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Development of a plasmid addicted system that is independent of co-inducers, antibiotics and specific carbon source additions for bioproduct (1-butanol) synthesis in *Escherichia coli*

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ABSTRACT

Synthetic biology approaches for the synthesis of value-based products provide interesting and potentially fruitful possibilities for generating a wide variety of useful compounds and biofuels. However, industrial production is hampered by the costs associated with the need to supplement large microbial cultures with expensive but necessary co-inducer compounds and antibiotics that are required for up-regulating synthetic gene expression and maintaining plasmid-borne synthetic genes, respectively. To address these issues, a metabolism-based plasmid addiction system, which relies on lipopolysaccharide biosynthesis and maintenance of cellular redox balance for 1-butanol production; and utilizes an active constitutive promoter, was developed in *Escherichia coli*. Expression of the plasmid is absolutely required for cell viability and 1-butanol production. This system abrogates the need for expensive antibiotics and co-inducer molecules so that plasmid-borne synthetic genes may be expressed at high levels in a cost-effective manner. To illustrate these principles, high level and sustained production of 1-butanol by *E. coli* was demonstrated under different growth conditions and in semi-continuous batch cultures, in the absence of antibiotics and co-inducer molecules.

1. Introduction

The advent and use of synthetic biology approaches has allowed the scientific community to engineer microorganisms for the production of a variety of value-based products, such as human insulin, proteases, and antibiotics (Chance and Frank, 1993; Adrio and Demain, 2014; Thykaer and Nielsen, 2003). Recently, the microbial production of biofuels, such as 1-butanol, has attracted great interest and gained momentum due to several environmental, economic and political factors. The chemical properties of 1-butanol, its high energy density and low hygroscopicity, and its compatibility with the current infrastructure, make it an attractive candidate for a transportation fuel, compared to ethanol (Peralta-Yahya and Keasling, 2010).

Clostridium acetobutylicum, a Gram-positive strict anaerobic bacterium, has historically been used for the microbial production of 1-butanol, as well as acetone and ethanol. The genes responsible for 1-butanol production in *C. acetobutylicum* were identified (Boynton et al., 1996; Fontaine et al., 2002) and heterologous expression of these genes has been accomplished in several different microorganisms, such as *Bacillus subtilis* (Nielsen et al., 2009), *Lactobacillus brevis* (Berezina et al., 2010), *Pseudomonas putida* (Nielsen et al., 2009), *Saccharomyces cerevisiae* (Steen et al., 2008), and *Escherichia coli* (Atsumi et al., 2008; Shen et al., 2011; Bond-Watts et al., 2011; Gulevich et al., 2012) with particular interest in *E. coli* due to its proven industrial usage and associated extensive genetic and biochemical knowledge. Early constructs resulted in low yields of 1-butanol produced by wild-type organisms (Atsumi et al., 2008). To overcome this, multiple metabolic and genetic factors were altered in efforts to increase 1-butanol production. For example, with *E. coli* changes in the growth conditions or medium, the use of plasmids or promoters for expression of the 1-butanol genes, the elimination of competitive pathways, and the use of homologous non-clostridial genes have all played a significant role in raising the level of 1-butanol production to impressive levels (Shen et al., 2011).

As opposed to chromosomal expression, plasmid-based systems are often used for up-regulated heterologous expression of synthetic genes in a non-native organism; this is the case for genes that are required for 1-butanol production. The use of a plasmid-based system has many advantages, as it allows for (1) an increase in enzyme pools by gene dosage, (2) control of gene expression if

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an appropriate promoter and co-inducer are chosen, and (3) rapid construction of different combinations of genes due to facile manipulation of plasmids, as opposed to insertion of genes on the chromosome. However, if one chooses to scale-up to industrial production levels, there are two major drawbacks with this laboratory bench method. The first is the combined cost associated with supplementing cultures with a co-inducer to induce gene expression and the use of antibiotics to maintain plasmid stability. Secondly, large scale-ups invite potential ecological issues associated with the usage of large amounts of antibiotics, such as the rise in antibiotic resistant bacterial strains.

To overcome reliance on antibiotics, the metabolism-based plasmid addiction system (PAS) was devised (Voss and Steinbuchel, 2006). Essentially, the PAS relies on the strict natural selection of plasmid-containing cells, due to the expression of a plasmid-encoded gene(s) that is required for the viability of the bacterium (Kroll et al., 2010). Therefore, cells that maintain a plasmid containing the essential gene(s) and a suite of value-based product gene(s) are viable and able to produce the desired product.

There are a few examples of the use of metabolism-based plasmid addicted systems for value-based product formation. The first example used *Ralstonia eutropha* strain H16. Plasmid expression of the essential 2-keto-3-deoxy-6-phosphogluconate (KDPG)-aldolase gene coupled with a cyanophycin synthetase gene, resulted in cyanophycin production with either fructose or gluconate as carbon source (Voss and Steinbuchel, 2006). A second example was cyanophycin production in *E. coli* (Kroll et al., 2011). Like the first example, plasmid addicted value-based product formation was medium- and carbon-source dependent. Another example was the plasmid-based expression of a synthetic 1-butanol operon in an *E. coli* mutant strain that restored anaerobic growth; as a consequence, 1-butanol was produced (Shen et al., 2011). However, in this instance, expression of the plasmid-based operon was dependent on co-inducer, isopropyl β-D-1-thiogalactopyranoside (IPTG), addition. Recently, the production of ethanol was accomplished in *E. coli* and, similarly to other examples, plasmid addiction was carbon source-dependent (Wong et al., 2014).

To date, all recently employed PAS systems negate the requirement of antibiotics for plasmid stability. However, each of these PAS systems relies on specific constraints; for example, the use of a specific carbon source or medium-specific growth condition, as well as the need for co-inducer supplementation for up-regulated gene expression. There is one exception that does not rely on any constraints; however, in this example the product titer was less than that of the control strain (Kroll et al., 2009). The aforementioned constraints limit the flexibility for industrial scale production. Addressing these issues, we now report the development of a plasmid addicted 1-butanol production system in *E. coli* that negates the need for expensive co-inducers and antibiotics, and is not limited by medium, carbon source, or growth condition. Without the constraints usually associated with metabolism-based plasmid addicted value-based product synthesis, this system produced significant yields of 1-butanol using a test strain of *E. coli* during semi-continuous batch culture.

2. Materials and methods

2.1. Reagents

The chemicals used were acquired from Sigma-Aldrich (St. Louis, MO) or Fisher Scientifics (Pittsburgh, PA). Oligonucleotides were purchased from Sigma-Aldrich (St. Louis, MO). Phusion High-Fidelity DNA polymerase and restriction enzymes were obtained from New England Biolabs (Ipswich, MA). Invitrogen T4 ligase was obtained from Life Technologies (Grand Island, NY).

2.2. Bacterial strains

E. coli strain JM109 (Yanisch-Perron et al., 1985) was used for propagation of plasmids and strain BW25113 (Datsenko and Wanner, 2000) was used for plasmid-addicted 1-butanol production.

2.3. Plasmid and strain construction

Plasmids and strains used in this study are listed in Table 1. To construct plasmids p62, p72, p91, p91BuOH(1), and p91BuOH(1)*lptB*, the multiple cloning site from pUC19, particularly the nucleotide sequence that spans the SacI and HindIII restriction sites, was subcloned into plasmid 3716 (a derivative of pLO11 (Schwarze et al., 2010) with a pBAD promoter and the *araC* gene (kindly provided by Dr. Oliver Lenz, Berlin, Germany)), replacing the existing nucleotide sequence between the SacI and HindIII restriction sites. As a result, the number of available restriction sites for cloning purposes was expanded, resulting in the construction of plasmid p62. A NcoI restriction site was introduced within the *araC* gene of p62 by site directed mutagenesis, thus resulting in plasmid p72. This allows one to replace the existing pBAD promoter with a promoter of choice flanked by NcoI and SacI restriction sites. The promoter sequence of the chromosomal *cbbL* gene from *R. eutropha* strain H16 was amplified from genomic DNA and cloned into pUC19. Site-directed mutagenesis was then performed on the *cbbL* promoter region. Specifically, the nucleotide sequence of the – 35 element within the *cbbL* promoter was changed to the consensus *E. coli* nucleotide sequence (TTGACA).

Table 1
Plasmids and strains used in this study.

Plasmid or strain	Relevant characteristics	Reference or source
Plasmids		
pUC19	General cloning vector	New England Biolabs
#3716	Derivative of pLO11, contains the pBAD promoter and *araC* gene	Oliver Lenz, Berlin, Germany
p62	#3716 Derivative with pUC19 multiple cloning site	This work
p72	p62 Derivative with NcoI restriction site in *araC* gene	This work
p91	p72 Derivative with modified *R. eutropha cbbL* promoter	This work
p91BuOH(1)	Synthetic butanol operon (*hbd*, *crt*, *ter*, *atoB*, *adhE2*)	This work
p91BuOH(1)*lptB*	Synthetic butanol operon with essential *lptB* gene	This work
Strains		
BW25113	Wild-type strain	ATCC, Manassas, Virginia
RKE01	BW25113 Δ*lptB* p91BuOH(1)*lptB*	This work
RKE03	BW25113 Δ*lptB* Δ*adhE* p91BuOH(1)*lptB*	This work
RKE05	BW25113 Δ*lptB* Δ*adhE* Δ*pta* p91BuOH(1)*lptB*	This work
RKE07	BW25113 Δ*lptB* Δ*adhE* Δ*pta* Δ*ldhA* p91BuOH(1)*lptB*	This work
RKE09	BW25113 Δ*lptB* Δ*adhE* Δ*pta* Δ*ldhA* Δ*frdABCD* p91BuOH(1)*lptB*	This work