animals (Nehlig 1996). Several studies have shown that R-3HB is neuroprotective. In fact, the application of R-3HB has been proposed as an effective treatment of neurodegenerative disorders, such as Huntington's disease and Parkinson's disease (Mejía-Toiber et al. 2006; Cheng et al. 2010). Interestingly, neurodegenerative disorders produce protein aggregation. Thus, further studies involving cell models of neurodegenerative disorders will show whether the protective effects of R-3HB on protein aggregation found in this work constitute a general characteristic of living organisms. Actually, we have recently demonstrated that the thiolase II, a gene involved in the R-3HB and mevalonate production, can be considered to be a conserved key element in the abiotic stress response in organisms from the Bacteria and Eukarya domains (Soto et al. 2011). Finally, manipulation of R-3HB production by genetic engineering may have a great impact on biotechnological developments to mitigate abiotic stress in highly divergent organisms.

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