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Expression Profile of *pha* Gene Cluster of *Pseudomonas putida* KT2442

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Summary: Polyhydroxyalkanoates (PHAs) are biodegradable polymers that many bacteria accumulate as carbon and energy storage when growth conditions are unbalanced. *Pseudomonas* strains belonging to the rRNA homology group I such as *P. putida* can accumulate medium-chain-length-PHA from monomers in the C8 to C10 range. Regulation of PHA synthesis and degradation in *P. putida* KT2442 has been studied using different molecular approaches. In this study six promoter regions located upstream of each *pha* gene were identified. The expression of the *pha* cluster have been analysed in the presence of octanoic acid *versus* glucose in the culture medium. Results demonstrated that the system is activated in the presence of octanoic acid as PHA precursor.

Keywords: PHA production; *pseudomonas*; transcriptional regulation

Introduction

Polyhydroxyalkanoates (PHAs) are degradable biopolyesters produced by a wide range of bacteria when the environmental conditions are not optimal for growing due to the limitation of a required nutrient and an excess of a carbon source.[1] Although various aspects of PHA production, including bacterial fermentation, isolation and physicochemical characterization of the polymer have been studied extensively during the past few decades, knowledge on the regulatory mechanisms at the molecular level is relatively limited. This can be partly due to the complexity of PHA metabolism, which implies an extremely intricate regulatory system. Thus, PHA synthesis in Pseudomonads comprises: i) central pathways, such as β-oxidation pathway and fatty acid *de novo* synthesis to convert fatty acid or carbohydrate intermediates, respectively, into different *(R)*-3-hydroxyacyl-CoAs, and ii) a specific or peripheral pathway encoded by the *pha* cluster of genes, which includes the genes encoding the synthases that transform the monomeric substrates *(R)*-hydroxyacyl-CoA thioesters into PHA.[2,3] The *pha* cluster is very well conserved along the medium-chain-length-PHA (mcl-PHA) producer strains. It is composed by (i) two synthase coding for genes (*phaC1* and *phaC2*) [4] involved in the PHA synthesis; (ii) a depolymerase (PhaZ) encoding gene (*phaZ*) responsible of PHA mobilization [5] and the *phaD* gene encoding a transcriptional regulator (PhaD protein).[6] The *phaF* and *phaI* genes are transcribed divergently to the other *pha* genes, and encode the phasins that play both regulatory and functional roles.[7] So far, very little is known about the regulatory system which drives the expression of the *pha* genes in *Pseudomonas* species. In this work we comparatively analyze the activity of the promoter regions of the *pha* genes in the model strain *Pseudomonas putida* KT2442.

Materials and Methods

β-galactosidase Assays in Micro-Well Assay

Upstream regions of *pha* genes of 200–300 bp were fused to the reporter gene *lacZ* of

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Escherichia coli using pUJ9 plasmid.[8] The resulting *Not*I cassettes of pUJ9 derivatives were subcloned into the mini-Tn*5* delivery plasmid pUT-Km and inserted into the chromosome of the strain *P. putida* KT2442 by the filter-mating technique.[6] 20 μL of the isolated transconjugant strains grown at an optical density (OD_{600}) of 0.2 were cultured in micro-well plates with 200 μL of minimal medium under different growth conditions in the presence of X-gal. Promoter activities were determined by measuring the development of blue colour.

Real-Time RT-PCR

Cells were grown in 0.1 N M63, which is a nitrogen-limited minimal medium (13.6 g of KH_2PO_4/L, 0.2 g $(NH_4)_2SO_4$/L, 0.5 mg $FeSO_4 \cdot 7\ H_2O$/L, adjusted to pH 7.0 with KOH), plus 15 mM octanoic acid or 20 mM glucose. For RNA purifications, 500 mL flasks containing 200 mL of culture medium were inoculated with *P. putida* KT2442 to reach a turbidity of 0.3, introduced in a rotary shaker operated at 220 rpm and incubated until the desired incubation times were reached. 5 mL of cells were collected by centrifugation and frozen at $-20\,^{\circ}C$. Cell pellet was resuspended in 200 ml of TE buffer and RNA was extracted using the RNeasy mini kit (QIAGEN). Reverse transcription of 1 μg of total RNA was performed using 200 units of Super Script Retrotranscriptase II (Invitrogen) using random hexamers as primers. Real-time PCR was performed using SYBR Green technology in an iQ^{TM}5 Real-Time PCR Detection System (BioRad). A standard curve was made using dilution series from 5×10^{-7} to 5 ng of the KT2442 genomic DNA in order to quantify the abundance of transcripts relative to total cDNA in each condition.

Results

In this work a tool for analysing the expression rate of the *pha* genes of *P. putida* KT2442 at different growth conditions was developed. The assay consists in culturing in micro-well plates recombinant strains of *P. putida* carrying in their chromosome a *β*–galactosidase reporter system, which allows the detection of each of the promoter driving the expression of the *pha* genes. By using this tool, it was determined that the *pha* locus in *P. putida* KT2442 is integrated by six functional promoters upstream the *phaC1*, *phaZ*, *phaC2*, *phaD*, *phaI* and *phaF* genes (Figure 1) which code for the synthases (PhaC), the depolymerase (PhaZ), the phasins (PhaF and PhaI) and the transcriptional regulator PhaD. All of them showed different transcription rates under different carbon sources (Figure 2) as

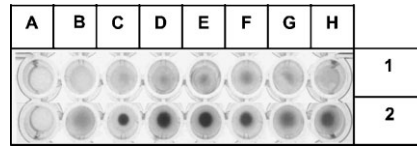

Figure 2.
Multiwell assay to measure the activity of the *pha* promoters of *P. putida* under different growth conditions. Lane 1, 0.1N M63 20 mM glucose; lane 2, 0.1N M63 15 mM octanoate. Rows: A, negative control; B, *P. putida* KT2442; C, KT2442 *Pd::lacZ*; D, KT2442 *Pf::lacZ*; E, KT2442 *Pi::lacZ*; F, KT2442 *Pz::lacZ*; G, KT2442 Pc_2::*lacZ*; H, Pc_1::*lacZ*.

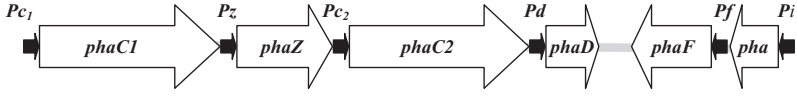

Figure 1.
The *pha* gene cluster of *P. putida*, which encodes the proteins involved in PHA metabolism, consists of four open reading frames (ORFs) transcribed in the same direction: *phaC1* and *phaC2* genes, encoding two PHA synthases; the *phaZ* gene, coding for PHA depolymerase; and the *phaD* gene, whose product is similar to members of TetR family of transcriptional regulators. In the opposite direction, there are two genes encoding the phasins (*phaF* and *phaI*), the major structural proteins of the PHA granule.

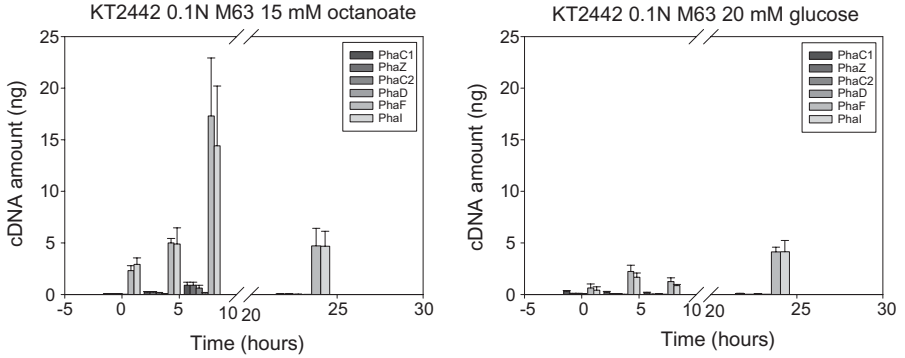

Figure 3.
Quantification of the expression rates of the *pha* genes by Real Time RT-PCR assays throughout the growth curve. Error bars represent standard deviation calculated from the results of at least three independent experiments.

seen by β-galactosidase multi-well assay. The micro-well assay allowed the comparison of promoter activities in a fast and straight system. Thus, up to 96 different conditions can be comparatively and qualitatively analyzed in a single plate, by measuring the development of blue coloured wells. It was determined that related sources such as octanoate increased the transcription driven by the *pha* cluster promoters. The strongest activities were directed by phasins promoters. The activities of all promoters are considerably reduced when glucose is added to the culture medium.

These results were also confirmed by real time RT-PCR (Figure 3). Gene expression is maximum at exponential phase, decreasing when stationary phase is reached. Results demonstrated a different expression rate of each gene under similar growth conditions.

Conclusion

The expression of the *pha* genes of the model strain *P. putida* KT2442 is driven by at least six promoters. The activities of these promoter regions are dependent on the carbon sources supplied into the culture medium. This is an important aspect that should be addressed when producing mcl-PHA from raw materials.

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