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Transcriptional feedback regulation of efflux protein expression for increased tolerance to and production of *n*-butanol

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ABSTRACT

Microorganisms can be engineered to produce a variety of biofuels and commodity chemicals. The accumulation of these products, however, is often toxic to the cells and subsequently lowers production yields. Efflux pumps are a natural mechanism for alleviating toxicity through secretion of the product; unfortunately, pump overexpression also often inhibits growth. Tuning expression levels with inducible promoters is time-consuming and the reliance on small-molecule inducers is cost-prohibitive in industry. We design an expression regulation system utilizing a native *Escherichia coli* stress promoter, P_{gntK}, to provide negative feedback to regulate transporter expression levels. We test the promoter in the context of the efflux pump AcrB and its butanol-secreting variant, AcrBv2. P_{gntK}-driven AcrBv2 confers increased tolerance to *n*-butanol and increased titers of *n*-butanol in production. Furthermore, the system is responsive to stress from toxic overexpression of other membrane-associated proteins. Our results suggest a use for feedback regulation networks in membrane protein expression.

1. Introduction

Microbial production of small molecules at high titer is often hindered by the toxicity of the product to the host organism. While multiple approaches exist to alleviate this toxicity through strain engineering, one of the most promising strategies is the use of efflux pumps to secrete the molecules out of the cell (Boyarskiy and Tullman-Ercek, 2015; Dunlop, 2011). This tactic has the benefit of relieving product toxicity with minimal alteration to the host cell, while also providing a way to increase titers through removal of product inhibition and potentially aiding in product separation from biomass. Indeed, efflux pumps have been used or engineered for the removal of multiple biofuel molecules including alkanes (Chen et al., 2013), olefins (Mingardon et al., 2015), alcohols (Foo and Leong, 2013; Yang et al., 2012), fatty acids (Lennen et al., 2013), and terpenes (Dunlop et al., 2011).

As is the case for many membrane proteins, however, the overexpression of efflux pumps often inhibits cell growth. Thus, in a biochemical production context, optimal expression is required to balance the toxicity associated with the overexpression of these membrane proteins with the advantages the pumps provide. The expression profiles that achieve this result depend not only on the pumps themselves, but also the host strains, and even the interactions between various pumps when combined in a single strain (Turner and Dunlop, 2015). Ideally, the cell would be able to sense the membrane environment and respond accordingly to express the efflux pumps at a maximal, nontoxic level in an inducer-independent context. This approach not only ensures maintaining optimal pump expression for maximizing productivity on a single-cell level, but also renders the use of externally provided inducers unnecessary – a major cost factor for industrial scale applications.

Genetic circuits offer a solution to these challenges. To design a system that is able to control membrane transporter levels, the circuit must respond to membrane protein overexpression stress while ignoring cell envelope stress from the product itself. Recently, dynamic regulation systems to control metabolic pathway enzyme expression have been engineered to sense metabolite concentration (Zhang et al., 2012), and detect the toxic build-up of intermediates (Dahl et al., 2013). Thus far, these systems have been designed to respond to either specific metabolites or to general cellular stress, rather than to a specific type of stress. Nonetheless, the concept holds promise for the regulation of membrane transporter levels.

In *Escherichia coli*, the multidrug efflux pump AcrB–AcrA–TolC natively effluxes antibiotics, surfactants, and hydrophobic solvents

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(Takatsuka et al., 2010; Yu et al., 2003). The inner membrane pump component, AcrB, was successfully targeted by multiple engineering efforts to secrete industrially important chemicals (Doshi et al., 2013; Foo and Leong, 2013; Mingardon et al., 2015), including the next-generation biofuel *n*-butanol (Fisher et al., 2014). Butanol is known to be toxic to the cell putatively through interactions with the cell membranes (Huffer et al., 2011; Rutherford et al., 2010). Evolved variants of the AcrB transporter were able to alleviate some of this toxicity through *n*-butanol secretion.

In this work, we utilize the native promoter of the gluconate metabolism operon, $P_{gntK,}$ to sense membrane stress specific to AcrB overexpression. Placing *acrB* under the control of this promoter allowed us to create a negative-feedback loop that autonomously controls AcrB expression to minimize cellular toxicity. Strains in which P_{gntK} controls the expression of a butanol-secreting mutant, AcrBv2, are as tolerant to *n*-butanol as strains for which AcrBv2 expression was optimized. Strikingly, these strains are also capable of producing up to 40% more *n*-butanol when co-expressing *n*-butanol production pathways. This regulation extends to other membrane-associated *E. coli* proteins and thus will be broadly useful in quickly finding ideal, non-toxic expression levels for membrane proteins for a range of applications.

2. Results

2.1. Identification of a responsive promoter to AcrB toxicity

To create a negative feedback loop capable of responding to AcrB-induced membrane stress, we first needed to identify a promoter that is negatively regulated specifically in response to this type of stress. To find such a regulator, we assembled a set of promising promoters known to respond to cell envelope stress signals as reported by Moen et al. (2009). We selected the promoter regions upstream of genes that had a two-fold or higher change in expression in response to stress and only responded to one or two classes of envelope stress (27 in total), in order to avoid promoters that are part of a general stress response. Many of the promoters are not yet well-characterized, thus we chose regions from 500 bases upstream to the start of these genes (or operons where appropriate) to represent the putative promoters of these systems. We then cloned the library of putative promoters upstream of the *mcherry* gene encoding a red fluorescent protein (RFP) in *E. coli* DH10-B cells. In the same strains we placed *acrB* on a second plasmid under the control of an arabinose-inducible P_{bad} promoter, and subjected cells to stress from increased AcrB expression by addition of different arabinose concentrations. The promoters responded in a variety of ways to this stress (Fig. 1 includes representative members of the library). Of the promoters not involved in overall cell stress, the putative promoter region from the *gntK* operon ($P_{gntK500\text{-}0}$) conferred the highest decrease in RFP expression. The tetracycline-inducible P_{tet} promoter, in both the induced and uninduced states, served as a control for general expression response to AcrB.

2.2. P_{gntK} is inhibited by AcrB overexpression but not n-butanol toxicity

We extended the use of our RFP reporter system to measure the response of $P_{gntK500\text{-}0}$ to varying stress levels from AcrB overexpression. We assessed the expression of RFP from the promoter as a function of AcrB overexpression under the control of a P_{bad} promoter from a second, high-copy (colE1 origin of replication) plasmid. We analyzed RFP fluorescence in strains with varying

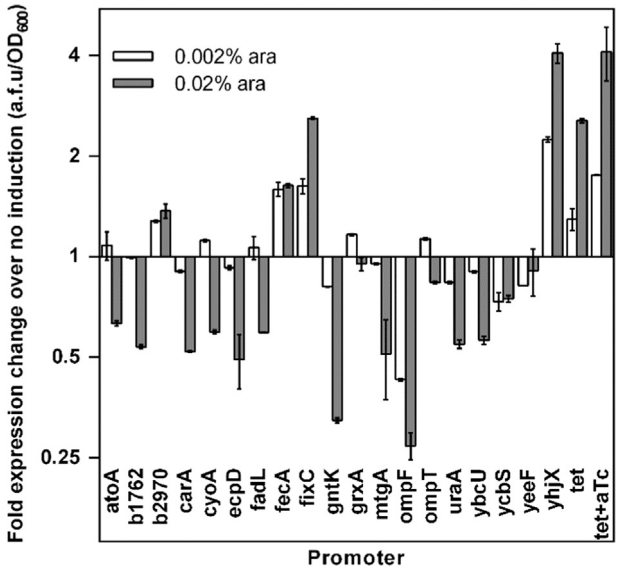

Fig. 1. Stress promoter response to AcrB overexpression. Putative stress promoters were cloned upstream of the gene encoding RFP and transformed into strains containing the P_{bad}-*acrB* vector. Expression is shown as relative change in RFP fluorescence compared to no inducer added after normalization to the number of cells (OD_{600}). Tet and tet+atc are control vectors containing the P_{tet} promoter without and with addition of the aTc inducer (100 ng/mL) respectively. These controls do contain the P_{bad}-*acrB* vector and arabinose, as indicated. Since the tetracycline promoter is not known to respond to arabinose or membrane protein toxicity, we use these controls as "low" and "high" constitutive promoters. Error bars denote one standard deviation, $n=3$.

arabinose induction levels. $P_{gntK500\text{-}0}$ has a uniformly negative response to *acrB* overexpression stress until it is fully repressed down to the basal level (Supplementary Fig. S1A). After full repression, additional increase in AcrB expression has no effect on the promoter. A similar experiment was performed with the addition of *n*-butanol as the stress inducer, as it is important in our scheme that the negative feedback response is limited to the stress associated with AcrB overexpression and not the toxicity from *n*-butanol (Supplementary Figure S1B). Since it is difficult to compare cell stress from diverse sources, we used cellular growth inhibition as a proxy for overall stress. We thus chose AcrB expression conditions and *n*-butanol concentrations corresponding to a range of final OD_{600} measurements that were comparable to one another. While there is some decrease in promoter activity due to *n*-butanol toxicity (~33% decrease), this response is not as severe as the one generated by AcrB overexpression (12-fold decrease).

2.3. P_{gntK} responds to AcrB overexpression through a unique repression mechanism

The regulation of the *gntK* promoter has been previously studied by Izu et al. (1997). P_{gntK} controls the expression of the gluconate kinase (GntK) with read-through to the gluconate importer (GntU). The promoter region was shown to begin at −169 bp from the start of *gntK*, and indeed, when we made a truncation to the putative promoter to include only 200 bp upstream of the start site rather than 500, the response to AcrB overexpression was the same. The region from −100 bp resulted in almost no constitutive expression and did not respond to AcrB overexpression (Supplementary Fig. S2). In this study we will refer to the −200 to 0 bp region upstream of *gntK* start site as P_{gntK}. The promoter is catabolically repressed (Hillen and Hueck, 1995) and is acted on by a repressor (GntR) which is constitutively expressed upstream of the *gntK/U* operon. In addition, evidence also suggests that GntK itself