

REPORT

Evolution, genomic analysis, and reconstruction of isobutanol tolerance in *Escherichia coli*

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Received 13.7.10; accepted 25.10.10

Escherichia coli has been engineered to produce isobutanol, with titers reaching greater than the toxicity level. However, the specific effects of isobutanol on the cell have never been fully understood. Here, we aim to identify genotype–phenotype relationships in isobutanol response. An isobutanol-tolerant mutant was isolated with serial transfers. Using whole-genome sequencing followed by gene repair and knockout, we identified five mutations (*acrA*, *gatY*, *tnaA*, *yhbJ*, and *marCRAB*) that were primarily responsible for the increased isobutanol tolerance. We successfully reconstructed the tolerance phenotype by combining deletions of these five loci, and identified glucosamine-6-phosphate as an important metabolite for isobutanol tolerance, which presumably enhanced membrane synthesis. The isobutanol-tolerant mutants also show increased tolerance to *n*-butanol and 2-methyl-1-butanol, but showed no improvement in ethanol tolerance and higher sensitivity to hexane and chloramphenicol than the parental strain. These results suggest that C4, C5 alcohol stress impacts the cell differently compared with the general solvent or antibiotic stresses. Interestingly, improved isobutanol tolerance did not increase the final titer of isobutanol production.

Molecular Systems Biology 6: 449; published online 21 December 2010; doi:10.1038/msb.2010.98

Subject Categories: metabolic and regulatory networks; cellular metabolism

Keywords: biofuel; genome sequencing; metabolic engineering; tolerance

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Introduction

To meet the escalating global demand for energy and reduce the negative environmental impact of petroleum-based fuels, increasing attention has been paid to higher alcohols as potential substitutes for gasoline. The chemical properties of these alcohols make them more suitable as a liquid fuel than ethanol, as they possess a higher energy density, lower vapor pressure, and lower hygroscopicity. In the past few years, the production of these alcohols, such as 1-propanol, 1-butanol, isobutanol, 2-methyl-1-butanol, and 3-methyl-1-butanol, have been demonstrated in *Escherichia coli* (Atsumi and Liao, 2008; Atsumi *et al.*, 2008; Cann and Liao, 2008; Connor and Liao, 2008; Shen and Liao, 2008). The production of isobutanol, in particular, has proven to be very successful with titers exceeding 20 g/l (Atsumi and Liao, 2008; Atsumi *et al.*, 2008;

Cann and Liao, 2008; Connor and Liao, 2008; Shen and Liao, 2008). Although isobutanol production continues long after growth stops (Atsumi *et al.*, 2008), isobutanol impairs cell growth at concentrations as low as 8 g/l in *E. coli* (Brynildsen and Liao, 2009). Since isobutanol was previously considered a trace microbial fermentation product, isobutanol cytotoxicity remains largely uncharacterized.

Many reports have been published on the cytotoxicity of an isomeric alcohol, *n*-butanol, in *Clostridium acetobutylicum* (Sinensky, 1974; Vollherbst-Schneck *et al.*, 1984; Bowles and Ellefson, 1985; Baer *et al.*, 1987). *n*-Butanol has been found to interact with the cell by altering the lipid composition and fluidity of the membrane (Vollherbst-Schneck *et al.*, 1984), decreasing the intracellular pH and ATP concentration, and inhibiting the uptake of glucose (Bowles and Ellefson, 1985). This toxicity is believed to limit current production of

n-butanol, and is one of the major concerns in industrial scale production. In *E. coli*, isobutanol causes similar effects, with growth retardation at 8 g/l, although the production continued up to 20 g/l (Atsumi *et al*, 2008). It is desirable, therefore, to characterize the responses and adaptations of *E. coli* to isobutanol stress in order to increase production by engineering isobutanol resistance into production strains.

Several transcriptional analyses have been performed to understand the stress caused by alcohols including ethanol, *n*-butanol, and isobutanol for *E. coli* (Gonzalez *et al*, 2003; Brynildsen and Liao, 2009; Rutherford *et al*, 2010) and *n*-butanol for *C. acetobutylicum* (Tomas *et al*, 2003, 2004). The isobutanol stress response in *E. coli* is qualitatively similar to that of *n*-butanol with respect to transcriptional levels, except for the increased repression of amino-acid biosynthesis by *n*-butanol (Rutherford *et al*, 2010). The response of ethanol, however, differs significantly from that of *n*-butanol and isobutanol, which is demonstrated by differential regulation of genes responsible for membrane potential management (Brynildsen and Liao, 2009). These results indicate that the cytotoxicity of longer chain alcohols is unlike ethanol cytotoxicity, despite the fact that these alcohols commonly disrupt the cell membrane. It was shown that isobutanol stress disrupts quinone–membrane interactions, which lead to respiratory distress and the activation of *arcA*, *fur*, and *phoB* (Brynildsen and Liao, 2009). Additionally, it has been reported that the levels of intracellular reactive oxygen species were increased after exposure to *n*-butanol in *E. coli*, which is common to other stress responses (Rutherford *et al*, 2010). Although both studies evaluated single knockout strains of genes with significantly perturbed expression levels to isobutanol or *n*-butanol stress, no single mutant significantly increased the tolerance level of *E. coli*. This result suggests that the complexity of a tolerance phenotype may require multiple coordinated changes (Gonzalez *et al*, 2003; Brynildsen and Liao, 2009; Rutherford *et al*, 2010), which may not be sufficiently revealed by gene-expression profiling techniques. One applicable approach to study such a cellular phenomena is the analysis of mutants, which are altered in particular functions. For example, the tolerance of *E. coli* and *Saccharomyces cerevisiae* for ethanol and *C. acetobutylicum* for *n*-butanol has been improved, and the mutants have been analyzed to determine the genotype–phenotype relationship for the alcohol tolerance (Yomano *et al*, 1998; Tomas *et al*, 2003; Alper *et al*, 2006). Moreover, recent improvements in whole-genome sequencing technologies facilitate our ability to determine genome diversity on a laboratory timescale (Smith *et al*, 2008; Srivatsan *et al*, 2008).

In this work, we applied a sequential transfer method to isolate a strain of *E. coli* (SA481) tolerant to increased levels of isobutanol after evolution from an isobutanol production host strain, JCL260 (Atsumi *et al*, 2008). To understand the genotype–phenotype relationship of isobutanol tolerance, we sequenced the whole genome of JCL260 and SA481 using the Illumina-Solexa sequencing platform and aligned the reads with the *E. coli* MG1655 sequence. To identify relevant mutations for the tolerant phenotype, we experimentally verified several essential mutations by individual deletions in JCL260 or repair in SA481. In addition, the isobutanol tolerance phenotype was successfully engineered into the

production host by introducing these select mutations into JCL260 to create a new tolerant host devoid of negative mutations commonly accumulated during evolution.

Results

Isolation and characterization of the isobutanol tolerance strain

To isolate an isobutanol-tolerant strain, we employed a sequential transfer method (Yomano *et al*, 1998) to the isobutanol production host strain, JCL260. JCL260 was initially inoculated into LB broth containing 4 g/l isobutanol. After 15 sequential transfers, the isobutanol concentration in the medium was increased to 6 g/l. The isobutanol concentration was then increased to 8 g/l after the next 15 transfers. After a total of 45 transfers, we isolated the largest single colony on an LB agar plate with 8 g/l isobutanol, denoted as SA481. To evaluate the isobutanol tolerance of SA481, we tested the growth of JCL260 and SA481 in the presence of 6, 8, 10 and 15 g/l isobutanol. SA481 showed increased growth compared with JCL260 in the presence of 6 and 8 g/l, while maintaining similar growth in the absence of isobutanol (Figure 1A and B) and did not grow in the presence of 10 and 15 g/l isobutanol (Figure 1A and B). To measure the viable cell count after isobutanol treatment, cells were plated on LB after 24 h of incubation with 6 and 8 g/l isobutanol. The cell count ratios (24 h/0 h) of SA481 were 13- and 5-fold higher than those of JCL260 in the presence of 6 and 8 g/l isobutanol, respectively (Figure 1C). These results indicate that evolutionary enrichment by serial dilution was successful for isolating isobutanol-tolerant strains of *E. coli*.

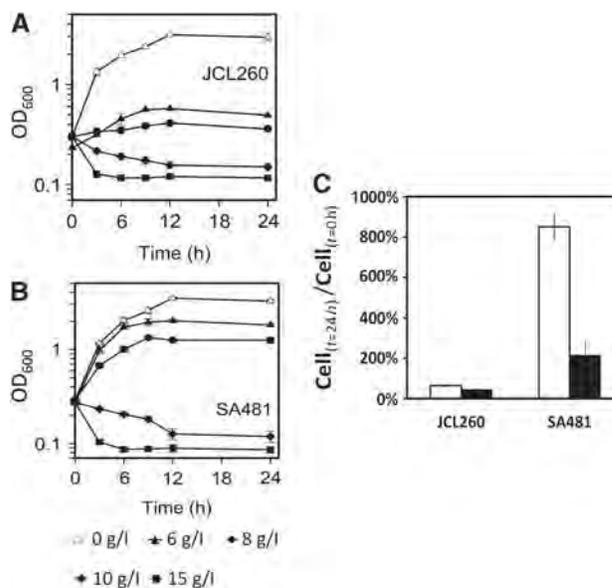


Figure 1 Comparison of growth with isobutanol stress. Cells were incubated in LB at 37°C. (A, B) Time courses for the growth of *E. coli* strain JCL260 (A) and SA481 (B) in the absence of isobutanol (open triangles) or in the presence of 6 g/l (closed triangles), 8 g/l (closed circles), 10 g/l (closed diamond), and 15 g/l (closed square) isobutanol (C). The ratio of viable cells at 0 and 24 h in the presence of 6 g/l (open bar) and 8 g/l (closed bar) isobutanol. All data were performed in triplicate.

Whole-genome sequencing of JCL260 and SA481

In order to identify specific mutations in SA481, the genomic DNA of JCL260 and SA481 was sequenced using the Solexa sequencing platform (Bennett, 2004). The sequenced reads were mapped to the reference genome, MG1655, using the short-sequence aligner, MAQ (Li et al, 2008), to generate consensus sequences of JCL260 and SA481 (Supplementary Figure S1). The coverage of reads across the genome allows us to identify structural variations on the genome including single-nucleotide polymorphisms (SNPs), deletions, insertions, and duplications (Figure 2). In summary, SA481 was found to contain one SNP (C1167G in *polA*) and 25 insertion sequence (IS) elements, of which 22 are contained within coding regions (Figure 2; Table I). In addition, we identified one deletion in between *hipA* and *flxA*, containing 62 genes (Figure 2; Table I). These mutations were subsequently targeted for further analysis.

Identification of key mutations

To identify mutations responsible for isobutanol tolerance, we systematically repaired each mutation in SA481 in individual strains. We hypothesized that if a mutation was important to

isobutanol tolerance, its repair in SA481 would decrease the tolerance. We created a total of 28 repaired mutants including a repair of the large deletion between *hipA* and *flxA* (Figure 3A). The repaired mutants were treated with 8 g/l of isobutanol for 24 h. To evaluate and compare the tolerance of each strain, we normalized the OD₆₀₀ values after 24 h of isobutanol treatment to the initial OD₆₀₀ value. Most of these repaired mutants did show reduced tolerance compared with SA481, suggesting that most of the mutations did contribute to the overall phenotype. No single repair completely abolished the tolerance phenotype. In particular, the repair of *acrA*, *yhbJ*, and the *hipA-flxA* fragment significantly decreased isobutanol tolerance, suggesting that these loci are particularly important for the tolerance phenotype.

Among the 62 genes in the *hipA-flxA* deletion, we focused on *marCRAB*, which are involved in multiple antibiotic resistance. To restore *marCRAB* without others in the *hipA-flxA* deletion, we inserted *marCRAB* in the intergenic region between *ybhC* and *ybhB*. However, the repair of *marCRAB* displayed a less dramatic decrease in isobutanol tolerance relative to the repair of the entire *hipA-flxA* deletion (Figure 3A), indicating that there may be additional genes in the *hipA-flxA* deletion that confer isobutanol tolerance upon deletion.

Next, we investigated the effect of multiple repairs on the isobutanol tolerance of SA481. We focused on the five mutants that showed significantly reduced isobutanol tolerance (*tnaA*, *gatY*, *acrA*, *yhbJ*, and the deletion *hipA-flxA*) for multiple repairs (Figure 3B). Three double-repair strains (*tnaA gatY*, *tnaA acrA*, and *gatY acrA*) showed a decreased isobutanol tolerance relative to their individual repairs (Figure 3B). A triple-repair strain (*tnaA*, *gatY*, and *acrA*), denoted as TW190, showed a significantly decreased tolerance to isobutanol relative to SA481 (Figure 3B). TW190 had an OD₆₀₀ ratio of 1.3 while SA481 had a ratio of 5.3 after being challenged with 8 g/l isobutanol. Upon repair of *marCRAB* in this strain (TW310), the OD ratio increased from 1.3 to 2.9. After the repair of *yhbJ* on this strain, the resulting strain containing all five repairs, denoted TW313, had an OD ratio of 1.9, which was slightly higher than that of JCL260, suggesting that there might be additional mutations that confer isobutanol tolerance in SA481.

Reconstruction of isobutanol tolerance

Having identified key mutations for isobutanol tolerance, we set out to reconstruct the phenotype by introducing specific

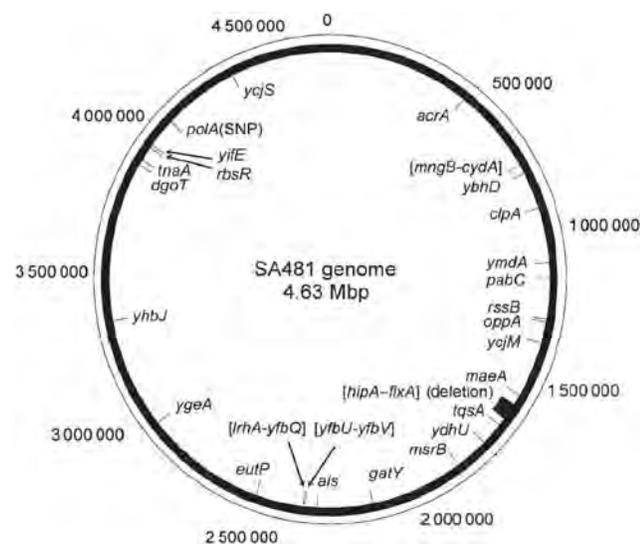


Figure 2 Summary of specific mutations on SA481 genome. Genes without annotation contain an IS insertion. Brackets represent an IS insertion in non-coding region or deletion (specified) between the two genes in the bracket.

Table I Summary of results of Solexa sequencing processed by MAQ

	JCL260	SA481
Total no. of paired-end reads	5 968 953	4 962 680
Total no. of mapped paired-end reads (%)	4 955 571 (83%)	4 232 277 (85.2%)
Genome coverage rate	98.77%	97.86%
Average depth (non-gap)	155.5	132.7
SNPs	0	1 (<i>polA</i>)
Indels	0	0
IS elements	0	25 (<i>acrA</i> , [<i>mngB-cydA</i>], <i>ybhD</i> , <i>clpA</i> , <i>ymdA</i> , <i>pabC</i> , <i>rssB</i> , <i>oppA</i> , <i>ycjM</i> , <i>maeA</i> , <i>tqsA</i> , <i>ydhU</i> , <i>msrB</i> , <i>gatY</i> , <i>ais</i> , [<i>lrhA-yfbQ</i>], [<i>yfbU-yfbV</i>], <i>eutP</i> , <i>ygeA</i> , <i>yhbJ</i> , <i>dgoT</i> , <i>tnaA</i> , <i>rbsR</i> , <i>yifE</i> , <i>ycjS</i>)
Deletion	0	1 (<i>hipA-flxA</i>)

For SNPs, indels, IS elements, and deletion, the number represents specific mutations after confirming by Sanger sequencing.

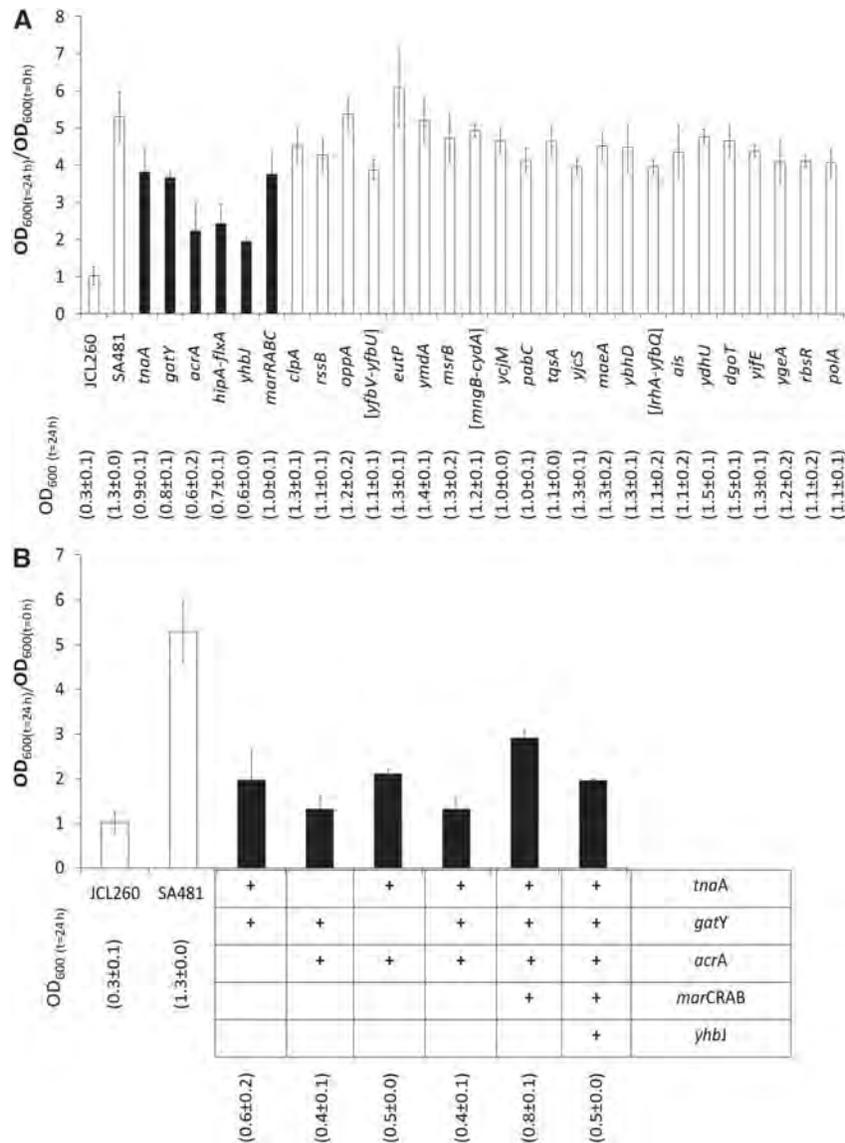


Figure 3 Effect of mutation repairs on isobutanol tolerance. **(A)** Single repair of mutations on SA481 and **(B)** multiple repairs. Each of the mutations on SA481, including SNP (*poIA*), deletions (*hipA-flxA*), IS mutations (the remaining), was repaired. Brackets represent IS insertions in intergenic regions. Cell was treated with 8 g/l isobutanol in LB for 24 h. The y axis indicates the ratio of OD₆₀₀ at 0 and 24 h. Gene names below the axis indicate repaired genes in SA481. The numbers below the gene names indicate the OD₆₀₀ values after 24 h isobutanol treatment. All data were performed in triplicate. The closed bars represent genes that were selected for multiple repairs.

mutations in the parental strain JCL260. The five key mutations (Figure 3A) responsible for isobutanol tolerance in SA481 were the result of four IS10 (Halling *et al*, 1982) insertions (*acrA*, *gatY*, *tnaA*, and *yhbJ*), and one deletion (*marCRAB*). The exact positions of these insertions are listed in Table II. The insertion of these IS elements can have three distinct effects: (1) inactivation of the gene in which it is inserted, (2) inactivation of the downstream genes through the polar effect, or (3) enhanced expression of upstream or downstream genes through additional promoters. All four of the insertion elements were inserted into coding regions, suggesting that each of these genes has been inactivated. For *acrA*, *gatY*, and *tnaA*, each of these genes is the first gene in an operon, further suggesting that the downstream genes

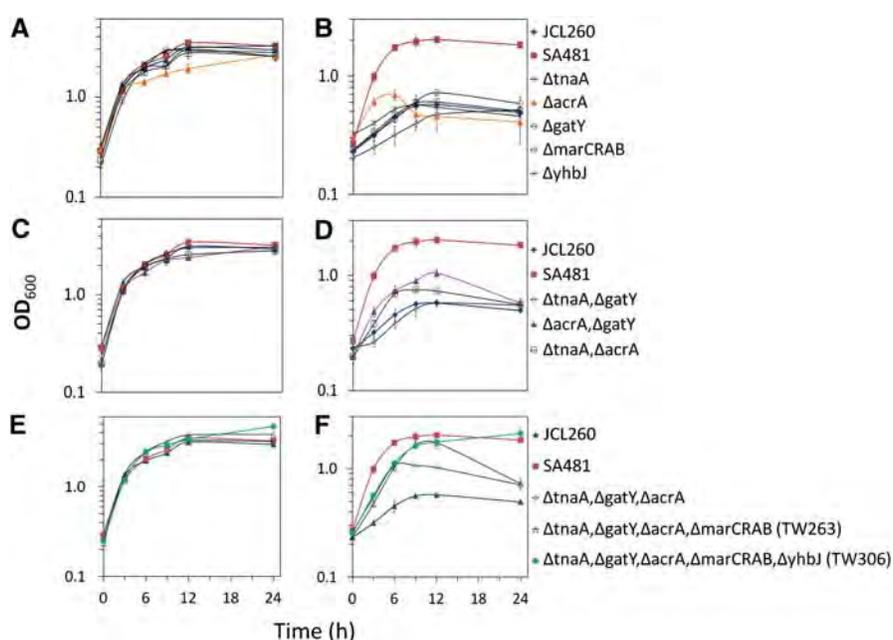
(*acrB*, *gatZABCD*, and *tnaB*) may also be inactivated. The *yhbJ* gene, which contains an IS10 insertion, is the fourth gene in the *rpoN* operon. The inactivation of YhbJ leads to the overexpression of GlmS (Li *et al*, 2008), which is responsible for the synthesis of glucosamine-6-phosphate (GlcN-6-P), a precursor to peptidoglycan and cell wall lipopolysaccharides (LPS) (Kalamorz *et al*, 2007).

To reproduce the tolerance phenotype of SA481 in JCL260, we deleted each of these five genes/clusters. The deletion mutants were treated with 6 g/l isobutanol and cell growth was monitored. Since JCL260 was virtually unable to grow at 8 g/l isobutanol, we used 6 g/l as a benchmark test to obtain a more observable difference in relative tolerance for this experiment. The individual deletion of *acrA*, *gatY*, *tnaA*, *yhbJ*, and

Table II Essential mutations responsible for isobutanol tolerance

Gene	Mutation type ^a	Position ^b (nucleotide)	Region	Coordinates	Function
<i>acrA</i>	IS10 (+)	1175	Coding	483 668	Membrane protein, subunit of AcrAB-TolC multidrug efflux transport system
<i>gatY</i>	IS10 (-)	2	Coding	2 125 224	Subunit of: tagatose-1,6-bisphosphate aldolase 2
<i>tnaA</i>	IS10 (+)	246	Coding	3 886 999	L-cysteine desulhydrase/tryptophanase
<i>yhbJ</i>	IS10 (+)	437	Coding	3 345 574	Predicted P-loop containing ATPase
<i>marCRAB</i>	Deletion, <i>hipA-flxA</i>		Coding + intergenic	1 588 791– 1 645 053	<i>marR</i> : DNA-binding transcriptional repressor <i>marA</i> : transcriptional dual regulator <i>marB</i> : multiple antibiotic resistance protein <i>marC</i> : predicted transporter

^aThe direction of the IS elements. '+' is forward, '-' is reverse strand.

^bStarting position of insertion sequence.

Figure 4 Reconstruction of tolerance phenotype in the parental strain (JCL260) by gene deletion. Significant mutations identified in Figure 3 were reconstructed in JCL260 to test isobutanol tolerance. Growth test of the *E. coli* strains in LB at 37°C without isobutanol (A, C, E) and with 6 g/l isobutanol (B, D, F). All data were performed in triplicate.

marCRAB did not improve isobutanol tolerance (Figure 4A and B), with the exception of the *acrA* strain, which showed better growth up to 6 h (Figure 4A and B). To uncover any synergistic effects, we combined these deletions. Two double-deletion strains ($\Delta\text{acrA } \Delta\text{gatY}$ and $\Delta\text{acrA } \Delta\text{tnaA}$) showed modest improvement in isobutanol tolerance (Figure 4C and D). By combining the deletions in these strains ($\Delta\text{tnaA } \Delta\text{gatY } \Delta\text{acrA}$), the tolerance to isobutanol increased further (Figure 4E and F). Additionally, we deleted *marCRAB* from the triple-deletion mutant, to create TW263. Although the additional deletion of *marCRAB* improved isobutanol tolerance, during stationary phase the tolerance was decreased (Figure 4F). Lastly, the additional deletion of *yhbJ* on TW263, denoted as TW306, showed a similar tolerance to SA481 (Figure 4F). In the presence of 6 g/l isobutanol, the initial growth of TW306 lagged behind that of SA481, suggesting that additional mutations beyond the five discussed here are responsible for

the complete phenotype seen in SA481. However, the tolerance phenotype seen in SA481 was largely reconstructed in TW306.

Effect of ΔyhbJ

Since ΔyhbJ had an important role in isobutanol tolerance, we investigated its role further. YhbJ negatively regulates the expression of GlmS by enhancing the degradation of the small RNA regulator *glmZ* (Kalamorz et al, 2007), which activates the transcription of *glmS*. Deletion of *yhbJ* increases the expression of GlmS (Kalamorz et al, 2007). To test the effect of GlmS activity toward isobutanol tolerance, we deleted *glmZ*, as GlmS is essential for cell growth. The *glmZ* gene encodes a non-coding RNA that activates transcription of *glmS* (Kalamorz et al, 2007). This deletion, however, did not have an effect on the tolerance to isobutanol (Supplementary Figure S2). Next, we overexpressed *glmZ* in JCL260, SA481, TW263,

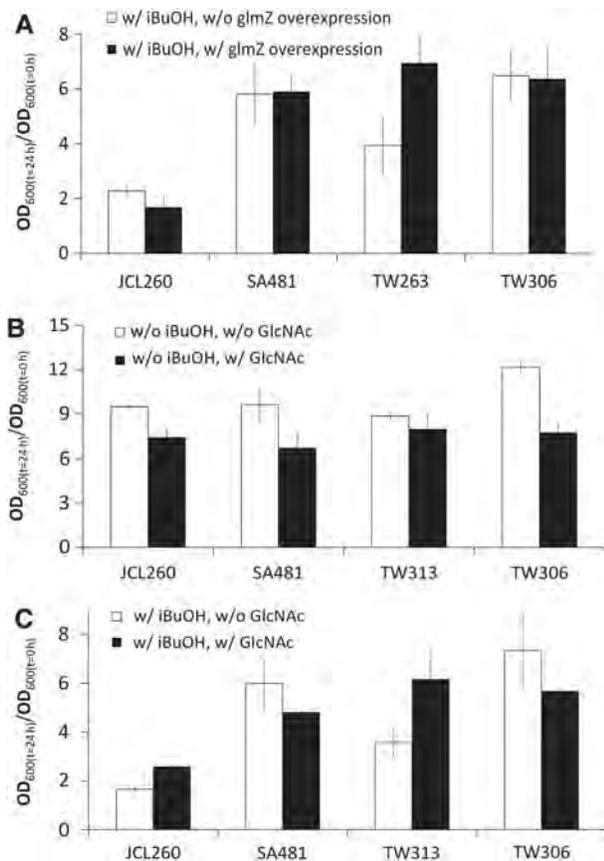


Figure 5 Analysis of $\Delta yhbJ$ effect. Cells were incubated in LB at 37°C. The y axis indicates the ratio of OD_{600} at 0 and 24 h. (A) Open and closed bars represent *E. coli* strains containing the control plasmid (pSA40) and the *glmZ* overexpression plasmid (pHW29), respectively, with 6 g/l isobutanol. (B, C) Open and closed bars represent without and with 10 g/l GlcNAc supplement, respectively, in the absence (B) of and the presence (C) of 6 g/l isobutanol. All data were performed in triplicate.

and TW306. In the presence of 6 g/l isobutanol, the tolerance of TW263 (*yhbJ*⁺) was increased to a similar level as that of TW306 ($\Delta yhbJ$). Conversely, the overexpression of *glmZ* had no effect on any $\Delta yhbJ$ strains (Figure 5A). This result suggests that GlnS activity may be saturated in the $\Delta yhbJ$ strains.

An alternative route for the production of GlcN-6-P in *E. coli* is from exogenous amino sugars such as *N*-acetylglucosamine (GlcNAc) and glucosamine (GlcN). To test whether increased GlcN-6-P improves isobutanol tolerance, we supplemented the growth medium with 10 g/l of GlcNAc. In the absence of isobutanol, the addition of GlcNAc slightly impaired cell growth (Figure 5B). In the presence of 6 g/l isobutanol, GlcNAc supplementation increased the tolerance of TW313 (*yhbJ*⁺, *acrA*⁺, *gatY*⁺, *tnaA*⁺, and *marCRAB*⁺) to a similar level as that of SA481. These data imply that an increase in the intracellular GlcN-6-P concentration can increase the isobutanol tolerance (Figure 5C).

Tolerance to other stresses

To test whether the isobutanol-tolerant mutants show a similar response toward other stresses, we measured the growth in

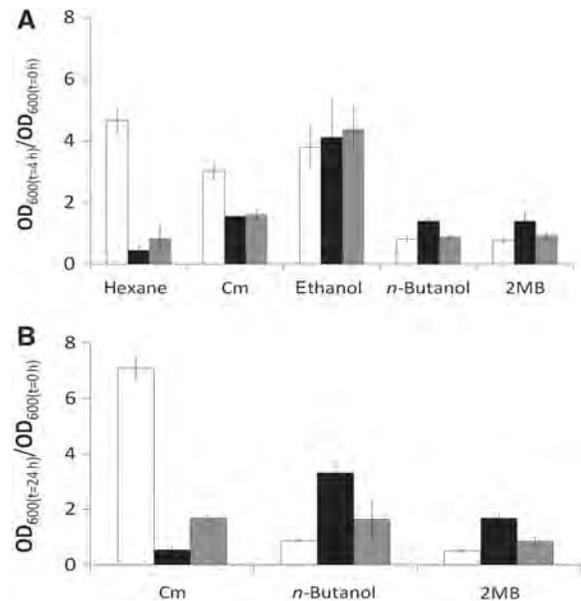


Figure 6 Effects of other solvents and antibiotics. Cells were incubated in LB at 37°C. The y axis indicates the ratio of OD_{600} at 0 and 4 h (A) and at 0 and 24 h (B) of JCL260 (white bar), SA481 (black bar), and TW306 (gray bar) under various types of solvent and antibiotics stress: hexane 3.3 g/l, chloramphenicol 5 mg/l, ethanol 32 g/l, *n*-butanol 6 g/l, 2-methyl-1-butanol 3 g/l. All data were performed in triplicate.

the presence of hexane, chloramphenicol (Cm), ethanol, *n*-butanol, and 2-methyl-1-butanol (2MB). Interestingly, SA481 and TW306 showed higher sensitivity to hexane and chloramphenicol than JCL260, which may be partially due to the inactivation of AcrAB/TolC system (Figure 6). This result suggests that the mechanism of isobutanol response is different from that of alkanes or antibiotics. Both SA481 and TW306 showed an increased tolerance to *n*-butanol and 2MB relative to JCL260. As expected, this result suggests that the mechanism of isobutanol tolerance is similar to that of other higher chain alcohols. However, SA481 and TW306 did not show increased tolerance to ethanol, suggesting that C4 and C5 alcohols induce a different stress to the cell than ethanol does.

Isobutanol production

To test whether the improved isobutanol tolerance enhances isobutanol production, the engineered strains were examined for their capacity to produce isobutanol. We compared isobutanol production levels from TW306, JCL260, and SA481 harboring pSA65 (*P_LlacO1::kivd adhA*) (Atsumi et al, 2010) and pSA69 (*P_LlacO1::alsS ilvC ilvD*) (Atsumi et al, 2008). The isobutanol production, cell growth, and glucose consumption rates of TW306 and SA481 were similar to those of JCL260 (Figure 7A–C). To keep the microaerobic condition, the data were collected every 24 h. However, we confirmed that all strains reached stationary phase (OD_{600} , ~10) around 8 h and produced ~4 g/l isobutanol at 8 h in pilot experiments. All production stopped after 120 h, demonstrating that improved isobutanol tolerance did not increase the final titer.

Next, 8 g/l isobutanol was added to the production media at induction to test the effect of isobutanol stress during growth

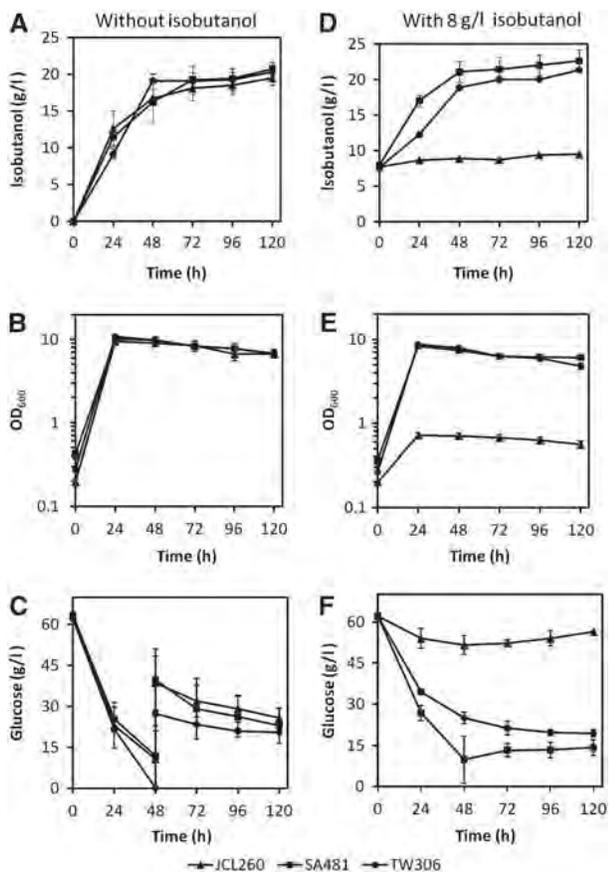


Figure 7 Isobutanol production from the engineered *E. coli*. (A–C) Isobutanol production in M9 medium containing 63 g/l glucose and 10 g/l yeast extract in a shake flask at 30°C (A), time profiles of cell growth (B), glucose concentration (C). At 48 h, 30 g/l glucose was added to the culture. (D–F) Isobutanol production with 8 g/l isobutanol supplement (D), time profiles of cell growth (E), glucose concentration (F) All data were performed in triplicate.

and protein production. After the addition of 8 g/l of isobutanol, growth and isobutanol production in JCL260 was nearly abolished, while SA481 and TW306 were still able to grow and produce isobutanol (Figure 7D–F). These results suggest that improved isobutanol tolerance can increase the growth and production of isobutanol under isobutanol stress, but is not essential for the high production of isobutanol during stationary phase.

Discussion

In this work, we demonstrated the *in vitro* evolution, genome analysis, and reconstruction of the isobutanol resistance phenotype. The use of serial dilution for evolving microorganisms has been reported previously (Yomano *et al*, 1998). However, this process accumulates multiple mutations, which are difficult to discern. High-throughput sequencing technologies have dramatically accelerated the identification of mutations on a genomic scale. In addition, advances in molecular genetic tools in *E. coli* further facilitate the identification of key mutations and reconstruction of the desired phenotype.

Here, we isolated an isobutanol-tolerant mutant, SA481, and sequenced the whole genome along with its parental strain JCL260. By comparing the sequences with the reference genome sequence, we identified specific mutations in SA481 including one point mutation, 25 IS insertions and one deletion between *hipA–flxA* containing 62 genes. JCL260 contains the Tn10 transposon on the F' plasmid, which itself contains the IS10 element found in all 25 IS insertions in SA481. We believe that the large number of IS10 insertions in SA481 is due to the self-duplication and random integration of these elements. By repairing individual mutations found in SA481 with wild-type sequences and reconstructing the mutations in the parental strain, we identified five key loci that are linked to isobutanol tolerance: *yhbJ*, *acrA*, *marCRAB*, *tmaA*, and *gatY*. The isobutanol tolerance phenotype can be largely attributed to the inactivation of these five loci, demonstrating that a complex phenotype can be systematically dissected and reconstructed.

The *yhbJ* gene encodes a predicted ATPase and current evidence shows that it negatively regulates the expression of *glmS* (Kalamorz *et al*, 2007), which catalyzes the synthesis of GlcN-6-P. An increase in GlcN-6-P enhances the synthesis of UDP-GlcNAc, which is a precursor for peptidoglycan and LPS. LPS is a major component of the outer membrane of bacteria, and the alteration of LPS constituents has been suggested as a barrier against solvent and antibiotic stress (Lee *et al*, 2009). Our data suggest that the deletion of *yhbJ* increased the tolerance to isobutanol by changing the LPS constituent of the outer membrane.

The *acrA* gene encodes a component of AcrAB–TolC multidrug efflux system, which is known to be induced by various types of stress (Ma *et al*, 1995). The physiological function of AcrAB–TolC is to protect the cell against various stresses including antibiotics and organic solvents by exporting these substances outside of the cell (White *et al*, 1997). Inactivation of any of the individual components diminishes the function of the whole complex (Takatsuka and Nikaido, 2009). Our results demonstrating an increased sensitivity to chloramphenicol and hexane in Δ *acrA* strains (SA481 and TW306) are consistent with these previous findings (Figure 6). The opposite effect, however, was seen for isobutanol tolerance (Figure 3A). Our previous work has suggested that isobutanol stress causes quinone depletion, which may be caused by membrane damage (Brynildsen and Liao, 2009). AcrAB–TolC, whose transcription is significantly induced by isobutanol and *n*-butanol (Brynildsen and Liao, 2009; Rutherford *et al*, 2010), can recognize a broad range of aromatic compounds as substrates (Diaz *et al*, 2001) and has an important function in the efflux of the electron shuttle anthraquinone-2,6-disulfonate in *Shewanella oneidensis* and *E. coli* (Shyu *et al*, 2002). The increased AcrAB–TolC production induced by isobutanol stress could enhance the excretion of quinones, whereas deletion of AcrAB–TolC could reduce the quinone depletion, which increases isobutanol tolerance.

It is interesting to note that AcrAB is induced by isobutanol and *n*-butanol according to microarray study (Brynildsen and Liao, 2009; Rutherford *et al*, 2010). Conventional wisdom would suggest overexpression of these proteins would increase tolerance. However, we found just the opposite: deletion of these genes increased tolerance.

The *marCRAB* locus also mediates several different types of stress including antibiotics and organic solvents. The *marA* and *marR* genes encode a transcriptional activator of many regulons and a repressor of the *marRAB* operon. The overexpression of *marA* or inactivation of *marR* has been previously shown to increase the tolerance of *E. coli* to multiple antibiotics, organic solvents, and oxidative agents by up-regulation of the AcrAB–TolC system and down-regulation of OmpF, which regulates membrane permeability (Alekhshun and Levy, 1999; Viveiros *et al*, 2007). DNA microarray analysis has shown that constitutive expression of *marA* during exponential growth induced 47 and repressed 15 genes responsible for energy metabolism, transport, protection response, and cofactor, amino-acid, nucleotide, and fatty-acid biosynthesis (Barbosa and Levy, 2000). Deletion of *marCRAB*, which decreases the expression of AcrAB–TolC, showed a similar effect on isobutanol tolerance with the deletion of *acrA*. Furthermore, the deletion of *marCRAB* in a Δ *acrA* strain increased isobutanol tolerance (Figure 4F), suggesting that additional factors regulated by MarA or MarR may also contribute to the isobutanol-tolerant phenotype.

The *tnaA* gene encodes L-cysteine desulfhydrase/tryptophanase (Watanabe and Snell, 1972; Awano *et al*, 2003), which is responsible for degradation of L-cysteine and L-tryptophan to pyruvate and indole, respectively. Indole is a putative extracellular signal (Wang *et al*, 2001), which has been shown to induce many xenobiotic exporter genes including *acrD*, *acrE*, *cusB*, *emrK*, *mdtA*, *mdtE*, and *ycel* (Hirakawa *et al*, 2004). Decreasing the intracellular concentration of indole by deletion of *tnaA* reduces the expression of the multidrug efflux.

The *gatYZ* genes encode tagatose-1,6-bisphosphate aldolase, which is involved in galactitol metabolism (Nobelmann and Lengeler, 1996), converting tagatose-1,6-bisphosphate to dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate. DNA microarray analysis revealed that isobutanol stress strongly induced expression of the *gat* operon (Brynildsen and Liao, 2009). The *gat* operon is activated by the global regulator CRP (Hollands *et al*, 2007), which is one of the most perturbed transcription factors by isobutanol (Brynildsen and Liao, 2009). Although the deletion of *gatY* increased isobutanol tolerance, it is unlikely due to the inability to degrade tagatose-1,6-bisphosphate, as KbaYZ can catalyze the same reaction (Brinkkötter *et al*, 2002). Thus, the deletion of *gatY* could alter the expression of the whole *gat* operon. Further analysis is required to determine the role of the *gatY* deletion in isobutanol tolerance.

The toxicity of end products has been suggested to limit the productivity of *n*-butanol, isobutanol, and other biofuel production processes (Tomas *et al*, 2003; Connor *et al*, 2010; Smith *et al*, 2010). Although the final titer of isobutanol of JCL260 reached beyond 20 g/l, which exceeded the limit of its tolerance level (8 g/l), we hypothesized that improving the tolerance of the host toward isobutanol could be an important key to increase the productivity. The productivities of our tolerant strains, SA481 and TW306, however, were similar to that of JCL260 in the absence of exogenously added isobutanol (Figure 7). This result suggests that isobutanol productivity may not be limited by isobutanol toxicity. Another possibility is that toxic effects of isobutanol during growth phase are

different than those in stationary phase. SA481 was isolated based on growth, whereas isobutanol production in growth phase was only 20% of the total production. In this case, a novel screening strategy will be required to isolate mutants, which demonstrate increased isobutanol tolerance during stationary phase. It will also be interesting to characterize the difference of isobutanol toxicity between growth and stationary phases. However, the isobutanol productivity of SA481 increased when the initial isobutanol concentration was not zero. This result is useful in scenarios such as continuous product removal where product concentration is kept non-zero.

The results of this study provide genetic information for the rational design of an isobutanol-tolerant strain of *E. coli*. The elucidation of phenotypically linked targets opens the possibility to uncover detailed mechanisms for the response to isobutanol stress by tolerant *E. coli*. By incorporating the genetic data with other genomic data such as gene-expression profiling, we expect that engineering an isobutanol-tolerant mutant with increased productivity should be attainable. This systems approach would rapidly improve production host such that variety of organisms can be used in metabolic engineering (Chao and Liao, 1993; Tomas *et al*, 2003; Alper *et al*, 2006; Atsumi *et al*, 2007, 2008, 2009).

Materials and methods

Reagents

All restriction enzymes and Antarctic phosphatase were purchased from New England Biolabs (Ipswich, MA). The Rapid DNA ligation kit was supplied by Roche (Mannheim, Germany). KOD DNA polymerase was purchased from EMD Chemicals (San Diego, CA). The chemicals used in this study were ordered from Sigma-Aldrich (St Louis, MO). Oligonucleotides were ordered from Integrated DNA Technologies (Coralville, IA).

Plasmids

The wild-type *glmZ* gene was amplified with primers HW119F (GCCGAATTCGT-AGATGCTCATTC) and HW119R (TAGCACGCGT AAAACAGGTCTGTA) from BW25113 genomic DNA and the PCR product was then digested with *EcoRI* and *MluI* and ligated into pSA40 (Atsumi *et al*, 2008) cut with the same enzymes, creating pHW29.

Culture conditions for evolution

To isolate an isobutanol-tolerant strain, we employed a sequential transfer method (Yomano *et al*, 1998) at 37°C in 5 ml aerobic LB broth in a 15-ml test tube with a plastic cap and shaking. The isobutanol production host strain, JCL260, was initially inoculated into LB broth containing 4 g/l isobutanol. Cultures were incubated for 48 h in a rotary shaker (150 r.p.m.). After 48 h, the cultures had reached stationary phase (OD₆₀₀, ~1–3). For the next round, 1% (vol/vol) of the 48-h culture was inoculated in 5 ml LB fresh medium. After 15 sequential transfers, the isobutanol concentration in the medium was increased to 6 g/l. The isobutanol concentration was then increased to 8 g/l after the next 15 transfers. After a total of 45 transfers, cells were plated on an LB agar plate containing 8 g/l isobutanol.

Isobutanol tolerance test

Isobutanol tolerance was determined using both viable cell count and OD₆₀₀ test. For OD₆₀₀ test, 1% (vol/vol) of the overnight culture was inoculated in 20 ml LB medium with 12 µg/ml tetracycline in 250 ml

baffled shake flasks and grew at 37°C until early exponential phase (OD₆₀₀, 0.2–0.35). We then distributed 5 ml of culture into a 15-ml test tube, followed by addition of isobutanol with the desired concentration. We used parafilm to wrap the cap of the test tube to reduce evaporation. The growth of cells was sampled and monitored by OD₆₀₀. The ratio of OD₆₀₀ at 24 and 0 h was used as measure of tolerance.

Viable cell counting was performed after OD₆₀₀ measurements. The cultures were diluted with LB broth, plated on an LB plate and incubated overnight at 37°C. The number of colonies formed was counted. All conditions were performed in three independent cultures.

Tolerance test of other solvents and antibiotics

The growth of JCL260, SA481, and TW306 was measured in the presence of hexane, chloramphenicol, ethanol, *n*-butanol, and 2MB. To find inhibitory concentrations, three or four concentrations were tested for each chemical. The concentrations in which at least one strain but not all three strains were able to grow were used for tolerance tests. The concentrations used in tolerance tests were 3.3 g/l hexane, 5 mg/l chloramphenicol, 32 g/l ethanol, 6 g/l *n*-butanol, and 3 g/l 2-methyl-1-butanol. For ethanol, we tested three concentrations (24, 32, and 40 g/l), but we could not find any significant difference between the three strains. Tolerance tests were carried out as described for the isobutanol tolerance test.

Culture conditions for isobutanol production

For isobutanol production, M9 medium containing 63 g/l glucose, 10 g/l yeast extract, 100 µg/ml ampicillin, 30 µg/ml kanamycin, and 1000th dilution of Trace Metal Mix A5 (2.86 g H₃BO₃, 1.81 g MnCl₂·4H₂O, 0.222 g ZnSO₄·7H₂O, 0.39 g Na₂MoO₄·2H₂O, 0.079 g CuSO₄·5H₂O, 49.4 mg Co(NO₃)₂·6H₂O per liter of water) was used for cell growth. In all, 1% (vol/vol) of the overnight culture was

inoculated into 20 ml fresh M9 medium in a 250-ml screw capped conical flask at 37°C for 2.5 h, followed by 0.1 mM IPTG induction. The induced culture was then transferred to a 30°C rotary shaker (250 r.p.m.). Samples were taken every 24 h until 120 h and the isobutanol media was quantified by a gas chromatograph described in Atsumi *et al* (2008). The glucose concentration of the culture broth was measured by a glucose analyzer, YSI Life Science 2300 (STAT Plus, OH).

Genomic DNA purification and Solexa sequencing

The genomic DNA of JCL260 and SA481 was purified by Qiagen DNA purification spin columns (Valencia, CA). Preparation of paired-end libraries and whole-genome sequencing was performed by the DNA microarray facility of the University of California, Los Angeles using the Illumina-Solexa sequencing platform (Bennett, 2004). A total of 5 968 953 and 4 962 680 paired-end reads (76 bp from each end) were obtained for JCL260 and SA481, respectively (Table I). The reads were consequently processed by the short-sequence aligner, MAQ (Li *et al*, 2008) for read alignment, SNP calling, and indel (short insertion or deletion) calling to detect the differences between two strains. Sequences generated in this study have been deposited in the NCBI sequence read archive under accession number SRA023550.2.

Alignment

The default setting of MAQ was used in the alignment. The maximum outer distance for a correct pair was set at 500 bp to conform to our paired-end library fragment size. Up to two mismatches were allowed for mapping the 76-mer reads against the reference genome. Of all the paired-end reads, 83.0% of JCL260 and 85.2% of SA481 reads were uniquely aligned in mate-pairs onto the reference genome, resulting in 155- and 132-fold coverage for JCL260 and SA481, respectively.

Table III List of strains

Strains	Relevant genotype	Reference
BW25113	<i>rrnB</i> _{T14} <i>ΔlacZ</i> _{WJ16} <i>hsdR514 ΔaraBAD</i> _{AH33} <i>ΔrhaBAD</i> _{LD78}	Datsenko and Wanner (2000)
JCL16	BW25113/F' <i>[traD36, proAB +, lacI^s ZΔM15 Tn10(Tet^R)]</i>	Atsumi <i>et al</i> (2007)
JCL260	JCL16 <i>ΔadhE ΔldhA ΔfrdBC Δfnr Δpta ΔpB</i>	Atsumi <i>et al</i> (2008)
SA481	Isobutanol tolerance strain, evolved from JCL260	This study
TW067	JCL260 <i>ΔmarR</i> (Kan ^R)	This study
TW068	JCL260 <i>ΔmarA</i> (Kan ^R)	This study
TW102	JCL260 <i>ΔacrA</i>	This study
TW118	SA481:: <i>tnaA</i> ⁺	This study
TW122	JCL260 <i>ΔtnaA</i>	This study
TW127	JCL260 <i>ΔgatY</i>	This study
TW129	SA481:: <i>gatY</i> ⁺	This study
TW139	SA481:: <i>acrA</i> ⁺	This study
TW157	SA481::(<i>hipA-flxA</i>) ⁺ <i>ΔydeS</i> (Kan ^R) <i>ΔydfW</i> (Cm ^R)	This study
TW175	SA481:: <i>tnaA</i> ⁺ <i>gatY</i> ⁺	This study
TW190	SA481:: <i>tnaA</i> ⁺ <i>gatY</i> ⁺ <i>acrA</i> ⁺	This study
TW200	JCL260 <i>ΔmarRAB</i>	This study
TW204	JCL260 <i>ΔtnaA ΔgatY</i>	This study
TW214	JCL260 <i>ΔtnaA ΔgatY ΔacrA</i>	This study
TW218	JCL260 <i>ΔmarC</i> (Kan ^R)	This study
TW219	JCL260 <i>ΔmarB</i> (Kan ^R)	This study
TW223	SA481:: <i>marCRAB</i> ⁺	This study
TW232	JCL260 <i>ΔmarCRAB</i>	This study
TW263	JCL260 <i>ΔtnaA ΔgatY ΔacrA ΔmarCRAB</i>	This study
TW281	JCL260 <i>ΔgatY ΔacrA</i>	This study
TW283	SA481:: <i>gatY</i> ⁺ <i>acrA</i> ⁺	This study
TW284	SA481:: <i>tnaA</i> ⁺ <i>acrA</i> ⁺	This study
TW285	JCL260 <i>ΔtnaA ΔacrA</i>	This study
TW305	SA481:: <i>yhbJ</i> ⁺	This study
TW306	JCL260 <i>ΔtnaA ΔgatY ΔacrA ΔmarCRAB ΔyhbJ</i>	This study
TW308	JCL260 <i>ΔyhbJ</i>	This study
TW310	SA481:: <i>tnaA</i> ⁺ <i>gatY</i> ⁺ <i>acrA</i> ⁺ <i>marCRAB</i> ⁺	This study
TW313	SA481:: <i>tnaA</i> ⁺ <i>gatY</i> ⁺ <i>acrA</i> ⁺ <i>marCRAB</i> ⁺ <i>yhbJ</i> ⁺	This study
TW315	JCL260 <i>ΔtnaA ΔgatY ΔacrA ΔmarCRAB ΔyhbJ ΔglmZ</i>	This study

Genomic manipulations

Host strain gene deletions were performed by P1 transduction using the appropriate Keio-collection strains (Baba *et al*, 2006) as the donor or using a DNA recombination method described in Datsenko and Wanner (2000). Mutant gene repairs were generated by P1 transduction or linear DNA recombination. In P1 transduction, specific strains in the Keio collection, which carries an antibiotic marker closed to the mutation site, were used as the donor to replace the mutated sequence in SA481. In linear DNA transformation, PCR-amplified BW25113 sequence flanking a kanamycin resistance cassette was used to transform SA481 using the recombination method described in Datsenko and Wanner (2000). Specifically, the fragment sequence of *hipA-flxA* in SA481 was repaired by P1 transduction using a BW25113-derived mutant in which *ydeS* and *ydfW* were replaced with kanamycin and chloramphenicol resistance genes, respectively. These two antibiotic resistance genes were used as selection markers in P1 transduction. Repaired strain details and primer information are summarized in the Supplementary information (Supplementary Tables I–IX). The kanamycin resistance gene was removed by the method described in Datsenko and Wanner (2000). To perform the repair of *marCRAB*, the DNA fragment *marCRAB* from BW25113 was inserted between *ybhC* and *ybhB* on SA481. The deleted and repaired fragments were verified by PCR and Sanger sequencing. The strain list is shown in Table III.

Supplementary information

Supplementary information is available at the *Molecular Systems Biology* website (<http://www.nature.com/msb>).

Acknowledgements

This work was supported in part by the UCLA-DOE Institute for Genomics and Proteomics, BioEnergy Science Research Center, and NSF grant MCB-0903955. TW and WH were supported by a Graduate Students Study Abroad Program from a National Science Council of Taiwan (ROC) (NSC-096-2917-1-007-102 and NSC97-2917-1-006-121). We are grateful to Michael R Connor for helpful comments on the manuscript.

Author contributions: SA, TW, and JCL conceived and designed the experiments. SA, TW, IMPM, and WH performed the experiments. SA, TW, PC, MP, and JCL analyzed the data. SA, TW, and JCL wrote the manuscript. All authors contributed to data interpretation, reviewed the manuscript, and approved the final version.

Conflict of interest

The authors declare that they have no conflict of interest.

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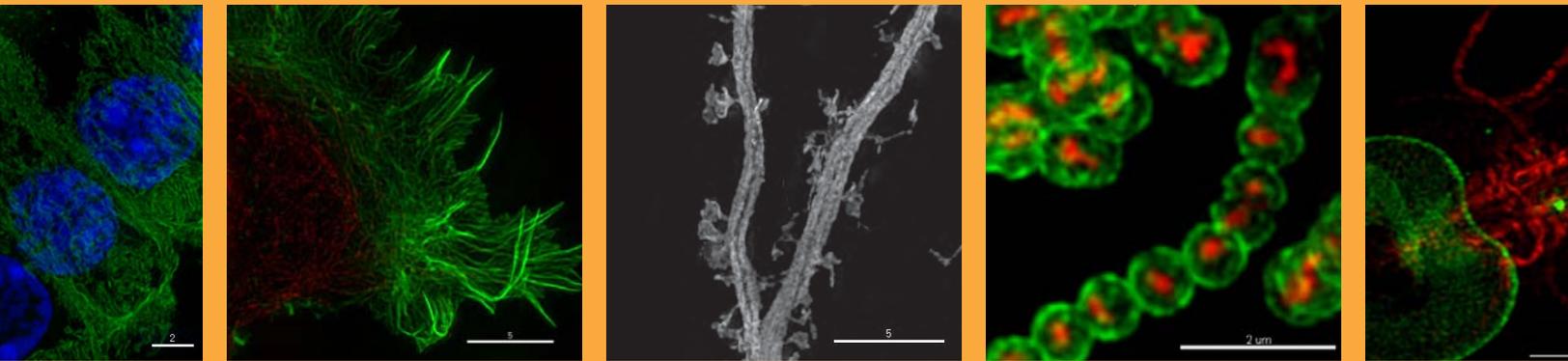
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