The alternative sigma factor, $\sigma^5$, affects polyhydroxyalkanoate metabolism in *Pseudomonas putida*

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**Keywords**

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**Abstract**

To determine whether the stationary sigma factor, $\sigma^5$, 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 $\sigma^5$ 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 1, 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* GP01 (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* GP01, upstream *phaC1*, consensus sequences similar to $\sigma^70$ and $\sigma^54$-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* GP01 have shown that polyhydroxyalkanoate degradation increased stress resistance and RpoS ($\sigma^5$) intracellular levels (Ruiz et al., 2004). This RNA polymerase $\sigma$ 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 $\sigma^5$ 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 μg 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 pUTPc₁::lacZ. This plasmid was transferred to *P. putida* KT2440 and SDM75 by mating with S17-1λpir. 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 *σ⁵* 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 *σ⁵* 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 ± 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 *Pc1*: 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 ± 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 μmol of o-nitrophenyl-β-D-galactopyranoside hydrolyzed per log CFU min$^{-1}$. The values expressed means ± 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 Pc1::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 pBBRpsoS 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/pBBRpsoS 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 (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/pBRR1MCS-5, SDM75/pBRR1MCS-5 and SDM75/pBBRrpoS cultured under polyhydroxyalkanoate and nonpolyhydroxyalkanoate accumulating conditions measured by disk inhibition assay

<table>
<thead>
<tr>
<th>Strain</th>
<th>Polyhydroxy-alkanoate accumulating condition*</th>
<th>Polyhydroxy-alkanoate accumulating condition†</th>
</tr>
</thead>
<tbody>
<tr>
<td>KT2440/pBRR1MCS-5</td>
<td>30.3 ± 0.6</td>
<td>31.2 ± 0.8</td>
</tr>
<tr>
<td>SDM75/pBRR1MCS-5</td>
<td>32.2 ± 0.6</td>
<td>38.7 ± 0.6</td>
</tr>
<tr>
<td>SDM75/pBBRrpoS</td>
<td>29.2 ± 0.7</td>
<td>32.3 ± 1.0</td>
</tr>
</tbody>
</table>

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

* Cultures grown during 30 h. In E\textsubscript{2} minimal medium supplemented with 15 mM sodium octanoate.
† Cultures grown during 30 h. In E\textsubscript{2} minimal medium supplemented with 10 mM sodium citrate.

Discussion

In this paper, we analyzed the role of the RNA polymerase stationary phase sigma factor ($\sigma^S$) 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 $\sigma^S$ (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 P\textsubscript{C1} 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\textsubscript{J}, cannot be excluded. The complementation with the rpoS gene in a plasmid of medium-copy-number silenced the expression driven by P\textsubscript{C1} 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 $\sigma^S$ 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 P\textsubscript{C1}. Taken together, the results suggest that $\sigma^S$ might be involved in the negative regulation of P\textsubscript{C1}, 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 Cupriavus 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 $\sigma^S$ 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 $\sigma^S$ 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 H\textsubscript{2}O\textsubscript{2} 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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### 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](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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