ENVIRONMENTAL BIOTECHNOLOGY 7 (1) 2011, 12-16

Analysis of DNA transcription termination sequences of gene coding for phaC1 polymerase in *Pseudomonas species*

Justyna Możejko, Małgorzata Królicka, Sławomir Ciesielski

Department of Environmental Biotechnology, University of Warmia and Mazury in Olsztyn, Słoneczna 45G, 10-718 Olsztyn, Poland
Corresponding author: Justyna Możejko, phone: (+48) (89) 5234144, FAX: (+48) (89) 5234131, E-mail: justyna.mozejko@uwm.edu.pl
Keywords: polyhydroxyalkanoates, *Pseudomonas*, sequence analysis, transcription terminator

ABSTRACT

Polyhydroxyalkanoates (PHAs) are natural polyesters that are synthesized by many bacteria as an intracellular carbon and energy compound. Medium-chain-length polyhydroxyalkanoates (mcl-PHAs) have gained much interest in research on microbial biopolymers because of their ease of chemical modification. Mcl-PHAs are naturally synthesized by *Pseudomonas* species by transformation of wide range of substrates. The physiological background of mcl-PHAs synthesis is known, and key genes engaged in this process are discovered already, but the knowledge about their molecular regulation is still limited. Especially, there is lack of information concerning the transcription termination of gene coding for PHA polymerase (*phaC1*). It is assumed that the main role could play Rho-independent termination, which is related to presence of palindromic sequences, which leads to formation of the hairpin structure and to dissociation of the ternary elongation complex (TEC). In this work, DNA sequences located after *phaC1* gene belonging to nineteen *Pseudomonas* strains were investigated. Among all analyzed strains, five had palindromic sequences, typical for Rho-independent terminators. Our results proved, that gene *phaC1*, coding for PHA polymerase, can be independently regulated only in some species.

INTRODUCTION

Problems related to the negative influence of petrochemical-derived plastic on global environment have generated interest in the development of biodegradable plastic polymers. Biopolymers are divided into three categories: chemically synthesized polymers, starch-based biodegradable plastics and polyhydroxyalkanoates The most commonly encountered microbial storage polymer is PHA. The last one attract more attention and interest from scientists and industrialists because they have similar physical and thermal properties with those of synthetic plastics.

Polyhydroxyalkanoates (PHAs) are a class of natural polyesters produced as discrete granules and used as a storage material for carbon and reducing equivalents. For that reason the PHA have useful properties: they are biodegradable, thermoplastic, biocompatible, non-toxic, and are considered as a replacement for petrochemical polymers. The wide range of procaryotic organisms, including Gram-negative and Gram-positive bacteria, show the ability to synthesize PHAs from numerous carbon sources such as alkanoic acids, alcohol and alkanes (Poirier et al. 1995). The amount of polymer accumulated by bacteria can reach levels as high as 90% of their cell dry weight (Madison et al. 1999). With few exception, PHAs are synthesized when an essential nutrient such as nitrogen, phosphate, magnesium or oxygen is available in limiting concentrations and the carbon source is present in excess (Schlegel et al. 1961). Classification of PHAs is based on the number of carbon atoms in the polymer.

Polyhydroxyalkanoates can consist of short-chain-length hydroxyalkanoic acids (PHA_{SCL}), medium-chain-length monomers (PHA_{MCL}) or long-chain-length (PHA_{LCL}), depending on the bacterial strain that produces the polymer and the culture condition available to the bacteria.

Medium-chain-length polyhydroxyalkanoates (mcl-PHAs), a group of PHAs with a monomer length of 6 to 12 carbon atoms, which are produced by *Pseudomonas* species, have attracted considerable attention in recent years because some of their functional groups can be modified by chemical reactions. Furthermore, due to its biocompatibility, biodegradability and thermo plasticity, mcl-PHAs are highly

valued in medical, agricultural and industrial applications. The proteins involved in the synthesis of PHAs are encoded by the *pha* gene cluster containing two polymerase genes *phaC1* and *phaC2*, a depolymerase gene *phaZ* and three regulatory genes *phaD*, *phaF* and *phaI*. Even though the key genes engaged in the process of PHAs synthesis are known, there is a lack of information about the regulation of the genes at the level of transcription termination.

In prokaryotes, the transcription is terminated by two major mechanisms (von Hippel 1998). One of them depends on Rho, which is a protein blocking RNA synthesis at specific sites. Rho, encoded by the gene *rho*, is a homohexamer, which has ATPase activity. The second one, called Rho-independent termination (or intrinsic termination), is related with presence of palindromic sequence that forms a stem-loop hairpin structure, which leads to the dissociation of the ternary elongation complex (TEC).

Based on recent analysis, only a minority of bacteria employ intrinsic termination, despite being an economical and efficient means for spatial regulation of gene expression (Ermolaeva et al. 2000; Washio et al. 1998). This is probably because they take into account the possibility that secondary structure alone could work as a terminator (Unniraman et al. 2001). However, the Rho-independent termination plays a large role in *Bacillus subtilis*, *Neisseria* and *Vibrio* genera or in the Pasteurellaceae (Kingsford et al. 2007). In *Pseudomonas corrugata*, a putative intrinsic transcription terminator consisting of a dyad symmetry (24bp) was discovered in the *phaC1-phaZ* intergenic region by Solaiman et al. (2008).

Characterization of transcription termination is the key to understanding the mechanism of mcl-PHA biosynthesis. The aim of this work was to characterize the way of *phaC1* gene transcription termination in various *Pseudomonas* strains (Figure 1).

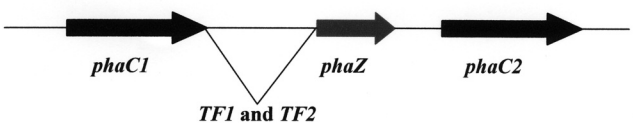

Figure 1. Schematic diagram of *pha* locus with the studied region. *phaC1*, *phaZ* and *phaC2* are the genes encoding PHA polymerase I, PHA depolymerase and PHA polymerase II, respectively. *TF1* and *TF2* are the palindromic sequences.

MATERIAL AND METHODS

The analysis was carried out based on the DNA sequences of bacteria belonging to *Pseudomonas* species and having *phaC1* and *phaZ* genes. All genomic sequences used in the present study have been downloaded from the genome database of the National Centre for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov).

Firstly, the sequences were aligned using a program Clustal W (Thompson et al. 1994). Next, the presence of open reading frame was analysed using ORF Finder (NCBI). The location of regulatory sequences on the stretch of DNA between *phaC1* and *phaZ* genes was identified using a knowledge of the structure's regulatory sequences in many bacteria across the genus *Pseudomonas*. Moreover, the fractional GC content of the *phaC1-phaZ* intergenic region was analysed based on the nucleotide content by using GeeCee program. Furthermore, the RNA secondary structure prediction program was used for the examination of the hairpin structure and for the calculation of the free energy of this formation. This procedure is based on Garnier-Robson algorithm modified by Brodsky et al. (1992). The homology is evaluated similarly to "nearest-neighbour" method (NNSSP). The probabilities of each secondary structure state (alpha-helix, beta-strand, coil) are assigned using parameters optimized for a training set.

RESULTS AND DISCUSSION

In this work a *phaC1-phaZ* intergenic region from nineteen *Pseudomonas* strains was investigated (Figure 2). These regions are vastly different. The length of this region ranged from 60 bp (*Pseudomonas putida* AF150670) to 225 bp (*Pseudomonas corrugata* AY910767). In case of *Pseudomonas* USM4-55, *Pseudomonas nitroreducens* and *Pseudomonas pseudoalcaligenes* the size of the intergenic region is the same (141 bp), and they are shorter in comparison with *Pseudomonas* sp. KBOS 04 (163 bp). More importantly, five of the analysed strains have a palindromic sequence, which is constituted of 24 bp with high GC content (Figure 2). The detailed analysis have indicated that this dyad symmetry is a transcription terminator.

The regulation of synthases *phaC1* and *phaC2* gene expression was examined in *Pseudomonas corrugata* (Conte et al. 2006). In the mentioned study it was shown that *phaC1* and *phaC2* genes were not co-transcribed, while they were independently regulated. These results are in accordance with data reported for *Pseudomonas putida* KT2440 and *Pseudomonas aeruginosa* (Hoffman and Rehm 2004). Unfortunately, despite of the fact that a transcriptional analysis of both PHA synthases *phaC1* and *phaC2* was performed, identification of sites at which termination events occur and promotion sites regulating mcl-PHA biosynthesis in these organisms has not yet been described.

Solaiman et al. (2008) have showed for the first time the influence of transcription terminator on PHAs biosynthesis. This study was focused on the transcription termination of *phaC1* gene in *Pseudomonas corrugata* 388. The complete analysis proved that an intrinsic transcription termination is located in the *phaC1-phaZ* intergenic region. Moreover, their results showed the influence of this characteristic region on the substrate-dependent expression of *phaC1* and *phaC2* genes. The function of the *phaC1-phaZ* intergenic region was investigated by the construction mutants. The results of the mentioned study showed that the transcription termination

Figure 2. The result of intergenic region DNA sequences alignment in studied *Pseudomonas* (*Ps.*) species. The palindromic sequences (*TF1* and *TF2*) and Rho-dependent terminators are indicated by grey and black colour, respectively.

sequence does not take part in the transcription regulation when the cells were grown on glucose as the carbon source, but on the contrary, when oleic acid was applied as the substrate.

Formation of a stable hairpin was considered as an important factor of intrinsic termination (Lesnik et al. 2001; Unnimaran et al. 2001; Schwartz et al. 2003; Wilson and von Hippel 1995; Yachie et al. 2006; Yarnell and Roberts 1999). Artsimovitch and Landick (1998) have verified that this structure may trigger the pause's TEC only for a time, and after a while the transcription process can further progress. However, Gusarov and Nudler (1999) have suggested that the hairpin destabilizes TEC complex. Moreover, it leads to dissociation of TEC into RNA, DNA and an RNA polymerase, what means the ending of transcription. In this study, the sequences located between *phaC1* and *phaZ* genes in *Pseudomonas* species were analysed to identify the bacteria for Rho-independent transcription terminator. Five of studied sequences (two strains of *Pseudomonas corrugata*, *Pseudomonas* sp. KBOS 03, *Pseudomonas* sp. KBOS 04, *Pseudomonas* sp. KBOS 17) were found to have this type of terminator. The phylogenetic analysis demonstrated that the strains KBOS 03, KBOS 04 and KBOS 17 were the nearest to *Pseudomonas fluorescens* (Ciesielski et al. 2006).

The strength and stability of the stem-loop structure is one of the factors of the termination efficiency by an intrinsic terminator. The free energy values had an effect on the shape of the predicted hairspin structures. The lower free energy value the stronger second structure, and thus more efficient pausing and termination (Mitra et al. 2008). As depicted in Table 1, the intergenic DNA sequence were studied paying special attention to free energy value. The obtained results indicate that a free energy of *TF1* and *TF2* of five species: *Pseudomonas* sp. KBOS17, *Pseudomonas* sp. KBOS03, *Pseudomonas corrugata*, *Pseudomonas corrugata* and *Pseudomonas* sp. KBOS04 was in the range of 1.8kcal·mol^{-1} and 2.0kcal·mol^{-1}, respectively, whereas these values were highest in *Pseudomonas* sp. KBOS04 (3.4 and 3.9 for *TF1* and *TF2*, respectively). The terminators analysed here have a stem-length of 6-8 base pairs, what is in accordance with the assumption of Wilson and von Hippel (1995), according to whom the optimal hairpins have a stem-length of 4-8 base pairs or even more. Analysis of *phaC1-phaZ* intergenic region revealed that it contains from 52% to 73% GC nucleotides. Research into nucleotide content of the *phaC1-phaZ* intergenic region showed that the GC pairs were from 52% to 73%. Recently it has been shown that GC-rich sequences located downstream of an intrinsic terminator cause more efficient termination than AT-rich structure (Epshtein et al. 2007).

The observations could suggest that *phaC1* gene, coding for PHA polymerase, can be independently regulated only in some species. Due to that it could be suggested that above mentioned species have Rho-independent termination

Table 1. The result of intergenic region analysis of 19 *Pseudomonas* species. The analyzed DNA sequences are given in Figure 2.

Strain	Accession number	Length [bp]	CG [%]	TF1 energy [kcal·mol^{-1}]	TF2 energy [kcal·mol^{-1}]
Pseudomonas putida	AY286491	62	66		
Pseudomonas putida	AF150670	60	62		
Pseudomonas sp. GL01	FJ214728	62	66		
Pseudomanas putida KT2440	EU604833	62	65		
Pseudomonas sp. USM4-55	EU275728	141	70		
Pseudomonas nitroreducens	AF336849	141	69		
Pseudomonas pseudoalcaligenes	AY043314	141	68		
Pseudomonas sp. GL06	FJ214729	142	69		
Pseudomonas mendocina	AF311979	142	67		
Pseudomonas sp. 61-3	AB014758	136	64		
Pseudomonas pseudoalaligenes HBQ06	AF336848	156	73		
***Pseudomonas* sp. KBOS17**	**AY790329**	**148**	**59**	**-1.8**	**-2.0**
***Pseudomonas* sp. KBOS03**	**AY790327**	**147**	**63**	**-1.8**	**-2.0**
Pseudomonas corrugata	**EF067339**	**224**	**60**	**-1.8**	**-2.0**
Pseudomonas corrugata	**AY910767**	**225**	**60**	**-1.8**	**-2.0**
***Pseudomonas* sp. KBOS04**	**AY790328**	**163**	**52**	**-3.4**	**-3.9**
Pseudomonas mediterranea	AY910768	103	56		
Pseudomonas auerofaciens	AB049413	95	60		
Pseudomonas sp. LDC-25	DQ910832	141	62		

of *phaC1* gene transcription. Results from this study conclusively show that the factor-dependent transcription termination of *phaC1* gene is characteristic for: *Pseudomonas* sp. USM4-55, *Pseudomonas nitroreducens*, *Pseudomonas pseudoalcaligenes*, *Pseudomonas pseudoalcaligenes* HBQ06, *Pseudomonas mendocina*, *Pseudomonas* sp. 61-3 and *Pseudomonas* sp. Gl06. However, in *Pseudomonas putida*, *Pseudomonas* sp. GL01, *Pseudomonas putida* KT2440, *Pseudomonas mediterranea*, *Pseudomonas* sp. LDC-25, *Pseudomonas auerofaciens* any terminators have been identified. These data imply that in the above mentioned species, transcription of gene *phaC1* takes place at the same time as transcription of gene *phaZ*.

CONCLUSIONS

The studied medium-chain-length polyhydroxyalkanoates synthesizing bacteria have shown different mechanisms of transcription termination of *phaC1* gene. Among nineteen analyzed DNA sequences, five have palindromic regions, typical for Rho-independent terminators, DNA sequences of seven species possess structures characteristic for Rho-dependent terminators, whereas analysis of the remaining intergenic regions suggests that *phaC1* gene is co-transcribed with *pha*Z gene. The observations could suggest that *phaC1* gene, coding for PHA polymerase, can be independently regulated only in some species.

Detection of transcription termination sites is a key to understanding the operon structure of mcl-PHA-synthesizing microorganisms. It gives us strong hints about gene function and the obtained information would be useful in further understanding of gene expression in mcl-PHA-producing bacteria.

ACKNOWLEDGEMENTS

This study was financed under Project No. 809.0801 of the University of Warmia and Mazury in Olsztyn, Poland.

REFERENCES

Artsimovitch, I., R. Landick. 1998. Interaction of a nascent RNA structure with RNA polymerase is required for hairpin-dependent transcriptional pausing but not for transcript release. Genes & Development 12: 3110-3122.

Brodsky, L.I., A.V. Vasilyev, Y.L. Kalaydzidis, Y.S. Osipov, A.R.L. Tatuzov, S.I. Feranchuk. 1992. GeneBee: the Program Package for Biopolymer Structure Analysis. Dimacs 8: 127-139.

Ciesielski, S., A. Cydzik-Kwiatkowska, T. Pokoj, E. Klimiuk. 2006. Molecular detection and diversity of medium-chain-length polyhydroxyalkanoates-producing bacteria enriched from activated sludge. Journal of Applied Microbiology 101: 190–199.

Conte, E., V. Catara, S. Greco, M. Russo, R. Alicata, L. Strano, A. Lombardo, S.D. Silvestro, A. Catara. 2006. Regulation of polyhydroxyalkanoate synthases (*phaC1* and *phaC2*) gene expression in *Pseudomonas corrugata*. Applied Microbiology and Biotechnology 72: 1054-1062.

Epshtein, V., C.J. Cardinale, A.E. Ruckenstein, S. Borukhov, E. Nudler. 2007. An allosteric path to transcription termination. Molecular Cell 28: 991-1001.

Ermolaeva, M.D., H.G. Khalak, O. White, H.O. Smith, S.L. Salzberg. 2000. Prediction of transcription terminators in bacterial genomes. Journal of Molecular Biology 301: 27–33.

Gusarov, I., E. Nudler. 1999. The mechanism of intrinsic transcription termination. Molecular Cell 3: 495-504.

Hoffman, N., B.H. Rehm. 2004. Regulation of polyhydroxyalkanoate biosynthesis in *Pseudomonas putida* and *Pseudomonas aeruginosa*. FEMS Microbiology Letters 237: 1-7.

Kingsford, C.L., K. Ayanbule, S.L. Salzberg. 2007. Rapid, accurate, computational discovery of Rho-independent transcription terminators illuminates their relationship to DNA. Genome Biology 8: R22.

Lesnik, E.A., R. Sampath, H.B. Levene, T.J. Henderson, J.A. McNeil, D. Ecker. 2001. Prediction of rho-independent transcriptional terminators in *Escherichia coli*. Nucleic Acids Research 29: 3583-3594.

Madison, L.L., G.W. Huisman. 1999. Metabolic engineering of poly (3-hydroxyalkanoates): from DNA to plastic. Microbiology and Molecular Biology Reviews 63: 21-53.

Mitra, A., K. Angamuthu, V. Nagaraja. 2008. Genome-wide analysis of the intrinsic terminators of transcription across the genus *Mycobacterium*. Tuberculosis 88: 566-575.

Poirier, Y., C. Nawrath, C. Somerville. 1995. Production of polyhydroxyalkanoates, a family of biodegradable plastics and elastomers, in bacteria and plants. Biotechnology 13: 142-150.

Schlegel, H.G., G. Gottschalk, R. von Bartha. 1961. Formation and utilization of poly-β-hydroxybutyric acid by Knallgas bacteria (*Hydrogenomonas*). Nature 191: 463-465.

Schwartz, A., A.R. Rahmouni, M. Boudvillain. 2003. The functional anatomy of an intrinsic transcription terminator. The EMBO Journal 22: 3385-3394.

Solaiman, D.K.Y., R.D. Ashby, G. Licciardello, V. Catara. 2008. Genetic organization of *pha* gene locus affects *phaC* expression, poly(hydroxyalkanoate) composition and granule morphology in *Pseudomonas corrugata*. Journal of Industrial Microbiology and Biotechnology 35: 111-120.

Thompson, J.D., D.G. Higgins, T.J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22: 4673-4680.

Unniraman, S., P. Ranjana, V. Nagaraja. 2001. Alternate paradigm for intrinsic transcription termination in Eubacteria. The Journal of Biological Chemistry 276: 41850-41855.

von Hippel, P.H. 1998. An integrated model of the transcription complex in elongation, termination and editing. Science 281: 660-665.

Washio, T., J. Sasayama, M. Tomita. 1998. Analysis of complete genomes suggests that many procaryotes do not rely on hairpin formation in transcription termination. Nucleic Acids Research 26: 5456–5463.

Wilson, K.S., P.H. von Hippel. 1995. Transcription termination at intrinsic terminators: The role of the RNA hairpin. Proceedings of the National Academy of Sciences of the United States of America 92: 8793-8797.

Yachie, N., K. Arakawa, M. Tomita. 2006. On the interplay of gene positioning and the role of Rho-independent terminators in *Escherichia coli*. FEMS Microbiology Letters 580: 6909-6914.

Yarnell, W.S., J.W. Roberts. 1999. Mechanism of intrinsic transcription termination and antitermination. Science 284: 611-615.