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Development of a high efficiency integration system and promoter library for rapid modification of *Pseudomonas putida* KT2440☆

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ABSTRACT

Pseudomonas putida strains are highly robust bacteria known for their ability to efficiently utilize a variety of carbon sources, including aliphatic and aromatic hydrocarbons. Recently, *P. putida* has been engineered to valorize the lignin stream of a lignocellulosic biomass pretreatment process. Nonetheless, when compared to platform organisms such as *Escherichia coli*, the toolkit for engineering *P. putida* is underdeveloped. Heterologous gene expression in particular is problematic. Plasmid instability and copy number variance provide challenges for replicative plasmids, while use of homologous recombination for insertion of DNA into the chromosome is slow and laborious. Further, most heterologous expression efforts to date typically rely on overexpression of exogenous pathways using a handful of poorly characterized promoters. To improve the *P. putida* toolkit, we developed a rapid genome integration system using the site-specific recombinase from bacteriophage Bxb1 to enable rapid, high efficiency integration of DNA into the *P. putida* chromosome. We also developed a library of synthetic promoters with various UP elements, −35 sequences, and −10 sequences, as well as different ribosomal binding sites. We tested these promoters using a fluorescent reporter gene, mNeonGreen, to characterize the strength of each promoter, and identified UP-element-promoter-ribosomal binding sites combinations capable of driving a ~150-fold range of protein expression levels. An additional integrating vector was developed that confers more robust kanamycin resistance when integrated at single copy into the chromosome. This genome integration and reporter systems are extensible for testing other genetic parts, such as examining terminator strength, and will allow rapid integration of heterologous pathways for metabolic engineering.

1. Introduction

Given the world's finite amount of fossil fuels, the need for renewable sources of fuels and commodity chemicals is becoming increasingly important. To address this issue, much work has gone into the production of these chemicals from biological sources. The majority of research to date has focused on the conversion of cellulosic biomass from plants into value added chemicals. However, recent reports have shown that biorefineries will need to valorize lignin streams to be economically viable (Davis et al., 2013). Lignin comprises 15–40% of terrestrial plant biomass (Ragauskas et al., 2014). Unlike cellulosic biomass, which is largely comprised of homogenous mixture of polysaccharides, lignin is a heterogeneous and interlinked polymer comprised of aromatic compounds which has limited its use in biorefineries. Currently, lignin streams in most biorefineries are slated for combustion for the generation of process heat and electricity. Several groups have used a combination of chemical and/or biological means to depolymerize lignin, and some have taken advantage of aromatic-consuming bacteria, such as *Pseudomonas putida* species, to transform lignin streams into value added chemicals (Linger et al., 2014; Rahimi et al., 2014; Vardon et al., 2015; Zakzeski et al., 2012).

A major limiting factor for rapid metabolic engineering of *Pseudomonas putida* is the availability of tools for sophisticated genetic engineering. The primary tools currently used for directed genetic engineering in *P. putida* KT2440, replicating plasmids such as derivatives of the pBBR family of plasmids (Kovach et al., 1995) and homologous recombination-based gene replacement technologies (Johnson and Beckham, 2015; Schafer et al., 1994), have many

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shortcomings. Replicating plasmids typically require constant selection for maintenance, have highly variable copy number (Lin-Chao and Bremer, 1986; Paulsson and Ehrenberg, 2001), and often come with a fitness cost (Mi et al., 2016). Current homologous recombination-based technologies rely on inefficient plasmid integration by host DNA repair enzymes, leading to low transformation rates. Recombination of these plasmids by single cross-over events leads to genome structures that are inherently unstable, allowing deletion or integration of DNA sequences, but simultaneously makes them unsuitable for rapid prototyping. In an attempt to address some of these shortcomings, *P. putida* genetic engineering tools have been expanded to include oligonucleotide (Aparicio et al., 2016), λ Red/Cre (Luo et al., 2016), and meganuclease (Martinez-Garcia and de Lorenzo, 2011) recombineering technologies.

Site-specific recombinases are enzymes that catalyze recombination between two specific sequences of DNA (for a review see (Brown et al., 2011)). Recombinases Flp and Cre are commonly used in molecular genetics, but these enzymes recognize identical sites and perform reversible recombination between the sites. Serine recombinases such as ΦC31 integrase, on the other hand, natively catalyze unidirectional phage genome integration through the recombination between *attP* (phage genome) and *attB* (bacterial genome) sequences, generating distinct *attL* and *attR* sequences in the process. Unlike the unidirectional λ phage integrase from *E. coli* that requires the *E. coli* protein IHF for recombination, these serine recombinases do not require any host factors and have been used for chromosomal insertion of heterologous DNA in organisms across the tree of life (Brown et al., 2011; Guss et al., 2008; Keravala et al., 2006; Siuti et al., 2014; Thomson et al., 2012; Xu and Brown, 2016). In addition to ΦC31 integrase, other serine recombinases have been identified and characterized, including the determination of the corresponding *attB* and *attP* sites enabling these systems to also be developed as genetic tools (Brown et al., 2011).

In other organisms, tuning protein expression can be very important for achieving increased yields from engineered pathways (Alper et al., 2005; Jones et al., 2015; Xu et al., 2013), but there are no well-characterized promoter or RBS libraries in *P. putida* to enable rational tuning of protein expression. Previous engineering efforts in *P. putida* have primarily relied on the lac family promoters (lac, lacUV5, tac, trc) from *E.coli* (Borrero-de Acuna et al., 2014; Johnson and Beckham, 2015; Meijnen et al., 2008, 2009; Nielsen et al., 2009; Vardon et al., 2015), with a few using native promoters such as Pm (Gemperlein et al., 2016), rrn (Wang et al., 2010), or PP_1099 promoter (Lin et al., 2016). The relative expression of the lac-family promoters in *P. putida* remains unknown, and the use of native promoters increases the likelihood of cryptic promoter regulation. To address these gaps in genetic tools, we tested serine recombinases to develop a high efficiency integration system to characterize a library of genetic elements to enable rational tuning of protein expression in *P. putida*.

2. Materials and methods

2.1. Plasmid construction

Phusion® High Fidelity Polymerase (Thermo Scientific) and primers synthesized by Integrated DNA Technologies (IDT) or Eurofins Genomics were used in all PCR amplifications for plasmid construction. Plasmids were constructed using NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs – NEB) or T4 DNA ligase (NEB) according to manufacturer's instructions. Plasmids were transformed into either competent Top10 (Life Technologies), NEB 5-alpha F'I^q (NEB), or Epi400 (Epicentre Biotechnologies) *Escherichia coli* according to manufacturer's instructions. Transformants were selected on LB (Miller) agar plates containing 50 mg/L kanamycin sulfate for selection and incubated at 37 °C. Plasmids were constructed using a combination of ligation of phosphorylated oligonucleotides, DNA synthesis by GenScript and IDT and Gibson Assembly. Sequences of all plasmids were confirmed using Sanger sequencing performed by GenScript or Eurofins Genomics. Annotated plasmid sequences are provided in Supplemental File 1.

2.2. Pseudomonas putida transformation and strain construction

2.2.1. Construction of hsdR deletion and integrase replacement strains by homologous recombination

P. putida KT2440 was used as a wild-type parent strain for all strains. *hsdR* deletion and integrase replacement strains were constructed using the pK18mob-sacB kanamycin resistance/sucrose resistance selection/counter-selection marker system (Marx, 2008) as described in detail previously (Johnson and Beckham, 2015). Briefly, transformations were carried out by electroporation of ~500–1000 ng of plasmid DNA into KT2440 cells. Transformed colonies were selected by growth overnight at 30 °C on LB agar medium with 50 μg/mL kanamycin sulfate. Resulting transformed colonies were single colony purified and incubated overnight at 30 °C on LB agar medium containing 50 μg/mL kanamycin sulfate to eliminate residual untransformed cells from being transferred. For counter-selection, colonies were streaked onto YT + 25% sucrose plates (10 g/L yeast extract, 20 g/L tryptone, 250 g/L sucrose and 18 g/L agar) and incubated overnight at 30 °C. Resulting colonies typically have recombined to remove *sacB* from the genome, as its expression causes a significant growth defect in the presence of sucrose. However, cells containing *sacB* can grow very slowly in the presence of sucrose, so colonies were streaked for isolation again on YT + 25% sucrose plates and incubated at 30 °C overnight to reduce the possibility of transferring cells in which *sacB* remains in the genome. The resulting colonies (typically 20) were cultured in LB broth overnight at 30 °C and screened for either the deletion of *hsdR* or replacement of *hsdR* with a P_{tac}-integrase/*attB* cassette by colony PCR and kanamycin sensitivity. *P. putida* integrase strains are listed in Table 1.

2.2.2. Plasmid transformation assays

Plasmid transformation assays were performed using a variation of the transformation procedure described in Section 2.2.1. 600 ng of each plasmid was transformed into either wild-type, JE90, JE1643-1646 (Table 1) electrocompetent cells. Following electroporation, cells were resuspended in 950 μl SOC, transferred to a microfuge tube, and incubated at 30 C to allow recovery. Due to the low efficiency of homologous recombination, all of the recovery volume was plated for pΔPP_0545. For the remaining plasmids, several fractions of the recovery cultures were plated and enumerated. For Fig. 1 assays, all samples were plated on LB agar supplemented with 50 μg/mL kanamycin sulfate, incubated for 24 h at 30 °C, then enumerated. For Fig. 3 assays, samples were plated on LB agar media supplemented with 15 or 50 μg/mL kanamycin sulfate, incubated for 16 h at 30 °C, enumerated, incubated for an additional 24 h (total 40 h) again at 30 °C, and enumerated a second time. Correct insertion of DNA into the *attB* site was confirmed using multiplex PCR as described in Supplemental Figs. 2 and 3. Analogous to the *E. coli* CRIM system (Haldimann and Wanner, 2001b), multiplex PCR using a 4 primer set (primers 1–4 Supplemental Figs. 2 and 3) were used to verify plasmid integration. Primers 1 and 2 flank the *attB* and primers 3 and 4 flank the *attP*. After site-specific integration, primers 1 and 4 flank the resulting *attL* and

Table 1
P. putida strains constructed for bacteriophage serine integrase testing.

Strain	Integrase/attB	Genotype
JE1646	None	Δ*hdsR*
JE1644	FBT1	Δ*hdsR*::Ptac:*ΦBT1int-attB*
JE1645	FC1	Δ*hdsR*::Ptac:*ΦC1int-attB*
JE1643	RV	Δ*hdsR*::Ptac:*RVint-attB*
JE90	BxB1	Δ*hdsR*::Ptac:*BxB1int-attB*