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Cell system engineering to produce extracellular polyhydroxyalkanoate depolymerase with targeted applications

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ABSTRACT

Novel platforms based on the application of bacterial cell systems as factories for production of new bioproducts open avenues and dramatically expand the catalogue of existing biomaterials. Herein, we designed the strategy based on *in vivo* production of extracellular *Pseudomonas fluorescens* GK13 ($PhaZ_{GK13}$) depolymerase to degrade previously biosynthesized polyhydroxyalkanotes (PHAs) or to obtain 3-hydroxyalkanoic acids (HAs). With this aim, extracellular $PhaZ_{GK13}$ was produced in recombinant strains and the optimal conditions for controlled release of HAs and oligomers by growing cells were set up with a particle suspension of ^{14}C-labelled PHA, being maximal after 24 h of incubation. Genetic modification of key factors involved in fatty acids metabolism revealed the influence of an active β-oxidation pathway on the extracellular degradation of PHA and subsequent HAs isolation. The highest HAs production was obtained using *Pseudomonas putida* KT2442 fadB mutant (0.27 mg/mL) due to the reduced ability of this strain to metabolize the degradation products. The system was applied to produce new added value HAs harboring thioester groups in the side chain from the functionalized mcl-PHA, PHACOS. Remarkably, hydrolyzed PHACOS showed greater potential to inhibit *Staphylococcus aureus*^{T} growth when compared to that of degradation products of non functionalized polyhydroxyoctanoate-*co*-hexanoate P(HO-*co*-HH).

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1. Introduction

The consumption of conventional petroleum based plastics far exceeds its recycle, although the latter has significantly increased since the 90s. These non-biodegradable plastics represent a serious environmental problem, and can be substituted by environmentally friendly bioplastics, as polyhydroxyalkanoates (PHAs) [1,2]. PHAs are biodegradable polyesters, synthesized by numerous microorganisms and accumulated in a form of carbon storage intracellular granules [3] for self-consumption, or released by producer microorganisms after death to the extracellular space as carbon source for the microorganisms living in the same niche [4]. Described PHAs are mainly linear, head-to-tail polyesters composed of over 150 different chiral hydroxy fatty acid monomers with the hydroxyl-substituted carbon atom at 3 position exclusively of the *R* configuration [5–7]. The number of carbon atoms forming *R* moiety can vary and, thus, short chain length PHAs (scl-PHAs) contain monomers with 4–5 carbon atoms, whereas medium chain length PHAs (mcl-PHAs) are composed of monomers with 6–14 carbon atoms [8]. One of the most promising applications of PHAs relies on these structural features, making the polymer a potential source of great diversity of *R*-3-hydroxyalkanoic acids (HAs), hereinafter used to define both monomeric and oligomeric building blocks of the polymer [9–11].

HAs are valuable starting materials for pharmaceutical and medical industries [12–14]. Thus, there is increasing demand for developing novel methods that allow efficient production of HAs. In parallel, there is growing awareness of the importance of the synthesis of enantiomerically pure mixture. This enantiomerical purity is a key factor for accomplishing biological function of HAs. Currently, two main strategies are used to produce HAs, one based on chemical synthesis and the other on biotransformation. However, the drawbacks that follow chemical approaches substantially limit their use. Namely, they tend to be expensive, not environmentally friendly, and the obtained yield of enantiomer pure HAs is often poor. Therefore, up-to-date tendency is to replace the conventional chemical processes by bio-based ones, which are less

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harmful for the environment and ensure the enantiomerical purity. This demand for more sustainable procedures led to the increased use of microorganisms for biotechnological processes [15].

Different approaches to promote PHAs biotransformation have been reported, mainly based on genetic engineering of enzymatic machinery involved in PHA turnover process, consisting in overexpression of PHA degradation enzymes or mutagenesis of PHA synthases [10,11,16]. Biotransformation strategies used to obtain PHA derived HAs are generally based on application of purified enzyme (*in vitro* approach) or cell system as a factory (*in vivo* approach). Biodegradation of PHAs can be driven by intracellular or extracellular depolymerases. The most extensively characterized, model enzyme in extracellular mcl-PHAs degradation is *Pseudomonas fluorescens* GK13 depolymerase ($PhaZ_{GK13}$) [17,18]. The main reaction products identified in *in vitro* systems of PHA depolymerization of $PhaZ_{GK13}$ are dimers [17,19], whereas in the case of *in vivo* intracellular polymer degradation by the PhaZ depolymerase of *Pseudomonas putida* KT2442, the main reaction products are monomers [10,13]. In contrast to costly methods involving enzyme purification, heterologous $PhaZ_{GK13}$ expressing microorganisms offer attractive approach regarding HAs production. In this respect, engineering of metabolic pathway can be used as a powerful tool to optimize the production process and increase the yields via evading HAs consumption by the producer.

Herein, we studied the effectiveness of recombinant $PhaZ_{GK13}$ producer strains to extracellularly degrade mcl-PHA, either aiming just to biodegrade it or with the goal of generating HAs. Specific fatty acids metabolism modifications have been addressed to improve the HAs production. After analyzing the influence of β-oxidation in the extracellular metabolism of PHA by *fadR* and *fadB* mutations, in *Escherichia coli* and *P. putida*, respectively, a new strategy has been designed to obtain enantiopure mixture of PHA derived monomers and oligomers. Finally, under this scheme we produced novel high added value HAs carrying thioester groups in the side chain and their antibacterial performance against *Staphylococcus aureus*^{T} has been evaluated.

2. Material and methods

2.1. Chemicals

Polyhydroxyoctanoate-*co*-hexanoate (P(HO-*co*-HH)) and poly-3-hydroxy-acetylthioalkanoate-*co*-3-hydroxyalkanoate PHACOS [20] were kindly supplied by Biopolis S.L. (Valencia, Spain). Chromatography media were obtained from GE-Healthcare (Uppsala, Sweden). Most chemicals were obtained from Sigma–Aldrich (St. Louis, MO). All other chemicals were purchased from Merck (Darmstadt, Germany).

2.2. Bacterial strains, media and growth conditions

E. coli MC4100 strain was used as host due to its mineral medium growth capability. *P. putida* KT2442 is a derivative strain of the parental strain KT2440 whose complete genome nucleotide sequence is accessible in the data bank [21]. Unless otherwise stated, *E. coli* and *P. putida* strains were grown in Lysogeny Broth (LB) medium [22] or in M63 mineral medium [23] at 37 °C and 30 °C, respectively. The carbon sources added to the mineral medium were glucose (20 mM) or glycerol (20 mM). Solid media were supplemented with 1.5% (w/v) agar; agar plates containing an opaque-polymer top layer were prepared by mixing equal volumes of P(HO-*co*-HH) (5–8 g/L) in water and LB medium agar or mineral medium agar at about 50 °C and pouring 7 mL onto a pre-warmed corresponding bottom layer. The appropriate selection antibiotics, ampicillin (100 μg/mL) and gentamicin (10 μg/mL) were added when needed.

2.3. DNA manipulation and plasmid construction

DNA manipulations and other molecular biology techniques were essentially performed as described previously [22]. Transformation of *E. coli* cells was carried out by using the RbCl method or by electroporation (GenePulser, Bio-Rad) [24]. Plasmid transference to the target *Pseudomonas* strains was done by the filter-mating technique [25]. DNA fragments were purified by standard procedures using Gene Clean (Bio 101, Inc., Vista, CA). To construct pLJ1, the 872 bp DNA fragment coding for the extracellular $PhaZ_{GK13}$ depolymerase was PCR-amplified by using the oligonucleotides PHAZGKF (5′ TCTAGAAGGAGATAAGTCATGC 3′) and PHAZGKR (5′ AAGCTTCCCGCGGTGGATCA 3′) using the total DNA of the strain *P. fluorescens* GK13 as template. For PCR amplification, we used 2 units of AmpliTaq DNA polymerase (PerkinElmer Life Sciences), 1 μg of DNA template, 1 μg of each deoxynucleoside triphosphate, and 2.5 mM $MgCl_2$ in the buffer recommended by the manufacturer. Conditions for amplification were chosen according to the G + C content of the corresponding oligonucleotides. The PCR product was digested with the engineered endonucleases XbaI and HindIII and cloned in pUC18.

For pIZPZ construction, pLJ1 plasmid was cut with SmaI and HindIII and the 882 bp fragment was subcloned in pIZ1016 plasmid cut with SalI, blunt extremes made with Klenow enzyme (according to manufacturer's instructions) and digested with HindIII.

E. coli MF4100 was obtained by spontaneous mutagenesis of *fadR* gene. Cells of parental strain MC4100 were grown on LB medium for 48 h, washed with saline solution, plated onto M63-agar plates with octanoate 15 mM as sole carbon source and incubated for 11 days. The colonies able to grow were sequenced using FADRR (5′ ATGGTCATTAAGGCGCAAAGC 3′) and FADRF (5′ TTATCGCCCCTGAATGGCTAA 3′) primers to confirm the *fadR* mutation.

Nucleotide sequences were determined directly with the same oligonucleotides used for cloning. All the constructions were confirmed by sequencing using an ABI Prism 3730 DNA Sequencer.

2.4. Depolymerase activity plates

For simple evaluation of enzyme activity, indicator plates were prepared in the same way as P(HO-*co*-HH) agar plates, except that the nutrient medium was replaced with Tris–HCl buffer (15 mM, pH 8). The enzyme solution was dropped onto wells punched in the plates and subsequently incubated at 37 °C. The diameters of the resulting clearing zones semiquantitatively indicated enzyme activity.

2.5. ^{14}C-labeled 3-hydroxyalkanoic release in growing cells system

MC4100, MC4100 (pLJ1), KT42FadB (pIZ1016) or KT42FadB (pIZPZ) cells were grown in M63 medium [23] with 20 mM glycerol (*E. coli* derived strains) or 20 mM glucose (*Pseudomonas* strains) as carbon source. This medium was supplemented with ^{14}C-labelled P(HO-*co*-HH)/water nano-particle emulsion (PHA*), prepared as described by de Eugenio et al. [26], resulting in 34 cpm/μg. Cells were incubated at 30 °C or 37 °C using an orbital shaker. Samples were taken at different time intervals, filtered and 200 μL of follow-throughs were analyzed using a scintillation counter.

2.6. 3-Hydroxyalkanoic release in growing cells system

MC4100 (pUC18), MC4100 (pLJ1), KTFadB (pIZ1016) or KT42FadB (pIZPZ) cells were grown in M63 medium with 20 mM glycerol (*E. coli* derived strains) or 20 mM glucose (*Pseudomonas* strains) as carbon source. P(HO-*co*-HH)/water particle emulsion

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