

The alternative sigma factor, σ^S , affects polyhydroxyalkanoate metabolism in *Pseudomonas putida*

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Abstract

To determine whether the stationary sigma factor, σ^S , influences polyhydroxyalkanoate metabolism in *Pseudomonas putida* KT2440, an *rpoS*-negative mutant was constructed to evaluate polyhydroxyalkanoate accumulation and expression of a translational fusion to the promoter region of the genes that code for polyhydroxyalkanoate synthase 1 (*phaC1*) and polyhydroxyalkanoate depolymerase (*phaZ*). By comparison with the wild-type, the *rpoS* mutant showed a higher polyhydroxyalkanoate degradation rate and increased expression of the translational fusion during the stationary growth phase. These results suggest that σ^S might control the genes involved in polyhydroxyalkanoate metabolism, possibly in an indirect manner. In addition, survival and oxidative stress assays performed under polyhydroxyalkanoate- and nonpolyhydroxyalkanoate- accumulating conditions demonstrated that the accumulated polyhydroxyalkanoate increased the survival and stress tolerance of the *rpoS* mutant. According to this, polyhydroxyalkanoate accumulation would help cells to overcome the adverse conditions encountered during the stationary phase in the strain that lacks RpoS.

Introduction

Fluorescent pseudomonads belonging to the rRNA–DNA homology group I, such as *Pseudomonas putida*, synthesize and accumulate medium-chain-length polyhydroxyalkanoates under conditions of nutrient limitation and carbon excess (Huisman *et al.*, 1989). The *pha* locus of *P. putida* KT2440 (Nelson *et al.*, 2002) and *P. putida* GPo1 (Prieto *et al.*, 1999) consist of seven genes. The genes *phaC1* and *phaC2*, which encode two polyhydroxyalkanoate polymerases, are separated by *phaZ*, which encodes a polyhydroxyalkanoate depolymerase. Downstream from these genes is *phaD*, which encodes a putative transcriptional regulator of the TetR family with an important role in polyhydroxyalkanoate biosynthesis (Klinke *et al.*, 2000). Finally, the genes *phaI* and *phaF*, which are transcribed in the opposite direction, encode polyhydroxyalkanoate granule-associated proteins (Prieto *et al.*, 1999). In *P. putida* GPo1, upstream *phaC1*, consensus sequences similar to σ^{70} - and σ^{54} -dependent promoters have been reported (Huisman *et al.*, 1991). According to previous results, *phaC1* and *phaZ* are transcribed in the same unit, and the

expression of these genes is subjected to negative regulation by PhaF in the absence of appropriate substrates for polyhydroxyalkanoate production (Prieto *et al.*, 1999). Recently, the results obtained in a very close strain named *P. putida* U suggested that the *pha* locus in this bacteria is integrated by six different functional units (*phaC1*, *phaZ*, *phaC2*, *phaD*, *phaI* and *phaF*) (Sandoval *et al.*, 2007).

At present, very little is known about the global regulatory mechanisms that affect *pha* gene expression in *Pseudomonas* (Prieto *et al.*, 2007; Velazquez *et al.*, 2007).

Previous results obtained with *P. putida* GPo1 have shown that polyhydroxyalkanoate degradation increased stress resistance and RpoS (σ^S) intracellular levels (Ruiz *et al.*, 2004). This RNA polymerase σ subunit is a global regulator that controls the expression of several genes involved in survival under adverse conditions. The above-mentioned findings led us to investigate whether σ^S was implicated in the regulation of *pha* genes expression in *P. putida*. In this work, we analyzed the role of RpoS in polyhydroxyalkanoate accumulation and in the expression of the promoter located upstream of the *phaC1* gene (*Pc₁*) in *P. putida*.

Materials and methods

Bacterial strains, plasmid and growth conditions

The plasmid, bacterial strains and primers used and constructed throughout this study are listed in supplementary materials. *Pseudomonas putida* strains were precultured ON in Luria–Bertani (LB) medium and used to inoculate Erlenmeyer flasks with E₂ minimal medium (Lageveen *et al.*, 1988) supplemented with 15 mM sodium octanoate or 10 mM sodium citrate as a carbon source. Cultures were incubated at 32 °C with orbital agitation (200 r.p.m.). When needed, antibiotics were added at the following concentrations in $\mu\text{g mL}^{-1}$: tetracycline 2.5, kanamycin 10 and gentamycin 10.

Oxidative stress experiments

The tolerance to hydrogen peroxide (H₂O₂) was assessed by a disk inhibition assay as described previously (Ayub *et al.*, 2004).

Construction of an *rpoS* knockout mutant of *P. putida* KT2440

Recombinant DNA techniques were performed according to standard procedures (Sambrook *et al.*, 1989) and the instructions of the manufacturers. An *rpoS* mutant of *P. putida* KT2440 was constructed by replacing a fragment within the *rpoS* gene with a kanamycin resistance cassette (for details see supplementary materials). The absence of the RpoS protein was confirmed by Western blot with a *P. putida* RpoS antiserum (data not shown). The resulting *rpoS* mutant was designated SDM75.

Construction of a *Pc₁::lacZ* translational fusion

A 908-bp fragment upstream of the *phaC1* gene of *P. putida* KT2440 containing the promoter region was amplified using PCR with primers pc1-Eco and pc1-Bam and cloned into the pUJ9 vector. The resultant *Pc₁::lacZ* translational fusion was confirmed by sequence analysis. An NotI/NotI fragment containing *Pc₁::lacZ* was subcloned into pUT-Tc to obtain pUT*Pc₁::lacZ*. This plasmid was transferred to *P. putida* KT2440 and SDM75 by mating with S17-1 λ pir. The transconjugants obtained were named KTPC1 and SDMPc1.

Complementation of the *rpoS* mutation

The *rpoS* gene of *P. putida* KT2440 and its promoter region were amplified with primers rpoSfor2 and rpoSrev2 and cloned into pBBR1MCS-5 to obtain pBBRrpoS. This plasmid was transferred to *P. putida* SDM75 and SDMPc1. The complementation of the *rpoS* mutation was corroborated using Western blot analysis with a *P. putida* RpoS antiserum.

Analytical determinations

The β -galactosidase activity was determined as described by Miller (1972). Polyhydroxyalkanoate was measured by GC (Braunegg *et al.*, 1978).

Results

The inactivation of the *rpoS* gene increases the polyhydroxyalkanoate degradation rate in *P. putida*

To analyze the role of σ^S in polyhydroxyalkanoate accumulation, we determined the time-dependent polyhydroxyalkanoate content in cultures of the *rpoS*-negative mutant SDM75 and of its parental strain KT2440 grown on E₂ minimal medium with sodium octanoate as a carbon source (Fig. 1). During the exponential growth phase, both strains accumulated similar amounts of polyhydroxyalkanoate, reaching a maximum of 24% (w/w) after 15 h of incubation. After entering into the stationary phase, the polyhydroxyalkanoate content of SDM75 diminished considerably, while KT2440 showed a minor reduction in the polymer content (Fig. 1b). By the end of the experiment (48 h of incubation), the accumulated polyhydroxyalkanoate was 0.74% for the mutant strain and 10.8% for the wild type.

The inactivation of the *rpoS* gene increases the expression of the *Pc₁* promoter region in *P. putida*

The higher decrease in the polyhydroxyalkanoate content of the *rpoS* mutant compared with the wild-type strain prompted us to analyze whether the absence of σ^S affects the expression of *phaC1* and *phaZ*, the genes involved in polyhydroxyalkanoate synthesis and degradation, respectively. Northern blot experiments performed by Prieto *et al.* (1999) demonstrated that *phaC1* and *phaZ* were part of the same transcription unit.

A *lacZ* translational fusion to the promoter region upstream of *phaC1* (*Pc₁*) was inserted into the chromosome of strains KT2440 and SDM75, to obtain KTPC1 and SDMPc1, respectively. The *rpoS* mutation was complemented by introducing the plasmid pBBRrpoS. Strains KTPC1 and SDMPc1 carrying the vector pBBR1MCS-5, and the complemented strain SDMPc1/pBBRrpoS were used to compare the β -galactosidase activity obtained during growth with sodium octanoate as a carbon source. The results are shown in Fig. 2a and b. The expression of the *lacZ* gene was similar for strains SDMPc1/pBBR1MCS-5 and KTPC1/pBBR1MCS-5 during the exponential phase, but differences were observed during the stationary phase. The β -galactosidase activity of SDMPc1/pBBR1MCS-5 increased progressively after the onset of the stationary phase,

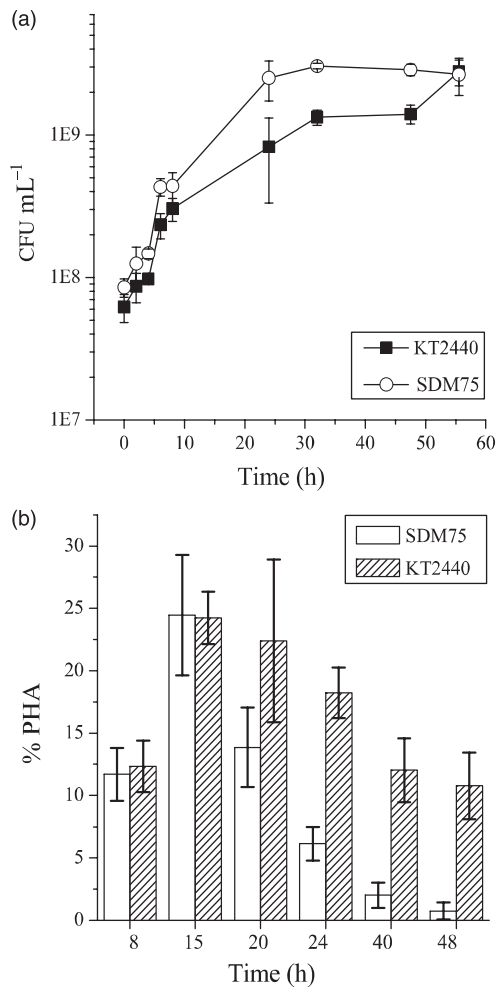


Fig. 1. Time-dependent polyhydroxyalkanoate content of *Pseudomonas putida* KT2440 and SDM75 cultures grown on octanoate as a carbon source. Bacteria were grown in E₂ minimal medium supplemented with 15 mM sodium octanoate. (a) Growth curves were performed by bacterial counts that are expressed as CFU mL⁻¹. The values represent means \pm SD of three replicated plate counts in LB agar plates, (b) polyhydroxyalkanoate content was measured by GC and is expressed as percentage of cellular dry weight. The data are means of three independent growth curves.

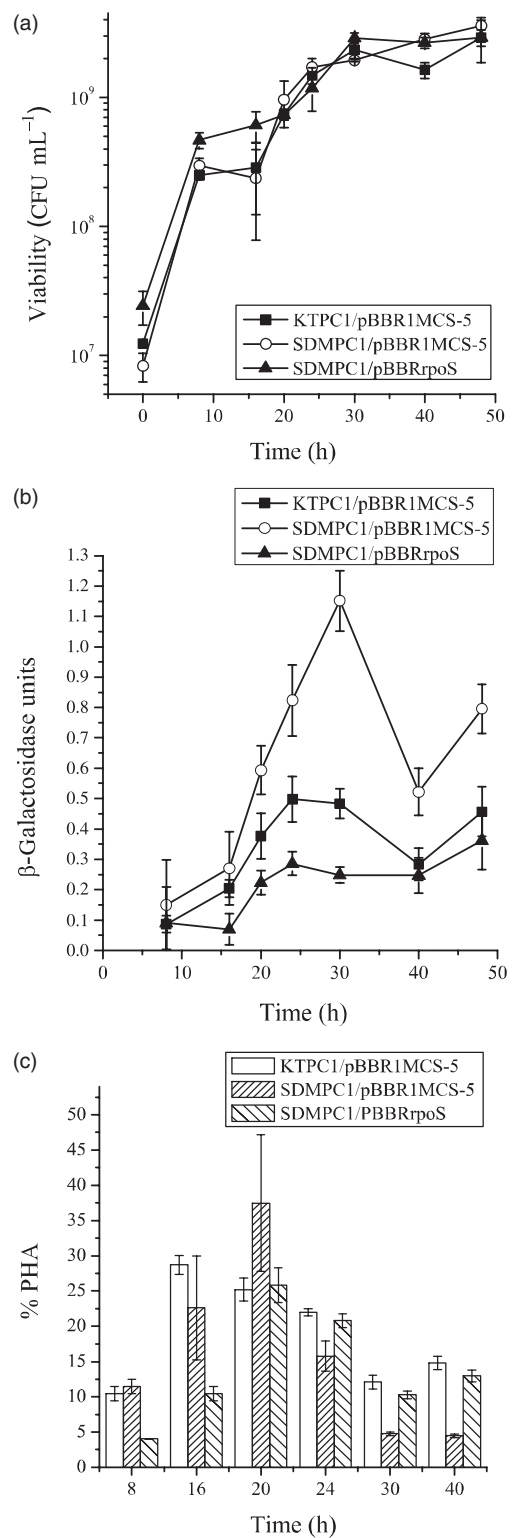


Fig. 2. Expression of $Pc_1::lacZ$ translational fusion and polyhydroxyalkanoate content of *Pseudomonas putida* KTPC1/pBBR1MCS-5, SDMPC1/pBBR1MCS-5 and SDMPC1/pBBRrpoS during growth with octanoate as a carbon source. Cells were cultured in E₂ minimal medium supplemented with 15 mM sodium octanoate. (a) Growth was estimated by bacterial counts (CFU mL⁻¹). The values represent means \pm SD of three replicated plate counts in LB agar plates. At different times, samples were withdrawn for determination of β -galactosidase activity (b) and polyhydroxyalkanoate content (c). One unit of β -galactosidase activity was defined as 1 μ mol of *o*-nitrophenyl- β -D-galactopyranoside hydrolyzed per log CFU min⁻¹. The values expressed means \pm SD of four independent experiments.

reaching fourfold the value observed in the exponential phase, after 30 h of incubation. After this time point, the β -galactosidase levels began to diminish. Meanwhile, for KTPC1/pBBR1MCS-5, the expression of the reporter fusion was almost constant during the stationary phase. In this growth phase, the $P_{C_1}::lacZ$ expression level of the *rpoS* mutant was always higher than that obtained for the wild-type strain. Complementation with a medium-copy-number plasmid carrying the *rpoS* gene produced a considerable decrease in β -galactosidase activity, yielding values that were inclusively lower than those observed for the wild-type. This is probably due to the higher intracellular content of σ^S in the complemented strain, according to Western blot experiments (not shown). Complementation of the *rpoS* mutation by inserting a single copy of the *rpoS* gene into the chromosome as described previously (Zuber *et al.*, 2003) restored the β -galactosidase levels to those observed in the wild type (not shown). Measurement of the polyhydroxyalkanoate intracellular content (Fig. 2c) showed an important decrease in the mutant during the stationary phase, coincidental with the increase in β -galactosidase levels (Fig. 2b). As expected, a minor polyhydroxyalkanoate depolymerization was observed in the wild-type strain. The polymer content of the complemented strain was lower than that obtained in the wild-type and the *rpoS* mutant strains during the exponential phase, but was the same as that observed for the wild type during the stationary phase. This indicates that the introduction of the *rpoS* gene into the mutant strain restored the wild-type phenotype regarding polyhydroxyalkanoate depolymerization in the stationary phase. The growth curve and the corresponding polymer content of the strains carrying pBBRrpoS and pBBR1MCS-5 (Fig. 2c) were different from that obtained with the strains used in Fig. 1b mainly due to the presence of gentamycin in the culture medium, which slowed the growth rate.

Polyhydroxyalkanoate- accumulating conditions increase survival and resistance to oxidative stress of the *P. putida rpoS* mutant

Several studies have demonstrated that the capability to synthesize and degrade polyhydroxyalkanoate had a positive effect on survival and stress resistance of bacteria in laboratory cultures, natural environments and biofilms (López *et al.*, 1998; Ruiz *et al.*, 1999, 2001, 2004; Kadouri *et al.*, 2003; Pham *et al.*, 2004). Taking this into account, we decided to compare the survival and stress resistance to H_2O_2 of the wild-type strain KT2440/pBBR1MCS-5, the *rpoS* mutant SDM75/pBBR1MCS-5 and the complemented strain SDM75/pBBRrpoS in laboratory cultures grown with a carbon source that allows polyhydroxyalkanoate accumulation (sodium octanoate) and with a carbon source that could not be used to synthesize the intracellular polymer

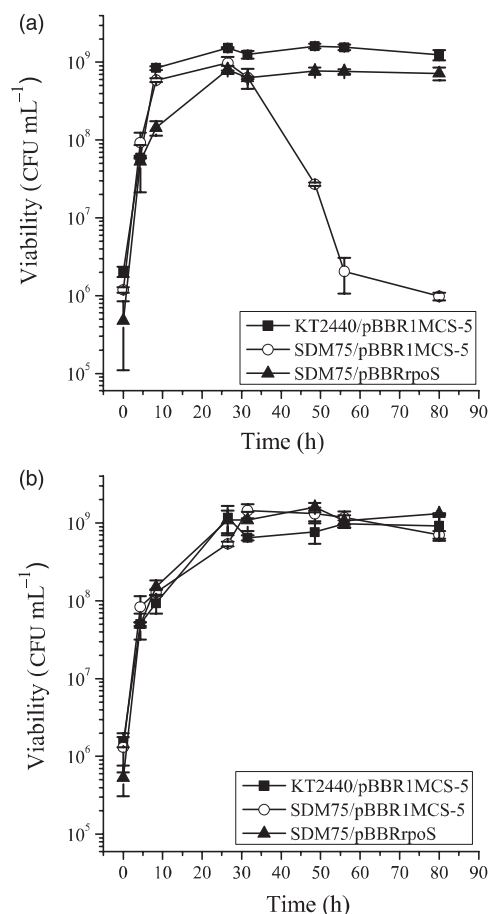


Fig. 3. Survival of *Pseudomonas putida* KT2440/pBBR1MCS-5, SDM75/pBBR1MCS-5 and SDM75/pBBRrpoS cultured under polyhydroxyalkanoate- and nonpolyhydroxyalkanoate-accumulating conditions. Cultures were grown in E_2 minimal medium supplemented with 10 mM sodium citrate (nonpolyhydroxyalkanoate-accumulating conditions) (a) and 15 mM sodium octanoate (polyhydroxyalkanoate-accumulating conditions) (b). Bacterial counts were expressed as $CFU\ mL^{-1}$. The experiment was repeated three times and a representative one is shown. Values represent means \pm SD of three replicated plate counts in LB agar plates.

(sodium citrate). As shown in Fig. 3a, when the cultures were grown with sodium citrate, the strain SDM75/pBBR1MCS-5 showed a pronounced decrease in CFU number after 48 h of incubation. This was not observed for the wild-type strain or the complemented strain, confirming that the decline in cell number is due to the *rpoS* mutation. Differences in cell survival were not observed during growth with octanoate as a carbon source (Fig. 3b). When the cells cultured with sodium octanoate were challenged with H_2O_2 , only minor differences were obtained in the resistance to this stress agent (Table 1). However, during growth with citrate, the *rpoS* mutant was more sensible than the wild type and the complemented strain. For strain SDM75/pBBR1MCS-5, the H_2O_2 inhibition zone was almost 25% larger than that observed for the other strains.

Table 1. Oxidative stress challenge of *Pseudomonas putida* KT2440/pBBR1MCS-5, SDM75/pBBR1MCS-5 and SDM75/pBBRrpoS cultured under polyhydroxyalkanoate and nonpolyhydroxyalkanoate accumulating conditions measured by disk inhibition assay

Strain	Polyhydroxy-alkanoate accumulating condition*	Nonpolyhydroxyalkanoate accumulating condition†
KT2440/pBBR1MCS-5	30.3 ± 0.6	31.2 ± 0.8
SDM75/pBBR1MCS-5	32.2 ± 0.6	38.7 ± 0.6
SDM75/pBBRrpoS	29.2 ± 0.7	32.3 ± 1.0

Values represent mean ± SD of triplicate disks. The zone of inhibition was measured in millimeters.

*Cultures grown during 30 h. In E₂ minimal medium supplemented with 15 mM sodium octanoate.

†Cultures grown during 30 h. In E₂ minimal medium supplemented with 10 mM sodium citrate.

Discussion

In this paper, we analyzed the role of the RNA polymerase stationary phase sigma factor (σ^S) in polyhydroxyalkanoate metabolism in *P. putida*. In *Pseudomonas*, besides stress resistance, RpoS is implicated in the production of polymers such as alginate, antibiotics and several virulence factors (Venturi, 2003). In *Azotobacter vinelandii*, transcription of the *phbBAC* biosynthetic operon is dependent on σ^S (Peralta-Gil *et al.*, 2002).

According to our experimental data, RpoS appears to affect polyhydroxyalkanoate metabolism after the onset of the stationary phase. Previous studies have reported that polyhydroxyalkanoate accumulation is a dynamic and simultaneous process of synthesis and degradation (Doi *et al.*, 1992). When the carbon source is exhausted, the polyhydroxyalkanoate is degraded to maintain cell metabolism, resulting in a decrease of the polymer content. The results obtained in this work show that, as a consequence of the *rpoS* mutation, there is an increase in the expression driven by the *Pc₁* promoter region in the stationary phase. At the same time, a dramatic reduction in the polymer content was observed. This enhanced depolymerization obtained in the mutant could be attributable to the higher depolymerase gene (*phaZ*) expression and the fact that during the stationary phase the carbon source for polyhydroxyalkanoate synthesis had been depleted. However, the presence of other promoters that drive the expression of *phaZ* in *P. putida* KT2440, as was demonstrated previously by Sandoval *et al.* (2007) in *P. putida* U, cannot be excluded. The complementation with the *rpoS* gene in a plasmid of medium-copy-number silenced the expression driven by *Pc₁* and regained the wild-type phenotype regarding polyhydroxyalkanoate content in the stationary phase. However, during the exponential phase, the polyhydroxyalkanoate

accumulation of the complemented strain was lower than that obtained in the wild type. It is known that the intracellular levels of σ^S are very low in the exponential phase and increase sharply at the onset of the stationary phase, but as the *rpoS* gene is in a medium-copy-number plasmid in the complemented strain, the lower polymer content observed during the exponential phase in this strain could be due to the presence of higher levels of RpoS that negatively affect the expression driven by *Pc₁*. Taken together, the results suggest that σ^S might be involved in the negative regulation of *Pc₁*, probably in an indirect manner. Some years ago, Klinke *et al.* (2000) reported that a mutation in the *phaD* gene of *P. putida* GPO1 lowered polyhydroxyalkanoate accumulation and caused an increase in polyhydroxyalkanoate depolymerization. The *phaD* gene has sequence similarities with members of the TetR family of transcriptional repressors (Ramos *et al.*, 2005). Moreover, according to two microarray analyses, the expression of the operon conformed by *phaC2* and *phaD* was reduced in *rpoS* mutants of *Pseudomonas aeruginosa* and *Pseudomonas chlororaphis* (Schuster *et al.*, 2004; Girard *et al.*, 2006). Therefore, *phaD* could have a regulatory effect on the *pha* gene cluster in *Pseudomonas*.

Given the role of polyhydroxyalkanoate as a sink of carbon and reducing equivalents that reflects the metabolic status of the cell, it is not surprising that several global regulatory networks are involved in the regulation of its metabolism. Pries *et al.* (1991) reported that mutants of *Cupriavidus necator* in genes homologous to components of the phosphoenolpyruvate-phosphotransferase (PEP-PTS) system of *Escherichia coli* showed a much more rapid decrease in polyhydroxybutyrate (PHB) content than the wild-type after depletion of the carbon source. Recently, it was shown that mutations in genes homologous to those of the nitrogen branch of the PTS system in *P. putida* affected polyhydroxyalkanoate accumulation (Velazquez *et al.*, 2007). The experiments described here show that in the absence of σ^S there is an increase in the expression of the genes involved in polyhydroxyalkanoate accumulation, which lead to a higher rate of polyhydroxyalkanoate degradation. This would provide more carbon and reducing equivalents for a cell that has a mutation in the alternative σ^S factors that activates a response to adverse conditions. In fact, our results indicate that under polyhydroxyalkanoate-accumulating conditions, the *rpoS* strain had similar survival and resistance to oxidative stress as the wild type. On the contrary, when the strains were cultured with a carbon source that does not allow polyhydroxyalkanoate accumulation, the mutant had lower survival and tolerance to H₂O₂ than the wild-type or the complemented strain. In *Pseudomonas*, it was suggested that other factors besides RpoS may be associated with stationary stress tolerance (Jorgensen *et al.*, 1999). These results support the idea that

polyhydroxyalkanoate plays a very important role in the stress response of bacteria.

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References

- Ayub ND, Pettinari MJ, Ruiz JA & López NI (2004) A polyhydroxybutyrate-producing *Pseudomonas* sp. isolated from antarctic environments with high stress resistance. *Curr Microbiol* **49**: 170–174.
- Braunegg G, Sonnleitner B & Lafferty RM (1978) A rapid gas chromatographic method for the determination of poly- β -hydroxybutyric acid in microbial biomass. *Eur J Appl Microb Biotechnol* **6**: 29–37.
- Doi Y, Kawaguchi Y, Koyama N, Nakamura S, Hiramitsu M, Yoshida Y & Kimura H (1992) Synthesis and degradation of polyhydroxyalkanoates in *Alcaligenes eutrophus*. *FEMS Microbiol Rev* **103**: 103–109.
- Girard G, van Rij E, Lugtenberg B & Bloemberg G (2006) Regulatory roles of *psrA* and *rpoS* in phenazine-1-carboxamide synthesis by *Pseudomonas chlororaphis* PCL 1391. *Microbiology* **152**: 43–58.
- Huisman GW, de Leeuw O, Eggink G & Witholt B (1989) Synthesis of poly-3-hydroxyalkanoates is a common feature of fluorescent pseudomonads. *Appl Environ Microbiol* **55**: 1949–1954.
- Huisman GW, Wonink E, Meima R, Kazemier B, Terpstra P & Witholt B (1991) Metabolism of poly(3-hydroxyalkanoates) (PHAs) by *Pseudomonas oleovorans*. Identification and sequences of genes and function of the encoded proteins in the synthesis and degradation of PHA. *J Biol Chem* **266**: 2191–2198.
- Jorgensen F, Bally M, Chapon-Herve V, Michel G, Lazdunski A, Williams P & Stewart GS (1999) RpoS-dependent stress tolerance in *Pseudomonas aeruginosa*. *Microbiology* **145**: 835–844.
- Kadouri D, Budman S, Jurkevitch E & Okon Y (2003) Involvement of the reserve material poly(β -Hydroxybutyrate) in *Azospirillum brasilense* stress endurance and root colonization. *Appl Environ Microbiol* **69**: 3244–3250.
- Klinke S, de Roo G, Witholt B & Kessler B (2000) Role of *phaD* in accumulation of medium-chain-length poly(3-hydroxyalkanoates) in *Pseudomonas oleovorans*. *Appl Environ Microbiol* **66**: 3705–3710.
- Lageveen RG, Huisman GW, Preusting H, Ketelaar P, Eggink G & Witholt B (1988) Formation of polyesters by *Pseudomonas oleovorans*: effect of substrates on formation and composition of poly-(R)-3-hydroxyalkanoates and poly-(R)-3-hydroxyalkenoates. *Appl Environ Microbiol* **54**: 2924–2932.
- López NI, Ruiz JA & Méndez BS (1998) Survival of poly-3-hydroxybutyrate producing bacteria in soils microcosms. *World J Microbiol Biotechnol* **14**: 681–684.
- Miller JH (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Nelson KE, Weinel C, Paulsen IT *et al.* (2002) Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ Microbiol* **4**: 799–808.
- Peralta-Gil M, Segura D, Guzman J, González LS & Espín G (2002) Expression of the *Azotobacter vinelandii* poly- β -hydroxybutyrate biosynthetic *phbBAC* operon is driven by two overlapping promoters and is dependent on the transcriptional activator PhbR. *J Bacteriol* **184**: 5672–5677.
- Pham TH, Webb JS & Rehm BH (2004) The role of polyhydroxyalkanoate biosynthesis by *Pseudomonas aeruginosa* in rhamnolipid and alginate production as well as stress tolerance and biofilm formation. *Microbiology* **150**: 3405–3413.
- Pries A, Priefert H, Kruger N & Steinbuechel A (1991) Identification and characterization of two *Alcaligenes eutrophus* gene loci relevant to the poly(β -hydroxybutyric acid)-leaky phenotype which exhibit homology to *ptsH* and *ptsI* of *Escherichia coli*. *J Bacteriol* **173**: 5843–5853.
- Prieto MA, Bühler B, Jung K, Witholt B & Kessler B (1999) PhaF, a polyhydroxyalkanoate-granule-associated protein of *Pseudomonas oleovorans* GPo1 involved in the regulatory expression system for *pha* genes. *J Bacteriol* **181**: 858–868.
- Prieto MA, de Eugenio LI, Galán B, Luengo JM & Witholt B (2007) Synthesis and degradation of polyhydroxyalkanoates. *Pseudomonas* (Ramos J-L & Filloux A, eds) pp. 397–428. Springer, The Netherlands.
- Ramos J, Martínez-Bueno M, Molina-Henares A *et al.* (2005) The TetR family of transcriptional repressors. *Microbiol Mol Biol Rev* **69**: 326–356.
- Ruiz JA, López NI & Méndez BS (1999) Polyhydroxyalkanoates degradation affects survival of *Pseudomonas oleovorans* in river water microcosms. *Rev Arg Microbiol* **31**: 201–204.
- Ruiz JA, López NI, Fernández RO & Méndez BS (2001) Polyhydroxyalkanoate degradation is associated with nucleotide accumulation and enhances stress resistance and survival of *Pseudomonas oleovorans* in natural waters microcosms. *Appl Environ Microbiol* **67**: 225–230.
- Ruiz JA, López NI & Méndez BS (2004) *rpoS* gene expression in carbon-starved cultures of the polyhydroxyalkanoate accumulating species *Pseudomonas oleovorans*. *Curr Microbiol* **48**: 395–400.
- Sambrook J, Fritsch E & Maniatis T (1989) *Molecular Cloning: a Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Sandoval A, Arias-Barrau E, Arcos M, Naharro G, Olivera ER & Luengo JM (2007) Genetic and ultrastructural analysis of

- different mutants of *Pseudomonas putida* affected in the poly-3-hydroxy-*n*-alkanoate gene cluster. *Environ Microbiol* **9**: 737–751.
- Schuster M, Hawkins A, Harwood C & Greenberg E (2004) The *Pseudomonas aeruginosa* RpoS regulon and its relation to quorum sensing. *Mol Microbiol* **51**: 973–985.
- Velazquez F, Pfluger K, Cases I, De Eugenio LI & de Lorenzo V (2007) The phosphotransferase system formed by PtsP, PtsO, and PtsN proteins controls production of polyhydroxyalkanoates in *Pseudomonas putida*. *J Bacteriol* **189**: 4529–4533.
- Venturi V (2003) Control of *rpoS* transcription in *Escherichia coli* and *Pseudomonas*: why so different? *Mol Microbiol* **49**: 1–9.
- Zuber S, Carruthers F, Kell C *et al.* (2003) GacS sensor domains pertinent to the regulation of exoproduct formation and to the biocontrol potential of *Pseudomonas fluorescens* CHAO. *Mol Plant–Microbe Interact* **634**: 634–644.

Supplementary material

The following supplementary material is available for this article online:

Appendix S1. Construction of an *rpoS* knockout mutant of *P. putida* KT2440.

Table S1. Bacterial strains, plasmids and primers used.

This material is available as part of the online article from: <http://www.blackwell-synergy.com/doi/abs/10.1111/j.1574-6968.2008.01203.x> (This link will take you to the article abstract).

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