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Utilizing a highly responsive gene, *yhjX*, in *E. coli* based production of 1,4-butanediol

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HIGHLIGHTS

- Two different *E. coli* strains were found to be sensitive, but to different extents, to 1,4-BDO.
- The *yhjX* gene, encoding a transporter, was highly upregulated during 1,4-BDO exposure
- The 1,4-BDO related *yhjX* response also differed in the two *E. coli* strains.
- Neither *yhjX* gene deletion nor overexpression impacted 1,4-BDO tolerance in *E. coli.*
- We demonstrated the use of the PyhjX promoter as an *E. coli* biosensor for 1,4 BDO.

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ABSTRACT

The role of *yhjX*, a predicted major facilitator superfamily protein, was examined in context of *E. coli* response to 1,4-butanediol (1,4-BDO). *E. coli* DH1 and MG1655, two commonly used metabolic engineering hosts, were both sensitive to the presence of 1,4-BDO in the growth medium, but to different extents. The strains also showed differences in the transcriptional response of the *yhjX* gene that was highly induced in response to 1,4-BDO. *yhjX* deletion improved growth of the *E. coli* strains in the control defined medium but did not significantly impact 1,4-BDO sensitivity. Overexpression of *yhjX* using a plasmid-borne copy and lactose-inducible promoter also did not result in an improvement in 1,4-BDO tolerance. However, the large differential expression of *yhjX* in response to this diol provided the foundation to develop a biosensor for the detection of 1,4-BDO using a fluorescent gene under the control of the *yhjX* promoter. A basic P_{yhjX}:GFP biosensor in *E. coli* DH1 allows the detection of 4–7% 1,4-BDO in the extracellular medium and provides a tool for high throughput engineering for improving 1,4-BDO production strains.

1. Introduction

Green production of chemicals using microbial platforms has become an achievable target. While metabolic engineering continues to advance microbial production of high value commodities such as pharmaceuticals (e.g. taxol, Ajikumar et al., 2010; Chang and Keasling, 2006), nutraceuticals (e.g. tyrosine, Juminaga et al., 2012; lycopene, Das et al., 2007) and other high value commodities (Bomgardner, 2012), recently, bulk products such as solvents (isobutanol, Atsumi et al., 2010), polymer precursors (styrene, McKenna and Nielsen, 2011) and biofuels (bisabolene, Peralta-Yahya et al., 2011; fatty acid ethyl esters, Steen et al., 2010; isopentenol, Chou and Keasling, 2012) are also being successfully targeted. Synthetic biology has begun to play an important role in key aspects of strain engineering including improved pathway regulation (Zhang et al., 2012a, 2012b), strategies and tools for chromosomal integration (Kuhlman and Cox, 2010), tolerance engineering (Dunlop et al., 2010) and pathway optimization (Moon et al., 2010), and is poised to become ubiquitous in many aspects of such efforts (Keasling, 2012). Strain development often requires an iterative process wherein numerous combinations of parts must be evaluated. Given the large number of parameters that require co-optimization, high-throughput strain generation is an essential aspect of strain engineering that must also be coupled with similarly paced measurements of strain performance. Synthetic biology can play a powerful role in the development of biosensors that aid in the development of high-throughput

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diagnostic assays to report on flux to intermediates (Pfleger et al., 2007) and final compounds (Dietrich et al., 2012).

Bulk chemicals, such as solvents or solvent-like compounds, are often toxic to microbes that may be used to produce them (Heipieper et al., 1994; Jarboe et al., 2010; Sangster, 1989). A complete description of 1,4-BDO production in *Escherichia coli* was recently published (Yim et al., 2011) and provides an excellent example of a toxic end product that was successfully engineered to obtain titers of almost 20 g L^{-1}. However, this report, along with previous commentaries from the authors of this research (Burk, 2010), estimate a production level of about 100 g L^{-1} of 1,4-BDO (10% in water) required for the process to be commercially viable. Thus, host engineering to improve production by at least 5 fold is still required in the case of this compound. The toxicity of this compound to *E. coli* is hypothesized to be a key factor that restricts the upper limit in production levels (Burk, 2010). Recognizing 1, 4-BDO as an important bio-product, we conducted tolerance studies on this compound. We examined the role of a highly responsive gene, *yhjX*, encoding a putative major facilitator superfamily 1 (MFS1) pump, in tolerance phenotypes and the applicability of the promoter of this locus for biosensor applications.

2. Materials and methods

2.1. Strain and plasmid construction

E. coli K-12 MG1655 and DH1 were used as the wild type strains for all experiments. The DH1 and MG1655 *yhjX* gene deletion strains were constructed using the *yhjX*-KO-Forward and *yhjX*-KO-Reverse primers and confirmed as described for *E. coli* BW25113 (Baba et al., 2006) (Supplementary Fig. S1). For the *yhjX* expression plasmid, the *yhjX* open reading frame was PCR amplified from *E. coli* BW25113 using the forward primer 5′-gctcaggaattcatgacaccttcaaattatca-3′ and the reverse primer 5′-gctcagggatccttaaagggagc-3′, and cloned using restriction enzymes (BamHI and EcoRI) into a pBba5k vector containing a P_{lac} promoter (Lee et al., 2011). Wild type and *yhjX* knockout strains were transformed with the P_{lac}:*yhjX* plasmid for overexpression assays. An empty plasmid, with only the P_{lac} promoter, transformed into the strains was used as the control in each case. For biosensor applications, a plasmid with the green fluorescent protein (GFP) driven by the *yhjX* gene promoter, P_{yhjX}:GFP was obtained from an existing library (Zaslaver et al., 2006) and was transformed into *E. coli* DH1 and MG1655. Control strains consisted of the same construct with the *yhjX* promoter removed using restriction enzymes (BamHI and XhoI) and blunt end cloning.

2.2. Adapting cells

All experiments were performed with strains adapted to M9 minimal media (per liter: 200 ml 5 × M9 salts, 2 ml 1 M $MgSO_4$, 50 ml 20% glucose, 20 ml 5% Casamino acids, 100 μl 0.5% Thiamine, 100 μl 1 M $CaCl_2$) and made into single use stocks using methods described previously (Dunlop et al., 2011).

2.3. Culturing methods

For toxicity assays, adapted single use glycerol stocks were used to inoculate fresh M9 medium supplemented with 50 μg/ml of kanamycin when necessary. To determine the effect of 1,4-BDO (J.T. Baker, Avantor Performance Materials, PA, USA) on *E. coli* growth, a wide range of concentrations were tested first (data not shown) and then narrowed to a range that caused stress but not significant cell death. Growth assays to test the effect of different concentrations of 1,4-BDO were performed using 24-well microtiter plates with low evaporation lids containing 800 μl of M9 medium and 3.75 μl of thawed single use stocks per well. Unless stated otherwise, all subsequent 1,4-BDO stress assays were conducted at 6% (v/v) 1,4-BDO.

2.4. Growth and fluorescence assays

For growth measurements, cultures were grown at 37 °C in triplicate and optical density (absorbance at 600 nm) was measured every 10 min using a plate reader (Synergy 4, BioTek, VT, USA). For combined growth and fluorescence measurements, cultures were grown at 37 °C in triplicate and optical density (absorbance at 595 nm) and fluorescence (Ex 470 nm/Ex 530 nm) were measured every 18 min using a plate reader (F200 Pro, Tecan, NC, USA).

2.5. Quantitative reverse transcriptase PCR (qPCR)

Overnight cultures of *E. coli* MG1655 and DH1 grown in M9 were diluted back into 6 separate cultures per strain at a dilution of 1:100 into fresh M9 medium. 1,4-BDO was added to 3 out of the 6 cultures per strain at mid-log phase (optical density at 600 nm [OD_{600}]), 0.35–0.40/ ml. The strains were allowed to grow another hour then harvested, centrifuged (8000*g*, 4 °C, 5 min) and the pellet stored at −80 °C. RNA was isolated using the Total RNA kit (Agilent, DE, USA) and any remaining genomic DNA contamination was removed using the Turbo DNA-free kit (Ambion, Life Sciences, NY, USA). cDNA was synthesized following the Superscript III Reverse Transcriptase protocol (Invitrogen, CA, USA). qPCR reactions were created using the PerfeCTa SYBR Green SuperMix ROX (Quanta Biosciences, MD, USA), cDNA template, the forward primer 5′-ccgctggctgacactcatcggtact-3′ and the reverse primer 5′-caagccgaaagagaaagcgacctg-3′ for *yhjX* and the forward primer 5′-gctgatgctggtgatgattg-3′ and the reverse primer 5′-agtcgcactttgccgtaatc-3′ for the control gene *hcaT*. qPCR was performed using the StepOne Plus Real-Time PCR System (Applied Biosystems, CA, USA). Fold change was calculated using a published mathematical model for relative quantification in real-time PCR (Pfaffl, 2001). For methods and results from the microarray data see supplementary section.

2.6. Overexpression assay

The Isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma Aldrich, MO, USA) inducible, P_{lac}:*yhjX* expression plasmid was compared to an empty control plasmid in the *E. coli* strains using leaky (0 μM IPTG) or 5 μM IPTG induction. To determine the effect of *yhjX* expression on tolerance, 24-well plates were prepared as described for growth assays, with 0%, 6% and 10% 1,4-BDO.

2.7. GFP reporter assays for 1,4-butanediol

The P_{yhjX}:GFP plasmid (Zaslaver et al., 2006) was transformed into *E. coli* MG1655, and DH1 and was assayed for fluorescence in response to varying levels of 1,4-BDO (0–12%).

3. Results

We examined the impact of 1,4-BDO on the growth of *E. coli* strains using an exogenous addition of 6% 1,4-BDO based on its published inhibitory levels (Burk, 2010). *E. coli* MG1655 was sensitive to 6% 1,4-BDO, showing about half maximal growth compared to the control without 1,4-BDO. However *E. coli* DH1 showed higher tolerance to 1,4-BDO compared to MG1655 (Fig. 1).

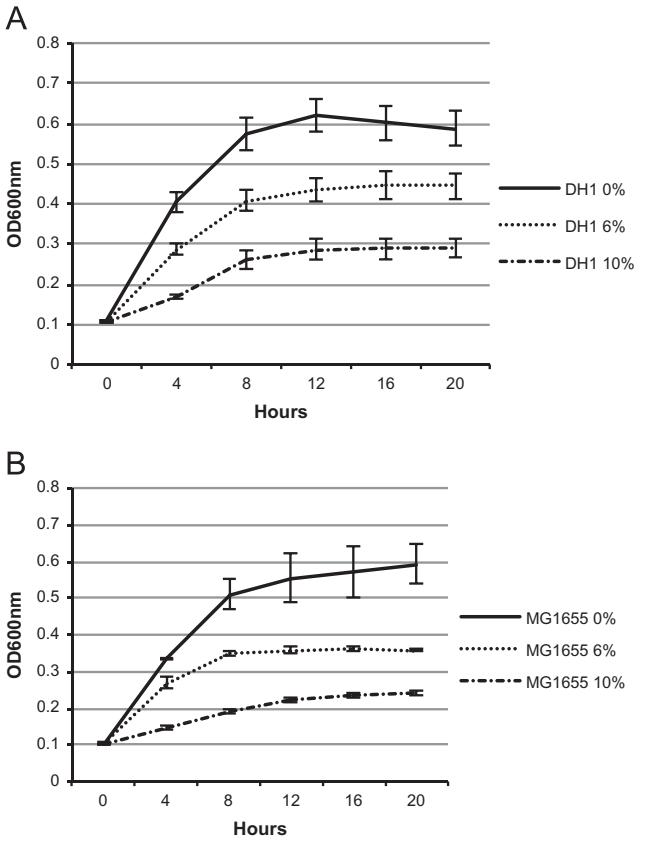

Fig. 1. Growth impact of 0%, 6% and 10% 1,4-BDO on *E. coli* strains DH1 (A), and MG1655 (B) measured as change in optical density over time.

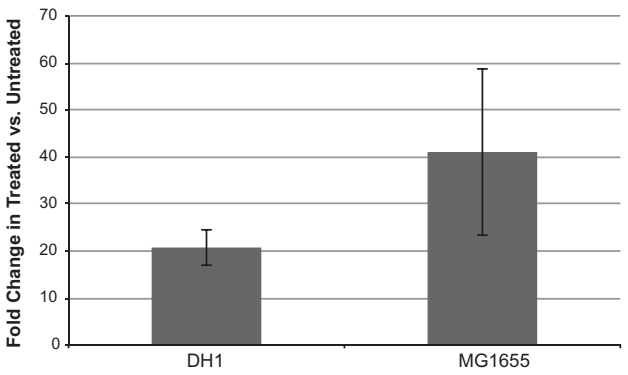

Fig. 2. Quantitative PCR measurement of the *yhjX* transcript in response to a one hour 6% 1,4-BDO exposure in *E. coli* strains DH1 and MG1655. Ratio shown is the level of *yhjX* cDNA for a given strain grown with or without 6% 1,4-BDO.

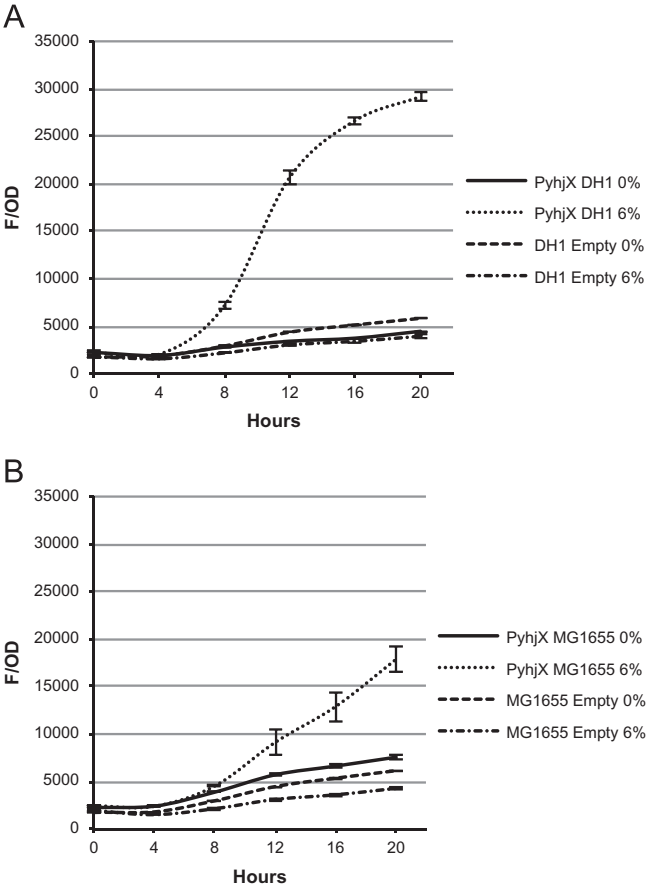

Fig. 3. Specific florescence in *E. coli* DH1 (A) and *E. coli* MG1655 (B) containing a P_{yhjX}: GFP construct using 1,4-BDO (0%, 6%) in the medium. Control vector in both strains is a P_{yhjX} only construct as described in the Section 2. Specific fluorescence (*F*/OD) is calculated as Fluorescence units/Abs at 595 nm.

Using quantitative RT-PCR we evaluated the differential expression of the most upregulated gene, *yhjX*, during 1,4-BDO exposure in *E. coli* DH1 in a preliminary gene expression experiment (Supplementary material). We confirmed the upregulation of *yhjX* upon exposure to 6% 1,4-BDO in both *E. coli* strains (Fig. 2). The *yhjX* transcript was upregulated to different levels the two strains in response to 1,4-BDO, 21 and 41 fold in DH1 and MG1655, respectively. Thus while MG1655 is more sensitive to 1,4-BDO, the transcriptional response of *yhjX* in DH1 appears to have greater dynamic range.

To examine if differences in the promoter or protein coding sequence of *yhjX* between DH1 and MG1655 could explain the different levels of upregulation, the *yhjX* coding sequence and 150 bp upstream of the start codon were amplified from each strain, cloned and sequenced. The sequences from the two strains were found to be identical to the sequences available on ecocyc. Thus, the same variant of the protein is being expressed to different levels using the same promoter sequences in response to 1,4-BDO in these strains. Downstream sequences and other regulatory mechanisms were not examined, but may also impact protein levels and the resulting function.

DH1 and MG1655 are two of the most commonly used *E. coli* strains for metabolic engineering, and *yhjX* was highly upregulated in the presence of 1,4-BDO in these strains. Thus, we tested the ability of the *yhjX* promoter to serve as a biosensor for 1,4-BDO. The promoter region of *yhjX* (P_{yhjX}) used to control expression of the gene encoding GFP in a plasmid-borne system was introduced into the wild type *E. coli* strains. Our results indicate that the P_{yhjX}: GFP construct generates a fluorescent signal that increases with increases in the levels of 1,4-BDO (Fig. 3). *E. coli* DH1 produced the best response from this plasmid based system, and the optimal range of the GFP based reporter is from 4% to 7% 1,4-BDO (Fig. 4).

Similar trends but slightly diminished reporter activity was observed in *E. coli* MG1655 (Fig. S2). A survey of other alcohols (e.g. 1,5-pentanediol, 1,3-propanediol) elicited variable fluorescent signals (Supplementary Fig. S3), suggesting that the P_{yhjX}:GFP construct may be useful for the detection of other end metabolites also.

We also evaluated the impact of *yhjX* on 1,4-BDO sensitivity in *E. coli*. We constructed gene deletion strains in MG1655 and DH1. Deletion of *yhjX* resulted in an improved growth in control medium relative to the wild-type strains, especially for *E. coli* MG1655 and marginally also for *E. coli* DH1 (Fig. 5). However, the

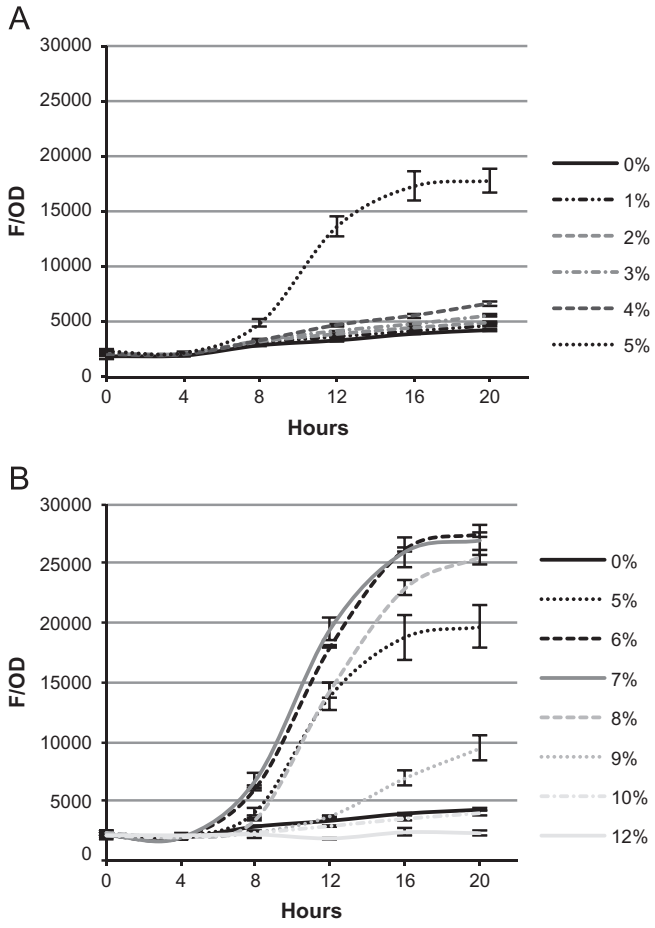

Fig. 4. Specific florescence in *E. coli* DH1 containing a P_{yhjX}: GFP construct with increasing levels of 1,4-BDO (0–5%) (A), and (5–12%) (B). Equivalent survey of the P_{yhjX}: GFP construct in *E. coli* MG1655 is provided in the supplementary section. Specific fluorescence (*F*/OD) is calculated as Fluorescence units/Abs at 595 nm.

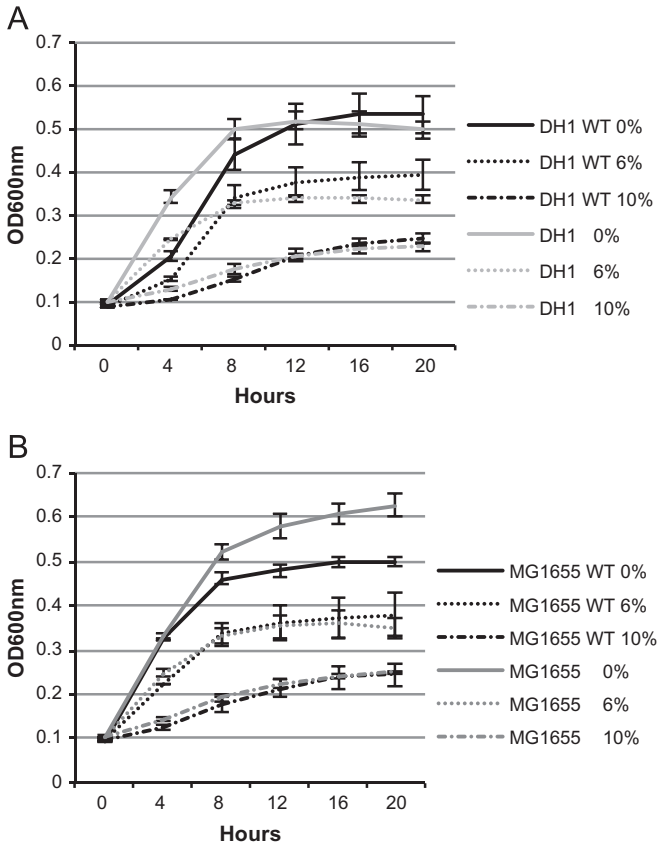

Fig. 5. Comparison of the *E. coli* DH1 to *E. coli* DH1Δ*yhjX* (A) and *E. coli* MG1655 to *E. coli* MG1655Δ*yhjX* (B) in the presence of 0%, 6% 10% 1,4-BDO in the medium.

response to 1,4-BDO remained similar to the wild type strains. Similarly, expression of the *yhjX* gene did not result in any improvement in 1,4-BDO tolerance (Fig. 6). Using IPTG levels greater than 5 μM during 1,4-BDO exposure resulted in additional reduction in growth (data not shown). Since the impact of *yhjX* overexpression may be better observed in a *yhjX* deleted strain, P_{lac}:*yhjX* constructs were evaluated with and without 1,4-BDO exposure in both wild type and *ΔyhjX* strains and compared with the same strains harboring a control vector (Fig. 6). Similar to the wild type strains with a native copy of *yhjX*, the *ΔyhjX* strains also showed no improvement in 1,4-BDO tolerance using *yhjX* expression.

4. Discussion

For compounds such as 1,4-BDO, measuring the production of the final metabolite is a key step in metabolic engineering efforts. However, in the successful employment of a combinatorial approach, testing a large number of gene alterations and growth parameters, analytical measurements such as GC–MS, though precise, can be the rate-limiting step. Genes that have high and specific upregulation in response to the final product can be invaluable in the development of biosensors and are amenable for use in a high throughput manner. A recent example is that of a biosensor for butanol, developed using a heterologous n-butanol-sensing transcription factor-promoter pair in *E. coli* (Dietrich et al., 2012), that enables production measurements that are intrinsically high throughput. A review of the process to manufacture 1,4-BDO in *E. coli* states that an economically viable process requires 10% or more production (Burk, 2010; Yim et al., 2011), while the current maximum reported is about 2% 1,4-BDO (Yim et al., 2011). As such, considerable additional strain engineering remains in the efforts to improve production above this current 2%. A 1,4-BDO biosensor provides a useful tool to expedite such engineering. The growth conditions used for 1,4-BDO production itself would have little or no impact on the P_{yhjX}:reporter performance, as the biosensor strain is likely to be used separate from the production strain. Under the growth conditions used in our study, the dynamic range of the generic GFP reporter is from 4% to 7% 1,4-BDO and is fairly specific to this diol relative to other components in the media. These limits are possibly defined by the toxicity of 1,4-BDO. While the toxicity may effectively induce genes only at concentrations of 4% or greater, the impact on cell growth beyond 7% places the upper limit on this detection. This range can be optimized using alternate reporter proteins that have greater sensitivity, reformulated media to reduce autofluorescence, different promoter strengths (such as by altering the RBS), and plasmid copy number. Such a biosensor can be used either within a production strain or via exogenous addition of extracted production cultures. A platform that uses exogenous detection allows both concentration and dilution of the production culture extract so as to further extend the 4–7% detection range. Further, an exogenous detection platform also eliminates the requirement of additional engineering of the production strain.

Since tolerance engineering for the development of robust hosts is a valuable goal we wanted to explore the potential role of *yhjX* in 1,4-BDO tolerance. *yhjX* is a putative major facilitator superfamily 1 transporter (Pao et al., 1998), suggested to be an oxalate-malate antiporter in most *E. coli* annotation databases

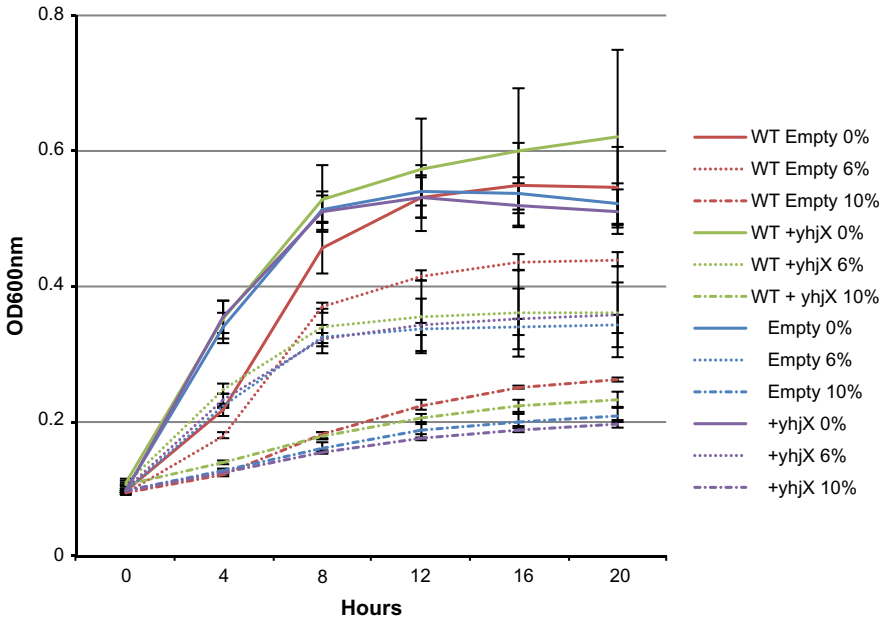

Fig. 6. 1,4-BDO exposure in *E. coli* DH1 wild type or DH1 Δ*yhjX* strains containing either a plasmid with a P_{lac} only (empty) or a P_{lac}: *yhjX* (yhjX) construct and grown in the presence of 0%, 6% 10% 1,4-BDO in the medium.

(Keseler et al., 2011). Very recently, in *E. coli* MG1655, *yhjX* was found to be the sole target of a two-component system YpdA/YpdB that was highly and specifically induced by pyruvate (Fried et al., 2013). Ecogene.org (Zhou and Rudd, 2013) lists other studies where the *yhjX* transcript shows upregulation. These include the study of a solvent-tolerant *E. coli* strain (TK31, derived from JA300) in a cyclohexane:*p*-xylene mix (Shimizu et al., 2005), *E. coli* W3110 exposed to oxygen limitation and pH change (Hayes et al., 2006), *E. coli* W3110 exposed to benzoate (Kannan et al., 2008), and MG1655 treated with small toxic peptides (Fozo et al., 2008). A factor that makes it challenging to compare results from these various studies is that they were conducted in different *E. coli* strains, using different growth conditions and measured using different measurement platforms. However, the large number of conditions and compounds evaluated in other reports (Fried et al., 2013) indicates *yhjX* to be very specifically regulated in *E. coli* MG1655.

In the present study, in both DH1 and MG1655, the deletion of this gene resulted in an improved growth profile under control growth conditions but neither strain showed any significant change in 1,4-BDO tolerance. Expression of this gene, using a plasmid borne expression system, provided no additional tolerance in either the wild type or the *ΔyhjX* strains. Higher induction of the *yhjX* gene expression resulted in growth impairment in both *E. coli* strains. Membrane protein overexpression often results in a decrease in growth (Wagner et al., 2007), and in host engineering applications very low induction is typically ideal (Dunlop et al., 2011). It is possible that high levels of the YhjX transporter is only beneficial under specific conditions but is otherwise burdensome. Such a burden may explain the growth improvement observed in the gene deletion strains in control medium, but not during 1,4-BDO exposure. None of the conditions we tested could identify YhjX levels that would provide better 1,4-BDO tolerance relative to the control strains.

Genes that show such high and relatively specific upregulation in engineered strains have also been used to develop dynamic sensory systems. Recent examples of such use include promoters that respond to pathway intermediates and resulted in 7.5 fold improvement in production (Zhang et al., 2012a, 2012b). While in these cases, the dynamic sensory system was used to drive a metabolic pathway; native regulation has also been used to express heterologous proteins in a robust, inducer free manner (Tsao et al., 2010). The P_{yhjX} promoter may also serve as a promoter to regulate 1,4-BDO export or tolerance mechanisms, so as to provide optimal levels of the corresponding proteins, reduce conflict with promoters being used for other engineered parts, and to avoid the use of costly inducers such as IPTG.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ces.2013.06.044.

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