Construction of a BioBrick™ compatible vector system for

Rhodococcus

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ABSTRACT

Throughout the past decade, the field of synthetic biology has grown rapidly. By using assembly platforms such as BioBricks™, scientists can quickly and easily build gene circuits or multi-step pathways. One limitation, however, is that most of these parts were designed and characterized with *Escherichia coli* as the target chassis. As a consequence, there exists a lack of standardized and well characterized or BioBrick™ compatible plasmid backbones that replicate in other potential non-model chassis organisms. The Gram-positive bacteria of the genus *Rhodococcus* represent an interesting chassis for biotechnological applications due to their tremendous metabolic capabilities. In this report we describe our progress toward developing a BioBrick™ compatible plasmid system for *Rhodococcus*. We demonstrate its utility for heterologous protein expression through flow cytometric analysis of the *lac* promoter in the oleaginous strain *Rhodococcus opacus* PD630.

Keywords

Synthetic biology, *Rhodococcus*, BioBrick™

1. INTRODUCTION

Efficiently and quickly assembling multi-step pathways or gene circuits using standardized genetic parts is one of the major principles of synthetic biology. The BioBrick™ paradigm, for example, facilitates rapid assembly of standardized biological parts, such as promoters, ribosome binding sites, and genes to produce functional genetic constructs (Knight, 2003). Additionally, composite-type parts like ‘Protein Generators’ (http://parts.igem.org/Help:Protein_Generators) can be joined into a single plasmid consecutively to produce pathways. Searching the Registry of Standard Biological Parts (<http://parts.igem.org>) however, reveals that the majority of bacterial synthetic biology parts were developed using *Escherichia coli* as the chassis. One factor leading to the lack of parts for non-model bacteria as potential chassis is the scarcity of standardized or BioBrick™ compatible plasmid backbones that can replicate in these bacteria.

Recently, several synthetic biology platforms have appeared for an increasing number of industrially or medically relevant Gram-positive and Gram-negative bacteria: *Acinetobacter baylyi* ADP1 (Murin et al., 2011), *Corynebacterium glutamicum* (Ravasi et al., 2012; Kang et al., 2014), *Ralstonia eutropha* (Bi et al., 2013), *Bacillus subtilis* (Radeck et al., 2013), *Rhodobacter sphaeroides* (Tikh et al., 2014), *Streptococcus pneumoniae* (Sorg et al., 2014), and *Synechocystis* sp. PCC 6803 (Pinto et al., 2015). Although not all of these systems are BioBrick™ compatible, they represent advances in standardization of platforms that fit into a synthetic biology framework.

Species of the genus *Rhodococcus* are Gram-positive bacteria capable of surviving on a wide range of carbon sources, including environmental pollutants, and hold

tremendous potential as whole cell biocatalysts (Bell et al., 1998; van der Geize and Dijkhuizen, 2004; Martínková et al., 2009).and more recently as robust chassis organism for lignin valorization (Kosa and Rgauskas, 2014, Yoneda et al., 2016). For example, the species *R. opacus* has been used for the stereoselective bioconversion of indene to indandiol, which can be used as a precursor for the production of the HIV protease inhibitor, Crixivan® (Chartrain et al., 1998). As another example, recombinant *R. opacus* has been used as a lipophilic enzyme capsule to reduce ketoisophorone to (6R)-levodione (89.2% enantiopurity) in an organic solvent solution (Tsuji et al., 2014).

Rhodococcus opacus PD630 is an oleaginous strain capable of accumulating between 76-80% of its cell dry weight as triacylglycerides (Alvarez et al., 1996; Gouda et al., 2008; Alvarez and Steinbüchel, 2010). The genome was recently sequenced and annotated, showing that this strain contains 9 plasmids and an extremely diverse set of metabolic pathways (Holder et al., 2011; Chen et al., 2013). This strain is also genetically tractable and easily engineered for heterologous protein expression. For example, while unable to naturally metabolize xylose, *R. opacus* PD630 engineered with xylose isomerase allowed for high-level production of fatty acids from xylose as the sole carbon source (Xiong et al., 2012; Kurosawa et al., 2013). Moreover, *R. opacus* PD630 can survive on lignocellulosic biomass such as crop residues while producing high yields of fatty acids, and therefore will serve as a suitable host for biodiesel or bio-based oleochemical production (Alvarez and Steinbüchel, 2002; Wang et al., 2014).

A handful of plasmids are currently available for engineering *Rhodococcus* as well as a thiostrepton inducible promoter system (Shao et al., 1995; Kalscheuer et al., 1999; Veselý et al., 2003; Dong et al., 2004; Nakashima and Tamura, 2004; Knoppová et al.,

2007). In this brief report we share our progress toward developing a BioBrick™ compatible platform for *Rhodococcus*.

2. MATERIALS AND METHODS

2.1. Enzymes and chemicals

Enzymes for general molecular biology were obtained from New England Biolabs (NEB) and used as specified by the suppliers. Site-directed mutagenesis PCR was conducted using the Agilent QuickChange™ Site-Directed Mutagenesis Kit following the supplied protocols. Chemicals were purchased from Sigma-Aldrich. Pre-made nutrient broth (NB) powder used to prepare the growth medium for *R. opacus* PD630 was purchased from HiMedia Laboratories.

2.2. Bacterial strains and cell growth conditions

Escherichia coli JM109 (DE3) from Promega was used for shuttle plasmid replication and for all molecular biology work. All cultures were grown in Luria-Bertani (LB) medium at 37°C containing 30 µg/ml kanamycin. *Rhodococcus opacus* PD630 (DSMZ strain No. 44193) was obtained from the German Type Strain Collection (DSMZ) and grown in NB (20 g/L) at 30°C containing kanamycin at 50 µg/mL (solid media) or 50-500 µg/mL (liquid media).

Preparation of electrocompetent *R. opacus* PD630 cells and electroporation protocol were as reported by Kalscheuer et al., 1999, except that a Bio-Rad Micropulser was used with a 0.2 cm cuvette pulsed at 2.0 kV. Time constants and transformation efficiency were similar to that reported by Kalscheuer et al., 1999.

2.3. Plasmids and vector construction

The *E. coli-R. erythropolis* shuttle vector pSRK21 was obtained from the Institute of Microbiology, Academy of Sciences of the Czech Republic (Veselý et al., 2003). To generate pSRKBB, all restriction sites incompatible with BioBrick™ [RFC10] and our optimized BioBrick™ compatible system (Vick et al., 2011) were removed via site-directed mutagenesis using the primers indicated in Table S1. Next, a linear fragment of the entire plasmid backbone flanked with *EcoRI* and *PstI* sites, but excluding the original multiple-cloning site and insert region of pSRK21, was created by PCR using the primers indicated in Table S1. The amplified product as well as the cassette from pUCBB-empty (Vick et al., 2011) were digested with *EcoRI* and *PstI*. The digested fragments were purified by DNA gel extraction and ligated using T4 DNA ligase, creating pSRKBB-empty. The same procedure was followed using pUCBB-eGFP (Vick et al., 2011) to create pSRKBB-eGFP. The sequence of the constructs was verified by DNA sequencing. pSRKBB-empty has been deposited in Addgene (#59449) and annotated plasmid maps are available as supplemental information (pSRKBB-empty.gb and pSRKBB-eGFP.gb).

2.4. Flow cytometry

Flow cytometry analysis was performed using a BD Biosciences FACScalibur™ equipped with a 488 nm argon laser and a 515-545 nm emission filter at a low flow rate. Samples from two independent cultures for each vector were taken from the cultures at 24 hours after inoculation (approximately mid-log phase) and were diluted 1000-fold into

phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂PO₄, 1.8 mM KH₂PO₄, pH 7.0) prior to analysis. Sample collection rate was maintained around 1000 events per second for a total of 50,000 events. Data collected were analyzed using FlowJo version 7.5 (<http://www.flowjo.com>).

To prepare the cultures, two individual colonies from freshly transformed *R. opacus* PD630 cells with either pSRKBB-empty or pSRKBB-eGFP were inoculated into 4 mL of NB medium containing 50 µg/mL kanamycin and grown at 30°C, 215 RPM until mid-log phase (OD₆₀₀ 0.5-0.8). The cultures were then transferred to 50 mL of NB medium in baffled flasks and incubated at 30°C, 215 RPM until mid-log phase, which required about 24 hours. The concentration of kanamycin varied from 50-500 µg/mL.

3. RESULTS

3.1. Vector features and construction

Currently, there is no platform available for *Rhodococcus* that facilitates rapid assembly of gene circuits or multi-step pathways. Therefore we constructed plasmid pSRKBB, which is compatible with BioBrick™ RFC[10] as well as the optimized BioBrick™ compatible system previously developed in our lab (Vick et al., 2011). Our basic cloning plasmid pSRKBB-empty (Figure 1) was derived from the backbone of pSRK21, an *E. coli*-*R. erythropolis* shuttle vector, which was itself derived from pSR1, the cryptic plasmid of *C. glutamicum* ATCC13058 (Yoshihama et al., 1985; Archer and Sinskey, 1993; Veselý et al., 2003). We removed all BioBrick™ incompatible restriction sites from the plasmid's backbone and confirmed that pSRKBB-empty replicates in *R. opacus* PD630 (strain DSMZ No. 44193). Cells electroporated with pSRKBB-empty

were selectable using kanamycin, which is also the resistance marker used for plasmid propagation in *E. coli*.

Figure 1

The plasmid pSRKBB-empty contains a modular cassette that our laboratory previously developed (Vick et al., 2011). The cassette is compatible with the BioBrick™ RFC[10] standard, although the *NotI* sequence normally found in the prefix and suffix has been moved downstream into a multiple cloning site. Briefly, using restriction digests this cassette allows users to easily swap promoters/RBS, genes, add 5' or 3' tags, and contains a C-terminal histidine-tag sequence (Figure 1). Because pSRKBB-based plasmids are BioBrick™ compatible, complex circuits or multigene pathways can easily be stacked onto this backbone. For more details regarding pSRKBB-empty we have provided the full sequence and annotated plasmid map in GenBank format (Supplemental file pSRKBB.gb). Furthermore, this plasmid has been deposited into the Addgene plasmid repository (plasmid ID 59449).

3.2. Characterization of the *lac* promoter

The *lac* promoter and derivatives such as the *tac* or *trc* promoter are commonly used constitutive promoters in synthetic biology and microbial engineering. These promoters were shown to function constitutively in *Rhodococcus* and have been used to drive expression of polyhydroxyalkanoic acid synthase, β -glucosidase and xylose isomerase (Kalscheuer et al., 1999; Veselý et al., 2003; Hetzler and Steinbüchel, 2013; Xiong et al., 2012; Kurosawa et al., 2013). However, quantitative data showing the strength of gene expression using the *lac* promoter in *Rhodococcus* does not exist.

Engineering well-regulated gene circuits and optimizing complex pathways requires detailed information regarding the level of gene expression. In this report we demonstrate the utility of pSRKBB as a tool for engineering *Rhodococcus* by characterizing the *lac* promoter. We inserted enhanced green fluorescent protein (eGFP) (Thastrup et al., 1997) downstream of the *lac* promoter in pSRKBB to generate pSRKBB-eGFP (Supplemental file pSRKBB-eGFP.gb). The new plasmid containing eGFP was electroporated into *R. opacus* PD630 and the fluorescent cell population was monitored by flow cytometry.

Previous work using the *lac* promoter to drive protein expression in engineered *Rhodococcus* was also conducted using plasmids that carried a kanamycin resistance marker (Vesely et al., 2003; Hetzler and Steinbüchel, 2013). In those studies, cell cultures were grown using a concentration of 50 $\mu\text{g mL}^{-1}$ kanamycin, therefore we chose that condition as our starting point. Using flow cytometry, we observed a bimodal distribution when cells were grown to the middle of exponential phase. Over 50% of the cell counts were observed in the autofluorescent range, as determined by comparison to *R. opacus* PD630 cells that did contain eGFP. The remainder of the cell population showed a strong, asymmetric fluorescent peak (Figure 2). The level of fluorescence intensity observed for *R. opacus* PD630 containing pSRKBB-eGFP was on the same order of magnitude as *E. coli* JM109 with the same plasmid (Figure S1). Similarly, we also noted an asymmetric fluorescent peak in *E. coli*.

Figure 2

In a prior study, it was reported that *R. erythropolis* grew robustly in concentrations of kanamycin as high as 250 $\mu\text{g mL}^{-1}$ (Kilbane II, 2006). We wondered if the population

distribution was a result of inadequate selection pressure, therefore we decided to see if we could shift the distribution of the fluorescent population toward a higher percentage by increasing the kanamycin concentration. We found that we could drive the fluorescent cell population as high as 70% by increasing kanamycin to 300 $\mu\text{g mL}^{-1}$ (Figure 2 B). At 400 and 500 $\mu\text{g mL}^{-1}$ kanamycin, cells grew well but we observed loss of fluorescence.

4. DISCUSSION

We successfully engineered a BioBrick™ compatible plasmid system for *Rhodococcus* enabling robust heterologous protein expression from the *lac* promoter in the oleaginous strain *Rhodococcus opacus* PD630. By increasing the kanamycin concentration in the culture medium to 300 $\mu\text{g mL}^{-1}$, the fluorescent cell population could be increased to 70% without impacting cell growth. Further increases in kanamycin concentration, however, resulted in a loss of fluorescence. This may be a response to selection pressure for a population that drives metabolic flux toward prioritization of other traits in response to kanamycin stress, thereby leading to a loss of observed fluorescence.

Hetzler and Steinbüchel engineered *Rhodococcus opacus* PD630 to survive on cellobiose as the sole carbon source by expression of β -glucosidase using the *lac* promoter (Hetzler and Steinbüchel, 2013). In this case, kanamycin was used as a selection marker, however the requirement for β -glucosidase essentially functioned as a second selection marker therefore creating pressure to maintain expression of this trait.

In our case the recombinant gene, eGFP, provided no selective pressure. As mentioned above, it is possible that in our experiments *R. opacus* has deprioritized metabolic flux toward synthesis of GFP in response to overcome stress by kanamycin. This could occur through the mobilization of insertion sequences (IS). Previous research has shown that in *E. coli*, chromosomal regions coding for IS or transposable elements can migrate into recombinant plasmids and disrupt gene or plasmid stability (Chen and Yeh, 1997; Prather et al., 2006). However deletion of these elements from the chromosome removed all incidents of plasmid disruption (Pósfai et al., 2006). The strain of *R. opacus* PD630 used in this work (and others) was an unmodified wild-type strain and likely has similar elements. Generation of an engineered strain with such elements deleted may therefore also increase maintenance of plasmid function under high stress conditions.

In conclusion, aside from the *lac* promoter and derivatives (e.g. *tac* or *trc* promoters), only a handful of others have been shown to function in *Rhodococcus*: *tipA* (Dong et al., 2004), *ace* (Hetzler et al., 2013), *dapA* (Knoppová et al., 2007), and *p45* (Veselý et al., 2003; Kilbane II, 2006). Using the developed modular plasmid pSRKBB, researchers can now easily expand the library of characterized promoters available for *Rhodococcus*. Furthermore, this can be accomplished in a manner that allows biological parts to be compatible with the BioBrick™ standard.

The *Rhodococci*, in particular *R. opacus* PD630, hold tremendous potential as industrial biocatalysts. We developed pSRKBB to allow us and others to quickly assemble enzymatic pathways in *Rhodococcus* using the BioBrick™ framework.

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FIGURE LEGENDS

Figure 1: Detailed diagram of pSRKBB-empty. The backbone was constructed from pSRK21 (Veselý et al., 2003). We removed all BioBrick™ incompatible restriction sites via site-directed mutagenesis. The original multiple cloning site was removed and flanking *EcoRI* and *PstI* sites added by PCR. The plasmid was reassembled with the highlighted insert, which was removed from our previously constructed plasmid, pUCBB-empty (Vick et al., 2011), using *EcoRI* and *PstI* prior to ligation with pSRKBB. pLac' is a modified, constitutive variant of the *lac* promoter (Schmidt-Dannert et al., 2000). The supplemental file pSRKBB-empty.gb contains the full sequence and annotated map.

Figure 2: Evaluation of the *lac* promoter in *R. opacus* PD630. **A.** Representative data from the flow cytometry analysis of GFP fluorescence in *R. opacus* PD630 containing pSRKBB-eGFP (Supplemental file pSRKBB-eGFP.gb). The colors correspond to kanamycin concentrations as shown in panel B. Cells were grown in nutrient broth (HiMedia Laboratories #M1680) to the middle of exponential phase, diluted 1000-fold into phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂PO₄, 1.8 mM KH₂PO₄, pH 7.0), and analyzed using a BD Biosciences FACScalibur™ equipped with a 488 nm argon laser and a 515-545 nm emission filter. The inset represents the spectral profile of control cells containing pSRKBB-empty. The arrows represent the fluorescence value at the peak maximums. **B.** Analysis of the percent fluorescent cell population at various concentrations of kanamycin. The percentages were calculated by gating out the autofluorescent cell population as determined by the spectral profile of pSRKBB-empty. Each column represents the average value of two independent experiments. The bars represent the range of values observed.

Figure 1

Figure 2

Note: Color figure for manuscript review and the online version. Figure will be changed to black-and-white for the print version.