Journal of Bioscience and Bioengineering
VOL. 122 No. 1, 64–69, 2016

ELSEVIER

www.elsevier.com/locate/jbiosc

Experimental evolution and gene knockout studies reveal AcrA-mediated isobutanol tolerance in *Ralstonia eutropha*

Amanda C. Bernardi,[1] Claudia S. Gai,[1] Jingnan Lu,[2] Anthony J. Sinskey,[1,3] and Christopher J. Brigham[1,4,*]

Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, USA,[1] Department of Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, USA,[2] Engineering Systems Division, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, USA,[3] and Department of Bioengineering, University of Massachusetts Dartmouth, 285 Old Westport Road, North Dartmouth, MA 02747, USA[4]

Received 14 September 2015; accepted 18 December 2015
Available online 19 January 2016

Isobutanol (IBT) has attracted much attention from researchers as a next generation drop-in biofuel. *Ralstonia eutropha* is a gram-negative bacterium which naturally produces polyhydroxybutyrate (PHB), and has been reported to produce IBT after metabolic engineering. Similar to other microbes, *R. eutropha* experiences toxicity from branched-chain alcohols and is unable to grow in the presence of IBT concentrations higher than 0.5% (v v^{-1}). Such low tolerance greatly limits the ability of *R. eutropha* to grow and produce IBT. In order to study toxicity to the cells, IBT-tolerant strains were developed by experimental evolution, revealing that two genes, previously described as being related to IBT tolerance in *Escherichia coli* (*acrA* and *acrA6*), also presented mutations in *R. eutropha* evolved strains. The effect on the physiology of the cells of in-frame deletions of each of these genes was assessed in wild type and engineered IBT-producing strains in an attempt to reproduce a tolerant phenotype. The mutant strains' ability to tolerate, consume, and produce IBT were also analyzed. Although deletions of *acrA6* and *acrA* did not significantly improve *R. eutropha* growth in the presence of IBT, these deletions improved cell survival in the presence of high concentrations of IBT in the extracellular milieu. Moreover, an in-frame *acrA* deletion in an engineered IBT-producing *R. eutropha* enhanced the strain's ability to produce IBT, which could potentially be associated with enhanced survival at high IBT concentrations.

[**Key words:** *Ralstonia eutropha*; Biofuel; Multidrug efflux pump; Isobutanol; Tolerance]

The demand for alternative fuel sources has increased in recent years due to the dwindling of fossil fuel supplies. Previous studies have described the production of branched-chain alcohols by a synthetic Ehrlich pathway (1,2) using genes from the branched-chain amino acid biosynthesis pathways of *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Ralstonia eutropha* and *Lactococcus lactis* (3–6). These branched-chain alcohols have a high potential as an alternative to fossil fuels, because of chemical attributes similar to gasoline (3,4,6–12).

A major challenge facing bioproduction of branched-chain alcohols is product toxicity to bacterial cells, which limits production potential. Recent studies have focused on understanding the toxicity of branched-chain alcohols in order to develop tolerance mechanisms that can be transferred into production strains (13–15). Toxicity effects of branched-chain alcohols are complex, often relating to general stress responses, and can vary greatly depending on the alcohols being produced and the microbial biocatalyst being used. Toxicity is usually correlated with breakdown of the cell membrane and increases with solvent hydrophobicity (16). Presence of IBT, one of the branched-chain alcohols that has been largely studied as a biofuel molecule, was shown to be toxic to *Escherichia coli* causing growth arrest at concentrations higher than 1% (v v^{-1}). This growth arrest is believed to be a consequence of changes in the respiratory machinery, as well as in iron and phosphate homeostasis. IBT is hypothesized to insert itself in the membrane of cells, thus dissociating and disrupting the interaction between quinones and the cell membrane. Quinones are anchored to the membrane via their isoprenoid chain and facilitate the necessary transfer of electrons. Changes in membrane–quinone interaction, due to IBT, cause quinone depletion specifically by dissociation or disruption of interaction of quinones with the cell membrane (15), and as a consequence the cellular response to redox state is affected. The major regulator of these quinone dependent respiratory changes is the AcrAB-TolC system. As a consequence of IBT-related quinone malfunction, AcrB is no longer inhibited by quinones and suffers autophosphorylation, which activates AcrA, resulting in excretion of quinones from the cell.

Much effort has been undertaken in developing tools to increase branched-chain alcohol tolerance in microbes, and different strategies are being implemented, such as membrane modification (17), employment of heat shock proteins (18) and increase in efflux pump activity (19–21). Previous studies in IBT tolerance (22,23) and *n*-butanol tolerance (24) used an experimental evolution approach to obtain strains capable of surviving in high concentrations of IBT due to a series of mutations in the genome. Among the detected mutations are five identified genes, common to both studies, that are either components of a multidrug efflux pump (*acrA6* and *acrA*) or part of its regulator, the *marCRAB* operon (*marA*,

* Corresponding author at: Department of Bioengineering, University of Massachusetts Dartmouth, 285 Old Westport Road, North Dartmouth, MA 02747, USA. Tel.: +1 508 999 9149; fax: +1 508 999 9139.
E-mail address: cbrigham@umassd.edu (C.J. Brigham).

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2015.12.015

TABLE 1. Strains used in this work with details of their genotype and plasmids.

Strain/plasmid	Genotype	Reference
R. eutropha		
H16	Wild type, gentamicin resistant (Gen^r)	ATCC17699
Re2061	H16Δ*phaCAB* (Gen^r)	32
Re2410	DJ21Δ*phaCAB*, Δ*ilvE*, Δ*bkdAB*(Gen^r)	32
CF106	H16 *adh*(Con) ethanol + 2,3-butanediol + (Gen^r)	33
DJ21	H16 *adh*(Con) ethanol + 2,3-butanediol + (Gen^r)	33
Re2432	IBT-tolerant strain evolved from H16 (Gen^r)	This work
Re2433	IBT-tolerant strain evolved from Re2061 (Gen^r)	This work
Re2405	CF106Δ*phaCAB* Gen^r	32
Re2425	DJ21Δ*phaCAB*, *ilvE*, *bkdAB*, *aceE* (Gen^r)	32
Re2438	H16 Δ*acrA6* (Gen^r)	This work
Re2439	Re2425 Δ*acrA6* (Gen^r)	This work
Re2442	H16 Δ*acrA* (Gen^r)	This work
Re2445	Re2425 Δ*acrA* (Gen^r)	This work
E. coli		
S17-1	Conjugation strain	34
Plasmid		
pJV7	pJQ200Kan with Δ*phaC1* allele inserted into *BamHI* restriction site, confers kanamycin resistance (Kan^r)	35
pJL26	pJL26 pBBR1MCS-2 with branched-chain alcohol production operon (*ilvBHCDkivd*) inserted into the multiple cloning site (Kan^r)	32
pΔ3357	pJV7 used for in-frame deletion of *acr*A6 gene (Kan^r)	This work
pΔ3729	pJV7 used for in-frame deletion of *acr*A gene (Kan^r)	This work

marC and yhbJ). These genes have had their role in alcohol tolerance (19,20,22,23,25–27) and organic solvents tolerance (28–31) widely described in the literature.

IBT can be produced by metabolically engineered *R. eutropha*, a gram-negative bacterium known to produce polyhydroxybutyrate (PHB), an intracellular carbon storage polymer, during nutrient starvation. Previous work by Lu et al. (32) describes that IBT production by *R. eutropha* is possible through the incorporation of an engineered biosynthetic pathway, which redirects the carbon flow from PHB to IBT production. Wild type *R. eutropha* however, is unable to grow in the presence of IBT concentrations higher than 0.5% (v v^{-1}), (32) which significantly limits its potential as an industrial IBT-production strain.

The main objective of this work is to identify genes involved in IBT tolerance in *R. eutropha* and to develop a strain capable of withstanding increased extracellular IBT concentrations.

Using similar approaches by Atsumi et al. (22) and Minty et al. (23), *R. eutropha* was evolved in the presence of increasing concentrations of IBT via sequential transfer. Five homologous genes previously described in *E. coli* for contributing to IBT tolerance were identified, sequenced, and analyzed in these evolved mutant strains. Once two important mutations were identified, in-frame deletions on those genes were constructed in both the wild type and IBT-producing *R. eutropha* strains in order to confirm their roles in IBT tolerance.

MATERIALS AND METHODS

Bacterial strains and plasmids Strains and plasmids used in the experiments described in this work are listed in Table 1.

Cultivation media and conditions *R. eutropha* strains were cultivated at 30°C in tryptic soy broth (TSB) (Becton Dickinson, Sparks, MD, USA) and minimal media formulated as described previously (32), using 2% (w v^{-1}) fructose or 0.2% IBT (v v^{-1}) as carbon source. Gentamicin was added in a 10 μg ml^{-1} final concentration, and for *R. eutropha* carrying plasmid, kanamycin was added to a final concentration of 200 μg ml^{-1}.

E. coli strains used for deletions were cultivated at 37°C in LB medium (36) with kanamycin added to a final concentration of 50 μg ml^{-1}.

Experimental evolution In order to develop and isolate an IBT-tolerant *R. eutropha* strain, we performed experimental evolution experiments using the sequential transfer method described previously (22,23,37). A single colony of *R. eutropha* strains H16 and Re2061 was transferred from a TSB agar plate into culture tubes containing 5 ml of TSB media. Tubes were incubated on a roller drum for 24 h at 30°C. A 2-ml aliquot of cultures was transferred to fresh rich media containing IBT. Over the course of the cultivation experiment, the IBT concentration was increased from 0.5% (v v^{-1}) to 2.0% (v v^{-1}) at a rate of 0.5% (v v^{-1}) every 15 days. Evolved IBT tolerant strains (Re2432 and Re2433) were isolated from the final bacterial population by plating on TSB with 2.5% IBT (v v^{-1}) after 75 days of sequential cultivation.

Identification of gene mutations in evolved strains Based on studies by Atsumi et al. (22) and Minty et al. (23) on IBT tolerance in *E. coli*, we selected the 5 common genes that had mutations identified in both prior studies and that had homologs with similar function described in *R. eutropha*, in order to screen for genetic lesions (Table 2).

These genes, along with 100 upstream and downstream base pairs, were sequenced from the evolved strains using primers listed in Supplementary material Table S1. Phusion DNA Polymerase (New England Biolabs, Ipswich, MA, USA) was used for DNA amplification; QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) was used for purifications of all DNA products before sequencing. The resulting sequences were compared to those from the parental strains using Mega5 software (38).

Plasmid and strains construction For DNA sequence amplification and extraction, materials were used as described above. Gel purifications of all DNA products and plasmid extractions were performed using QIAprep Spin Miniprep Kit (Qiagen). Restriction enzymes used in this study were from New England Biolabs.

Plasmids for deletion of genes were constructed according to Lu et al. (32). For the deletion of the target genes, standard procedures were used as previously described (39). All plasmids used for in-frame gene deletions were constructed using the Gibson Assembly method (40). Gene deletion plasmids were introduced into *R. eutropha* strains and in-frame deletions were identified as described previously (41). Gene-specific primers for determining the presence of a deletion are listed in Supplementary Table S1.

Isobutanol tolerance evaluation Wild type and evolved *R. eutropha* strains (Table 1) were subjected to two different assays in order to assess their tolerance to IBT. The first assay determines the effect of extracellular IBT on *R. eutropha* growth. Strains were cultivated in minimal medium with 2% fructose (w v^{-1}), 0.05% NH_4Cl (w v^{-1}), and different concentrations of IBT (0%, 0.5%, and 1% (v v^{-1})). Growth was monitored by measuring optical density of the culture at 600 nm (OD_{600nm}).

The second assay, which is hereby referred to as the survival assay, determines *R. eutropha* cell viability after exogenous IBT exposure. Cells were grown on TSB media overnight, diluted in fresh media to OD_{600nm} of 1.0, and IBT was added to

TABLE 2. Potential genes related to IBT tolerance and their respective location and description.

E. coli gene	*R. eutropha* homologue/locus tag	Description
marC	H16_A3413	Putative transporter
marA	H16_A3378	AraC family transcriptional regulator
acrA6	H16_A3357	Cation/multidrug efflux system, membrane-fusion component
acrA	H16_A3729	Acriflavine resistance protein A
yhbJ	H16_A0381	Hypothetical protein