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

Grazia Licciardello[a], Giulia Devescovi[b], Patrizia Bella[c], Corinne De Gregorio[a], Antonino F. Catara[a], Salvatore P.P. Guglielmino[d], Vittorio Venturi[b], Vittoria Catara[c].

[a]Parco Scientifico e Tecnologico della Sicilia s.c.p.a., z.i. Blocco Palma I, Str.le Lancia 57, 95121 Catania, Italy
[b]Bacteriology Group, International Centre for Genetic Engineering and Biotechnology, Area Science Park, Padriciano, Trieste, Italy
[c]Dipartimento di Scienze delle Produzioni Agrarie e Alimentari, Università degli Studi di Catania, Via Santa Sofia 100, 95131 Catania, Italy
[d]Dipartimento Scienze Biologiche ed Ambientali, Università di Messina
glicciardello@pstsicilia.it

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 (*glpF, glpK, glpD, glpR*). Absence of the *glpF* gene, which codifies for the "glycerol uptake facilitator protein", was highlighted. In order to understand the expression of the *pha* gene cluster, we investigated the promoter activity of *phaC1, phaC2, phaZ, phaD* and *phaI* genes. When glycerol was present as the carbon source, P_I was found to be the most active promoter. Expression analysis of the knock-out mutant of the *phaD* gene, which is a transcriptional regulator belonging to the TetR family, showed that PhaD acts as an activator of the *phaI* promoter which, in turn, triggers the transcription of the *phaIF* operon. The activation of P_{C1}, which controls the *phaC1ZC2D,* by PhaD, was less efficient than 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 *pha* gene cluster responsible for PHA metabolism, well conserved among *Pseudomonas* spp., consists of genes encoding two synthases (*phaC1* and *phaC2*), a depolymerase (*phaZ*) responsible for PHA mobilization (type II biosynthetic locus) and the *phaD* gene encoding a putative transcriptional regulator (Klinke et al*.,* 2000). In addition, the *phaF* and *phaI* genes, transcribed divergently to the other *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, β-oxidation and fatty acid *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 *phaJ* or *phaG* genes, key link enzymes between β-

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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).
P. mediterranea pha locus has only been partially described (Solaiman et al*.,* 2005; Bella et al., 2007). A comparison with the taxonomic related strains *P. corrugata* 388 and CFBP 5445, demonstrated that this locus lacks a 24 bp sequence in the *phaC1–phaZ* intergenic region codifying for a putative *rho* independent terminator responsible for a slight variation in the PHA composition from oleic acid (Solaiman et al*.,* 2008). Our recent draft genome sequence of *P. mediterranea* CFBP 5447 (submitted in Genbank) helped us to investigate the central metabolic pathways involved in mcl-PHA synthesis, the peripheral pathway encoded by the *pha* cluster and genes involved in glycerol metabolism (*glpF, glpK, glpD, glpR*). The activity of the promoter regions of *pha* genes was monitored 24 hrs and 48 hrs after inoculation. The role of PhaD as an activator of *pha* cluster was demonstrated by mutagenesis analyses.

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	CFBP
VVD	*phaD*:: pKnock, Km^r	This study
Plasmids		
pMP220	Promoter probe vector, IncP Tc^r	Spaink et al*.,* 1987
pKnock-Km^r	Mobilizable suicide vector, Km^r	Alexeyev, 1999
pGEM-T	Cloning vector; Amp^r	Promega
pMPPhaC1	*phaC1 promoter* cloned in pMP220	This study
pMPPhaZ	*phaZ promoter* cloned in pMP220	This study
pMPPhaC2	*phaC2 promoter* cloned in pMP220	This study
pMPPhaI	*phaI promoter* cloned in pMP220	This study
pKMPhaD	pKnock containing an internal fragment of *phaD* gene	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 (ATGGCAAGGAACCCCTTGTC) and PhaDintRev (AACAGCAACGTCAGGGTGAT). It was cloned first in pGEM and then as an *Eco*RI fragment in the corresponding site in pKNOCK-Km, generating pKMPhaD. 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 P_{C1}, P_{C2}, P_Z, P_D, P_I *lacZ* 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).

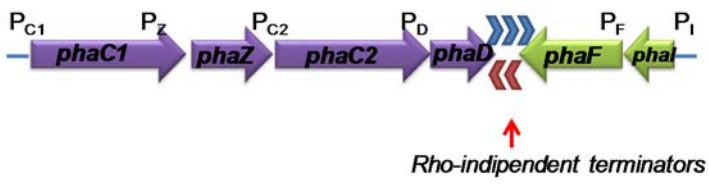

Figure 1. The Pha gene cluster of P. mediterranea CFBP 5447 is organized in two operons - phaC1ZC2D and phaIF - as revealed by the presence of rho independent terminators.

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, the transcriptional expression levels of *phaC1, phaC2, phaZ, phaD* and *phaI* were investigated. We constructed five *lacZ* promoter fusions with the upstream region of each gene (named P_{C1}, P_{C2}, P_Z, P_D, P_I promoter regions), which were transferred into *P. mediterranea* CFBP 5447 by triparental mating. *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_I*::lacZ* fusion. It was more than 5-fold higher than that observed in the strain carrying the P_{C1}*::lacZ* fusion after 24 hrs and 24-fold after 48 hrs. Expression levels of the strain carrying the P_{C1}*::lacZ* fusion and P_{C2}*::lacZ* fusion were essentially similar, with a slight decrease after 48 hrs. No reporter activity was detected for P_Z and P_D promoters.

3.3 PhaD activates transcription of P_{C1} and P_I

In order to describe the role of the *phaD* gene, it was insertionally inactivated thereby creating the VVD genomic mutant of *P. mediterranea*. The P_{C1}, P_{C2}, P_Z, P_D, P_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_I was strongly reduced in the VVD mutant. A 3-fold lower expression level than the Wt strain was observed for the P_{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_{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 polycistronic 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 *phaI-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_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 *phaIF* operon by PhaD, whereas no detectable difference in *phaC1ZC2D* operon expression was assessed. These results need to be confirmed by real-time PCR.

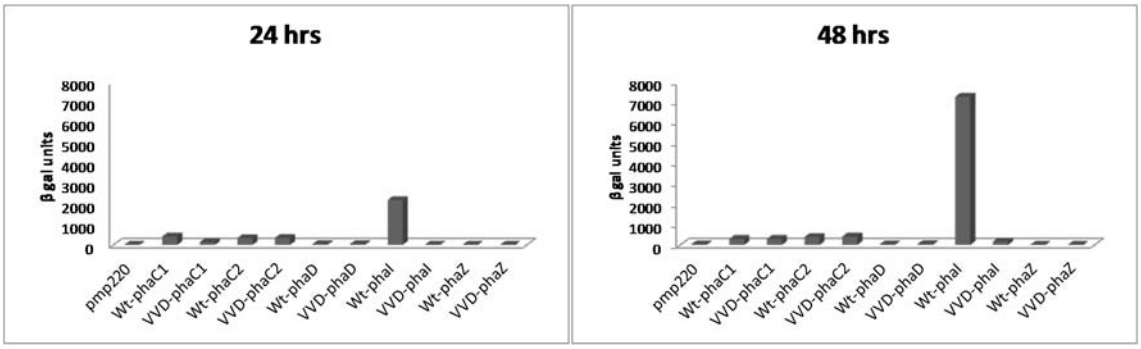

Figure 2. Promoter activities of phaC1, phaZ, phaC2, phaD and phaI in the parent strain P. mediterranea *CFBP 5447 and VVD mutant derivative. The values are averages of at least three independent experiments.*

4. Conclusions

The improvement in PHA bacterial productivity using genetic engineering of our model *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 *P.*

mediterranea 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 the *pha* genes in *P. mediterranea* CFBP 5447. We found that P_{C1} and P_I are the most active and are responsible for the transcription of *phaC1ZC2D* and *phaIF* operons, respectively. The P_I operon was the most active and drives the expression of *phaF* and *phaI* 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 P_I promoter is strongly controlled by PhaD which acts as a transcriptional activator. In this growth condition, the less efficient activation of *phaC1ZC2D* 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 the *glpF* gene 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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