Transcriptional Analysis of Pha Genes in *Pseudomonas Mediterranea* CFBP 5447 Grown on Glycerol

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We analysed the draft genome sequence of *Pseudomonas mediterranea* CFBP 5447 in order to identify firstly the central metabolic pathways that convert fatty acids or carbohydrate intermediates into mcl-PHA and secondly the genes involved in glycerol metabolism (\textit{glpF, glpK, glpD, glpR}). Absence of the \textit{glpF} gene, which codifies for the "glycerol uptake facilitator protein", was highlighted. In order to understand the expression of the \textit{pha} gene cluster, we investigated the promoter activity of \textit{phaC1, phaC2, phaZ, phaD} and \textit{phaI} genes. When glycerol was present as the carbon source, \textit{P_I} was found to be the most active promoter. Expression analysis of the knock-out mutant of the \textit{phaD} gene, which is a transcriptional regulator belonging to the TetR family, showed that PhaD acts as an activator of the \textit{phaI} promoter which, in turn, triggers the transcription of the \textit{phaIF} operon. The activation of \textit{P_Cr}, which controls the \textit{phaC1ZC2D}, by PhaD, was less efficient than \textit{P_I}.

1. Introduction

Polyhydroxyalkanoates (PHAs) are biodegradable polymers naturally produced by bacteria as carbon storage granules, even from renewable resources. Because of they are biodegradable and recyclable, they are considered as a valid green alternative to conventional plastics in order to manufacture frequently used products (Luengo et al., 2003). The large-scale production of PHAs involves high costs due to the fermentation and separation process. Such costs can be reduced by using appropriate low-cost carbon sources and optimized growth conditions (Solaiman et al., 2006). In addition, genome analysis and metabolic engineering represent good strategies to increase PHA production, by providing efficient cell factories.

Most *Pseudomonas* species are able to produce medium-chain-length-PHAs (mcl-PHAs) containing monomers from 6 to 14 carbon atoms (Timm and Steinbuchel, 1990). The entire \textit{pha} gene cluster responsible for PHA metabolism, well conserved among *Pseudomonas* spp., consists of genes encoding two synthases (\textit{phaC1} and \textit{phaC2}), a depolymerase (\textit{phaZ}) responsible for PHA mobilization (type II biosynthetic locus) and the \textit{phaD} gene encoding a putative transcriptional regulator (Klinke et al., 2000). In addition, the \textit{phaF} and \textit{phaI} genes, transcribed divergently to the other \textit{pha} genes, encode the phasins, thus playing regulatory and functional roles (Prieto et al., 1999).

Knowledge of the molecular mechanisms regulating mcl-PHA synthesis and degradation is relatively limited (Prieto et al., 2007, Sandoval et al., 2007). PHA metabolism comprises two central pathways, \( \beta \)-oxidation and fatty acid \textit{de novo} synthesis, depending on whether the carbon source is related (oleic acid, vegetable oils, fatty acids) or unrelated (glucose, glycerol), respectively. Precursors for mcl-PHA polymerases, derived from these pathways, are provided by \textit{phaJ} or \textit{phaG} genes, key link enzymes between \( \beta \)-
oxidation or fatty acid biosynthesis and mcl-PHA biosynthesis, respectively (Rehm and Steinbuchel, 1999). According to *P. aeruginosa* studies, glycerol uptake and metabolism is mediated first of all by the outer membrane OprB, and then by the glycerol facilitator GlpF, which is involved in glycerol transport, as well as the glycerol kinase (GlpK) and a citoplasmic-membrane-associated G3P dehydrogenase (GlpD) (Schweizer et al., 1997). The *glp* operon and the key role of GlpR in the optimization of PHA production from glycerol, has been recently demonstrated (Escapa et al., 2013). Within the *Pseudomonas fluorescens* group (Solaiman et al., 2002), *P. mediterranea* CFBP 5447 (9.1) is able to bioconvert refined and biodiesel-glycerol into a mcl-PHA (Palmeri et al., 2012), with an approximate molecular weight of 56 KDa, and which produces a transparent odourless film (Pappalardo et al., 2013).

**2. Materials and Methods**

**2.1 Bacterial strains, media and growth conditions**

The strains and plasmids used in this study are listed in Table 1. *P. mediterranea* CFBP 5447 was routinely grown at 28°C in both nutrient agar (Oxoid, Milan, Italy) supplemented with 1% dextrose (NDA) and Luria-Bertani (LB) agar (Sambrook et al., 2001). Antibiotics were added as required, with the following final concentrations: ampicillin, 100 µg mL⁻¹; tetracycline, 15 µg mL⁻¹ (*E. coli*), or 40 µg mL⁻¹ (*Pseudomonas*); kanamycin, 50 µg mL⁻¹ (*E. coli*) or 100 µg mL⁻¹ (*Pseudomonas*).

DNA manipulations, including digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with T4 ligase, and *E. coli* transformation were performed as described by Sambrook et al. (2001). Triparental matings from *E. coli* to *P. mediterranea* CFBP 5447 were carried out with the helper strain *E. coli* DH5α (pRK2013) (Figurski and Helinski, 1979). For PHA production, strains were grown in E-medium supplemented with reagent grade glycerol 2% (v/v), as previously reported (Palmeri et al., 2012).

| Table 1. Strains and plasmids used in this study. |
|----------------------------------|-----------------------------|
| *P. mediterranea* strains        |                             |
| CFBP 5447                        | Wild type                   |
| VVD                             | *phaD*: pKnock, Km'<br>CFBP<sup>+</sup> |
| Plasmids                        |                             |
| pMP220                          | Promoter probe vector, IncP <br>Tc'<br>Spaink et al., 1987 |
| pKnock-Km'                      | Mobilizable suicide vector, Km'<br>Alexeyev, 1999 |
| pGEM-T                          | Cloning vector; Amp'<br>Promega |
| pMPPhaC1                        | *phaC1* promoter cloned in pMP220<br>This study |
| pMPPhaZ                         | *phaZ* promoter cloned in pMP220<br>This study |
| pMPPhaC2                        | *phaC2* promoter cloned in pMP220<br>This study |
| pMPPhaI                         | *phaI* promoter cloned in pMP220<br>This study |
| pKMPphaD                        | pKnock containing an internal fragment of *phaD* gene<br>This study |

**2.2 Construction of *P. mediterranea* VVD mutant**

The *phaD* genomic mutant was created as follows. An internal part of *phaD* was amplified by PCR as a 339-bp fragment, using primer PhaDintFw (ATGCCAAGGACCCCTTGTGC) and PhaDintRev (AACAGCAGCTACGGGTGAT). It was cloned first in pGEM and then as an *EcoRI* fragment in the corresponding site in pKNOCK-Km, generating pKMPphaD. This plasmid was then used as a suicide delivery system in order to create a *phaD* knockout mutant through homologous recombination in strain CFBP 5447, as de-
scribed by Alexeyev (1999), thus generating *P. mediterranea* VVD. The fidelity of the marker exchange events was confirmed by PCR.

### 2.3 Reporter gene fusion assay

Transcriptional fusion plasmids for *phaC1*, *phaC2*, *phaZ*, *phaD* and *phaI* promoter regions based on the pMP220 promoter probe vector were constructed. Fragments containing regions upstream the starting codon of each gene were amplified by PCR using genomic DNA of *P. mediterranea* CFBP 5447 as the template and specific primer sets. The DNA fragments were then cloned into pGEM-T (Promega), removed as EcoRI/XbaI, and cloned in pMP220 yielding *Pc1, Pc2, Pz, Pd, PlacZ* promoter fusions. β–galactosidase activities were determined during growth in E-medium supplemented with reagent grade glycerol 2% (v/v) following Miller (1972), with the modification of Stachel and associates (1985). All experiments were performed in triplicate and the mean value is given.

### 2.4 RT-PCR analysis

*P. mediterranea* CFBP 5447 and VVD mutant derivative were grown in E-medium supplemented with reagent grade glycerol 2% (v/v) at the exponential growth phase (14 hours after inoculation). Total RNA was isolated using a commercial RNA extraction kit (RiboPure-Bacteria Kit, Ambion), as recommended by the manufacturer in triplicate. RNA samples were quantitatively analyzed by Nanodrop. Following a DNAse purification step by Turbo DNA-free kit (Ambion), 200 ng of total RNA was used for the RT reaction using a Transcription System kit (Promega). The reverse transcription reaction was performed at 24°C for 10 min, followed by 15 min at 42°C, and inactivation at 95 °C for 5 min. PCR reactions were performed under the following conditions: an initial 94°C for 3 min, followed by 33 cycles of 94°C for 30 sec, 52°C for 30 sec and 72°C for 1 min, and a final extension of 72°C for 10 min. Eight primers were used to amplify *phaC1-phaZ, phaZ-phaC2, phaC2-phaD* and *phaF-phaI* overlapping regions. As a negative control, PCR reactions with the same primer sets were performed using RNA samples that had not been reverse transcribed.

### 3. Results

#### 3.1 Genome mining on metabolic pathways involved in PHA biosynthesis from glycerol

Using the pFAM search domain and tBlastn applications, genes that codify for enzymes involved in β-oxidation (*fad*) and fatty acid *de novo* synthesis (*fab*), able to convert fatty acids or carbohydrates into precursors for PHA biosynthesis were mapped. These genes were dispersed in different loci along the entire genome.

The entire type II PHA locus consisting of six genes in 6725 bp (Figure 1) was identified. Five putative rho independent terminators were mapped within the locus, three downstream of *phaD* and in the same direction and two downstream of *phaF*. They represent sites of transcription termination and act as gene regulators (Solaiman et al., 2008). We found that there was no putative rho independent terminator in the *phaC1-phaZ* intergenic region, thus confirming Solaiman et al. (2008).

The *glp* operon responsible for glycerol catabolism was 4240 bp in length and comprised only the genes putatively coding for GlpK, GlpD and GlpR. The “glycerol uptake facilitator protein” coded by *glpF* gene was not identified in this strain, thus explaining the prolonged lag growth phase in media containing glycerol. The same result has been observed in *P. corrugata* CFBP 5454 genome, recently obtained (Licciardello et al., 2014).

![Figure 1. The Pha gene cluster of *P. mediterranea* CFBP 5447 is organized in two operons - *phaC1ZC2D* and *phaIF* - as revealed by the presence of rho independent terminators.](image-url)
3.2 Transcriptional analysis of the genes involved in PHA synthesis from glycerol

In order to determine the best molecular strategies for the optimization of the PHA biosynthetic process, transcriptional expression levels of phaC1, phaC2, phaZ, phaD and phal were investigated. We constructed five lacZ promoter fusions with the upstream region of each gene (named P\textsubscript{C1}, P\textsubscript{C2}, P\textsubscript{Z}, P\textsubscript{D}, P\textsubscript{I}) promoter regions), which were transferred into \textit{P. mediterranea} CFBP 5447 by triparental mating. \textit{P. mediterranea} strains carrying each lacZ fusion were cultured using reagent grade as a carbon source. The β-galactosidase activity was monitored after 24 and 48 hrs (Figure 2). The highest level of reporter expression was detected in the strain carrying the P\textsubscript{I}::lacZ fusion. It was more than 5-fold higher than that observed in the strain carrying the P\textsubscript{C1}::lacZ fusion after 24 hrs and 24-fold after 48 hrs. Expression levels of the strain carrying the P\textsubscript{C1}::lacZ fusion and P\textsubscript{C2}::lacZ fusion were essentially similar, with a slight decrease after 48 hrs. No reporter activity was detected for P\textsubscript{Z} and P\textsubscript{D} promoters.

3.3 PhaD activates transcription of P\textsubscript{C1} and P\textsubscript{I}

In order to describe the role of the phaD gene, it was insertionally inactivated thereby creating the VVD genomic mutant of \textit{P. mediterranea}. The P\textsubscript{C1}, P\textsubscript{C2}, P\textsubscript{Z}, P\textsubscript{D}, P\textsubscript{I} lacZ promoter fusions were transferred to the VVD mutant strain and β-galactosidase activity monitored after 24 and 48 hrs. Promoter activity quantification data showed that P\textsubscript{I} was strongly reduced in the VVD mutant. A 3-fold lower expression level than the Wt strain was observed for the P\textsubscript{C1} in the VVD mutant after 24 hrs, whereas no reduction was detected after 48 hrs (Figure 2).

Only a basal expression level was detected in the phaC2 gene in the VVD mutant, suggesting that it is probably driven by an internal promoter P\textsubscript{C2}. The absence of promoter activity in the upstream regions of phaZ and phaD genes suggests that their expression is controlled by an upstream promoter. The presence of a polycistrionic transcription unit (operon) comprised of phaC1, phaZ, phaC2 and phaD was demonstrated by RT-PCR amplification of overlapping phaC1-phaZ, phaZ-phaC2, phaC2-phaD regions. In addition, the presence of three putative transcriptional rho terminators downstream phaD (Figure 1), confirmed this hypothesis. Similarly, the presence of the phal-F overlapping region and the identification of two putative rho terminators located downstream of phaF highlighted that these two genes are organized as an operon with P\textsubscript{I} as a promoter directly controlled by PhaD. Preliminary semiquantitative expression of these genes by RT-PCR in the Wt and VVD mutant confirmed the control of phal-F operon by PhaD, whereas no detectable difference in phaC1ZC2D operon expression was assessed. These results need to be confirmed by real-time PCR.

![Figure 2](image-url)  
*Figure 2. Promoter activities of phaC1, phaZ, phaC2, phaD and phal in the parent strain P. mediterranea CFBP 5447 and VVD mutant derivative. The values are averages of at least three independent experiments.*

4. Conclusions

The improvement in PHA bacterial productivity using genetic engineering of our model \textit{P. mediterranea} strain entails not only defining the genes involved in the bioconversion but also regulating these genes and understanding the links between the metabolic pathways. In this study, the draft genome sequence of \textit{P.}
medierranea CFBP 5447 was mined enabling us to identify the complete PHA gene cluster and also the glycerol uptake and metabolism genes. We defined the activity of promoter regions of thepha genes in P. mediterranea CFBP 5447. We found that PC1 and PI are the most active and are responsible for the transcription ofphaC1ZC2D andphaIF operons, respectively. The PI operon was the most active and drives the expression ofphaF andphaI genes.

Our results are in accordance with findings for P. putida KT2442 (de Eugenio et al., 2010). In the presence of glycerol as a carbon source, the PI promoter is strongly controlled by PhaD which acts as a transcriptional activator. In this growth condition, the less efficient activation ofphaC1ZC2D operon, in the Wt compared to the VVD mutant strain, is similar to findings observed for P. putida KT2442 cultured in glucose. In fact for P. putida KT2442 it has already been demonstrated that PhaD controls the carbon source dependence of the transcription profile of this operon by an intermediate of fatty acid β-oxidation which acts as a PhaD inducer (de Eugenio et al., 2010).

The discovery that P. mediterranea CFBP 5447 lacks theglpFgene contributes to understanding its prolonged lag growth phase in the presence of glycerol as a carbon source. This gene is present in other Pseudomonas spp. that produce PHAs, such as P. aeruginosa (Schweizer et al., 1997) and P. putida (Escapa et al., 2013). However, this is the first time to our knowledge that the absence of glpF has been reported in a Pseudomonas strain. Our recent acquisition of the draft genome sequence of the closely related bacteria P. corrugata CFBP 5454 (Licciardello et al., 2014), similarly revealed the absence of this gene, prompting us to further investigation.

We plan new strategies for culture optimization in order to improve the efficiency in P. mediterranea CFBP 5447 of the glycerol bioconversion into PHAs which can then be used as coatings for paper materials and plasticizers thus widening the range of blends.

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