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RESEARCH ARTICLE

Improving Microbial Biogasoline Production in *Escherichia coli* Using Tolerance Engineering

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ABSTRACT **Engineering microbial hosts for the production of fungible fuels requires mitigation of limitations posed on the production capacity. One such limitation arises from the inherent toxicity of solvent-like biofuel compounds to production strains, such as *Escherichia coli*. Here we show the importance of host engineering for the production of short-chain alcohols by studying the overexpression of genes upregulated in response to exogenous isopentenol. Using systems biology data, we selected 40 genes that were upregulated following isopentenol exposure and subsequently overexpressed them in *E. coli*. Overexpression of several of these candidates improved tolerance to exogenously added isopentenol. Genes conferring isopentenol tolerance phenotypes belonged to diverse functional groups, such as oxidative stress response (*soxS*, *fpr*, and *nrdH*), general stress response (*metR*, *yqhD*, and *gidB*), heat shock-related response (*ibpA*), and transport (*mdlB*). To determine if these genes could also improve isopentenol production, we coexpressed the tolerance-enhancing genes individually with an isopentenol production pathway. Our data show that expression of 6 of the 8 candidates improved the production of isopentenol in *E. coli*, with the methionine biosynthesis regulator MetR improving the titer for isopentenol production by 55%. Additionally, expression of MdlB, an ABC transporter, facilitated a 12% improvement in isopentenol production. To our knowledge, MdlB is the first example of a transporter that can be used to improve production of a short-chain alcohol and provides a valuable new avenue for host engineering in biogasoline production.**

IMPORTANCE **The use of microbial host platforms for the production of bulk commodities, such as chemicals and fuels, is now a focus of many biotechnology efforts. Many of these compounds are inherently toxic to the host microbe, which in turn places a limit on production despite efforts to optimize the bioconversion pathways. In order to achieve economically viable production levels, it is also necessary to engineer production strains with improved tolerance to these compounds. We demonstrate that microbial tolerance engineering using transcriptomics data can also identify targets that improve production. Our results include an exporter and a methionine biosynthesis regulator that improve isopentenol production, providing a starting point to further engineer the host for biogasoline production.**

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Isopentenol (3-methyl-3-buten-1-ol) is an important target compound as a precursor to pharmaceuticals, flavor compounds, such as prenol and isoamyl alcohol, and industrial chemicals, such as isoprene, and also for its tremendous potential as an immediately usable biofuel (1, 2). However, the microbial production of such compounds in bulk from renewable biomass is challenging, especially when considering the required yields for an economically viable process. Isopentenol production in *Escherichia coli* has been reported to use two different metabolic routes: via the amino acid biosynthetic pathway (3) and by refactoring the *Saccharomyces cerevisiae* mevalonate pathway in *E. coli* (4–6). The reported titers for isopentenol production in *E. coli* using the heterologous yeast-based mevalonate pathway now range in the gram-per-liter scale (4, 6). The inherent toxicity of these solvent-like compounds to the host microbe may limit the titer as production titers approach toxic concentrations. The lower limit of short-chain-alcohol toxicity in *E. coli* ranges from 2 to 8 g liter^{-1} (7), concentrations too low to be industrially relevant. In order to achieve economically viable product titers, it is necessary to engineer production strains to tolerate high titers of the product. To our knowledge, no tolerance candidates for C_5 alcohols have been reported to date.

Here we determined if improved isopentenol tolerance would lead to increased titers. Several recent reports of the microbial

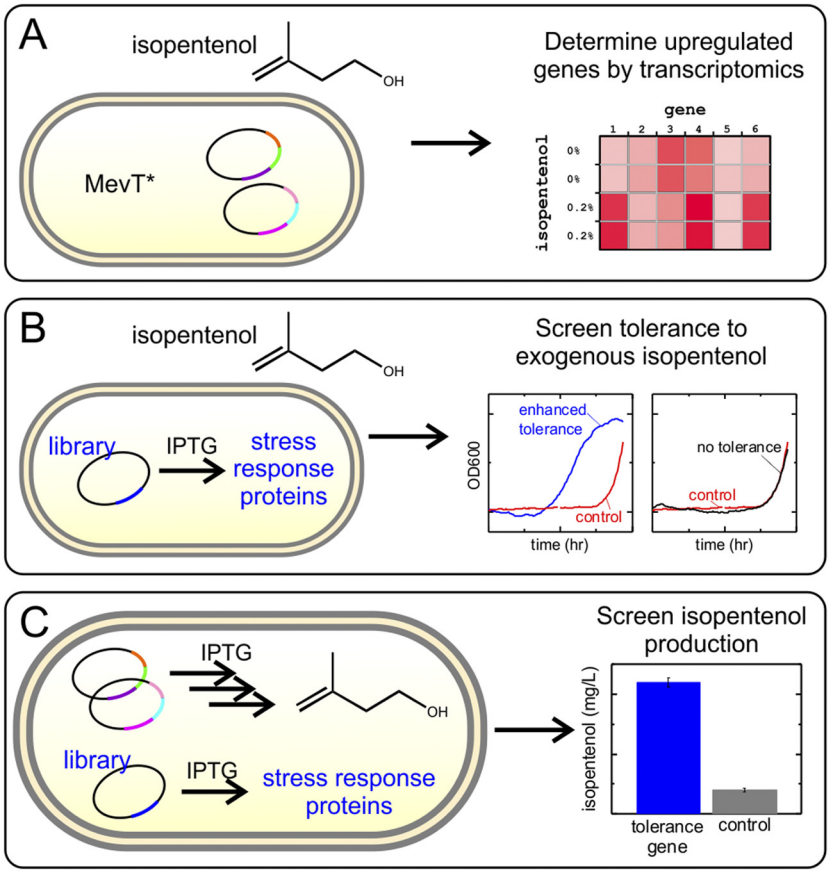

FIG 1 Scheme for screening tolerance and increasing titer of isopentenol. (A) First, transcriptomics of *E. coli* DH1 expressing an inactive mevalonate pathway (MevT*; see Materials and Methods) in the presence of exogenous isopentenol was used to determine what genes are upregulated in response to isopentenol stress. (B) A library of those genes was overexpressed in the presence of exogenous isopentenol. Growth curves were used to measure enhanced tolerance. (C) Finally, genes that conferred tolerance were tested for their ability to increase the isopentenol titer in a production strain.

production of solvent-like compounds indicate the toxicity of these compounds to be a key limitation in obtaining desirable production levels (8, 9). Cellular transport specifically has emerged as a direct yet effective mechanism for improved tolerance and, where pertinent, production of solvent-like compounds. We have shown in previous studies that a highly effective strategy of using RND (resistance-nodulation-division) efflux pumps for solvent tolerance may not be applicable to shorter-chain alcohols (7). Other classes of pumps have been discovered and described for tolerance engineering of aromatic acids, terpenes, and ionic liquids (10–12) but have not been evaluated for short-chain alcohols.

Functional genomics measurements reveal a large number of responses to toxic compounds, including many other modes of alleviating solvent stress (13, 14). Due to the promise of utilizing systems biology to engineer alcohol-tolerant strains (15), we used transcriptomics data to identify genes that may serve as candidates for tolerance engineering for isopentenol. Whole-genome transcriptomics revealed several genes that were responsive to exogenous isopentenol (Fig. 1A). We then tested the efficacies of the most highly induced candidates to improve tolerance to exogenous isopentenol (Fig. 1B). The candidates that successfully alleviated growth lag when overexpressed in the presence of isopentenol were then integrated with an isopentenol production strain (Fig. 1C) (6). Several candidate genes improved isopentenol tolerance, and one candidate, MetR, improved the isopentenol titer by 55%. The putative ATP binding cassette (ABC) transporter MdlB increased the titer by 12% and is, to our knowledge, the first example of an exporter that has improved the titer of a short-chain alcohol. The knowledge of even one such transporter for a desirable final product can lead to considerable additional discoveries, such as bioprospecting for homologs (7) and utilizing directed evolution (12, 16). These results demonstrate the importance of utilizing transcriptomics data for improving short-chain alcohol production as a guide for host engineering.

RESULTS

Identification of *E. coli* genes that confer tolerance to isopentenol. An *E. coli* isopentenol production strain with an inactive mevalonate-based isoprenoid biosynthetic pathway due to a mutation in the 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) synthase (17, 18) was treated with 0.2% (wt/vol) isopentenol. Changes in transcriptional levels of the genes in response to isopentenol stress were determined by microarray analysis. The distribution of COGs (clusters of orthologous groups) and the top 100 up- and top 100 down-regulated genes are listed in Table S1 in the supplemental material. Forty of the top hundred significantly upregulated genes ($\log_2$ value $>$ 1.9) were selected for further

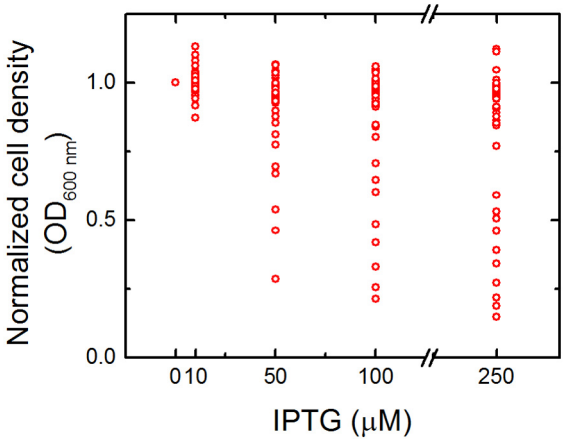

FIG 2 Induction of tolerance genes is toxic above 10 μM IPTG. *E. coli* DH1 harboring each of the 40 genes cloned into pBbA5k was induced with 0, 10, 50, 100, and 250 μM IPTG and grown for 21 h. For each strain, the OD_{600} was measured and normalized to growth without IPTG. The only concentration of IPTG that did not decrease growth across all strains was 10 μM IPTG; thus, all subsequent tolerance assays were performed with 10 μM IPTG. For OD_{600} data for a specific strain, see Fig. S1 in the supplemental material.

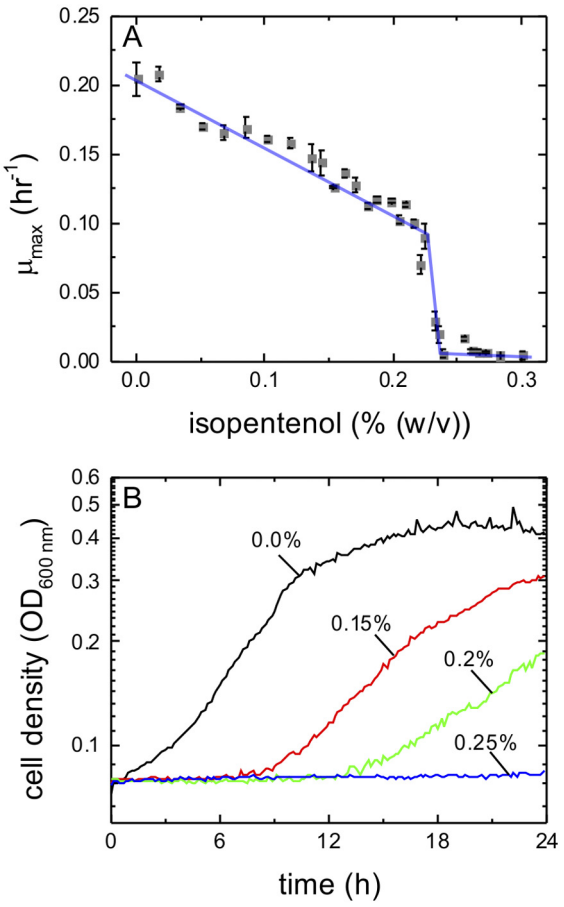

FIG 3 Addition of isopentenol decreases the growth rate and causes growth lag. (A) Maximum growth rates (μ_{max}) of DH1 pBbA5k-*rfp* induced with 10 μM IPTG were determined with exposure to 0 to 0.3% (wt/vol) isopentenol. Error bars represent standard errors from at least 4 replicates. The MIC under these growth conditions is 0.24% (wt/vol) isopentenol. (B) Representative growth curves of *E. coli* DH1 harboring pBbA5k-*rfp* induced with 10 μM IPTG and grown in the presence of 0% (wt/vol), 0.10% (wt/vol), 0.15% (wt/vol), and 0.20% (wt/vol) isopentenol. The addition of 0.15% exogenous isopentenol caused a sufficient lag in growth recovery, and thus 0.15% isopentenol was chosen as the concentration of isopentenol for tolerance assays.

evaluation in improving isopentenol tolerance (see Table S2). The gene candidates were cloned into pBbA5k for overexpression using the P_{lac} promoter to investigate their potential in improving isopentenol tolerance in *E. coli* DH1.

The expression burden of the 40 candidates expressed using medium-copy-number pBbA5k (see Table S3 in the supplemental material) was evaluated to avoid potential protein expression toxicity in the host. At 50 μM isopropyl-β-D-thiogalactopyranoside (IPTG) and greater, a subset of strains showed decreased final cell density after 21 h of growth, indicating expression toxicity (Fig. 2; see also Fig. S1). Thus, 10 μM IPTG was chosen as a suitable induction concentration to reduce potential protein toxicity in subsequent tolerance analysis.

The control strain, *E. coli* DH1 harboring pBbA5k-*rfp* (red fluorescent protein [RFP]), was used to determine the concentration of isopentenol to be used for growth impact assays. The growth rate of the control strain was measured with exposure to 0 to 0.3% (wt/vol) isopentenol. The MIC was determined to be 0.24% (wt/vol) isopentenol for DH1 pBbA5k-*rfp* (Fig. 3A). In addition to slowing the growth rate, isopentenol lengthened the growth lag (Fig. 3B). The addition of 0.15% (wt/vol) exogenous isopentenol caused a sufficient change in growth rate and lag, and thus 0.15% (wt/vol) exogenous isopentenol was used for all subsequent growth impact assays.

Growth impact assays of the 40 strains overexpressing the tolerance candidates revealed many strains with reduced growth lag compared to that of the control strain in the presence of 0.15% (wt/vol) isopentenol (see Fig. S2 in the supplemental material). The strains that demonstrated shortened growth lag in the initial screen were assayed again in triplicate (Fig. 4). The eight genes that reduced the isopentenol-related growth lag were *metR*, *ibpA*, *nrdH*, *soxS*, *mdlB*, *fpr*, *gidB*, and *yqhD*.

The majority of tolerance-conferring genes increase isopentenol production. Next, we tested the potential of the tolerance-conferring genes to improve isopentenol production. To avoid plasmid compatibility issues upon cotransformation with the isopentenol pathway plasmids (specifically, pJBEI-6830 [6]), the confirmed tolerance genes were subcloned into a low-copy-number plasmid, pBbS5k, prior to transformation into the production strain. The isopentenol production pathway is induced with a high IPTG concentration (500 μM IPTG) (6); however, the tolerance-conferring genes expressed on the medium-copy plasmid pBbA5k had demonstrated toxicity when induced with as little as 50 μM IPTG (Fig. 2). DH1 harboring only the tolerance-enhancing genes on the low-copy-number plasmid pBbS5k was tested for toxicity when induced with 500 μM IPTG (see Fig. S3 in the supplemental material). These strains grew similarly to the RFP-expressing control strain and thus allowed the use of the 500 μM concentration of IPTG required for isopentenol induction without the previously measured toxicity that had been observed during candidate tolerance protein expression (see Fig. S3).

The eight genes with confirmed tolerance phenotypes were individually expressed in an isopentenol production strain, specifically *E. coli* DH1 harboring the isopentenol production plasmids

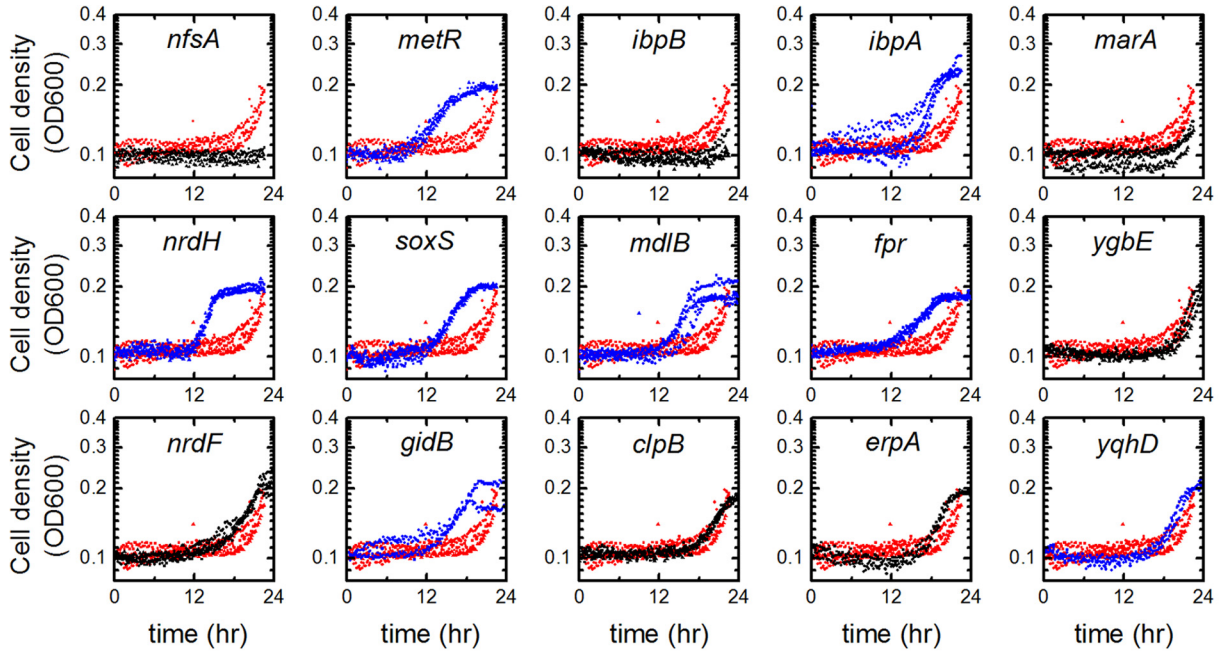

FIG 4 Eight gene candidates demonstrated tolerance to 0.15% (wt/vol) isopentenol. Strains that demonstrated isopentenol tolerance in the initial screen were screened in triplicate for their tolerance to 0.15% (wt/vol) isopentenol (black or blue) in comparison to an *rfp*-expressing strain (red traces). The eight genes that conferred the greatest growth lag shortening, shown in blue, are *metR*, *ibpA*, *nrdH*, *soxS*, *mdlB*, *fpr*, *gidB*, and *yqhD*.

pJBEI-6830 and pJBEI-6833 (6), here referred to as the production strain (PS). Production strains coexpressing a given tolerance gene on pBbS5k are here referred to as PS+[gene product]. These strains were induced over a range of IPTG concentrations to determine the dose-response effect of induction on titer. The maximum titer for most strains plateaued after 500 μM IPTG; thus, all subsequent production strains were induced with 500 μM IPTG (see Fig. S4 in the supplemental material). The production of isopentenol was quantified in triplicate for all PS strains at 24, 48, and 72 h after 500 μM IPTG induction (Fig. 5). The PS strain coexpressing RFP on the pBbS5k plasmid (PS+RFP) was used as a titer control. Production of isopentenol was highest at 48 h after induction, after which the quantity of isopentenol in the cultures decreased slightly, likely due to volatility (Fig. 5). At the 48-h time point, the isopentenol titer in the PS+SoxS and PS+NrdH strains were statistically similar to that of PS+RFP. The remaining 6 strains show an increase in the isopentenol titer over that of the PS+RFP control, producing 12 to 55% more isopentenol after 48 h (Fig. 5 and Table 1).

Transporters have emerged as a powerful and simple mechanism to engineer tolerance toward solvent-like molecules (7, 16). The putative ABC transporter MdlB (expressed by PS+MdlB) increased isopentenol titer by 12% over that of the control strain (PS+RFP). To determine if the effect of MdlB was titratable, the titer was measured for PS+MdlB and PS+RFP over a range of IPTG concentrations after 25 h. In this time frame, all IPTG con-

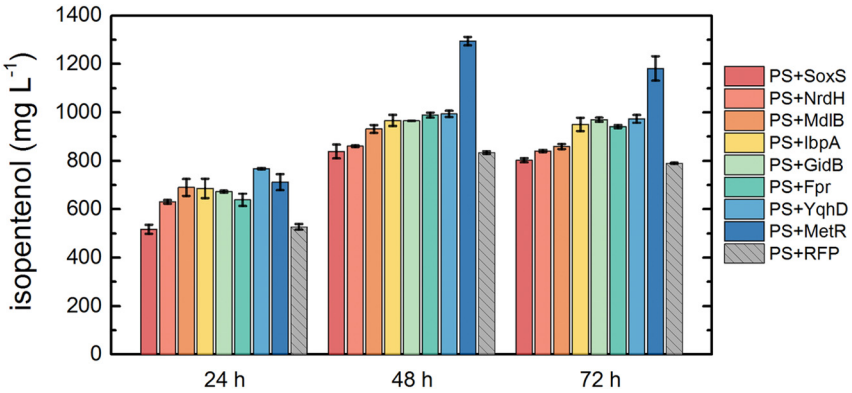

FIG 5 MetR increases production of isopentenol by 55%. The eight tolerance genes were individually coexpressed with the isopentenol production pathway. The tolerance genes and the production pathway were simultaneously induced with 500 μM IPTG in triplicate, and the isopentenol concentration was measured 24, 48, and 72 h after induction. The production strain coexpressing *metR* (PS+MetR) improved the isopentenol titer 55% over that for the *rfp*-expressing strain (PS+RFP).

TABLE 1 Isopentenol tolerance-enhancing genes

Gene	Description	Microarray $\log_2$, *z* score	Isopentenol production (mg liter^{-1})[a]	Improvement in titer (%)[b]	Reference(s)
soxS	DNA-binding transcriptional dual regulator	3.51, 2.81	838 ± 29	0	28, 42, 43
nrdH	Glutaredoxin-like protein	2.17, 2.08	860 ± 3	3	44
mdlB	Predicted multidrug ABC transporter	3.75, 2.31	931 ± 16	12	25
ibpA	Heat shock chaperone	4.80, 2.82	967 ± 23	16	26, 27
gidB	Glucose-inhibited division protein B	5.32, 2.64	965 ± 1	16	29, 45, 46
fpr	Ferredoxin-NADP reductase	2.30, 2.07	989 ± 10	19	28
yqhD	Alcohol dehydro-genase, NAD(P) dependent	2.40, 2.39	994 ± 13	19	14, 30, 31
metR	DNA-binding transcriptional activator	2.57, 2.55	1,290 ± 20	55	32, 33

[a] Averages from triplicates after 48 h.
[b] Percent improvement in titer is in comparison to result for PS+RFP at 48 h (834 ± 5 mg liter^{-1}).

centrations produced statistically similar titers; thus, we infer that any change in the titer produced by PS+MdlB is due to increasing levels of MdlB. PS+MdlB produced more isopentenol than the control strain at all IPTG concentrations (Fig. 6), increasing the titer over that for the control strain by about 20%, 50%, and 60% when induced with 100 μM, 250 μM, and 500 μM IPTG, respectively.

***mdlB* but not *metR* is specific to isopentenol tolerance.** MdlB and MetR are particularly interesting because both methionine biosynthesis regulation and pumps have been implicated as candidates to improve tolerance to other alcohols (7, 19–21). To determine specificity, we investigated if MdlB or MetR conferred tolerance to other short-chain alcohols. While MdlB and MetR expression reduced the growth lag in the presence of isopentenol, the alternate alcohols did not cause a growth lag, and thus the growth rate was used as a metric for tolerance (see Fig. S5 in the supplemental material). Expression of MdlB did not improve the growth rate or final cell density for any other alcohol tested. Expression of MetR slightly improved the growth rate in the presence of ethanol and butanol by 2.5 and 15% (see Fig. S5). This suggests that the mechanism for MdlB improving isopentenol tolerance is specific to isopentenol but that MetR may have broader applications in improving alcohol tolerance.

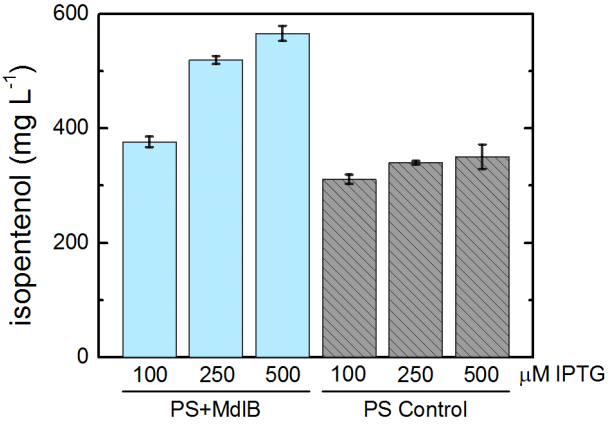

FIG 6 The putative ABC transporter MdlB increases isopentenol production. The isopentenol titer was measured in triplicate in PS+MdlB and PS+RFP over a range of IPTG concentrations to determine if the increase in production is titratable. Isopentenol titer is significantly increased in PS+MdlB at 100 μM, 250 μM, and 500 μM IPTG (P value $=8 \times 10^{-4}$, 2×10^{-6}, and 1×10^{-4}, respectively, two-sided t test).

DISCUSSION

Systems biology can play an important role in metabolic and host engineering. In the past decade, a growing number of reports have successfully used genome-wide methods to discover and utilize pathways, genes, and regulatory mechanisms to obtain superior host platforms or to optimize metabolic pathways. Ranging from genome-wide response changes implemented using altered (22) or heterologous (23) regulators to recombineering-based genome editing (24), there are powerful methods to develop host microbes with a desirable phenotype that complements bioproduction pathways. Traditional transcriptomics remains part of this toolbox and has been used extensively to understand cellular responses to final products that are also stressors (13–15). However, candidates that show high differential expression in response to these compounds may not necessarily improve tolerance (15). Increased tolerance also may not correspond invariably with increased production. Therefore, most candidates shortlisted from such efforts must be examined further for their efficacy in improving tolerance and production.

Studies of *E. coli* stress responses to other alcohols, such as *n*-butanol, have shown that short-chain alcohols primarily cause protein misfolding, cell envelope stress, and oxidative stress (14). The most highly responsive candidates during isopentenol exposure spanned several functional categories. Upregulated genes were dominated by chaperones (*ibpA* and *ibpB*), redox and energy balance genes (*soxS*, *frmB*, *nrdH*, and *yqhD*), and membrane proteins (*mdlB*, *tauC*, and *mdtC*). Several transcriptional factors, some of which are known to regulate genes in response to solvent tolerance, were also upregulated (*marA*, *yqhC*, and *metR*). The transcriptomics in response to exogenous isopentenol are summarized in the supplementary section (see Table S1 in the supplemental material). Of the 100 upregulated candidates ($\log_2$ value > 1.9), 40 candidates, distributed over several functional groups, were selected for further evaluation. The selected candidates included proteins involved in oxidative stress relief, RNA/DNA repair and synthesis (e.g., ribonucleotide reductase and redox coenzymes), the prevention and refolding of misfolded protein (e.g., heat shock proteins and chaperones), transport, metabolism, and growth, as well as several hypothetical proteins (see Table S2 in the supplemental material).

Of the 40 candidates screened, 8 candidates were found to confer tolerance to exogenously added isopentenol: *metR*, *ipbA*, *nrdH*, *soxS*, *mdlB*, *fpr*, *gidB*, and *yqhD*. Table 1 and also Text S1 in the supplemental material briefly describe the native functions of

these genes. These genes were then overexpressed in an isopentenol production strain (6) to determine if any genes that conferred tolerance also improved the isopentenol titer (Fig. 5). Since the tolerance phenotype was selected using the exogenous addition of isopentenol, *a priori*, it is challenging to predict the candidates that may also have an impact on production.

The tolerance genes that did not increase production of isopentenol were *soxS* and *nrdH*. The overexpression of these genes allowed the strain to recover from the sudden high concentration of exogenously added isopentenol (Fig. 4), which suggests that the resulting proteins are directly or indirectly involved in ameliorating isopentenol stress. However, the lack of corresponding change in production suggests that the mechanisms by which these candidates function may not impose a net impact on the efficiency of the pathway or the flux of carbon toward this final product (Fig. 5).

The genes that increased both tolerance and isopentenol production were *mdlB*, *ibpA*, *gidB*, *fpr*, *yqhD*, and *metR* (Fig. 4 and 5). Broadly, these genes have been implicated in stress response or cellular repair mechanisms. MdlB is a predicted multidrug transporter subunit of the ABC transporter superfamily (25). IbpA is a heat shock protein and a molecular chaperone (26, 27). GidB is glucose-inhibited division protein B and is induced under stress conditions known to stimulate ppGpp accumulation (28, 29). Fpr is a ferredoxin-NADP reductase and contributes to damage repair during the SoxRS response of *E. coli* (28). YqhD is an NADPH-dependent alcohol dehydrogenase that has been implicated in increased titers of other biofuel candidates (14, 30, 31). The most notable increase in titer was observed with the overexpression of MetR. MetR is an activator of MetE and MetH of the methionine synthesis pathway (32). Inactivation of MetE by oxidative stress has been reported to result in methionine auxotrophy (33). Based on the reported roles of these genes, no one function may be universally applied to engineering strains to increase the isopentenol titer. Rather, this subset of proteins demonstrates the importance of screening libraries of potential candidates for improving both tolerance and titers.

MdlB is, to our knowledge, the first native pump that has been shown to increase both tolerance and production of short-chain alcohols (Fig. 6) (7). The native function and regulation of MdlB are largely unknown. A survey of existing gene array data for *E. coli* (Ecogene 3.0 [34]) indicates that MdlB is typically upregulated in response to antibiotics such as novomycin or mutations such as Δ*gyrB* that uncoil DNA (35). Short-chain alcohols may passively diffuse through the membrane, making them difficult targets for active export. Further investigation is required to determine the mechanism of MdlB improving the isopentenol titer, perhaps as a pump.

The availability of methionine is known to be limited under stress conditions, such as heat shock, oxidative stress, and solvent stress (19, 33, 36). Here, we have shown that MetR expression increases both isopentenol tolerance and titers (Fig. 5) and MetR slightly improves growth in the presence of butanol (see Fig. S5 in the supplemental material). Methionine biosynthesis was shown to be upregulated in the presence of exogenous furfural, and it was proposed that inhibition of sulfur assimilation limited cysteine and methionine biosynthesis (19). Additionally, ethanol was shown to stall translation at nonstart AUG codons, proposed to be the cause of methionine auxotrophy under solvent stress (20). Furthermore, a deletion in the methionine biosynthesis repressor MetJ increased ethanol tolerance (20). Given that methionine biosynthesis regulation is altered under solvent stress, future work is required to determine amino acid dependence in an isopentenol production strain.

In conclusion, we used transcript level measurements to screen gene candidates that respond to a key biogasoline candidate, isopentenol. Using tolerance and production studies, we identified several genes that enhanced isopentenol tolerance and in some cases production. This strategy improved the isopentenol titer by up to 55%, and subsequent host engineering will focus on combining these genes for further improvement in the titer.

MATERIALS AND METHODS

Strains, plasmids, oligonucleotides, chemicals, and culture media. See Table S3 in the supplemental material for a complete list of plasmids and strains used in this study. The host strain used for plasmid construction was *E. coli* DH10B (Invitrogen). For microarray studies of isopentenol tolerance, the MevT* strain [*E. coli* DH1 harboring pMevT(C159A), pMevB, and pC9b] was used. The MevT* strain expresses an inactive version of the mevalonate-based isoprenoid biosynthetic pathway due to a mutation in HMG-CoA synthase on pMevT(C159A) (17, 18). The MevT* strain also harbors pMevB (17, 18) and pC9b (5). All vectors in the MevT* strain are expressed on an IPTG-inducible *lacUV5* promoter. For growth assays and isopentenol production, *E. coli* DH1 (ATCC 33849) was used. Gene candidates that could potentially improve isopentenol tolerance were cloned into the BioBrick vectors pBbA5k and pBbS5k (37). Both vectors have a *lacUV5* promoter and kanamycin resistance marker, but the replication origins are p15A and SC101, respectively. The isopentenol production strains all harbor plasmids pJBEI-6830 and pJBEI-6833 (6). The mevalonate pathway genes were cloned into vectors pBbA5c and pTrc99A. This strain was transformed with tolerance-conferring genes cloned into vector pBbS5k for production assays. Oligonucleotides were synthesized by Integrated DNA Technologies (USA) (see Table S4). All strains used in this study are listed in Table S3. All chemicals were purchased from Sigma-Aldrich (USA) unless otherwise stated.

Luria broth (LB) supplemented with the appropriate antibiotics was used during cell cultivation for plasmid construction and preparation of precultures for cell adaptation in minimal medium. Growth assays were performed in M9 minimal medium, which consisted of 1× M9 salt (Difco), 2 mM $MgSO_4$, 100 μM $CaCl_2$, 0.5 mg liter^{-1} thiamine, and 0.4% glucose. Isopentenol production strains were grown in a modified M9 3-morpholinopropane-1-sulfonic acid (MOPS) minimal medium (MM9), which consisted of 1× M9 salt (Difco), 75 mM MOPS (pH 7.40, 2 mM $MgSO_4$, 10 μM $CaCl_2$, 10 μM $FeSO_4$, 1× micronutrient, and 1% glucose. The 1× micronutrient was composed of 4 μM boric acid, 0.8 μM manganese chloride, 0.3 μM cobalt chloride, 0.15 μM cupric sulfate, 0.1 μM zinc sulfate, and 0.03 μM ammonium molybdate.

Where appropriate, chloramphenicol (50 μg ml^{-1}), carbenicillin (100 μg ml^{-1}), and kanamycin (15 μg ml^{-1} for isopentenol production or 30 μg ml^{-1} otherwise) were added to the medium.

Microarray sample preparation and data analysis. *E. coli* DH1 MevT* was used to determine genes upregulated in the presence of exogenous isopentenol. The strain was grown in MM9 with 1% glucose, 2 mM Mg, and 0.5 mM IPTG and treated with 0% or 0.2% isopentenol added exogenously. Samples were collected at 150 min and 390 min after isopentenol addition. mRNA preparation and microarray analysis for whole-genome transcript response were performed as described previously (38).

Cloning and expression of genes. Gene candidates to be tested for improving isopentenol tolerance were PCR amplified using *E. coli* MG1655 genomic DNA purified using the DNeasy blood and tissue kit (Qiagen, Life Bio, New York) as the template. The vector backbones and the sequences of the oligonucleotides used for amplifying the genes are listed in Table S4 in the supplemental material. J5 was used to design the assembly of the gene candidates into the vector backbones (39). The vector backbones were prepared by PCR amplification from pBbA5k-RFP

and pBbS5k-RFP (37), using the oligonucleotides pBb5-F and pBb5-R. iProof high-fidelity DNA polymerase (Bio-Rad, California) was used for the PCRs according to the manufacturer's recommendation.

The genes were cloned into the selected vectors by following an adapted Golden Gate protocol (40, 41). Specifically, for each cloning reaction, 50 ng of the vector backbone was mixed with an equimolar amount of the amplified gene fragment in a solution consisting of 1.5 μl 10× T4 DNA ligase reaction buffer (NEB), 0.15 μl bovine serum albumin (10 mg/ml; NEB), 1 μl BsaI (Fermentas), and 1 μl T4 DNA ligase (2000 cohesive end units/μl; NEB) and made up with deionized water to a final volume of 15 μl. The mixture was subjected to the following program for ligation: 25 cycles at 37°C for 3 min and 16°C at 4 min, followed by 1 cycle at 50°C for 5 min and 80°C for 5 min. DH10B chemically competent cells were transformed with 5 μl of the ligation reaction mixture and recovered in SOC (super optimal broth with catabolite repression) for 1 h at 37°C. The transformants were plated on LB-kanamycin agar plates and incubated overnight at 37°C. Single colonies were inoculated into LB-kanamycin and grown overnight at 37°C with shaking at 200 rpm. Plasmids were isolated, and the genes were verified by DNA sequencing.

Transformation of cells and adaptation of cells to M9 minimal medium. Plasmids expressing tolerance gene candidates were transformed into chemically competent *E. coli* DH1 containing the plasmids for isopentenol production, pJBEI-6830 and pJBEI-6833 (6), resulting in a three-plasmid-containing strain. The transformants were recovered in SOC medium (Invitrogen) for 1 h at 37°C and plated on LB agar supplemented with the appropriate antibiotics.

The strains were adapted to defined M9 minimal medium by subcultivating single colonies in minimal medium. Single colonies were inoculated into 5 ml LB medium supplemented with the appropriate antibiotics and were grown overnight at 37°C with shaking. The overnight cultures were diluted 100-fold in 5 ml minimal medium (M9 for the single-plasmid strains and MM9 for the production strains) supplemented with the appropriate antibiotics and grown for 24 h at 37°C with shaking. Subcultivation was repeated twice more to fully adapt the cells. The adapted cells were centrifuged (3,000 × *g*, 2 min), resuspended in the respective minimal medium with 10% glycerol added, and stored at −80°C in 50-μl aliquots.

Effect of gene overexpression on strain growth. For each strain harboring a single plasmid with the tolerance gene candidate cloned into pBbA5k, a 50-μl aliquot of adapted cells was inoculated into 2 ml M9 supplemented with kanamycin. The cultures were grown overnight at 37°C with shaking at 225 rpm. The cells were diluted to an optical density at 600 nm (OD_{600}) of ~0.05 in fresh M9-kanamycin with 0, 10, 50, 100, or 250 μM IPTG. The plates were sealed with breathable clear film (Thermo Scientific) and grown for 21 h at 37°C with shaking at 225 rpm. The OD_{600} of the cultures was read on a microplate reader (SpectraMax M2; Molecular Devices).

Impact of gene candidates on isopentenol tolerance. Precultures were prepared as described in the previous section and diluted to an OD_{600} of ~0.05 in fresh M9-kanamycin with 10 μM IPTG and 0 to 0.3% (wt/vol) isopentenol, as indicated. All growth curves were measured in 24-well plates sealed with breathable clear film (Thermo Scientific), and the OD_{600} was measured every 10 min while the cells were grown for 24 h at 37°C with shaking in a microtiter plate reader (Tecan Infinite F200). The MIC of isopentenol was determined by calculating the maximum growth rate for at least 4 growth curves per concentration of isopentenol. Subsequent isopentenol growth impact experiments were performed using 0.15% (wt/vol).

Isopentenol production titer in whole culture. Precultures of the production strains were prepared as described in the previous section in MM9 minimal medium supplemented with the appropriated antibiotics and diluted to an OD_{600} of ~0.08 in 5 ml of fresh medium. The cultures were grown at 37°C with shaking until an OD_{600} of ~0.4 was reached. Expression of the production and tolerance genes was then simultaneously induced with 500 μM IPTG. The cultures were subsequently grown at 30°C with shaking for isopentenol production. After 24, 48, and 72 h of induction, 200-μl aliquots of the cultures were extracted using 200 μl ethyl acetate (with 10 mg liter^{-1} *n*-butanol as an internal standard), and the organic extracts were diluted 5-fold with ethyl acetate before they were analyzed by gas chromatography, as described previously (2), to quantify the isopentenol production titer. IPTG dose-response experiments were performed similarly using 100, 250, 500, or 1,000 μM IPTG.

Tolerance to other alcohols. Precultures were prepared as described in previous sections and diluted to an OD_{600} of ~0.08 in fresh M9-kanamycin with 10 μM IPTG and an alcohol of interest. The alcohols chosen and the concentrations used were as follows: ethanol at 3% (vol/vol), *n*-butanol at 0.7% (vol/vol), *n*-pentanol at 0.15% (vol/vol), isopropanol at 2% (vol/vol), isobutanol at 0.8% (vol/vol), and isopentanol at 0.25% (vol/vol). The inhibitory concentrations were derived from previous reports (7, 16). The plates were sealed with breathable clear film (Thermo Scientific), and the OD_{600} was measured every 20 min while the cells were grown for 18 h at 37°C with shaking in a microtiter plate reader (Tecan Infinite F200). Maximum growth rates were calculated for each growth curve.

Microarray data accession number. Complete data obtained in this work are available in the GEO database under accession number GSE53138 and at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE53138.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01932-14/-/DCSupplemental.

Text S1, DOCX file, 0.03 MB.
Figure S1, TIF file, 4 MB.
Figure S2, TIF file, 12.5 MB.
Figure S3, TIF file, 1.5 MB.
Figure S4, TIF file, 0.7 MB.
Figure S5, TIF file, 6.7 MB.
Table S1, PDF file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.
Table S3, DOCX file, 0.02 MB.
Table S4, DOCX file, 0.02 MB.

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