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In-silico-driven metabolic engineering of *Pseudomonas putida* for enhanced production of poly-hydroxyalkanoates

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ABSTRACT

Here, we present systems metabolic engineering driven by in-silico modeling to tailor *Pseudomonas putida* for synthesis of medium chain length PHAs on glucose. Using physiological properties of the parent wild type as constraints, elementary flux mode analysis of a large-scale model of the metabolism of *P. putida* was used to predict genetic targets for strain engineering. Among a set of priority ranked targets, glucose dehydrogenase (encoded by *gcd*) was predicted as most promising deletion target. The mutant *P. putida* Δ*gcd*, generated on basis of the computational design, exhibited 100% increased PHA accumulation as compared to the parent wild type, maintained a high specific growth rate and exhibited an almost unaffected gene expression profile, which excluded detrimental side effects of the modification. A second mutant strain, *P. putida* Δ*pgl*, that lacked 6-phosphogluconolactonase, exhibited a substantially decreased PHA synthesis, as was also predicted by the model. The production potential of *P. putida* Δ*gcd* was assessed in batch bioreactors. The novel strain showed an increase of the PHA yield (+80%), the PHA titer (+100%) and cellular PHA content (+50%) and revealed almost unaffected growth and diminished by-product formation. It was thus found superior in all relevant criteria towards industrial production. Beyond the contribution to more efficient PHA production processes at reduced costs that might replace petrochemical plastics in the future, the study illustrates the power of computational prediction to tailor microbial strains for enhanced biosynthesis of added-value compounds.

1. Introduction

Bio-based plastic materials are among the most important products to overcome the shortage of fossil resources and the environmental burden linked to conventional plastics produced from petroleum. Particularly, poly-hydroxyalkanoates (PHAs) have proven valuable to replace common oil-based polymers because of their similar mechanical and physical properties (Madison and Huisman, 1999). Through the past decades, short-chain-length PHAs such as poly(3-hydroxybutyrate) and related co-polymers have been commercialized in the biopolymer market because of a well-established production process (Ryu et al., 1997; Wang and Lee, 1997), supported by knowledge on the underlying biosynthetic pathway at the molecular level (Rehm, 2003). However, these short-chain-length polymers reveal rather poor physical properties which restrict them to the production of soft plastic such as packaging films (Chen, 2009). This has recently shifted the interest to medium-chain-length PHAs with superior properties that promise a broader spectrum of applications (Kim et al., 2007) in the chemical industry (Chen and Wu, 2005), but also as material for medical purposes (Zinn et al., 2001), protein purification (Rehm, 2007), and drug delivery (Grage et al., 2009). *Pseudomonas putida* KT2440 and related strains produce such medium-chain-length PHAs (Huijberts et al., 1992). Most of the investigations with this bacterium have focused on the use of fatty acids as carbon source, since these promote high accumulation of the biopolymer up to 80% of the cell dry weight (Poblete-Castro et al., 2012a, 2012b). The production of medium-chain-length PHAs on fatty acids has meanwhile been complemented by the identification of PHA regulatory factors (de Eugenio et al., 2010a, 2010b), the generation of new co-polymers (Liu et al., 2011) and process development (Kellerhals et al., 2000; Sun et al., 2007). As the raw material accounts for the most part of the production costs for PHAs (Yamane, 1993), one step ahead towards a cost-effective process is the use of substrates, such as

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glucose, which are cheaper than fatty acids. Nevertheless, a previous report has shown that glucose-grown *P. putida* accumulates much less of the desired biopolymer as compared to other substrates, including fatty acids (Huijberts et al., 1992), which places sugar-based production as economically not viable against the conventional oil-based route.

In this regard, systems metabolic engineering now opens novel avenues to enhance the synthesis of value-added products. As the genomic repertoire of *P. putida* comprises the entire set of enzymes to synthesize PHAs, there seems no direct need to insert heterologous genes in first instance. In fact, a re-engineering of its metabolic pathway network towards improved PHA production appears more straightforward. Hereby, the sequenced genomes of different *P. putida* strains (Nelson et al., 2002; Tang et al., 2011; Tao et al., 2011) and the genome-scale networks created (Nogales et al., 2008; Puchalka et al., 2008; Sohn et al., 2010) now enable a next level of strain design, i.e., design-based systems metabolic engineering, which recruits systems-wide network modeling to predict genetic targets. The beauty of this approach was successfully demonstrated by model-based design of a bacterial strain that then accumulated 120 g L^{-1} lysine with only 12 defined genomic traits (Becker et al., 2011). Design-based strain engineering is supported by powerful software tools such as Minimization of Metabolic Adjustments (MOMA) (Segre et al., 2002), and Optknock (Burgard et al., 2003), and more recently FluxDesign (Melzer et al., 2009). The latter is based on elementary flux modes and enables the simultaneous prediction of amplification, attenuation and deletion targets for any biological system of interest (Melzer et al., 2009). Here, we rationally re-designed *P. putida* KT2440 towards enhanced production of medium-chain-length PHAs using glucose as carbon source. Translating the computational design guided by FluxDesign into the laboratory, the metabolically engineered strain that carried only one predicted key mutation, revealed a 100% increase in the final PHA titer, a 50% increase of the cellular PHA content and a 80% in the PHA yield, relative to its parent strain, *P. putida* KT2440. This displays an important step towards industrial production of medium-chain-length PHAs using *P. putida* as a cell factory.

2. Materials and methods

2.1. Strains and plasmids

The parent strain *P. putida* KT2440 (DSM 6125) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). All PCR fragments generated for the construction of plasmids were sub-cloned into the plasmid pCR®2.1 (Invitrogen, CA, USA), transformed into *Escherichia coli* DH5α (Invitrogen, CA, USA), and validated by sequencing. The plasmid pEMG (Martinez-Garcia and de Lorenzo, 2011) was used for the construction of pEMG_ΔPP1023 and pEMG_ΔPP1444. In the first case, about 600 bp of the upstream and downstream regions of the *P. putida* gene PP1023, i.e., *pgl* that encodes for 6-phosphogluconolactonase, were amplified using Taq DNA polymerase (Qiagen, Venlo, The Netherlands) with primers UP1023XbaIF and UP1023ClaIR, as well as DOWN1023ClaIF and DOWN1023XmaIR (Table 1) and genomic DNA from strain *P. putida* KT2440. Both PCR fragments were inserted into the *Xma*I-*Xba*I restriction sites of pEMG, which generated pEMG_ΔPP1023. In the case of vector pEMG_ΔPP1444, the 600 bp upstream and downstream regions of the gene PP1444, i.e., *gcd* that encodes glucose dehydrogenase, were amplified using the primers UP1444KpnIF and UP1444R, as well as DOWN1444F and DOWN-1444EcoRIR (Table 1). Both PCR fragments were fused by PCR (Shevchuk et al., 2004). The resulting fragment was integrated into pEMG via the *Eco*RI-*Kpn*I restriction sites to generate pEMG_ΔPP1444. pEMG derivatives were then transformed into *E. coli* CC118λ*pir* (Herrero et al., 1990) to generate donor cells for the later tri-parental mating with *P. putida* KT2440 (Bagdasarian et al., 1981; Regenhardt et al., 2002) and the helper strain *E. coli* HB101 as described by (de Lorenzo and Timmis, 1994).

2.2. Genetic engineering of P. putida

To generate single and double deletion mutants of *P. putida* KT2440, genome editing was applied (Martinez-Garcia and de Lorenzo, 2011). For this purpose, the pEMG derivatives were first co-integrated by a single crossover into the chromosome of *P. putida* KT2440 using tri-parental mating with *E. coli* HB101 as helper strain, as well as the donor strains *E. coli* CC118λ*pir* pEMG_ΔPP1023 and *E. coli* CC118λ*pir* pEMG_ΔPP1444, respectively. Successful homologous integration of the vector DNA was confirmed by PCR (data not shown). The successful genomic deletions were confirmed by PCR.

2.3. Medium

P. putida strains were grown in a defined mineral medium (M9) consisting of (per liter) 12.8 g $Na_2HPO_4 \cdot 7H_2O$, 3 g KH_2O_4, 1 g NH_4Cl, and 0.5 g NaCl. This basic solution was autoclaved and subsequently supplemented with 0.12 g of $MgSO_4 \cdot H_2O$, trace elements (mg L^{-1}): 6.0 $FeSO_4 \cdot 7H_2O$, 2.7 $CaCO_3$, 2.0 $ZnSO_4 \cdot H_2O$, 1.16 $MnSO_4 \cdot H_2O$, 0.37 $CoSO_4 \cdot 7H_2O$, 0.33 $CuSO_4 \cdot 5H_2O$, 0.08 H_3BO_3, and 18.5 g L^{-1} glucose (all filter-sterilized).

2.4. Cultivations

2.4.1. Flask experiments

P. putida strains, kept as frozen stock in 25% glycerol at −80 °C, were streaked on Luria Bertani agar plates and incubated for one day at 30 °C. Single colonies were then picked from the plate and inoculated it into a 50 mL shake flask containing 10 mL of the above described medium and incubated overnight under aerobic

Table 1
Primers used in this study for the construction of defined *P. putida* deletion strains.

Primer	Sequence (5′-3′)
UP1023XbaIF	TCTAGACGGCCAGTACATTGCCGGCT
UP1023ClaIR	ATCGATGGTCCGCCAGTTCATGAGCCTT
DOWN1023ClaIF	ATCGATGGCCTGTCGATGACCCGTTCGCT
DOWN1023XmaIR	CCCGGGGGCGGTAGCCCAGGGCATAGC
UP1444KpnIF	GGTACCGGTTTCAAGCTCAGCGGCAG
UP1444R	CGACCGAAACGCGACACAAGGGTTAGAAACTGCTCTGGATCTTCAGG
DOWN1444F	GATCCAGAGCAGTTTCTAACCCTTGTGTCGCGTTTCGGTCGCGCAGC
DOWN1444EcoRIR	GAATTCGACCTCGTCGGTCGGCTCGG