[BIO28] Regulation studies of *phaC*(C1 and C2) genes in *Pseudomonas* sp. USM 4-55

Kamariah Hasan, Mohd Nazalan Najimudin, Mohd Razip Samian

School of Biological Sciences, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia.

Introduction

Among the various biodegradable plastics available, polyhydroxyalkanoates (PHAs) attract a lot of attention because these polymers are produced by bacteria and have thermoplastic properties. They are biodegradable, biocompatible, moisture resistant, versatile, have long shelf life and are made from renewable source materials (Pouton et al., 1996).

The Pseudomonad group synthesize mainly medium-chain-length PHAs, which consist of monomers of 6 to 14 carbon long. The polymers are flexible and can be formed into pliable sheets and rubbers. Genes involved in PHA metabolism encode three PHA synthases, a PHA depolymerase and several phasins-protein that bind to the surface of PHA granules. Of these, only PHA synthases are absolutely essential for the synthesis of PHA in non-PHA producers such as *Escherichia coli*. In previous project the *phaC1* and *phaC2* structural genes that are involved in the last step in PHA polymerization and the gene for intracellular PHA depolymerase have been cloned and sequenced (Baharuddin. A., 2002). The aim of this project is to investigate the regulation of the *pha*C genes. To better understand the regulatory of the expression of the *pha* genes and the principle role of each of the PHA syntase, we need asses to the effect of different growth conditions on the expression of *pha* genes of *Pseudomonas* sp. USM4-55 and identify the regulatory proteins involved in the regulation of *pha* genes expression.

Materials and methods

Bacterial strains, plasmid and growth conditions

The bacterial strains and plasmids used in this study are listed in table 1. *Pseudomonas* sp. USM4-55 and *E. coli* were cultivated at 37 ºC on Luria-Bertani (LB) medium (Sambrook et al., 1989). When needed, ampicillin (50µg/ml), kanamycin (50µg/ml) and streptomycin (50µg/ml) were added to the medium.

DNA manipulation and plasmid construction

Isolation of plasmids, PCR amplification, digestion of restriction endonucleases, subcloning, agarose gel electrophoresis, transformation of *E. coli* and other molecular techniques were carried out by standard procedures (Sambrook et al., 1989) or as recommended by manufacturers. DNA restricton fragments were isolated from agarose gels by using a QIAEX II Gel Extraction Kit (QIAGEN). Recombinant plasmids containing *phaC1* and *phaC2* were purified using Wizard® Plus SV miniprep DNA purification system (Promega). All other DNA-manipulating enzymes were used as recommended by the manufacturers

Construction of phaC1/C2::lacZ-kan^r.

pJRD215 was digested with Xho1 and Sal1 to remove *kan^r* genes, resulting in 8.57 kb pKEM100. A 1.6 kb *phaC1* and 1.8 kb *phaC2* were amplified by PCR using 9H clones (Baharuddin. A., 2002) as a template. The amplified fragments were cut with EcoR1 and Xba1 and ligated into pKEM100, to create plasmid pKEM101 and pKEM102 containing *phaC1* and *phaC2* respectively. Since there are no unique site in *phaC1* for subcloning of *LacZ-kan^r*, a new restriction site, BspE1 was introduced in the sequence using a linker. The resulting plasmid is pKEM101B

A 1 kb *kan^r* gene was amplified by PCR using pJRD215 as a template. The amplified fragment was cut with Kpn1 and Sal1 and ligated into pLKL201 to create pKEM103 and pKEM104. A 3.1 kb PCR product of promoterless *LacZ* was cloned next to *kan^r* gene to produce plasmid pKEM203 and pKEM204. Plasmid pKEM301 and pKEM302 was constructed by subcloning the *LacZ- kan^r* cassette into the pKEM101B and pKEM102.

Homologous recombination

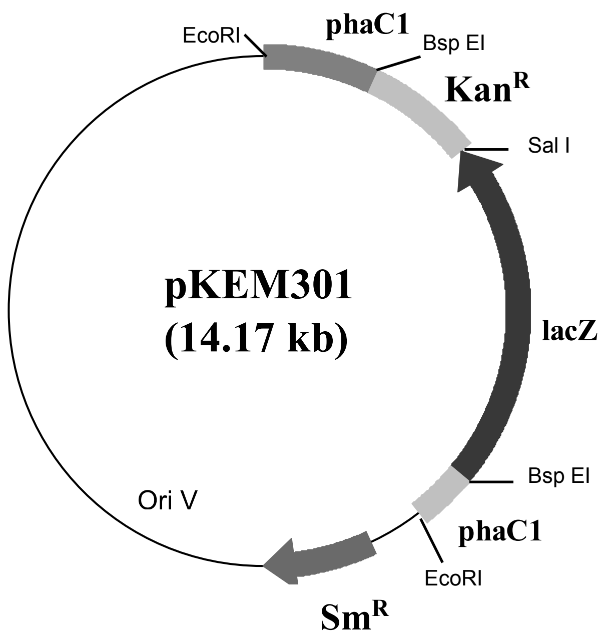
Both plasmid pKEM301 and pKEM302 were transformed into *Pseudomonas* sp.

USM4-55 via electroporation. The mutant *Pseudomonas* sp. 4-55, referred to hereinfter as *Pseudomonas* sp. USM4-551 and *Pseudomonas* sp. USM4-552 for both *C1* and *C2* respectively. Plasmid curing were done by culturing the mutants in LB without antibiotic for 16 hours at 42 °C and subculture for three times.

TABLE 1 Bacterial strains and plasmids used in this study

Strain or Plasmid	Relevent characteristics	source or reference
Strains		
Pseudomonas sp. USM4-55	Wild type	Few L. L., 2000
P. USM4-551	*phaC1* mutant	This study
P. USM-552	*phaC2* mutant	This study
E. coli JM109	F' *rec*A1 *end*A1*hs*dR17(r_K- m_K+) *rel*A *sup*E	
Plasmids		
pJRD215	Cosmid: Km^r Sm^r RSF1010 replicon Mob^+	Davison et. al 1987
pLKL201	Amp^r	Lau K. L., 2002
pKEM100	pJRD215 derivative: Km^s	This study
pKEM101	pJRD215 derivative: Km^s, *phaC1*	This study
pKEM101B	pJRD215 derivative: Km^s, *phaC1*, linker BspE1	This study
pKEM102	pJRD215 derivative: Km^s, *phaC2*	This study
pKEM103	pLKL201 derivative: Km^r	This study
pKEM104	pLKL201 derivative: Km^r	This study
pKEM203	pLKL201 derivative: Km^r, promoterless *LacZ*	This study
pKEM204	pLKL201 derivative: Km^r, promoterless *LacZ*	This study
pKEM301	pJRD215 derivative: Km^r, *phaC*1, linker BspE1, promoterless *LacZ*	This study
pKEM302	pJRD215 derivative: Km^r, *phaC2*, promoterless *LacZ*	This study

a)



b)

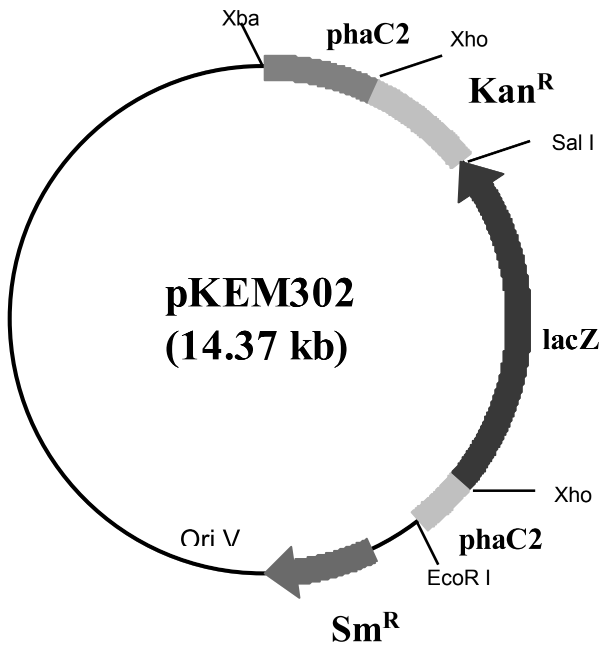


FIGURE 1 The resulting plasmids fusion, a) pKEM301 b) pKEM302

Result and Discussion

In order to investigate the contribution of each PHA synthase to the biosynthesis of PHAs in *Pseudomonas* sp. USM4-55, isogenic *phaC1* or *phaC2* mutants were generated by insertional inactivation of the corresponding chromosomal gene. For this purpose, two suicide vectors pKEM301 and pKEM302 based on plasmid pJRD215 were constructed as shown in fig.1. The resulting plasmids have a *lacZ* reporter gene (*lacZ* devoid of its promoter) that was fused to the *pha* operon by inserting it in the same orientation, within the *pha* promoter or the *pha* open reading frame(ORF) without interfering transcription of the *pha* genes. The plasmids were subsequently transferred into *Pseudomonas* sp. USM4-55 by electroporation. Plasmid curing was done to promote homologous recombination between chromosomal *pha* region and *phaC::LacZ* cassette (*kan^r* gene allowing direct selection). The mutants were cultured for a few generation in LB plus kanamycin to loose the the plasmid that did not form recombination. Kanamycin-resistant and streptomycin-sensitive transformants, most probably representing homogenotes carrying the respective interrupted PHA synthase were selected. Southern hybridization was carried out for every clones using *phaC1* and *phaC2* from *Pseudomonas* sp. USM4-55 as probes. The effectiveness of this approach is currently being studied.

In the future, the mutant *Pseudomonas* sp. cells will be incubated under various condition known to influence the types of polyester produced and expression of *phaC*1 and *phaC2* is assayed by measuring the β-galactosidase activity.

Acknowledgements

The authors wished to thank the Ministry of Science, Technology and Innovation (MOSTI), Malaysia for the National Science Fellowship awarded to Kamariah Hasan. This work was supported by research grant from MOSTI and USM short-term grant

References

Baharuddin, A., *Pengklonan dan pencirian gen polihidroksialkanoat (PHA) dari Pseudomonas* sp. USM4-55.(2002). Thesis Ijazah Sarjana Sains. Universiti Sains Malaysia, Pulau Pinang, Malaysia.

Davison, J., Heusterspreute, M., Chevalier, N., Vinh, H.T., and Brunel, F., (1986). *Vectors with restriction site banks V.Pjrd215, a wide-host-range cosmid vector with multiple cloning site*. *Gene*:275-280.

Few, L.L., *Pencirian, penghasilan poli-3-hidroksialkanoat(P-3HA) dalam*

Pseudomonas sp. (2001). Thesis Ijazah Doktor Falsafah Sains. Universiti Sains Malaysia, Pulau Pinang, Malaysia.

Lau, K.L., (2002). *Pencirian dan pengawalaturan homolog cutF (nlpE) Salmonella typhi.*Thesis Ijazah Doktor Falsafah Sains. Universiti Sains Malaysia, Pulau Pinang, Malaysia.

Pouton, C. W., and S. Akhtar, (1996). *Biosynthetic polyhydroxyalkanoates and their potential in drug delivery*. Adv. Drug Delivery Rev.18:133-162.

Sambrook, J., E.F. Fritch, and T. Maniatis. (1989*). Molecular cloning : a laboratory manual*, 2nd edition. Cold Spring Harbor, NY : Cold Spring Harbor Laboratory Press.