Metabolic Engineering 39 (2017) 128–140

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben

Original Research Article

Novel T7-like expression systems used for *Halomonas*

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ARTICLE INFO

Keywords:
Part mining
Expression system
Morphological engineering
PHB
Synthetic biology
Non-model microorganism

ABSTRACT

To engineer non-model organisms, suitable genetic parts must be available. However, biological parts are often host strain sensitive. It is therefore necessary to develop genetic parts that are functional regardless of host strains. Here we report several novel phage-derived expression systems used for transcriptional control in non-model bacteria. Novel T7-like RNA polymerase-promoter pairs were obtained by mining phage genomes, followed by *in vivo* characterization in non-model strains *Halomonas* spp TD01 and *Pseudomonas entomophila*. Three expression systems, namely, MmP1, VP4, and K1F, were developed displaying orthogonality (crosstalk < 0.7%), tight regulation (3085-fold induction), and high efficiency (2.5-fold of P_{tac}) in *Halomonas* sp. TD01, a chassis strain with a high industrial value. The expression under the corresponding T7-like promoter libraries persisted with striking correlations (R^2 > 0.94) between *Escherichia coli* and *Halomonas* sp. TD01, implying suitability of broad-host range. Three *Halomonas* TD strains were then constructed based upon these expression systems that enabled interchangeable and controllable gene expression. One of the strains termed *Halomonas* TD-MmP1 was used to express the cell-elongation cassette (*minCD* genes) and polyhydroxybutyrate (PHB) biosynthetic pathway, resulting in a 100-fold increase in cell lengths and high levels of PHB production (up to 92% of cell dry weight), respectively. We envision these T7-like expression systems to benefit metabolic engineering in other non-model organisms.

1. Introduction

Engineering of microorganisms has enabled the production of various chemicals including platform molecules (Steen et al., 2008; Yang et al., 2016a), amino acids (Yu et al., 2015), biofuels (Lee et al., 2015), pharmaceuticals (Breitling and Takano, 2015) and biomaterials (Yang et al., 2016b). Synthetic biology, as a new methodology for microorganism engineering (Breitling and Takano, 2016; Jensen and Keasling, 2014; Stephanopoulos, 2012), allows control of biological processes in a more efficient, reliable, and predictable manner (Brophy and Voigt, 2014; Gu et al., 2016; Rajkumar et al., 2016; Venturelli et al., 2016). Advances in engineering genetic control circuits, especially those with intriguing spatial and temporal control functions, have been made in several model organisms especially *Escherichia coli* (Bonnet et al., 2012; Daniel et al., 2013; Ke et al., 2016; Kotula et al., 2014; Nielsen et al., 2016; Wang et al., 2014). However, in metabolic engineering practices, non-model organisms are often used (Hammar et al., 2015; Tran and Charles, 2016); these include soil bacteria in the plant rhizosphere, probiotics in the human gut, and industrial bacterial strains in open or closed bioreactors (Cardinale and Arkin, 2012; Venturelli et al., 2016).

Non-model bacteria usually preserve some unique pathways for their unique products. Their values could be further improved by introducing new pathways and adding new genetic control (Li et al., 2016a). However, when a control circuit is transferred from one species/strain to another, substantial efforts are usually required to rebuild the circuit to preserve its functions, and unexpected failures often occur (Cardinale and Arkin, 2012; Nevozhay et al., 2013; Prindle et al., 2012; Venturelli et al., 2016). This is largely because biological parts in a circuit are often influenced by host strains, and altered activity of biological parts frequently causes failures in process control (Brophy and Voigt, 2014; Kittleson et al., 2012). For example, when a well-built transcriptional circuit, an AND gate (Anderson et al., 2007), was transferred from *E. coli* strain MC1061 to *E. coli* DH10B, the gate function unexpectedly failed (Moser et al., 2012). Another example is that TetR homologues as transcriptional regulators sourced from their

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http://dx.doi.org/10.1016/j.ymben.2016.11.007
Received 17 September 2016; Received in revised form 21 October 2016; Accepted 21 November 2016
Available online 24 November 2016

native hosts resulted in growth defects and failed in its regulatory functions in *E. coli* (Stanton et al., 2014). These observations indicate that novel biological parts that are operational regardless of chassis strains are highly in demand; with these parts, engineering genetic control in non-model bacteria would be much more efficient and reliable.

In this study, we used *Halomonas* sp. TD01 as a representative industrial strain to address the development of broad-host biological parts for genetic control in non-model microorganisms. *Halomonas* sp. TD01 is a halophile able to grow in highly saline medium under unsterile conditions (Tan et al., 2011). It can utilize glucose as a single substrate to intracellularly produce large amounts of polyhydroxyalkanoates (PHAs), a family of degradable bioplastics with diverse structures and properties (Fu et al., 2014; Tan et al., 2014). A problem in the engineering of this strain is the lack of robust, high-performance genetic parts for implementing biological process control. Previous studies developed several constitutive and inducible promoters applicable in *Halomonas* (Li et al., 2016b; Tan et al., 2014). However, the number and performance of these transcriptional parts are rather limited. For instance, P_{porin}, the strongest promoter developed thus far for *Halomonas* spp., is constitutive and often aggravated genetic instability. Meanwhile for an inducible gene expression in *Halomonas*, there is only one available promoter, namely, IPTG-dependent P_{trc}. Therefore, new genetic parts with a wide host range, tight regulation, and high efficiency represent a much-needed and enabling step toward fully realizing the potential of *Halomonas* and other non-model microorganisms.

In our initial attempts to develop inducible expression system for efficient transcriptional control in *Halomonas* sp. TD01, the conventional T7 expression system was used (Elroy-Stein and Moss, 1990; Kushwaha and Salis, 2015; Studier and Moffatt, 1986; Temme et al., 2012). However, the T7 system inexplicably failed despite multiple troubleshooting attempts. Instead of continued debugging, we turned to part mining (Martinez-Garcia et al., 2015b; Nielsen et al., 2013; Rhodius et al., 2013; Stanton et al., 2014) to source novel T7-like expression systems from a phage genome database. Computational and *in vivo* experimental results revealed six T7-like RNA polymerase-promoter pairs with cross-species activities (three species, *E. coli* S17-1, *Halomonas* sp. TD01, and *Pseudomonas entomophila* LAC31). Based upon these six pairs, three expression systems with a high efficiency, mutual orthogonality, strict regulation, genetic stability, and variable transcriptional levels in *Halomonas* were developed. The subsequent cross-species evaluation showed that the functional characteristics of these expression systems persisted with strikingly high correlations when the systems were transferred from *E. coli* to *Halomonas* sp. TD01, highlighting their potentials for broad-host-range applications. Subsequently, the components of expression systems including RNA polymerases (RNAPs) were integrated into the chromosome of *Halomonas* sp. TD01 to stabilize the T7-like function, thus yielding three *Halomonas* platform strains. Further, a platform strain harboring a representative T7-like system, MmP1, was used to control the bacterial cell shapes (Tan et al., 2014) and polyhydroxybutyrate (PHB) biosynthetic pathway (Pohlmann et al., 2006), respectively. Results showed that the MmP1 system was extraordinarily efficient and robust to the host context. Together, these T7-like novel expression systems and the idea of part mining provide intriguing design flexibility for transcriptional control of biological process in *Halomonas* and enable the transfer of transcriptional control from a model bacterium to an industrially interesting one with reliability and predictability.

2. Material and methods

2.1. Strains, media, and chemicals

E. coli strain S17-1 was used for molecular cloning and plasmid propagation throughout this study. *E. coli* strain S17-1, *Halomonas* sp. TD01, and *Pseudomonas entomophila* LAC31 were used for the characterization of T7-like RNAP-promoter pairs. Luria–Bertani (LB) medium (g/L: 10 tryptone, 5 yeast extract, and 10 NaCl) was used for culturing *E. coli* and *P. entomophila*, while LB medium supplemented with 60 g/L NaCl (termed "60LB" hereafter) was employed for *Halomonas* sp. TD01. For PHA production, LB and 60LB media with 20 g/L glucose (called "LBG" and "60LBG" hereafter) were used. Antibiotics, including (mg/L) 25 chloramphenicol, 100 spectinomycin, or 100 ampicillin, were added as needed. The inducer for P_{tac} promoter was isopropyl β-D-1-thiogalactopyranoside (IPTG). All chemicals were purchased from Sigma-Aldrich unless otherwise indicated.

2.2. Computational methods for part mining

The protein sequence of T7 RNAP was used to search the NCBI database with the tblastn program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for bacteriophages carrying homologues. The program was run against the NCBI genome (chromosome) database with default algorithm parameters. For the identification of T7-family RNAP-specific promoters, PHIRE (phage *in silico* regulatory elements) software package (Lavigne et al., 2004; Temme et al., 2012) was used. The parameter settings were string length (L), 20; degeneracy (D), 4; dominanNum, 4; and window size (W), 30. The window size used was larger than the default because we intended to *a priori* identify conserved sequence motifs across the promoter region whenever possible. WebLogo (Crooks et al., 2004) was used to generate the sequence logos for each set of putative promoters. The putative transcription start site (+1) and the −7 to −12 bp region of phage promoters were manually identified.

2.3. Plasmid construction and coarse characterization

T7-family RNAPs and promoters ($P_{T7\text{-}like}$) were *de novo* synthesized at GenScript (Nanjing, China), cloned into RNAP-module plasmid (p15A origin, Amp^+) and promoter-module plasmid (pSC101 origin, Cm^+) respectively, via the Golden Gate Assembly method (see Table 1 for plasmid properties and Supplementary Figure 1 for plasmid maps). For coarse characterization, each RNAP module and its corresponding promoter module (superfolder green fluorescent protein, sfGFP, as the reporter gene) were assembled together on the plasmid backbone pSEVA 321 using the Gibson Assembly method to create an RNAP-promoter-p321 plasmid series and then were transferred into *E. coli, Halomonas* sp. TD01, and *Pseudomonas entomophila* LAC31.

For the orthogonality test, the chosen RNAP modules were exhaustively assembled with the corresponding $P_{T7\text{-}like}$-p321 modules (3 RNAPs ×3 promoters =9 combinations in detail) on pSEVA 321. Orthogonality was characterized using the same method as that for the coarse characterization described above. The concentration of IPTG was 1 mM.

Flat-bottom 96-well plates (Corning) covered with air permeable sealing films (Corning, BF-400-S) were used for the characterization studies. For coarse characterization, *E. coli* strain S17-1, *Halomonas* sp. TD01, and *Pseudomonas entomophila* LAC31 harboring T7-like systems were first inoculated into a plate containing LB or 60LB medium, respectively, for overnight cultivation (37 °C for *E. coli* and *Halomonas* while 30 °C for *Pseudomonas entomophila*, 1000 rpm, mB100-40 Thermo Shaker, Aosheng). Then, cell cultures were diluted 500-fold using fresh LB medium in a new plate, supplemented with IPTG of appropriate concentrations, and cultivated for 18 h. Subsequently, an appropriate aliquot of each cell culture (1 μL for *E. coli*; 0.5 μL for *Halomonas* sp. TD01) was transferred to another plate containing 200 μL/well phosphate-buffered saline (PBS) for analysis by flow cytometry.