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Genomic Library Screens for Genes Involved in n-Butanol Tolerance in *Escherichia coli*

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Abstract

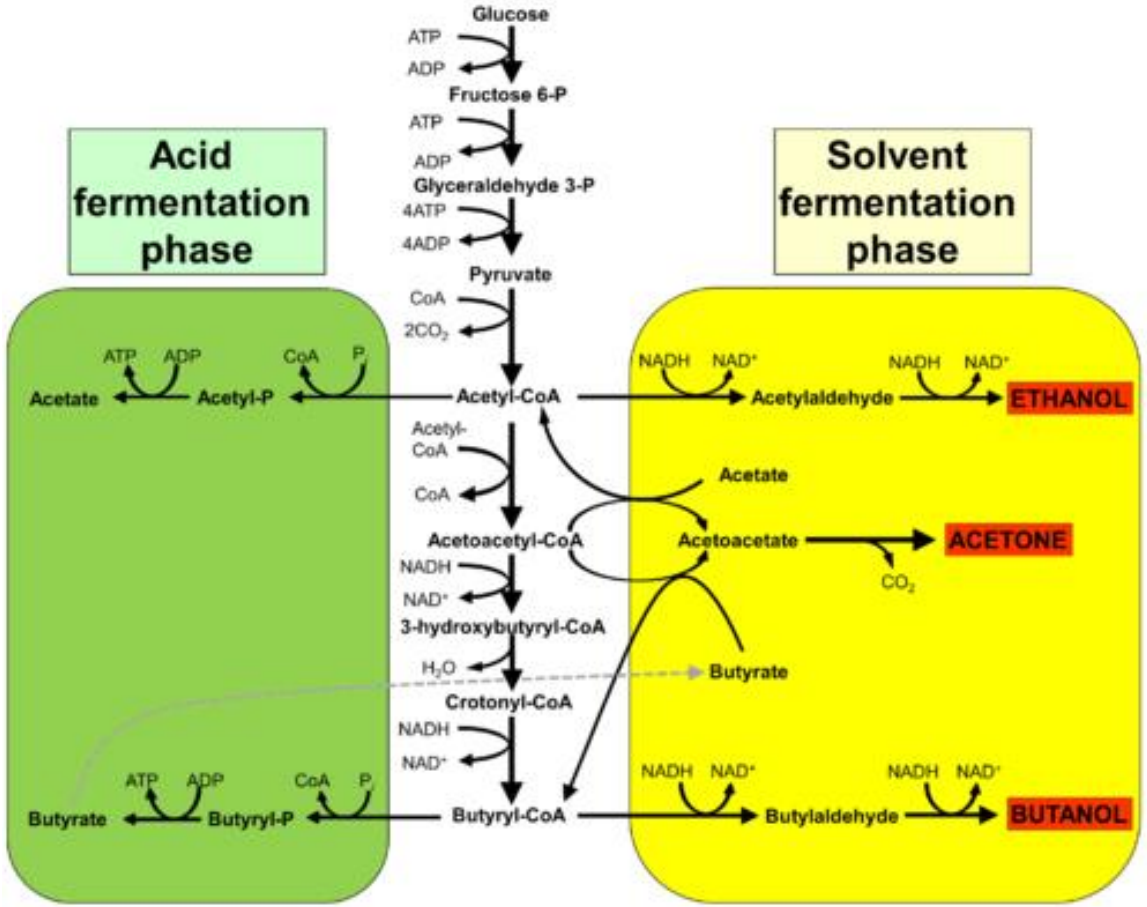
Background: n-Butanol is a promising emerging biofuel, and recent metabolic engineering efforts have demonstrated the use of several microbial hosts for its production. However, most organisms have very low tolerance to n-butanol (up to 2% (v/v)), limiting the economic viability of this biofuel. The rational engineering of more robust n-butanol production hosts relies upon understanding the mechanisms involved in tolerance. However, the existing knowledge of genes involved in n-butanol tolerance is limited. The goal of this study is therefore to identify *E. coli* genes that are involved in n-butanol tolerance.

Methodology/Principal Findings: Using a genomic library enrichment strategy, we identified approximately 270 genes that were enriched or depleted in n-butanol challenge. The effects of these candidate genes on n-butanol tolerance were experimentally determined using overexpression or deletion libraries. Among the 55 enriched genes tested, 11 were experimentally shown to confer enhanced tolerance to n-butanol when overexpressed compared to the wild-type. Among the 84 depleted genes tested, three conferred increased n-butanol resistance when deleted. The overexpressed genes that conferred the largest increase in n-butanol tolerance were related to iron transport and metabolism, *entC* and *feoA*, which increased the n-butanol tolerance by 32.8±4.0% and 49.1±3.3%, respectively. The deleted gene that resulted in the largest increase in resistance to n-butanol was *astE*, which enhanced n-butanol tolerance by 48.7±6.3%.

Conclusions/Significance: We identified and experimentally verified 14 genes that decreased the inhibitory effect of n-butanol tolerance on *E. coli*. From the data, we were able to expand the current knowledge on the genes involved in n-butanol tolerance; the results suggest that an increased iron transport and metabolism and decreased acid resistance may enhance n-butanol tolerance. The genes and mechanisms identified in this study will be helpful in the rational engineering of more robust biofuel producers.

n-Butanol has been proposed as a substitute for diesel fuel and gasoline. It is produced in small quantities in nearly all fermentations (see fusel oil), but species of Clostridium produce much higher yields of butanol, and research is currently underway to increase the ultimate yield of biobutanol from biomass.

Glycolysis and fermentation of pyruvic acid by Clostridia yield the end products butyric acid, butanol, acetone, isopropanol, and carbon dioxide.[7]



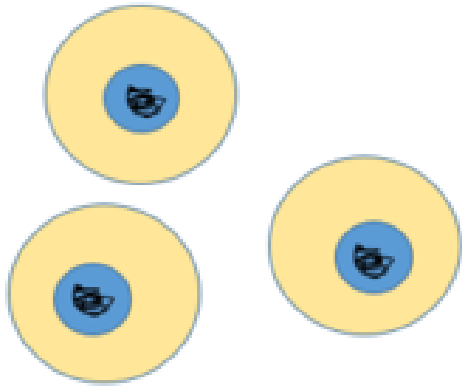
“The complex growth and production phases and the strict anaerobic nature of the native producers have prompted researchers to pursue heterologous hosts for biobutanol production. In the last few years, with the advances in metabolic engineering, non-native producers of n-butanol such as Escherichia coli [4–6], Saccharomyces cerevisiae [7], Lactobacillus brevis [8], Pseudomonas putida [9] and Bacillus subtilis [9], have been demonstrated as potential hosts for use in n-butanol production.”

“However, n-butanol is highly toxic to microorganisms [10–12]… Understanding the mechanisms involved in n-butanol response can help to facilitate the engineering of production **hosts** for improved tolerance.”

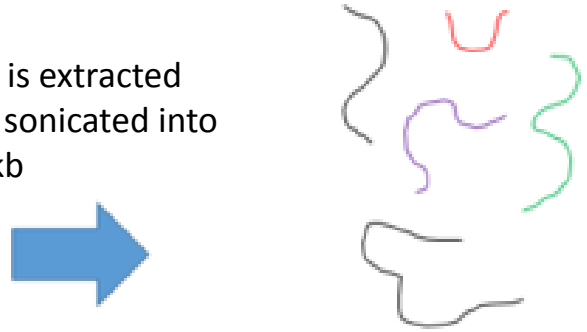
-Reyes LH, Almario MP, Kao KC (2011) Genomic Library Screens for Genes Involved in n-Butanol Tolerance in Escherichia coli. PLoS ONE 6(3): e17678. doi:10.1371/journal.pone.0017678

Genomic Library Construction

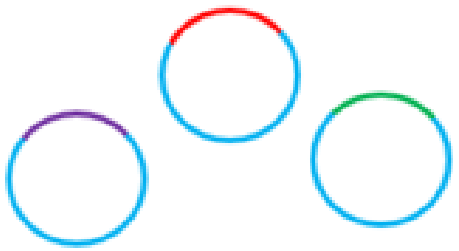
E. Coli K-12 Strain



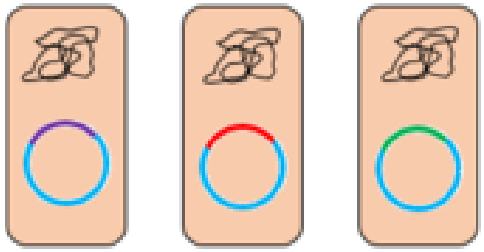
Dna is extracted and sonicated into 2-3kb



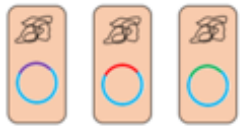
DNA fragments are inserted into cloning vectors



Bacterial cells are transformed with vectors



Gene Enrichment Strategy and Comparative Genome Hybridization Array

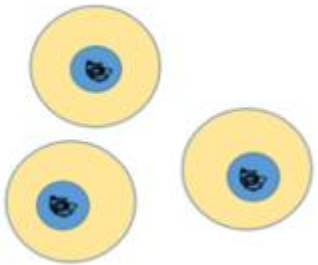


Untreated, transformed bacterial cells

Increasing n-butanol, transformed bacterial cells

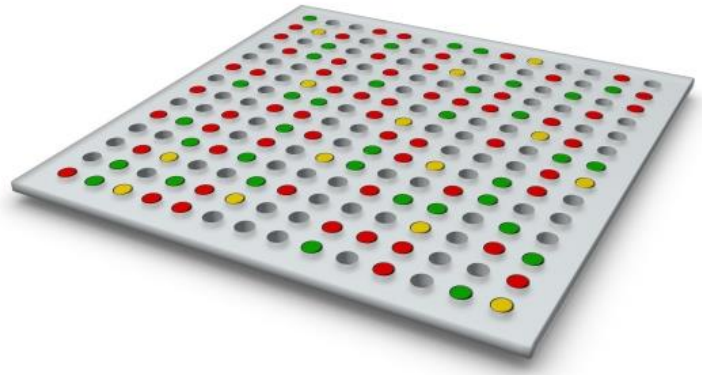
Take transformed cells and treat with respective conditions

Identify Enriched/Depleted Genes

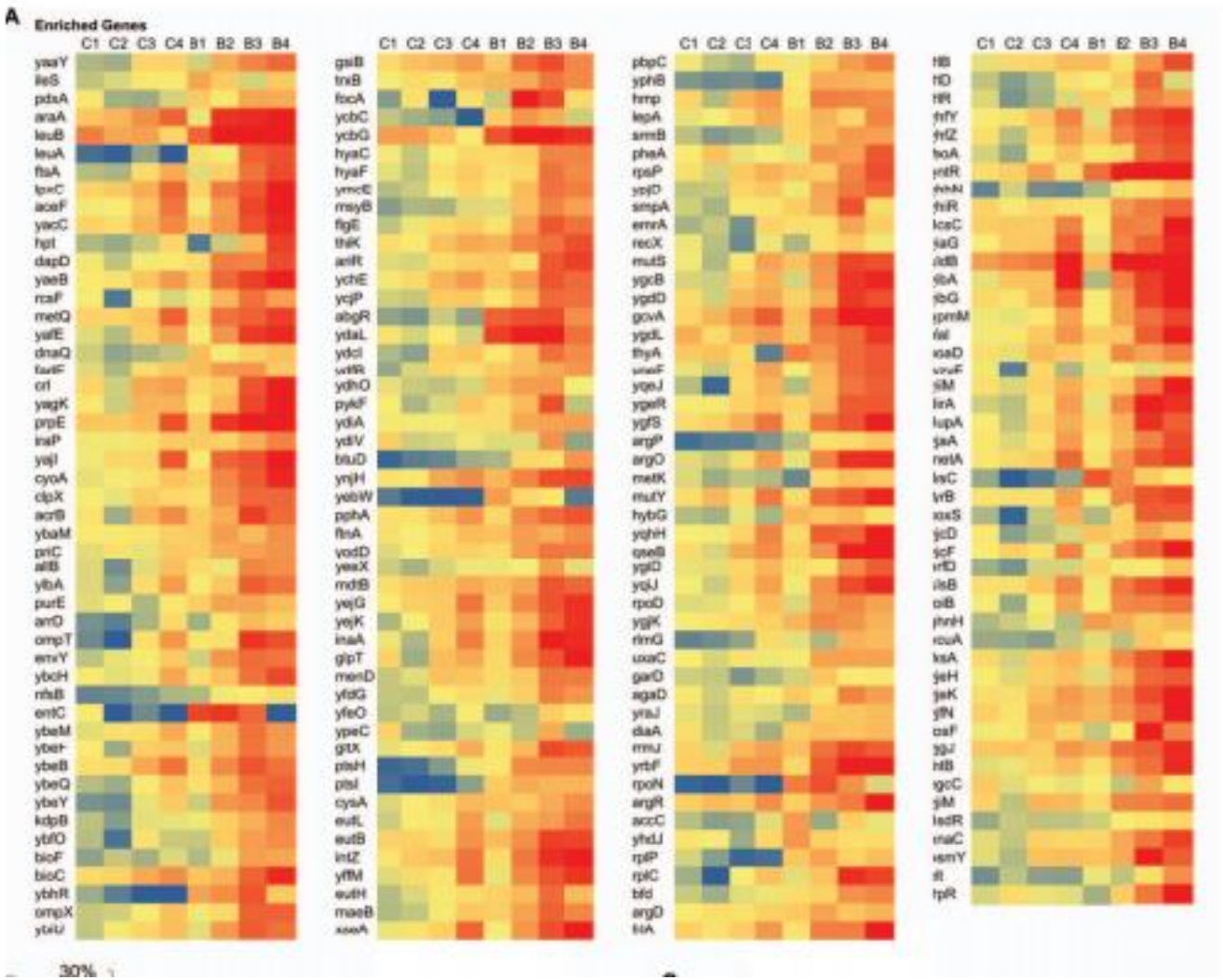


DNA is extracted from cells and digested with a restriction enzyme

Hybridize DNA to microarray chip with reference to E. Coli Genome and measure fluorescent intensity (ratio of n-butanol treated to untreated bacterial cells)



Enriched Genes



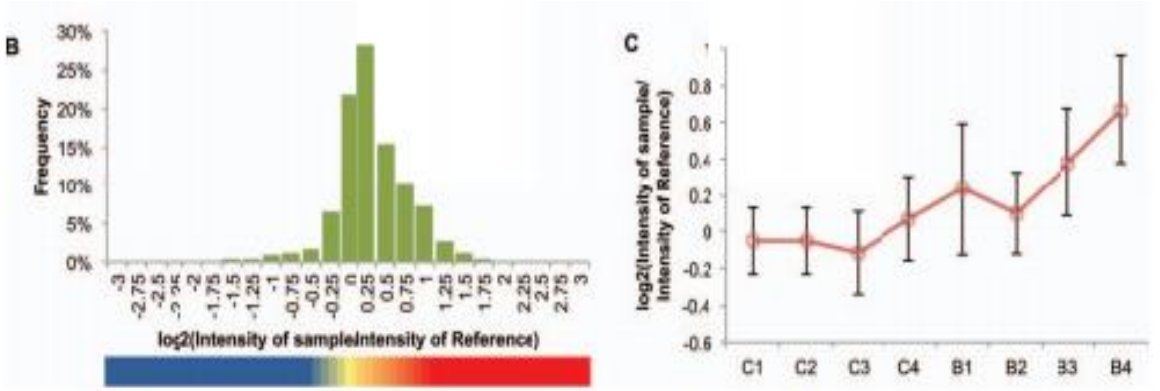


Figure 1. Profiles of genes significantly enriched in the n-butanol challenge. A. Heat map of all genes enriched. **B.** Histogram of the range of normalized log_2*(Intensity of sample/Intensity of reference)*. The colored bar at the bottom part of the figure is the legend for Figure 3A. **C.** The averaged profile.

doi:10.1371/journal.pone.0017678.g001

Enriched Genes Functions/Gene Ontology

Table 1. Membrane related genes enriched in the n-butanol challenge.

Function	Genes enriched
Efflux pump and anti-porters	*acrB, argO, emrA, focA* and *ybhR*
Amino acid and sugar transporter systems	*agaD, akgK, btuD, dcuA, frlA, glpT, gsiB, kdpB, metQ, sgcC, ycjP* and *yjeH*
Membrane lipoproteins	*cyoA, eutH, eutL, hyaC, ompT, ompX, rfaI, smpA, yajI, yfdG, ygdD, yjcD* and *yqjD*
Multidrug resistance	*acrB, emrA, mdtB* and *ychE*
Stress response	*ompT, yjaA* and *yodD*

doi:10.1371/journal.pone.0017678.t001

Table 2. Gene Ontology terms enriched in the enriched set of genes.

GO ID	Term	Log odd-ratio	Corrected p-value
GO:0003700	Sequence-specific DNA binding transcription factor activity	0.62	0.07
GO:0016564	Transcription repressor activity	0.90	0.07
GO:0050897	Cobalt ion binding	1.71	0.06
GO:0030145	Manganese ion binding	1.03	0.09
GO:0006525	Arginine metabolic process	1.71	0.06
GO:0009085	Lysine biosynthetic process	2.64	0.01
GO:0019867	Outer membrane	0.97	0.07
GO:0009102	Biotin biosynthetic process	2.93	0.01
GO:0030955	Potassium ion binding	2.20	0.02
GO:0046912	Transferase activity, transferring acyl groups, acyl groups converted into alkyl on transfer	2.93	0.02
GO:0009098	Leucine biosynthetic process	3.20	0.02
GO:0006352	Transcription initiation	2.93	0.02
GO:0016987	Sigma factor activity	2.71	0.03
GO:0044011	Single-species biofilm formation on inanimate substrate	3.52	0.02
GO:0070301	Cellular response to hydrogen peroxide	3.10	0.02

doi:10.1371/journal.pone.0017678.t002

Overexpression of Enriched Genes

Table 3. Genes that significantly increase n-butanol tolerance when they are overexpressed using ASKA collection. *

Clone	IIE	RSGR	p-Value
ampI	10.8±0.9%	−13.3±0.7%	0.01
entC	32.8±4.0%	−0.8±0.1%	0.05
ybiA	12.7±0.8%	−8.4±0.3%	0.02
metA	14.9±0.9%	−7.2±0.2%	0.01
ahlI	13.9±1.0%	−12.2±0.6%	0.02
phnH	42.4±3.0%	18.4±0.4%	0.01
fecA	49.1±3.3%	3.6±0.1%	0.00
focA	4.3±0.2%	−15.4±0.3%	0.02
hyaF	20.8±2.1%	15.4±0.6%	0.03
ymcE	13.2±0.4%	−11.1±0.2%	0.02
yfdG	20.3±1.4%	4.9±0.2%	0.00

doi:10.1371/journal.pone.0017678.t003

$$IIE = \frac{\left(\frac{\mu_{\text{ASKA or Keio @ 0.5\% n-Butanol}}}{\mu_{\text{ASKA or Keio @ 0\% n-Butanol}}\right) - \left(\frac{\mu_{\text{WT @ 0.5\% n-Butanol}}}{\mu_{\text{WT @ 0\% n-Butanol}}\right)}{\left(\frac{\mu_{\text{WT @ 0.5\% n-Butanol}}}{\mu_{\text{WT @ 0\% n-Butanol}}\right)}$$

$$RSGR = 1 - \left(\frac{\mu_{\text{ASKA or Keio @ 0\% n-Butanol}}}{\mu_{\text{WT @ 0\% n-Butanol}}}\right)$$

IIE = measures the increase in the n-butanol tolerance growth rate in comparison with the specific growth rate in absence of the solvent

RSGR = measures the change of the specific growth rate due to the overexpression of the gene

*ASKA collection is a set of ORF clones of E. Coli under an inducible lac promoter

**μ is maximum growth rate

Highlights for Overexpression of Enriched Genes

entC/feoA – involved in iron metabolism; novel finding

yibA, metA, ymcE – heat shock related genes under control of σ^{32}

ompT – outer membrane protease that is active under denaturing conditions

focA – formate transporter; efflux pump that regulates intracellular formate pool

Depleted Genes

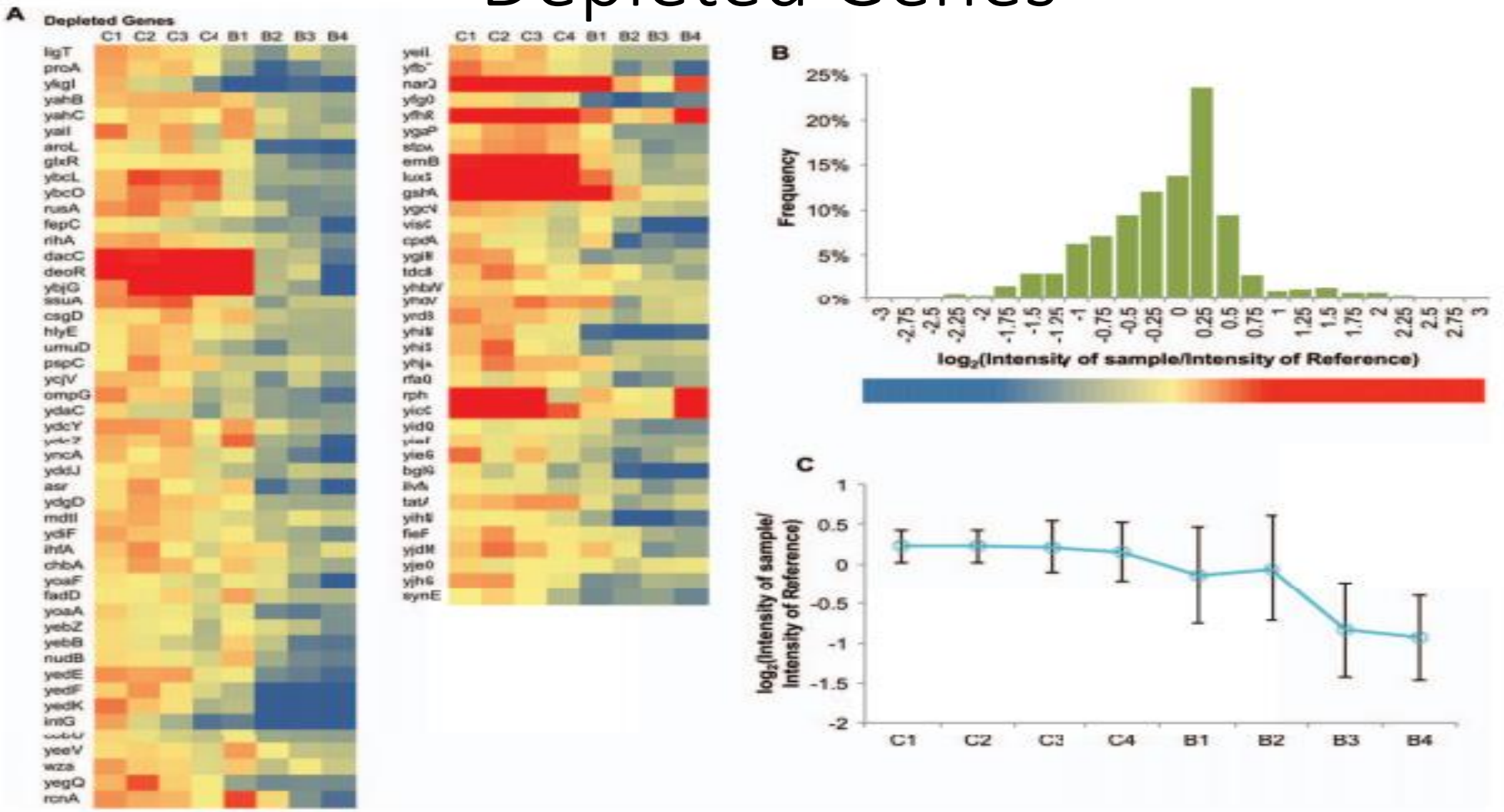


Figure 2. Profiles of genes significantly depleted in the n-butanol challenge. A. Heat map of all genes depleted. **B.** Histogram of the range of normalized *log2(Intensity of sample/Intensity of Reference.* The colored bar at the bottom part of the figure is the legend for Figure 4A. **C.** The averaged profile.

doi:10.1371/journal.pone.0017678.g002

Depleted Genes Functions/Gene Ontology

Table 4. Gene Ontology terms enriched in the depleted gene set.

GO ID	Term	Log odd-ratio	Corrected p-value
GO:0006508	Proteolysis	2.54	0.00
GO:0008360	Regulation of cell shape	1.94	0.09
GO:0008658	Penicillin binding	3.42	0.09
GO:0008236	Serine-type peptidase activity	3.57	0.00
GO:0009081	Branched chain family amino acid metabolic process	2.42	0.05
GO:0009405	Pathogenesis	3.42	0.10
GO:0003984	Acetolactate synthase activity	3.42	0.10
GO:0046654	Tetrahydrofolate biosynthetic process	3.42	0.04
GO:0046930	Pore complex	2.94	0.02
GO:0043190	ATP-binding cassette (ABC) transporter complex	1.89	0.09
GO:0009432	SOS response	2.42	0.05
GO:0015774	Polysaccharide transport	2.57	0.09

doi:10.1371/journal.pone.0017678.t004

Deletion of Enriched Genes

Table 5. Genes that significantly enhance n-butanol tolerance when deleted from the *E. coli* genome.

Mutant	IIE	RSGR	p-value
astE	48.7±6.3%	−3.3±0.3%	0.00
ygiH	14.8±1.2%	12.3±0.6%	0.02
rph	48.4±4.1%	−10.2±0.6%	0.01

doi:10.1371/journal.pone.0017678.t005

*Using Keio Knockout collection E. Coli K-12 with single gene knockouts using flp-frt recombination

$$IIE = \frac{\left(\frac{\mu_{\text{ASKA or Keio @ 0.5\% n-Butanol}}}{\mu_{\text{ASKA or Keio @ 0\% n-Butanol}}\right) - \left(\frac{\mu_{\text{WT @ 0.5\% n-Butanol}}}{\mu_{\text{WT @ 0\% n-Butanol}}\right)}{\left(\frac{\mu_{\text{WT @ 0.5\% n-Butanol}}}{\mu_{\text{WT @ 0\% n-Butanol}}\right)}$$

$$RSGR = 1 - \left(\frac{\mu_{\text{ASKA or Keio @ 0\% n-Butanol}}}{\mu_{\text{WT @ 0\% n-Butanol}}}\right)$$

IIE = measures the increase in the n-butanol tolerance in comparison with the specific growth rate in absence of the solvent

RSGR = measures the change of the specific growth rate due to the overexpression of the gene

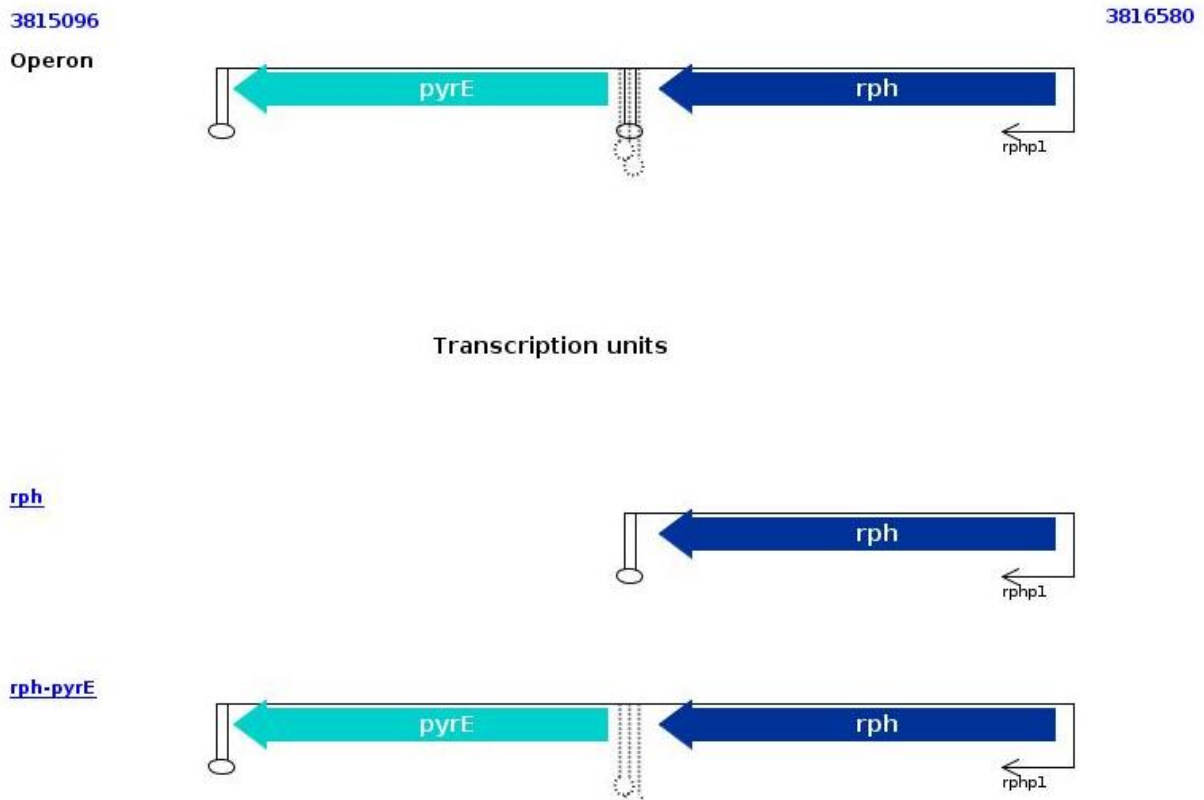
Highlights for Knockout of Depleted Genes

astE – hydrolyzes N-succinylglutamate into succinate and L-glutamate; L-glutamate involved in acid stress response (so decreased L-glutamate associates with decreased acid stress response which associates with increased n-butanol resistance)

ygiH – encodes inner membrane protein; regulates intracellular levels of acyl-ACP

Highlights for Knockout of Depleted Genes

rph – RNase PH gene; however E.Coli strain BW25113 (strain used for genomic library) has innate frameshift, so suggests other downstream products may be at work



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Transcriptional Analysis of *Lactobacillus brevis* to N-Butanol and Ferulic Acid Stress Responses

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Abstract

Background: The presence of anti-microbial phenolic compounds, such as the model compound ferulic acid, in biomass hydrolysates pose significant challenges to the widespread use of biomass in conjunction with whole cell biocatalysis or fermentation. Currently, these inhibitory compounds must be removed through additional downstream processing or sufficiently diluted to create environments suitable for most industrially important microbial strains. Simultaneously, product toxicity must also be overcome to allow for efficient production of next generation biofuels such as n-butanol, isopropanol, and others from these low cost feedstocks.

Methodology and Principal Findings: This study explores the high ferulic acid and n-butanol tolerance in *Lactobacillus brevis*, a lactic acid bacterium often found in fermentation processes, by global transcriptional response analysis. The transcriptional profile of *L. brevis* reveals that the presence of ferulic acid triggers the expression of currently uncharacterized membrane proteins, possibly in an effort to counteract ferulic acid induced changes in membrane fluidity and ion leakage. In contrast to the ferulic acid stress response, n-butanol challenges to growing cultures primarily induce genes within the fatty acid synthesis pathway and reduced the proportion of 19:1 cyclopropane fatty acid within the *L. brevis* membrane. Both inhibitors also triggered generalized stress responses. Separate attempts to alter flux through the *Escherichia coli* fatty acid synthesis by overexpressing acetyl-CoA carboxylase subunits and deleting cyclopropane fatty acid synthase (*cfa*) both failed to improve n-butanol tolerance in *E. coli*, indicating that additional components of the stress response are required to confer n-butanol resistance.

Conclusions: Several promising routes for understanding both ferulic acid and n-butanol tolerance have been identified from *L. brevis* gene expression data. These insights may be used to guide further engineering of model industrial organisms to better tolerate both classes of inhibitors to enable facile production of biofuels from lignocellulosic biomass.

Citation: Winkler J, Kao KC (2011) Transcriptional Analysis of *Lactobacillus brevis* to N-Butanol and Ferulic Acid Stress Responses. PLoS ONE 6(8): e21438. doi:10.1371/journal.pone.0021438