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Genetic programming of catalytic *Pseudomonas putida* biofilms for boosting biodegradation of haloalkanes

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ABSTRACT

Bacterial biofilms outperform planktonic counterparts in whole-cell biocatalysis. The transition between planktonic and biofilm lifestyles of the platform strain *Pseudomonas putida* KT2440 is ruled by a regulatory network controlling the levels of the trigger signal cyclic di-GMP (c-di-GMP). This circumstance was exploited for designing a genetic device that over-runs the synthesis or degradation of c-di-GMP – thus making *P. putida* to form biofilms at user's will. For this purpose, the transcription of either *yedQ* (diguanylate cyclase) or *yhjH* (c-di-GMP phoshodiesterase) from *Escherichia coli* was artificially placed under the tight control of a cyclohexanone-responsive expression system. The resulting strain was subsequently endowed with a synthetic operon and tested for 1-chlorobutane biodegradation. Upon addition of cyclohexanone to the culture medium, the thereby designed *P. putida* cells formed biofilms displaying high dehalogenase activity. These results show that the morphologies and physical forms of whole-cell biocatalysts can be genetically programmed while purposely designing their biochemical activity.

1. Introduction

Pseudomonas putida is an environmental, Gram-negative bacterium that ubiquitously colonizes soil and metabolizes a broad range of natural and synthetic organic compounds (Martins dos Santos et al., 2004; Nelson et al., 2002; Nikel, 2012; Nikel et al., 2014a, (2015a); Timmis, 2002). Because of their remarkable ability to degrade pollutants, *P. putida* strains are being extensively studied for a number of industrial and environmental uses (Martínez-García et al., 2014a, 2014b, 2014c, 2014d; Poblete-Castro et al., 2012). In particular, strain KT2440, a derivative of the toluene-degrading wild-type *P. putida* strain mt-2 (Bagdasarian et al., 1981), exhibits unusual (and attractive) traits such as growth on a broad range of recalcitrant compounds, tolerance to solvents, antibiotics, and heavy metals, and shows high reducing power availability. These qualities make *P. putida* a platform of choice for engineering harsh redox reactions on organic substrates (Nikel, 2012). A large variety of genetic tools have been developed over the years to analyze, clone, and manipulate the genes (and even the whole genome) of this bacterium for a suite of environmental and industrial applications (Martínez-García and de Lorenzo, 2012; Martínez-García et al., 2015; Nikel and de Lorenzo, 2013a, 2013b). Still, any catalytic activity engineered in a given bacterial host has to be delivered to the cognate substrate in a specific physical format that often determines the whole effectiveness of the process. Planktonic cells are usually very sensitive to the stressful conditions prevalent in a stirred tank bioreactor (Delvigne and Goffin, 2013), or other catalytic scenarios where cells are dispersed in a much larger volume of an aqueous medium. In contrast, biofilms of catalytic bacteria are advantageous over suspended cells not only in that physical proximity limits the unwanted diffusion of intermediates (Rosche et al., 2009), but also in that they exhibit a superior tolerance to physicochemical insults and harsh reaction conditions (D'Alvise et al., 2010; Karande et al., in press). Moreover, bacteria within a biofilm are known to display lower cell-to-cell variability than their planktonic counterparts and endure different types of stresses (Ackermann, 2013; Nikel et al., 2014b). Finally, cells adhered to hard surfaces, e.g., on porous solid carriers such as Raschig rings, allow for the operation of catalysts in packed column reactors (Halan et al., 2012; 2014).

Alas, most Laboratory-adapted *P. putida* strains produce thin and somewhat weak biofilms (Gjermansen et al., 2005, 2010). It is often the case that recurrent re-isolation of the same strain from liquid media inadvertently results in the enrichment of mutants that stick less to surfaces. Furthermore, the occurrence of biofilms is subject to a complex regulatory network that rotates around controlling intracellular levels of the secondary messenger cyclic diguanosine monophosphate (c-di-GMP). This small molecule governs the transition from planktonic to biofilm lifestyle (and

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back) in a wide range of bacteria (Römling et al., 2013; Schirmer and Jenal, 2009), including *P. putida* and other Pseudomonads (Österberg et al., 2013; Tolker-Nielsen et al., 2000; Ueda and Saneoka, 2014). This situation happens by regulating cell adhesiveness through inhibition of flagellar movement and production of sticky exopolymeric substances (EPS) (Fazli et al., 2014; Flemming and Wingender, 2010). More than 40 proteins encoded in the *P. putida* genome contain GGDEF domains (diguanylate cyclase needed for c-di-GMP formation) or EAL domains (c-di-GMP phosphodiesterase) (Ausmees et al., 2001; Gjermansen et al., 2006; Simm et al., 2004), making biofilm formation an archetypal polygenic phenotype. When the merged cyclase activity is high, c-di-GMP increases, matrix material and EPS are produced and biofilm is formed (Hengge, 2009; Liang, 2015). In contrast, elevated phosphodiesterase activity leads to dispersal of the surface-stuck bacteria. This regulatory complexity results on very limited options for artificially controlling biofilm development (Caly et al., 2015; Schuster and Markx, 2014), as it would be desirable for engineering predictable catalysts and their formulation for industrial bioprocesses.

In this work, we have engineered two genes from *Escherichia coli*, encoding active GGDEF and EAL domains, to be transcribed in *P. putida* under the control of a cyclohexanone-inducible, tightly-regulated expression system purposely designed to suit this objective (Benedetti et al., 2015). We show below that the resulting construct altogether took over the endogenous regulatory network of c-di-GMP and allowed for controlled biofilm formation according to specific catalytic needs, e.g., for biodegradation of the environmental pollutant 1-chlorobutane. Specifically, we demonstrate that dehalogenation of the haloalkane increased by >2-fold by delivering the biochemical activity from the thereby induced biofilm as compared to the same setup with planktonic cells. These results accredit not only that the catalytic efficiency of a whole-cell biotransformation can change dramatically with its planktonic versus biofilm lifestyle, but also that the physical form and the morphology of the active biomass can be genetically programmed by implanting synthetic devices on endogenous regulatory networks.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Bacterial strains used in this study are listed in Table 1. *E. coli* HB101, carrying plasmid pRK600, was used as a helper for triparental mating, and it was routinely grown in LB medium (Green and Sambrook, 2012). *P. putida* was incubated at 30 °C in M9 minimal medium (Green and Sambrook, 2012) added with $MgSO_4$ at 2 mM and either glucose or succinate at 0.4% (w/v) as the sole carbon source. Kanamycin (Km, 50 μg ml^{-1}), streptomycin (Sm, 80 μg ml^{-1}), cloramphenicol (Cm, 30 μg ml^{-1}), gentamicin (Gm, 10 μg ml^{-1}), and/or tetracycline (Tc, 15 μg ml^{-1}) were supplemented to the culture media when required. For haloalkane dehalogenase assays, cells were grown in low-chloride M9 minimal medium (M9C medium), containing $(NH_4)_2SO_4$ instead of NH_4Cl and Na_2SO_4 instead of NaCl (Nikel and de Lorenzo, 2013a). Growth was estimated by measuring the optical density at 600 nm (OD_{600}) after diluting the culture whenever needed. Flask cultures were set in 125-ml Erlenmeyer flasks containing culture medium up to one-fifth of their nominal volume. Static cultures were incubated with no shaking, and shaken-flask cultures were agitated at 170 rpm. Unless stated otherwise in the text, cyclohexanone and isopropyl-β-D-1-thiogalactopyranoside (IPTG) were added to the cultures to induce the ChnR- and LacI^Q-based expression systems, respectively, at 1 mM.

2.2. Assessment of colony morphology in Congo Red medium plates

The colony morphology of selected *P. putida* recombinant strains was assessed in Congo Red plates, i.e., M9 minimal medium

Table 1
Bacterial strains and plasmids used in this study.

Bacterial strain	Relevant characteristics[a]	Reference
Escherichia coli		
HB101	Helper strain; F$^-$ λ^- *hsdS20*(r_B– m_B–) *recA13 leuB6*(Am) *araC14* Δ(*gpt-proA*)*62 lacY1 galK2*(Oc) *xyl-5 mtl-1 thiE1 rpsL20*(Sm^R) *glnX44*(AS)	Boyer and Roulland-Dussoix (1969)
Pseudomonas putida		
KT2440	Wild-type strain derived from *P. putida* mt-2, cured of the pWW0 TOL plasmid	Bagdasarian et al. (1981)
KT2440 Δall-Φ	*P. putida* KT2440 derivative with prophage 1, prophage 4, prophage 3, and prophage 2 deleted	Martínez-García et al. (2014c)
KT2440 Δall-Φ GFP	*P. putida* KT2440 Δall-Φ derivative constitutively expressing *gfp2* as a chromosomal Tn*7* insertion ($P_{A1/04/03}$→ *gfp2*), Gm^R	Martínez-García et al. (2014c)
KT-BG	*P. putida* KT2440 derivative carrying a BCD2-*msf●GFP* transcriptional fusion as a chromosomal Tn*7* insertion, Gm^R	Zoebel et al. (in press)
KT-BG-PelA	*P. putida* KT-BG derivative carrying a P_{pelA}-BDC2-*msf●GFP* transcriptional fusion as a chromosomal Tn*7* insertion (cyclic diguanosine monophosphate reporter strain), Gm^R	This study
Plasmids		
pRK600	Cm^R; *oriV*(ColE1), *tra*$^+$ *mob*$^+$ functions from plasmid RK2	Keen et al. (1988)
pSEVA2311	Km^R; *oriV*(pBBR1), *chnR*, P_{chnB}	Benedetti et al. (2015)
pSEVA2311M	Km^R; *oriV*(pBBR1), *chnR*, P_{chnB} → *msf●GFP*	Benedetti et al. (2015)
pSEVA424	Sm^R; *oriV*(RK2), *lacI^Q*, P_{trc}	Silva-Rocha et al. (2013)
pYedQ	Tc^R; vector pRK404 (Scott et al., 2003) carrying *yedQ* from *E. coli*	Ausmees et al. (2001)
pYhjH	Tc^R; vector pBBR1-MCS3 (Kovach et al., 1995) carrying *yhjH* from *E. coli*	Gjermansen et al. (2006)
pSYedQ	Km^R; *oriV*(pBBR1), *chnR*, P_{chnB}→*yedQ*	This study
pSYhjH	Km^R; *oriV*(pBBR1), *chnR*, P_{chnB}→*yhjH*	This study
pSLacH	Km^R; *oriV*(pBBR1), *lacI^Q*, P_{trc}→*yhjH*	This study
pBG	Km^R Gm^R; *oriV*(R6K), carries the Tn*7* ends Tn7L and Tn7R and a BCD2-*msf●GFP* fusion	Zoebel et al. (in press)
pBG-PelA	Km^R Gm^R; pBG carrying the P_{pelA} promoter as a P_{pelA}-BDC2-*msf●GFP* transcriptional fusion	This study
pSEVA4413	Sm^R; *oriV*(ColE1), P_{EM7} promoter	This study
pAHDO	Sm^R; *oriV*(RK2), *xylS*, *Pm*→AHDO	Nikel and de Lorenzo (2013a)
pSAHDO	Sm^R; *oriV*(ColE1), P_{EM7}→AHDO	This study

[a] Abbreviations used in this table are as follows: Cm, chloramphenicol; Km, kanamycin; Gm, gentamycin; Rif, rifampicin; Sm, streptomycin; Tc, tetracycline; *gfp2*, gene encoding an enhanced green fluorescent protein; *msf●GFP*, gene encoding the monomeric and superfolder green fluorescent protein (msf●GFP); BCD2, optimized translational coupler; AHDO, alkyl halide degradation operon.