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Recent progress in development of synthetic biology platforms and metabolic engineering of *Corynebacterium glutamicum*

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ABSTRACT

The paradigm of synthetic biology has been evolving, along with relevant engineering, to achieve designed bio-systems. Synthetic biology has reached the point where it is possible to develop microbial strains to produce desired chemicals. Recent advances in this field have promoted metabolic engineering of *Corynebacterium glutamicum* as an amino-acid producer for use in intelligent microbial-cell factories. Here, we review recent advances that address *C. glutamicum* as a potential model organism for synthetic biology, and evaluate their industrial applications. Finally, we highlight the perspective of developing *C. glutamicum* as a step toward advanced microbial-cell factories that could produce valuable chemicals from renewable resources.

1. Introduction

Recent developments in synthetic biology that have re-defined biological systems based on engineering concepts with computer-aided tools have enabled construction of efficient biological systems under precise control by gene expression (Keasling, 2008; Lee, 2012). Metabolic engineering combined with synthetic biology has been aimed at identifying target chemicals that could replace petroleum-based chemicals either by production of identical chemicals, or by producing bio-chemicals with identical properties (Lee et al., 2012). A logical outcome of these designed systems and intentions would be to construct highly efficient microbial-cell factories to produce those desired products from renewable resources; without being subject to the metabolic imbalances inevitable in a typical host.

Microbial cell factories require functional components including synthetic DNA and RNA; as well as artificial network circuits for genetic regulation. These are needed in order to redirect carbon flux, construct novel metabolic pathways and make structural constituents such as bacterial compartments. The engineering of *Escherichia coli* and *Saccharomyces cerevisiae* has been favored by scientists because they are relatively versatile hosts with numerous genetic tools available. As a result of synthetic biology, variants of such microbial species are now capable of producing advanced biofuels (Choi and Lee, 2013; Schirmer et al., 2010; Steen et al., 2010), materials (Jensen et al., 2010) and pharmaceuticals (Paddon et al., 2013). Besides *E. coli* and *S. cerevisiae*, new opportunities in synthetic biology have been explored with *Bacillus subtilis* (van Dijl and Hecker, 2013), *Pichia pastoris* (Vogl et al., 2014), *Streptomyces avermitilis* (Komatsu et al., 2013), and cyanobacteria (Atsumi et al., 2009; Oliver et al., 2013). *Corynebacterium glutamicum* is another microbial host attractive as a target for the tools and concepts of synthetic biology.

C. glutamicum is a Gram-positive, facultative anaerobic, non-pathogenic soil bacterium (GRAS: Generally Recognized As Safe) that is used for large-scale industrial production of the flavor enhancer L-glutamate (2.93 million tons in 2012) and the food additive L-lysine (1.95 million tons in 2012). Recent studies using metabolic engineering have shown that *C. glutamicum* is also capable of producing a variety of other commercially interesting chemicals and materials. Other reviews covering *C. glutamicum* have been focused on biological production of chemicals and

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materials (Becker and Wittmann, 2012; Wendisch et al., 2006; Wieschalka et al., 2013); and its systems biology and physiology (Bott, 2007; Bott and Niebisch, 2003).

In this review, we focus on recent advances and developments involving *C. glutamicum*; based on synthetic biology and metabolic engineering. We have divided the review into two sections. In the first section, we describe recent advances of synthetic DNA elements and devices for *C. glutamicum*. Next, we cover new approaches for developing microbial-cell factories that can utilize non-conventional carbon sources and produce novel chemicals for use in foods and pharmaceuticals.

2. Recent development of synthetic elements and technologies for *C. glutamicum*

2.1. Controllable gene expression systems with DNA parts

Engineered-microbial-cell factories are able to produce the target chemicals by re-constructing metabolisms to include synthetic pathways. Engineering a synthetic pathway in a host requires expression of genes encoding heterologous metabolic enzymes; under transcriptional or translational control (Seo et al., 2013). For controlling gene expression, genetic elements in the form of DNA parts and devices, are necessarily defined and assembled with the coding sequence of target enzymes in a self-replicating vector; or onto a chromosome. In this section, we will discuss current and potential DNA parts for *C. glutamicum* and the development of plasmids as synthetic platforms for gene expression.

2.1.1. Synthetic biology of DNA parts for C. glutamicum

DNA parts are usually considered DNA sequences with biological features. DNA parts deposited at the main distributor Registry of Standard Biological Parts (RSBP, 68% shared; parts.igem.org); supporting iGEM competition, are classified as promoters, ribosome binding sites, protein coding sequences that encode a particular protein, and terminators for each element of transcription and translation of target gene expression. These also include plasmids that are a self-replicating DNA backbone compatible with the DNA parts. Many DNA parts at RSBP have worked in *E. coli*, *B. subtilis*, Yeast, and bacteriophage T7. Based on the keyword search of 'glutamicum', only four DNA parts of *C. glutamicum* were shown: BBa_K525121/BBa_K525123 (promoter, RBS, signal peptide and protein encoding sequence for the surface layer protein, CspB); BBa_K822001 (promoter region associated with the *ldhA* operon); BBa_K1170003 (promoter region of P-atp2 in F^0F^1 ATPase); and BBa_K1124012 (anti-trpD mRNA; antisense mRNA of anthranilate phosphoribosyltransferase). Since application of synthetic biology to *C. glutamicum* is strongly recommended for development of microbial-cell factories, the community of government and private institutions working with *C. glutamicum* has been building a library of *C. glutamicum*-specific DNA parts through iGEM completions and increasing publications (Rytter et al., 2014; Yim et al., 2013).

Because the promoter is one of the crucial DNA parts essential for gene transcription, the series of promoters in *C. glutamicum* have been studied with sigma subunits of RNA polymerase and gene expression profiles; using fluorescent proteins. Fifty-three endogenous promoters were first selected for the consensus sequence analysis. This work showed that those have well-conserved −10 regions (as tgngnTA(c/t)aaTgg), and less conserved −35 regions (binding sites for RNA polymerase). Their transcriptional promoters also function in *E. coli* and other bacteria (Pátek et al., 2003) (Fig. 1a). In addition, σ-factor-dependent promoters were dissected into seven groups with multiple promoters (Pátek and Nesvera, 2011). For instance, 159 promoters subjected to σ^A; which is an essential primary sigma factor belonging to the σ^{70}-family for the transcription of housekeeping genes, were analyzed with consensus from alignment of TTGNCA and GN**TA**NANTNG (−35 and extended −10 region with high similarity sequence in bold). The other sigma factors are significantly related to gene transcription under various stress conditions (e.g., heat and oxidative stress), and slower growth in the transition phase. Those endogenous promoters were useful for understanding the transcriptional system of *C. glutamicum*. Recently, comprehensive transcriptome analysis using improved RNAseq technique has been identified numerous endogenous promoters from intergenic and antisense regions in the genome of *C. glutamicum* (Pfeifer-Sancar et al., 2013).

Strong constitutive promoters such as the *cspB* (BBa_K525121), *gapA* promoter (encoding glyceraldehyde 3-phospahte dehydrogenase), *tuf* promoter (encoding the translational elongation factor EF-Tu), and *sodA* (encoding manganese superoxide dismutase) were extensively used for gene expression in an expression-plasmid or for replacement of the native promoters. These strategies have been applied to many biotechnological applications that were discussed in a recent review (Pátek et al., 2013). However, engineering of synthetic pathways in *C. glutamicum* needs predictable promoters to adjust gene expression strengths for optimal gene expression of target genes. To develop new promoters, twenty fully-synthetic promoters of *C. glutamicum* have been developed based on the level of fluorescent intensity of green fluorescent protein (GFP). The library of synthetic promoters with leaderless transcripts led to the constitutive gene expression of the target gene and was applied to high-level production of endoxylanase and antibody fragments (Yim et al., 2013). Other DNA parts such as 5'-UTRs and terminators could also be used to construct controllable gene expression in *C. glutamicum*. Recently, modification of the stop codon in the sequence of ornithine cyclodeaminase (OCD) has led higher specific activity of OCD (0.71 μmol/min/mg of protein) and higher accumulation of L-proline (10.9 g/L), compared to native OCD of none of activity and accumulation of L-proline) (Jensen and Wendisch, 2013).

2.1.2. Synthetic biology of potential DNA parts for C. glutamicum

Small RNA (sRNA) transcripts have been identified through the next-genome sequencing technique. The sRNAs are able to influence transcriptional and translational efficiencies of gene expression. Some antisense sRNAs regulate metabolisms by modulating the levels of metabolic proteins by interfering and attenuating mRNA transcription, cleaving RNA, or blocking translation (Thomason and Storz, 2010). Therefore, sRNAs with those functions can be useful to regulate gene expression in a host. Those small DNA and RNAs parts have been developed and have improved nucleic-acid-based general techniques. These have led to successes in the use of synthetic biology and metabolic engineering by modulating gene expressions using a tunable 5′-UTR library (Pfleger et al., 2006), synthetic ribo-switches (Yang et al., 2013) and combinatorial gene knockdown based on synthetic small rRNAs (Na et al., 2013).

A small antisense RNA of *C. glutamicum* in the reverse transcription of the genes cg1934 and cg1935 has been reported (Zemanová et al., 2008), and *odhA*-antisense RNA expression with overexpression of the *odhA* gene was found to trigger glutamate production (Kim et al., 2009). A recent transcriptome-sequencing technique, with read-mapping, allowed detection of 815 small RNAs of *C. glutamicum*; including 69 sRNAs of Rho-independent terminator (Mentz et al., 2013) (Fig. 1b and d). After analysis of those small RNAs mapped with promoter sites, a 298 mRNA leader sequence, 262 trans-encoded sRNAs, 63 antisense 5′-UTRs, 464 antisense RNAs, and 16 antisense 3′-UTRs were classified. Their lists were open to the public and could be used as repertoires of