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An evolutionary strategy for isobutanol production strain development in *Escherichia coli*

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ABSTRACT

Higher alcohols such as isobutanol possess several physical characteristics that make them attractive as biofuels such as higher energy densities and infrastructure compatibility. Here we have developed a rapid evolutionary strategy for isolating strains of *Escherichia coli* that effectively produce isobutanol from glucose utilizing random mutagenesis and a growth selection scheme. By selecting for mutants with the ability to grow in the presence of the valine analog norvaline, we obtained *E. coli* NV3; a strain with improved 24-h isobutanol production (8.0 g/L) in comparison with a productivity of 5.3 g/L isobutanol obtained with the parental wild type strain. Genomic sequencing of NV3 identified the insertion of a stop codon in the C-terminus of the RNA polymerase σ^S-factor, RpoS. Upon repair of this inhibitory mutation (strain NV3r1), a final isobutanol titer of 21.2 g/L isobutanol was achieved in 99 h with a yield of 0.31 g isobutanol/g glucose or 76% of theoretical maximum. Furthermore, a mutation in *ldhA*, encoding D-lactate dehydrogenase, was identified in NV3; however, repair of LdhA in NV3r1 had no affect on LdhA activity detected from cell extracts or on isobutanol productivity. Further study of NV3r1 may identify novel genotypes that confer improved isobutanol production.

1. Introduction

Efforts aimed at deriving energy and chemicals from biorenewable resources have intensified dramatically due to concerns of the diminishing supply of fossil fuel reserves and increasing environmental concerns (Stephanopoulos, 2007). Much of this attention has focused, in particular, on the production of ethanol by fermentation. For example, ethanol production reached 9.2 billion gallons in the United States in 2009, a 40% increase in comparison with 2008 (Seiferlein, 2009). However, several physical characteristics of ethanol make it less attractive as a biofuel in comparison with other higher alcohols (C3 or higher) such as its lower energy density and high hygroscopicity, making it incompatible with current infrastructure. Recently, the production of several higher alcohols that do not share these shortcomings has been demonstrated using *Escherichia coli* (Atsumi et al, 2008, 2009; Atsumi and Liao, 2008a; Cann and Liao, 2008; Connor and Liao, 2008; Hanai et al., 2007; Shen and Liao, 2008; Withers et al., 2007; Zhang et al., 2008). In addition to *E. coli*, isobutanol has also been produced using the well-known amino acid producer, *Corynebacterium glutamicum* (Smith et al., 2010), and with *Synechococcus elongatus* through CO_2 fixation via photosynthesis (Atsumi et al., 2009; Lan and Liao, 2011).

Metabolic engineering is a strategy often used to increase the microbial production of valuable products (Stephanolpoulos and Stafford, 2002). Typically, a rational design approach is employed to increase the production of a target compound by overexpression of the biosynthetic pathway and/or inactivation of other competitive pathways. Although this method has been proven to be useful for a wide variety of target compounds (Atsumi et al., 2008; Atsumi and Liao, 2008b; Causey et al., 2004; Lee et al., 2009; Lehmann and Lutke-Everslosh, 2011; Yazdani and Gonzalez, 2008; Yu et al., 2011), further improvement of production often defies biochemical reasoning and physiological insight.

On the other hand, random mutagenesis and selection have been proven to be powerful tools to rapidly isolate a strain with a desired phenotype such as improved amino acid production (Ikeda, 2003). This method allows one to consider an enormous number of genotypes if a proper selective pressure is applied. Using this method, strain development can be quite rapid, although unnecessary and/or inhibitory mutations are often introduced. Therefore, it is highly desirable to identify the beneficial mutations and introduce them into a strain with a clean background.

Random mutagenesis is often done using chemicals that introduce mutations throughout the entire genome such as N′-nitro-N-nitrosoguanidine (NTG) or ethylmethane sulfonate (EMS). Exposing the cell to an analog of the target amino acid

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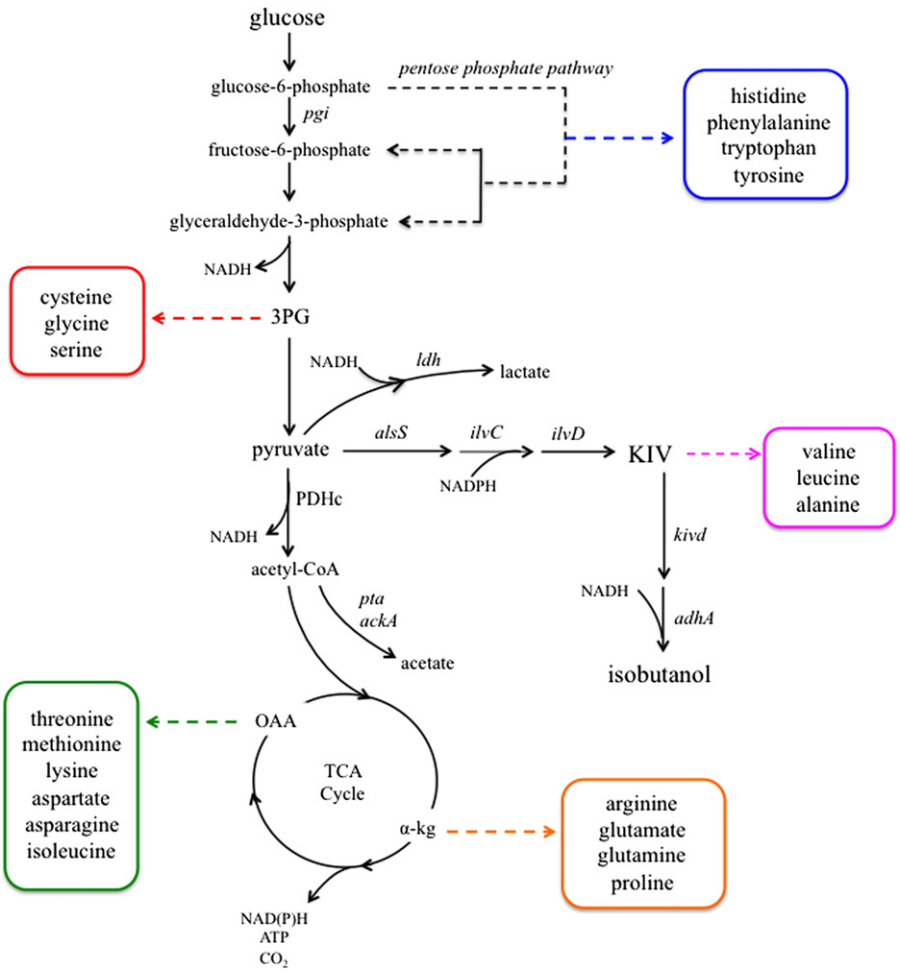

Fig. 1. The isobutanol and amino acid biosynthesis pathways from glucose in *E. coli*. KIV, 2-ketoisovalerate; 3PG, 3-phospho-D-glycerate; α-kg, alpha-ketoglutarate; OAA, oxaloacetate.

can be used as a selective pressure for amino acid strain development. After mutagenesis, mutants are selected for the ability to grow in the presence of these toxic analogs. A minimum concentration of the analog is used such that a cell must be harboring beneficial mutations to survive by deregulating natural biosynthesis or outcompeting the analog for incorporation into growing polypeptides. It is noteworthy to say, however, that better growth in the presence of the analog does not guarantee improved amino acid production. Various resistance mechanisms may be introduced (i.e. improved valine synthesis, improved norvaline efflux, removal of allosteric regulation in key enzymes) and so measuring amino acid production is required. This method has been shown to be successful for engineering strains of *corynebacteria* for valine and lysine production (Oh et al., 1993; Tsuchida and Yoshinaga, 1974), and in *E. coli* for increased valine synthesis (Uemura et al., 1972). This can also be applied towards isolating strains with improved higher alcohol production being that the higher alcohols are derived from the same 2-keto acid precursors of native amino acids. Indeed, the successful isolation of an *E. coli* strain with improved 3-methyl-1-butanol production has been demonstrated using random mutagenesis and selection for growth in the presence of a leucine analog, 4-aza-D,L-leucine (Connor et al., 2010).

Isobutanol production in *E. coli* has been successful using a strain engineered through rational design (Atsumi et al., 2008, 2009) by expression of the heterologous isobutanol pathway from plasmid (Fig. 1). Similarly, the strains presented in this study do not naturally produce isobutanol and are thus harboring plasmids for expression of the isobutanol pathway. This study demonstrates the application of an established strain construction method, commonly used to isolate improved amino acid producers, to generate an improved isobutanol production strain of *E. coli*. Isolation and sequencing of such a strain may then lead to the discovery of new genotypes related to improved higher chain alcohol production.

2. Materials and methods

2.1. Reagents

KOD DNA polymerase was purchased from EMD Chemicals (San Diego, CA). Oligonucleotides were ordered from Integrated DNA Technologies (Coralville, IA). DL-norvaline was purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Strains and plasmids

All *E. coli* strains and plasmids used are listed in Table 1. *E. coli* XL1-Blue (Stratagene) was used to propagate all plasmids. The mutation identified in the *rpoS* gene of strain NV3 (C910T) was repaired by amplification of the C-terminus of *rpoS* from *E. coli* MG1655 genomic DNA (gDNA) using primers K902 (5′ CATACGCAACCTGGTGGATTCGC 3′)+K903 (5′ CAGATGCTTACTTACTCGCGGAACAG 3′) and fusing the resulting PCR fragment by SOE with

Table 1
Strains and plasmids used in this study.

Name	Relevant genotype	Reference
Strains		
E. coli BW25113	$rrnB_{T14}$ Δ*lacZ*WJ16 *hsdR514* Δ$araBAD_{AH33}$ Δ$rhaBAD_{LD78}$	(Datsenko and Wanner, 2000)
E. coli XL1 Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB* $lacI^qZ$Δ*M15 Tn10* (Tet^R)]	Stratagene
JCL16	BW25113/F′ [traD36, proAB+, lacIq ZΔM15 (Tet^R)]	(Atsumi et al., 2008)
JCL260	as BW25113 but Δ*ldhA*Δ*frd*Δ*adhE*Δ*pta*Δ*fnr*Δ*pflB*	(Atsumi et al., 2008)
NV1	NTG-created mutant from JCL16	this study
NV2	mutant evolved from NV1	this study
NV3	NTG-created mutant from NV2	this study
NV3r1	as NV3 but with wild type *rpoS*	this study
NV3r2	as NV3r1 but with wild type *ldhA*, kan^R	this study
Plasmids		
pSA55	ColE1 ori; Amp^R; P_LlacO_1::*kivd–ADH2*	(Atsumi et al., 2008)
pSA65	ColE1 ori; Amp^R; P_LlacO_1::*kivd–adhA*	(Atsumi et al., 2009)
pSA69	p15A ori; Kan^R; P_LlacO_1::*alsS–ilvC–ilvD*	(Atsumi et al., 2008)

a kanamycin cassette amplified from pKD13 (Datsenko and Wanner, 2000) using primers K904 (5′ CGCGAGTAAGTAAGCATCTGCTGTCAAACATGAGAATTAA 3′)+K905 (5′ CACAGAAAAGGCCAGCCTCGCTTGAGACTGGCCTTTCTGAGTGTAGGCTGGAGCTGCTTC 3′). Similarly, *ldhA* was cloned from *E. coli* MG1655 gDNA with primers K1212 (5′ ATTTTTCAATATCGCCATAGCTTTCAATTAAATTTG 3′)+K1213 (5′ TTAAACCAGTTCGTTCGGGCAG 3′) and fused with a kanamycin cassette constructed with primers K1214 (5′ AATCTGGAAAAAGGCGAAACCTGCCCGAACGAACTGGTTTAActgtcaaacatgagaattaa3′)+K1215 (5′ ATTGGGGATTATCTGAATCAGCTCCCCTGGAATGCAGGGGAGCGGCAAGAgtgtaggctggagctgcttc 3′). The *rpoS-Km*^{r} repair cassette was transformed into NV3 harboring pKD46 (Datsenko and Wanner, 2000) and plated on LB+kanamycin (50 μg/ml). Presence of the kanamycin cassette was confirmed by PCR and repair of the *rpoS* mutation to wild type sequence was confirmed using DNA sequencing (Genewiz, La Jolla, CA). Strain NV3r1 was thus obtained by removal of the kanamycin marker using plasmid pCP20 (Datsenko and Wanner, 2000) and is identical to NV3 but with a wild type allele of *rpoS* and a small scar downstream the *rpoS* open reading frame. Likewise, strain NV3r2 was obtained by transforming strain NV3r1 harboring pKD46 (Datsenko and Wanner, 2000) with the constructed *ldhA-Km*^{r} repair cassette. Introduction of the kanamycin cassette was confirmed by PCR and repair of the *ldhA* mutation was confirmed by sequencing.

2.3. Genomic sequencing

The entire genome of NV3 was sequenced at the Joint Genome Institute (Walnut Creek, CA) using Solexa DNA sequencing. Genomic DNA of NV3 was isolated using a Qiagen DNEasy Blood and Tissue kit.

2.4. Media and cultivation

All production cultures were started by 1% (v/v) inoculation from 3 ml LB overnight cultures shaken at 37 °C in a rotary shaker (250 rpm). All production strains were cultured in 3.6% glucose+0.5% yeast extract+M9+1000X Trace Metal Mix (27 g $FeCl_3 \cdot 6H_2O$, 2 g $ZnCl_2 \cdot 4H_2O$, 2 g $CaCl_2 \cdot 2H_2O$, 2 g $Na_2MoO_4 \cdot 2H_2O$, 1.9 g $CuSO_4 \cdot 5H_2O$, 0.5 g H_3BO_3, 100 ml HCl per liter water) and shaken at 37 °C (250 rpm). After an OD_{600} of 0.4–0.5 was reached, production cultures were induced with 0.1 mM IPTG and moved to 30 °C for the remainder of the fermentation. Unless otherwise stated, all production fermentations were performed in 20 ml media in 250 ml screw cap shake flasks. For long-term fermentations (> 24 h), glucose and nutrients (feeding solution) were added as needed to supply additional carbon source. Feeding solution was composed of 1X M9 minimal media+500 g/L glucose+5 g/L yeast extract. pH was monitored every 24 h and 10 M NH_4OH was added as needed to maintain a pH=6.5. Antibiotics were added appropriately to all cultures (50 μg/ml kanamycin, 100 μg/ml ampicillin)

2.5. LdhA enzyme assays

For preparation of cell extract, JCL16, JCL260, NV3, NV3r1 and NV3r2 were inoculated into 3 ml LB test tube cultures and incubated overnight at 37 °C in a rotary shaker (250 rpm). These overnight cultures were then used to inoculate 1% (v/v) 20 ml cultures of production media (3.6% glucose+0.5% yeast extract+M9) in 250 ml screw cap shake flasks. These cultures were then incubated for 24 h at 30 °C in a rotary shaker (250 rpm). 5 ml of each overnight cultures was harvested, concentrated 5-fold to 1 ml in 0.1 M phosphate buffer (pH 7.0) and lysed with 0.1 mM glass beads.

D-lactate dehydrogenase activity of crude lysates was assayed as previously described (Mat-Jan et al., 1989). LdhA activity was detected by measuring NADH oxidation at 340 nm in 1 ml of 100 mM phosphate buffer (pH 7.0), 200 μM NADH, 30 mM pyruvate and 10 μL crude extract. Total protein concentrations were measured by the Bradford assay.

2.6. NTG mutagenesis, selection and screening

N′-nitro-N-nitrosoguanidine (NTG) mutagenesis was performed with wild type *E. coli* JCL16 by amending a method previously described (Miller, 1972). 475 μl of cell suspension in 0.1 M citrate buffer (pH 5.5) was transferred to a 1.5 ml microcentrifuge tube, 25 μl NTG (1 mg/ml) was added and cells were incubated at 37 °C for 30 min to obtain a kill percentage of ~50%. The cells exposed to NTG were washed twice with 1 ml phosphate buffer (pH 7.1), plated on norvaline selection plates (M9 minimal media+0.5% glucose+norvaline) and incubated at 37 °C for 48 h. Of the 2×10^8 cells that were subjected to NTG mutagenesis and plated for growth on selection plates, 119 colonies showed good growth. For isolation of E. coli NV1, the colonies displaying the best growth in 2 g/L norvaline were streaked onto plates of identical selection media and incubated at 37 °C for 48 h to confirm the norvaline resistance phenotype. 96 of the confirmed norvaline resistant mutants displaying the best growth (based on colony size) were then inoculated from colony into 200 μl GlcM9 media (0.5% glucose+M9 minimal media) in a 96-deep-well-plate (Corning Inc., Corning, NY, USA) and shaken at 37 °C in a rotary shaker (250 rpm). After 16 h of incubation, the mutants were challenged in 200 μl GlcM9 media plus 2 g/L norvaline by

transferring 1 μl of overnight culture (0.5% v/v) into a fresh 96-well plate and incubated for 24 h at 37 °C in a rotary shaker (250 rpm). Mutants displaying the best growth in the presence of norvaline were transformed with pSA55 ($P_L lacO_1$::*kivd-ADH2*) and their abilities to produce isobutanol in comparison with JCL16 were assayed in 20 ml production media in a 250 ml screw cap shake flask as previously described, yielding strain NV1.

Strain NV1 was inoculated from colony in 3 ml LB media and incubated for 16 h at 37 °C in a rotary shaker (250 rpm). The overnight culture was then inoculated at different dilutions (0.1% and 1.0% v/v) into 3 ml GlcM9+2 g/L norvaline media in test tubes and incubated at 37 °C in a rotary shaker (250 rpm). Good growth was witnessed in 1% inoculums in 24 h; however, no growth was witnessed in the tubes inoculated at 0.1% until suddenly after 48 h of incubation, indicating a spontaneous mutation. This culture was streaked onto selective plates (GlcM9+2 g/L norvaline) to isolate individual colonies. Isolated colonies were then picked and the phenotype displaying strong 24 h growth in GlcM9+2 g/L norvaline media from low inoculation (0.1% v/v) was confirmed, giving strain NV2.

For construction of NV3, NV2 was subjected to NTG mutagenesis, as previously described for mutagenesis of JCL16, and the exposed cells were then plated on norvaline selective media (GlcM9+2.5 g/L norvaline) for 48 h at 37 °C. Mutants growing on the selective plates were pooled together in 10 ml 0.1 M phosphate buffer (pH 7.0) for inoculation (1% v/v) of 11 ml norvaline screening media (3.6% glucose+0.5% yeast extract+5 g/L norvaline+M9) in sealed microaerobic tubes (12.5 ml capacity, BD Vacutainer). Mutants were enriched for growth in a microaerobic environment to simulate the culture condition used to perform isobutanol production experiments. After inoculation, the microaerobic tubes were incubated at 37 °C in a rotary shaker (250 rpm). Every 24 h, cells with the ability to grow in the selective condition were further challenged by 1% (v/v) inoculation into a fresh tube of the same media composition but with a higher norvaline concentration (8 g/L followed by 10 g/L). Mutants from the microaerobic culture (10 g/L norvaline) were isolated by dilution and plating on 3.6% glucose+0.5% yeast extract+12 g/L norvaline+M9 plates at 37 °C. After 48 h of incubation, colonies showing the best growth on the selective plates were then screened for improved isobutanol production in comparison with JCL16 and NV2 in 10 ml production media in 125 ml screw cap flasks.

2.7. Metabolite detection

Alcohol compounds produced were quantified using gas-chromatography (GC) equipped with flame ionization as previously described (Atsumi et al., 2008). All other compounds were measured by applying filtered fermentation broth to an Agilent 1100 high-performance liquid chromatograph equipped with an autosampler (Agilent Technologies) and a Bio-Rad Aminex HPX87 column (5 mM H_2SO_4; 0.6 ml/min; column temperature, 35 °C; Bio-Rad Laboratories, Hercules, CA).

3. Results

3.1. Random mutagenesis and selection with norvaline

A schematic of the methods used for the construction of the isobutanol producing strain of *E. coli* is shown in Fig. 2. JCL16, the parent strain, was treated with NTG and spread on minimal media plates containing glucose and norvaline (2 g/L). 96 mutants displaying the best resistance to norvaline, based on colony size, were inoculated into a 96-well plate to screen for mutants with significantly improved growth in comparison with JCL16. Mutants showing the most improvement in growth in the presence of 2 g/L norvaline were then screened for improved isobutanol production by transformation with pSA55 ($P_L lacO_1$::*kivd-ADH2*) (Fig. 2).

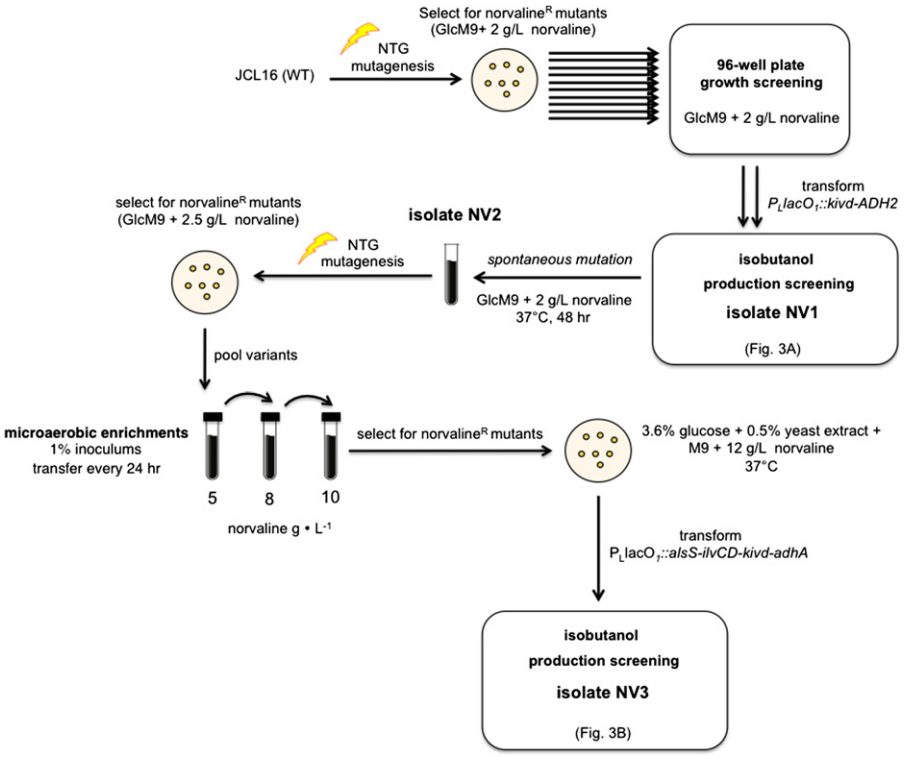

Fig. 2. Schematic of random mutagenesis, selection and screening scheme used to construct strain NV3 from wild type strain JCL16. 'GlcM9' denotes 5 g/L glucose+M9 minimal media.

As shown in Fig. 3A, norvaline resistant mutant G2 (renamed NV1) produced 590 mg/L isobutanol whereas JCL16 produced 200 mg/L isobutanol. In assaying the extent of norvaline resistance of NV1 in 2 g/L norvaline GlcM9 liquid media, we isolated NV2 from a tube showing spontaneous, strong growth from a 0.1% inoculation, which was previously not seen with NV1 (data not shown).

To obtain a more resistant strain and improve isobutanol production further, strain NV2 was subjected to NTG mutagenesis and mutants were selected for growth on minimal media plates with an increased concentration of norvaline (2.5 g/L). After enrichment for fast growing mutants in microaerobic liquid cultures and isolation of colonies on selective plates, ten mutants displaying the best growth on plate were screened for improved isobutanol production (Fig. 3B). Mutant number 10 (renamed NV3) performed the best (6.1 g/L isobutanol) in comparison with NV2 (4.1 g/L) and JCL16 (3.1 g/L; Fig. 3B).

3.2. Isobutanol production with NV3

We performed long-term fermentations of JCL16, NV3 and JCL260 harboring pSA69 ($P_L$lacO1::*alsS–ilvC–ilvD*) and pSA65 ($P_L$lacO_1::*kivd–adhA*) to compare their isobutanol production profiles (Fig. 4A). During the first 24 h, NV3 produced 8.0 g/L isobutanol, while JCL260 produced 7.7 g/L, and JCL16 produced 5.3 g/L. As shown in Fig. 4B, NV3 displayed dramatically increased cell growth. After entry into the stationary phase, JCL260 continued to produce isobutanol, with production reaching 21.0 g/L in 99 h. On the other hand, the isobutanol productivity of NV3 dramatically stalled during the stationary phase of growth, with isobutanol accumulating to only 13.6 g/L. In addition, NV3 began to die much more rapidly in comparison with JCL260 or JCL16 (Fig. 4B). JCL16 exhibited similar growth to JCL260 and ultimately produced 12.5 g/L isobutanol after 99 h.

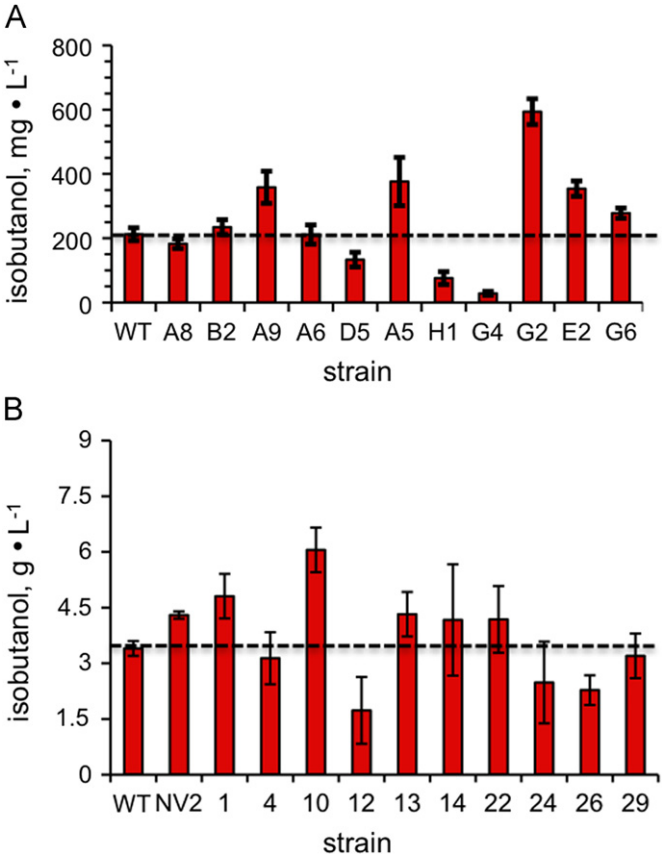

Fig. 3. Comparison of isobutanol production. (A) Comparison of 24 h isobutanol titers obtained by 11 mutants isolated from growth screening and the parental strain, JCL16 (WT). Strains are harboring pSA65 ($P_L$lacO_1::*kivd–adhA*). Strain G2 was renamed NV1. (B) Comparison of 24 h isobutanol titers obtained with 10 mutants isolated by random mutagenesis of NV2 and norvaline enrichment and selection. Strain number 10 was renamed NV3. Strains are harboring pSA65 and pSA69 ($P_L$lacO_1::*alsS–ilvC–ilvD*) for expression of the heterologous isobutanol pathway. Values obtained with JCL16 (WT) in these experiments are indicated by the dotted lines.

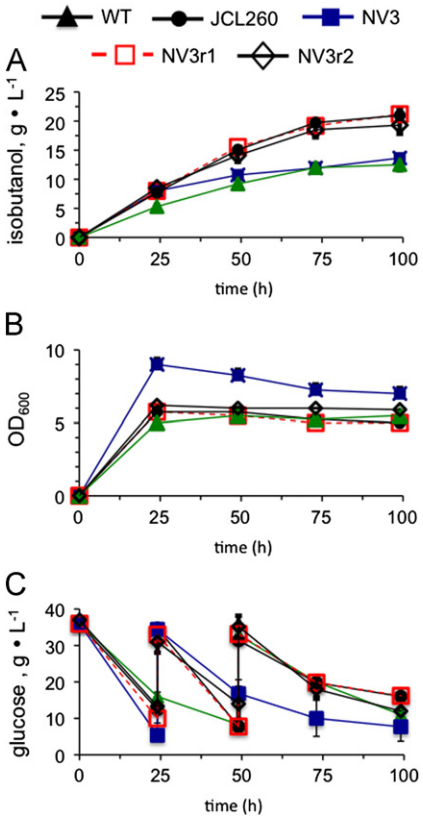

Fig. 4. Comparison of (A) isobutanol production, (B) growth and (C) glucose consumption profiles obtained with JCL16, JCL260, NV3, NV3r1 and NV3r2 harboring both pSA65 ($P_L$lacO_1::*kivd–adhA*) and pSA69 ($P_L$lacO_1::*alsS–ilvC–ilvD*) for expression of the heterologous isobutanol pathway.

3.3. Genomic sequencing of NV3 and repair of rpoS

Genomic sequencing of strain NV3 was performed to identify the mutations introduced from mutagenesis and to elucidate underlying genotype-phenotype relationships in regards to isobutanol production. Sequencing with a depth of 31X coverage identified 208 total mutations, 62 of which are synonymous mutations in ORFs, using *E. coli* MG1655 as the reference genome. Of the 208 total mutations, 62 of them were located in non-coding regions. Mutations were found throughout metabolism in a variety of amino acid pathways and enzymes related to gene regulation and expression (Table 3). Because of the high number of mutations found in NV3, it is more realistic to identify the beneficial mutations and move them into a strain with a clean background. Following this rationale, we first pursued mutations that fell on intuitive targets. In particular, sequencing identified a mutation on *rpoS* (C910T) that introduced a stop codon in place of the Q304 residue. This mutation resulted in the truncation of the last 26 amino acids of the stationary phase RNA polymerase σ^s factor. To determine the effect of this mutation in *rpoS*, we replaced the mutant nucleotide with the wild type

Table 2
Comparison of metabolites produced or consumed and isobutanol yields obtained using JCL16, JCL260, NV3, NV3r1 and NV3r2 harboring isobutanol production plasmids pSA69 and pSA65. Data shown are final (99 h) metabolite concentrations (g/L) in triplicate. Isobutanol yield is given as grams isobutanol produced per gram glucose consumed.

Strain	Concentration ($g\ L^{-1}$)							
	Ethanol	Succinate	Lactate	Acetate	Formate	Isobutanol	Total glucose consumed	Isobutanol yield (g/g)
JCL16	3.7	1.2	10.0	7.1	0.4	12.0	50	0.24
JCL260	0.7	0.0	0.4	0.7	0.0	21.0	66	0.32
NV3	2.3	1.3	0.7	3.2	0.1	13.6	57	0.24
NV3r1	0.8	0.9	1.1	1.0	0.1	21.2	68	0.31
NV3r2	1.3	0.8	1.3	1.5	0.2	19.3	64	0.30

Table 3
Missense mutations of NV3 identified in genes related to major metabolic pathways, transcriptional/translational regulation, DNA/RNA manipulation, and membrane transport.

Enzyme	Pathway/annotation	Mutation
Metabolism		
ProC	proline biosynthesis	A98T
Dxs	isoprenoid biosynthesis	G235S
AcnA	TCA cycle	S522G
DapE	lysine biosynthesis	M107I
XylB	xylose degradation	G370S
GpmM	glycolysis	G72S
Tdh	threonine dehydrogenase	R108C
FsaB	glycolysis	G24E
MetH	methionine biosynthesis	T216I
LdhA	D-lactate dehydrogenase	R118C
PfkB	glycolysis	P243L
AnsB	asparagine/aspartate biosynthesis	T157I
Regulation		
RpoS	RNA polymerase σ^S-factor	Q304STOP
RelA	ppGpp biosynthesis	P532S
OsmY	stress response factor	G164D
DNA/RNA manipulation		
GltX	glutamyl-tRNA synthetase	A313V
PolA	DNA polymerase I	L18F
RplA	50S ribosomal subunit	E190K
RplL	50S ribosomal subunit	A42V
RpoB	RNA polymerase β subunit	A1043V
RpoC	RNA polymerase β′ subunit	R311H, T1024I
DbpA	RNA helicase	P28L
RecB	recombinase	P212L
TufB	elongation factor Tu	T29I
PheT	phenylalanine-tRNA synthetase	D423N
Transport		
FucP	fucose transport	A233T
XylH	xylose transport	P212S
GltS	glutamate Transport	S28F
BtuB	outer membrane porin	A162G
EamA	cystine transport	A95T
AcrD	RND transporter component	N939K
AcrB	RND transporter component	G740D, G625S
Fpr	fructose transport	R133H

sequence using chromosomal recombination with linear PCR product in a fashion similar to a gene knockout method (Datsenko and Wanner, 2000). The resulting strain with a wild type *rpoS* allele was named NV3r1.

3.4. NV3r1 Is an effective isobutanol producer

We tested the effect of the *rpoS* repair on isobutanol production using NV3r1 (Fig. 4A). As indicated by the improved isobutanol production profile of NV3r1, the repair of *rpoS* restored NV3's ability to produce isobutanol effectively during stationary phase. Final production reached 21.2 g/L isobutanol after 99 h, a significant improvement in comparison with JCL16. Furthermore, NV3r1 displayed a more typical growth profile in comparison with NV3 (Fig. 4B). Using JCL260, we achieved an overall isobutanol yield of 0.32 g/g glucose, whereas using JCL16 and NV3 resulted in identical yields of 0.24 g/g (Table 2). Upon repairing the RpoS mutation in NV3, strain NV3r1 produced a yield of 0.31 g/g or ~76% of theoretical maximum.

Table 4
Comparison of D-lactate dehydrogenase (LdhA) activities of JCL16, JCL260, NV3, NV3r1 and NV3r2 obtained from crude cell extracts. Data shown are the average of three independent experiments. 'ND' denotes no activity detected.

Strain	Relevant genotype	Specific activity ($U\ mg^{-1}$)
JCL16	wild type *ldhA*	2.1
JCL260	Δ*ldhA*	ND
NV3	$LdhA^{R118C}$	0.1
NV3r1	$LdhA^{R118C}$	0.1
NV3r2	wild type *ldhA*	0.1

3.5. Investigation of the LdhA mutation

Genomic sequencing of NV3 identified a mutation in LdhA (R118C) that may be contributing to the improved isobutanol production phenotype of our mutant strain. Interestingly, *ldh* is the only common gene between NV3 and the rationally designed strain (JCL260) that contains mutation. Because LdhA is in direct competition with ALS for pyruvate, we sought to determine the mutation's effect on LdhA activity in NV3r1 and compare with JCL16, JCL260 and NV3 activities. As shown in Table 4, an LdhA activity of 2.1 $U\ mg^{-1}$ was obtained from JCL16, whereas LdhA activity from JCL260 was not detectable (ND). NV3 and NV3r1 showed very low LdhA activities (0.1 $U\ mg^{-1}$) in comparison to the parent strain, JCL16, indicating that the mutation may be detrimental to the enzyme's activity. To address this, we repaired the LdhA mutation in strain NV3r1, yielding strain *E. coli* NV3r2, and assayed for LdhA activity using NV3r2 cell extracts. As shown in Table 4, repair of LdhA did not improve LdhA activity detected from NV3r2. Furthermore, isobutanol (Fig. 4A) and lactate production (Table 2) obtained with NV3r2 harboring the isobutanol pathway (pSA65 + pSA69) were not significantly affected indicating that other mechanisms may be contributing to low LdhA activity and improved isobutanol production. Although NV3r1 and JCL260 perform similarly, they do not have any other mutations in common; suggesting the evolutionary strategy presented in this work can identify strains that perform as well as the rationally designed strain through different genotypes.

4. Discussion

This study demonstrates a method for the rapid construction of *E. coli* strains with improved isobutanol production using random mutagenesis and growth selections with a valine analog, norvaline. With this method, we did not rely on known information or gene targets and we were able to consider a tremendous number of genotypes using growth selections in norvaline. There have been many studies regarding the construction of amino acid producing strains by random mutagenesis and selection with amino acid analogs (Ikeda and Nakagawa, 2003; Leuchtenberger et al, 2005). Because these higher alcohol production pathways utilize the 2-keto acid precursors of native amino acids, this well developed selection strategy poses to be a powerful tool for the construction of higher alcohol production strains. Random mutagenesis of JCL16 coupled with a growth selection with norvaline allowed for the isolation of NV1, which showed the best growth when challenged with norvaline. As shown in Fig. 3A, not all strains that exhibited improved norvaline resistance displayed improved alcohol production, indicating that cells have many ways of resisting norvaline toxicity, such as blocking the uptake of norvaline or improved ability to distinguish valine and norvaline in feedback inhibition and protein synthesis. For this reason, candidate strains also needed to be screened for improved isobutanol production. NV1 exhibited improved performance in regards to isobutanol production (Fig. 3A) after overexpression of the two terminal steps of the isobutanol pathway (KDC and ADH2), drawing 2-ketoisovalerate away from valine, leucine and alanine synthesis. To obtain a strain with increased resistance to norvaline in an environment similar to the condition we use for isobutanol production, strain NV3 was isolated by random mutagenesis of NV2 followed by a series of liquid enrichments for mutants with norvaline resistance in a microaerobic condition (Fig. 2). The best isobutanol producer obtained from screening the isolated variants, NV3, demonstrated improved production for the first 24 h (8.0 g/L). However, production stalled during stationary phase (Fig. 4).

NV3 was found to have a total of 208 mutations throughout the entire genome located in central metabolic pathways as well as a wide variety of amino acid pathways (lysine, proline, methionine, asparagine/aspartate). Many mutations were also found in enzymes related to cell regulation and DNA/RNA modification such as DNA polymerase I (PolA), the 50S ribosomal subunits (RplA and RplL), RNA polymerase subunits (RpoB and RpoC), and stress response enzymes (RelA and OsmY). In addition, a mutation in the stationary phase RNA polymerase sigma factor, RpoS, which resulted in the truncation of the last 26 residues of the C-terminus was discovered in NV3. This mutation was predicted to have a high probability of significance because RpoS is a master regulator of transcription during the cell's transition to stationary phase, and this region of the protein is known to play a role in the recognition of promoters with sub-optimally spaced promoter regions (Checroun et al, 2004; Typas and Hengge, 2006). Indeed, repair of the *rpoS* mutation seemed to return the cell's ability to transition into stationary phase and produce isobutanol to elevated titers (21.2 g/L isobutanol; Fig. 4A) with a yield equal to ~76% of theoretical maximum (Table 2).

Interestingly, no mutations were found in the valine pathway from pyruvate or in genes that were disrupted in JCL260 (Δ*ldhA*Δ*frd*Δ*adhE*Δ*pta*Δ*fnr*Δ*pflB*), with the exception of LdhA (R118C). Upon discovering this mutation it seemed likely that LdhA is inactivated in NV3, allowing for more pyruvate availability and improving isobutanol production. Further, enzymatic assays confirmed that strains possessing the mutant LdhA (strains NV3 and NV3r1) have very low LdhA activity in comparison with JCL16. Surprisingly, repair of the LdhA mutation to yield strain NV3r2, did not improve LdhA activity or have any significant effect on isobutanol production. It is not clear why repair of the R118C mutation did not rescue LdhA activity. Perhaps there are other mutations that alter the expression of *ldhA* or improve the heterologous expression of ALS, driving more pyruvate towards isobutanol synthesis. Further studies may be done to address these questions.

Here we have constructed an isobutanol producing strain of *E. coli*, using random mutagenesis and selection with norvaline. Wild type JCL16 was chosen as the parental strain to (1) demonstrate that the method introduced is an effective strain development strategy and (2) allow for the discovery of novel genotypes that are beneficial for isobutanol production in *E. coli*. Because the evolutionary strategy presented here utilizes random mutagenesis, negative mutations, such as the $RpoS^{Q304STOP}$ mutation discovered in NV3, may be introduced when generating strains with desired phenotypes. In future studies, using a lower mutagenic frequency and increasing the number of mutants considered during the selection and screening process may help to address this issue. The final isobutanol titers and yields obtained with NV3r1 and NV3r2 are significant improvements in comparison with JCL16, and similar to those obtained with an effective isobutanol producer, JCL260 (Atsumi et al, 2008), highlighting the effectiveness of this strategy. Similar to JCL260, NV3r1 was not engineered or evolved to tolerate over 20 g/L isobutanol (Atsumi et al, 2010). Therefore, we cannot expect the final isobutanol titer from NV3r1 to significantly surpass JCL260. However we did expect, and witnessed from NV3r1 and NV3r2, a dramatic improvement in the rate of isobutanol production in comparison with the parental strain, JCL16. Valine and KIV were not detected in the supernatants of JCL16, NV3 or NV3r1 when cultured in 20 ml production media and sampled at 24 and 72 h (data not shown). However, improved valine synthesis does not necessarily correlate with an increased KIV pool size or efflux of valine or KIV as we did not screen for these compounds.

Other efforts to obtain better production strains utilizing random mutagenesis and selection using structural analogs have proven to be successful (Connor et al., 2010; Hirao et al., 1989; Ohnishi et al., 2002). Of particular relevance, the genotype of a lysine hyperproducing strain of *Corynebacterium glutamicum* resistant to S-(2-aminoethyl)-L-cysteine was analyzed to identify positive genotypes relevant to lysine production (Ikeda et al., 2006). Here, the authors discovered mutations in enzymes that are proximal to or in the target pathway that reduced the activities of carbon competitive pathways; however, the mutations were also found to increase the activity of the lysine pathway by alleviating allosteric regulation of a key enzyme, aspartokinase, and increasing NADPH supply (Ikeda et al., 2006). Although the beneficial mutations contributing to the improved production phenotype of NV3r1 are not in intuitive genes, they might apply to other higher alcohols and compounds beyond isobutanol. These beneficial mutations may be identified in NV3r1 through further study and introduced into JCL260 or an *E. coli* strain evolved to tolerate high isobutanol concentrations (Atsumi et al., 2010) to produce a host with superior isobutanol productivity.

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