Engineering Alternative Butanol Production Platforms in Heterologous Bacteria

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Abstract

Alternative microbial hosts have been engineered as biocatalysts for butanol biosynthesis. The butanol synthetic pathway of *Clostridium acetobutylicum* was first re-constructed in *Escherichia coli* to establish a baseline for comparison to other hosts. Whereas polycistronic expression of the pathway genes resulted in the production of 34 mg/L butanol, individual expression of pathway genes elevated titers to 200 mg/L. Improved titers were achieved by co-expression of *Saccharomyces cerevisiae* formate dehydrogenase while overexpression of *E. coli* glyceraldehyde 3-phosphate dehydrogenase to elevate glycolytic flux improved titers to 580 mg/L. *Pseudomonas putida* and *Bacillus subtilis* were also explored as alternative production hosts. Polycistronic expression of butanol biosynthetic genes yielded butanol titers of 120 mg/L and 24 mg/L from *P. putida* and *B. subtilis*, respectively. Production in the obligate aerobe *P. putida* was dependent upon expression of *bcd-etfAB*. These results demonstrate the potential of engineering butanol biosynthesis in a variety of heterologous microorganisms, including those cultivated aerobically.

Keywords: biofuel, butanol, *E. coli*, *P. putida*, *B. subtilis*, tolerance, product inhibition.

Introduction

With applications as a feedstock in plastic manufacturing and as an industrial solvent, the current butanol market in the United States alone is about 2.9 billion lbs per annum. However, chemical synthesis of butanol relies upon propylene feedstock, a petroleum-based substrate (Ezeji et al., 2007). Alternatively, butanol (biobutanol) can be naturally synthesized by solventogenic bacteria of the genus *Clostridium* through fermentation of renewable substrates, such as glucose. The once prosperous Acetone-Butanol-Ethanol (ABE) fermentation has garnered resurgent interest as a result of unprecedented economic and political concerns associated with increasing demand of nonrenewable energy resources. In this regard, biobutanol has also emerged as a promising renewable liquid transportation fuel. With thermodynamic and physical properties that are highly akin to those of gasoline, biobutanol can be used either as a blending agent or direct fuel replacement in conventional vehicles (Antoni et al., 2007). More specifically, butanol possesses a nearly 50% higher energy density than ethanol, representing about 95% of the energy density of gasoline (Cascone, 2008). Since it is nearly 12-times more hydrophobic than ethanol, butanol can also be distributed and utilized within existing transportation fuel infrastructures without corrosive consequences.

The suitability of Clostridial biocatalysts for use in industrial fermentations suffers from several phenotypic disadvantages, including spore formation that can result in the loss of butanol forming abilities. Additionally, stresses caused by butanol toxicity have been attributed to the loss of pSOL1, a mega-plasmid encoding several essential solvent-forming genes (Borden and Papoutsakis, 2007). Furthermore, the metabolic shift from acidogenesis to solventogenesis in *Clostridium* presents additional complications

for continuous culture (Antoni et al., 2007). Overall, despite efforts to improve the biobutanol production efficiency of *Clostridium* (Harris et al., 2000; Mermelstein et al., 1994; Sillers et al., 2008; Tomas et al., 2003), relatively poor characterization and a lack of compatible genetic tools remain as central obstacles impeding natural biocatalyst progression.

In addition to phenotypic instabilities associated with Clostridial biocatalysts, the productivity of butanol fermentations is routinely limited by the effects of product cytotoxicity. Butanol has been found to accumulate primarily within the cytoplasmic membrane of *Clostridium* (Bowles and Ellefson, 1985) where it leads to disruption of the ordered structure of the phospholipid bilayer. This phenomenon produces an increase in membrane fluidity (Osborne et al., 1990b) which results in the loss of intracellular molecules (including proteins, RNA, and ATP), as well as an inability to maintain transmembrane ion gradients (Isken and de Bont, 1998). The resultant feed-back inhibition precludes butanol accumulation in culture media to titers above ~13 g/L for wild-type strains (Jones and Woods, 1986).

In *C. acetobutylicum*, butanol biosynthesis begins with the condensation of two molecules of acetyl-CoA to yield acetoacetyl-CoA. This reaction is catalyzed by a thiolase which is encoded by *thil* (Figure 1). The genes encoding for enzyme activities for the step-wise conversion of acetoacetyl-CoA to butyryl-CoA are clustered together in the polycistronic BCS operon. This operon is comprised of the genes *crt*, *bcd*, *etfAB*, and *hbd*, encoding for crotonase, butyryl-CoA dehydrogenase, electron transfer proteins, and 3-hydroxybutyryl-CoA dehydrogenase, respectively. A bi-functional aldehyde/alcohol dehydrogenase, encoded by either *adhE1* or *adhE2*, catalyzes the final steps of butanol

87 synthesis from butyryl-CoA (Figure 1). Although *C. acetobutylicum adhE1* can catalyze
88 the conversion of butyryl-CoA to butyraldehyde and then butanol with a single enzyme, it
89 can also mediate the synthesis of ethanol from acetyl-CoA (through acetaldehyde). *C.*
90 *acetobutylicum* also possesses two distinct butanol dehydrogenase isozymes, encoded by
91 *bdhA* and *bdhB* which have been found to have a high specificity for the conversion of
92 butyraldehyde to butanol (Welch et al., 1989). In that same study, it was also reported
93 that *bdhB* provided a significantly higher catalytic turn over rate of butyraldehyde than
94 *bdhA*. Recently, different groups have successfully re-constructed the butanol
95 biosynthetic pathway of *C. acetobutylicum* using heterologous microorganisms, including
96 *E. coli* (Atsumi et al., 2008a; Inui et al., 2008) and *S. cerevisiae* (Steen et al., 2008).
97 Although *S. cerevisiae* has been found to possess favorable industrial attributes, including
98 moderate butanol tolerance (Fischer et al., 2008; Knoshaug and Zhang, 2008),
99 preliminary attempts to engineer butanol biosynthesis in yeast have resulted in the
100 production of merely 2.5 mg/L (Steen et al., 2008). Meanwhile, butanol titers in *E. coli*
101 engineered to express the *Clostridium* butanol pathway have been reported as high as 552
102 mg/L (Atsumi et al., 2008a). The biosynthesis of butanol, as well as other higher
103 alcohols of interest, from keto-acid precursors (typically used in amino acid biosynthesis)
104 has also been explored as an alternative route towards biofuel production (Atsumi et al.,
105 2008b). In subsequent studies it was shown that through this non-natural pathway,
106 butanol could be produced in excess of 800 mg/L as a co-product with n-propanol (Shen
107 and Liao, 2008). Although the effects of product inhibition were likely to have remained
108 unnoticed given the relatively low titers achieved in each of these previous studies, the
109 butanol toxicity threshold of *E. coli* is known to be below that of *Clostridium* (Fischer et

al., 2008; Knoshaug and Zhang, 2008). Thus, it is anticipated that modest inhibitory thresholds of these strains will ultimately limit their achievable outputs as their productivity is further engineered to that which is required of production-level strains.

Solvent tolerant phenotypes consist of evolved mechanisms by which many opportunistic microorganisms have developed the means to survive in extreme environments. Notable naturally solvent tolerant bacteria include species of *Rhodococcus*, *Bacillus*, and *Pseudomonas* (de Bont, 1998). For example, *P. putida* S12 derives its solvent tolerance from an increased proportion of *trans*-unsaturated fatty acids in its cytoplasmic membrane (Heipieper and Debont, 1994), as well as through the use of active efflux pump systems. Such mechanisms permit maintenance of cytoplasmic membrane integrity in the presence of high concentrations of organic solvents, and have allowed *P. putida* S12 to demonstrate moderate tolerance to butanol in previous studies (de Carvalho et al., 2004). For these same reasons, *P. putida* S12 has also previously been employed as an engineered host strain for the biosynthesis of phenol (Wierckx et al., 2005) and cinnamic acid (Nijkamp et al., 2005). Meanwhile, solvent tolerant species of *Bacillus* have also been isolated that can tolerate butanol concentrations as high as 2.5-3.7% (wt./vol.) (Sardessai and Bhosle, 2002), by incorporating tolerance mechanisms that can include adaptations to the cell wall composition and through the use of stress response proteins (Kang et al., 2007). In an effort to explore an alternative paradigm towards the engineering of robust biocatalysts, we have re-constructed the butanol biosynthesis pathway in heterologous hosts with known natural solvent tolerance and high industrial utility (Schmid et al., 2001). More specifically, we have engineered functional pathway expression strategies to allow biobutanol synthesis by both

Pseudomonas putida and *Bacillus subtilis*. To provide a baseline for comparison, our study begins by also engineering butanol biosynthesis in *E. coli*. In contrast to previous works, we apply alternative strategies for functional pathway construction and continue on to explore the effects of the overexpression of enzymes involved in increasing glycolytic flux or regenerating NADH on butanol production.

Materials and Methods

Microbial strains

C. acetobutylicum ATCC 824 and *P. putida* S12 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). *B. subtilis* KS438, a sporulation deficient strain, was kindly provided by Dr. Alan Grossman of the Department of Biology at the Massachusetts Institute of Technology, USA. *E. coli* DH10B and XL1-Blue (Stratagene, La Jolla, CA) were used for cloning and plasmid maintenance. *E. coli* BL21Star(DE3) (Invitrogen, Carlsbad, CA) was used as the host strain to allow the expression of genes under the T7*lac* promoter.

Plasmid construction

Genes derived from *C. acetobutylicum* ATCC 824 (*thil*, *hbd*, *crt*, *bcd*, *etfAB*, *adhE1*, *adhE2*), *E. coli* K-12 (*atoB* and *gapA*), and *P. putida* KT2440 (*acd*) were obtained via polymerase chain reaction (PCR) using genomic DNA (gDNA) templates. All gDNA samples were prepared using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). *S. cerevisiae fdh1* chromosomal DNA (cDNA) fragment was obtained from mRNA using SuperScript One-Step RT-PCR (Invitrogen, Carlsbad, CA).

RNA was isolated from *S. cerevisiae* grown overnight in YPD medium (Difco, Franklin Lakes, NJ). Custom oligonucleotides (primers) were purchased for all PCR amplifications (Sigma-Genosys, St. Louis, MO).

The natural butanol biosynthesis pathway of *C. acetobutylicum* (Figure 1) was first re-constructed using two broad host range compatible expression vectors, pMMB206G and pRK415 ((Keen et al., 1988a); kindly donated by Dr. Keith Poole, Department of Microbiology and Immunology, Queen's University, Canada), which possess the RSF1010 and RK2 replicons, respectively. pMMB206G contains a *taclac* (*tac*-*lac*UV5 in tandem) promoter and a gentamycin resistance selective marker while pRK415 contains a *lac* promoter and a tetracyclin resistance marker. pMMB206G was constructed by ligating the Klenow-filled, *Mlu*I-*Mlu*I gentamycin resistance cassette from pBSL141 (ATCC; (Alexeyev et al., 1995)) into the *Xmn*I site of pMMB206 (ATCC; (Morales et al., 1991)), thereby disrupting the original chloramphenicol resistance marker. To construct pmT, *thil* was first ligated into the *Bam*HI site of pMMB206G. pmTA1 was then generated by ligation of *adhE1* between the *Sal*I – *Pst*I sites of pmT. *bdhB* was then ligated into the *Sal*I site of pmTA1 to yield pmTBA1. prBCS was constructed by inserting the 4.7-kb BCS operon (containing *crt*, *bcd*, *etfAB*, and *hbd*) into the *BamH*I site of pRK415. To investigate the effects of background butyryl-CoA dehydrogenase activity in *P. putida* strains, prCCS was constructed by first digesting prBCS with *Apa*LI and *Dra*III. The linearized, 14.4 kb fragment was gel purified then Klenow-filled to generate a blunt-end product. This truncated fragment was then re-ligated with itself to create prCCS (a *bcd*$^-$ and *etfB*$^-$ derivative of prBCS).

Compatible vectors pETDuet-1, pCDFDuet-1, pACYCDuet-1, and pCOLADuet-1 (Novagen, Darmstadt, Germany) were used to provide individual expression of each gene under a T7*lac* promoter and a ribosome binding site (RBS). *thil* was inserted between the *Nde*I and *Xho*I sites of pETDuet-1 to create peT plasmid. *atoB* was inserted between the *Bgl*II and *Xho*I sites of pETDuet-1 to form peA plasmid. Plasmid pcHC was prepared by inserting *hbd* and *crt* between the *EcoR*I and *Pst*I, and *Nde*I and *Xho*I sites, respectively, in pCDFDuet-1 vector. *bcd* and *etfAB* fragments were inserted between the *BamH*I and *Sal*I and *Xho*I and *Pac*I sites, respectively, in pCOLADuet-1 to create pkBE. As an alternative to *bcd, acd,* encoding an acyl-CoA dehydrogenase from *P. putida* was inserted between the *SalI* and *HindIII* sites in vector pCOLADuet-1 to create plasmid pkA. Additionally, the gene encoding for *Streptomyces collinus* crotonyl-CoA reductase (*ccr*) was synthetically constructed (DNA2.0, Menlo Park, CA) with codon usage optimized for expression in *E. coli.* The synthetic *ccr* fragment was inserted between the *EcoR*I and *Hind*III sites in vector pCOLADuet-1 to create plasmid pkC. The *adhE1* fragment was inserted into pACYCDuet-1 vector between the *EcoR*I and *Pst*I sites to create plasmid paA1. The *adhE2* fragment was inserted into pACYCDuet-1 vector between the *BamH*I and *Sal*I sites to create plasmid paA2. Three plasmids containing genes encoding enzymes to promote greater glycolytic flux or NADH regeneration were constructed. Plasmid peAG was created by inserting *gapA* into the *BamH*I and *Sac*I sites of peA. Cloning *fdh1* between the *Nco*I and *Pst*I sites of peA resulted in plasmid peAF. Together with the T7*lac* promoter fragment, *fdh1* was inserted into peAG between the *Sac*I and *Pst*I sites to create plasmid peAGF.

To re-construct the butanol biosynthetic pathway in *B. subtilis*, the BCS operon was first ligated between the *Nhe*I and *Sph*I sites of pDR111 ((Britton et al., 2002); donated by Dr. Alan Grossman, MIT) to create pdBCS. pJBN1 and pDRPyr-Kan were each constructed by ligating the 1.8-kb *Eco*RI – *Bam*HI fragment containing the hyper-spank promoter, multi-cloning site, and *lacI* from pDR111 with *Eco*RI – *Bam*HI linearized pDG1664 ((Guerout-Fleury et al., 1996); obtained from the *Bacillus* Genetic Stock Center at The Ohio State University) and pPyr-Kan ((Middleton and Hofmeister, 2004); obtained from the *Bacillus* Genetic Stock Center), respectively. *thil* was cloned into the *Nhe*I site of pJBN1 resulting in pjT while *adhE2* was ligated between the *Sal*I and *Sph*I sites of pDRPyr-Kan, yielding ppA2. Plasmid construction and cloning was performed using *E. coli* DH10B.

In all cases, the Expand High Fidelity PCR System (Roche, Basel, Switzerland) or Phusion High Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) was used for DNA amplification. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). All positive constructs were identified via restriction digest and nucleotide sequencing. Plasmids constructed in the present work are listed in Table 1.

Strain development and culture conditions

E. coli ED1.0 was obtained by transforming *E. coli* DH10B with prBCS and pmTA1 whereas ED2.0 resulted from the transformation of *E. coli* DH10B with prBCS and pmTBA1. Positive transformants were isolated on LB plates containing gentamycin (20 mg/L) and tetracycline (10 mg/L). To create *E. coli* strain EB1.0, BL21Star(DE3)

was transformed with plasmids peT, pcHC, pkBE, and paA1. To test the utility of *acd* and *ccr*, EB2.A and EB2.C were created by replacing pkBE in strain EB1.0 with either pkA or pkC, respectively. Replacement of peT with peA in strain EB1.0 yielded strain EB3.0. Strain EB4.0 was created by replacing paA1 with paA2 in EB1.0 to compare the effect of *adhE2* expression with *adhE1*. In order to explore the possibility of improving butanol synthesis by increasing intracellular NADH or glycolytic flux towards precursors, strains EB4.F and EB4.G were created by replacing peA in EB4.0 with peAF and peAG, respectively. To investigate the effects of both glyceraldehyde 3-phosphate dehydrogenase and formate dehydrogenase expression on butanol biosynthesis, strain EB4.GF was created by replacing peA in EB4.0 with peAGF. Cells containing all compatible plasmids were isolated on LB plates containing ampicillin (30 mg/L), streptomycin (25 mg/L), kanamycin (25 mg/L), and chloramphenicol (8 mg/L). Deletions of *adhE* and *ldhA* in *E. coli* BL21Star(DE3) strains were performed according to a previously reported method (Datsenko and Wanner, 2000). All recombinant strains developed in this study are listed in Table 2.

Fermentation experiments using ED and EB strains were initiated by culturing the recombinant strains in 6 mL TB using 15-mL tubes overnight at 37°C, in a shaker rotating at 225 rpm. The preinoculum was used to seed 150 mL TB medium supplemented with 5 g/L glucose or glycerol in 250-mL screw-capped flasks, at an initial optical density at 600 nm (OD_{600}) of 0.05. Both aerobic and anaerobic culture conditions were studied. Anaerobic cultures were first grown under aerobic conditions in sealed shake flasks to promote biomass production. With a limited headspace volume, these closed cultures became naturally depleted of oxygen after 3-5 h (as indicated by the

addition of 5 mg/L resazurin to the culture medium). Cultures were incubated at 37°C in a rotary shaker until OD_{600} reached 0.8. At this point, 0.1 mM IPTG was added to the cultures to induce recombinant protein expression. Following induction, cells were cultivated at 30°C. In all cases, TB medium was supplemented with 20 mg/L gentamycin and 10 mg/L tetracycline (ED strains), or 30 mg/L ampicillin, 25 mg/L streptomycin, 25 mg/L kanamycin, and 8 mg/L chloramphenicol (EB strains). The addition of formic acid to cultures at an initial concentration of 1 g/L was also investigated to promote greater cofactor regeneration in cultures of EB4.F and EB4.0 (control). Culture media were sampled at 24 h intervals for up to 72 h. Samples were centrifuged to pellet cells while the aqueous supernatant was collected for HPLC analysis.

P. putida S12 was co-transformed with prBCS and pmTA1 to construct PS1.0, or with prBCS and pmTBA1 to yield PS2.0. Meanwhile, construction of strains PS1.A and PS2.A was accomplished by co-transformation of *P. putida* S12 with prCCS or pmTA1 or pmTBA1, respectively. Selection of these strains was performed using LB plates containing 20 mg/L gentamycin and 10 mg/L tetracycline. Butanol production in both PS strains was performed at 30^{o}C in 250 mL shake flasks containing 50 mL TB medium with 0.5% (wt./vol.) glucose or glycerol. Induction protocols were performed as described for *E. coli*, though using 1 mM IPTG. All media were supplemented with gentamycin (20 mg/L) and tetracycline (10 mg/L). Aerobic conditions were promoted throughout the study due to the obligately aerobic nature of *P. putida*.

Competent *B. subtilis* KS438 cells were sequentially transformed with pdBCS, pjT, and ppA2 where they were integrated into the chromosome at the *amyE*, *thrC*, and *pyrD* loci, respectively, via double-crossover homologous recombination. The resultant

strain, BK1.0, was *amyE*$^{-}$ *thrC*$^{-}$ *pyrD*$^{-}$. LB plates containing 100 mg/L spectinomycin,12.5 mg/L lincomycin,0.5 mg/L erythromycin, and 5 mg/L kanamycin were used for selection of transformants, as appropriate. Butanol fermentation experiments with BK1.0 were performed as described above for *E. coli* strains, except that induction was performed using 1 mM IPTG. Antibiotics were not required for the maintenance of BK1.0, and thus were not added to the fermentation medium.

Butanol challenge

50 mL of TB medium was inoculated with preinoculum of *E. coli* BL21Star(DE3), *P. putida* S12, or *B. subtilis* KS438 to give an initial OD_{600} of 0.05. Cultures were incubated at 30°C while shaking at 250 rpm. After reaching mid-exponential growth stage ($OD_{600} \approx 1.5$), challenges were applied by butanol addition to a final concentration between 0 and 2% (wt./vol.). Growth and viability were then monitored for 24 hours post butanol addition through optical density measurements and plate counts, respectively. Culture samples were diluted in phosphate buffer (pH 7.0) prior to absorbance readings to yield an average OD_{600} measurement of 0.5. Culture samples were serially diluted by up to 10^{-7} in phosphate buffer prior to plating 100 μL of each dilution on LB agar and incubated at 30°C overnight. Counts were made on all plates yielding a countable number of distinct colony forming units (CFUs), and expressed as CFU/mL of original culture. Error was estimated at one standard deviation of all plates counted for each sample at each time point (typically 2-3).

Metabolite analysis

Solvents and fermentation products were analyzed via HPLC using an Agilent 1100 series instrument equipped with a refractive index detector (RID). Analyte separation was achieved using an Aminex® HPX-87H anion exchange column (Bio-Rad Laboratories, Hercules, CA) according to the method of Buday et al. (Buday et al., 1990) using 5mM H_2SO_4 as the mobile phase. External standards provided calibration for titer determination.

Results

Construction of butanol-producing *E. coli*

Butanol synthesis in *E. coli* was first investigated via polycistronic expression of the Clostridial genes. DH10B was transformed with prBCS and pmTA1 (strain ED1.0) or with prBCS and pmTBA1 (strain ED2.0). Both strains were cultured in TB medium supplemented with either 0.5% (wt./vol.) glucose or glycerol to assess their ability to synthesize butanol. Following induction with IPTG, butanol was detected in the culture broth of both ED1.0 and ED2.0 after about 48 h, but only when the TB medium was supplemented with glycerol (a more reduced substrate than glucose). Specifically, butanol synthesis by ED1.0 and ED2.0 reached up to 34 ± 5 mg/L and 33 ± 2 mg/L, respectively (Figure 2). These results are comparable with earlier efforts to reconstruct the butanol pathway in *E. coli* (Atsumi et al., 2008a). Co-expression of *bdhB* with *adhE1* in strain ED2.0 had no impact on butanol titer.

The establishment of *E. coli* strains expressing polycistronic constructs provides a baseline for comparison of productivity with the other heterologous hosts. However, we hypothesized that butanol biosynthesis in *E. coli* could also potentially be improved by

promoting greater expression of the heterologous Clostridial enzymes. The effects of different strategies of expressing a heterologous multi-gene biosynthetic pathway on metabolite production in *E. coli* have been explored (Hwang et al., 2003). Specifically, the polycistronic expression in *E. coli* of a heterologous pathway was compared with the simultaneous individual expression of each gene under a promoter and a ribosome binding site. By using the latter strategy, it was demonstrated that the production of each recombinant protein in the biosynthetic pathway was improved, translating into increased production of non-native metabolites.

We tested the effect of individual expression of *thil*, *hbd*, *crt*, *bcd*, *etfAB*, and *adhE1* under a T7*lac* promoter and a ribosome binding site in *E. coli* EB1.0. Since butanol production was not improved by expression of *bdhB* in strain ED2.0 (relative to ED1.0), *bdhB* was excluded in the construction of all EB strains. Butanol production after 48 h from EB1.0 was 200 mg/L, which is approximately a five-fold improvement over the production from strain ED1.0 (Figure 2). It has repeatedly been demonstrated that butyryl-CoA dehydrogenase, isolated from either *C. acetobutylicum* or from recombinant *E. coli*, failed to exhibit enzymatic activity *in vitro* (Atsumi et al., 2008a; Boynton et al., 1996; Hartmanis and Gatenbeck, 1984). Furthermore, *in vivo* butyryl-CoA dehydrogenase activity is also dependent upon the coordinated functional expression of electron transfer flavoproteins *etfA* and *etfB*. Since the functionality of the butyryl-CoA dehydrogenase complex could possibly be a rate-limiting step in the butanol synthesis pathway, we explored the utility of crotonyl-CoA reductase (*ccr*) derived from *Streptomyces collinus* to mediate the conversion of crotonyl-CoA to butyryl-CoA (strain EB2.C). *ccr* has been previously characterized and functionally expressed in *E. coli*

(Wallace et al., 1995). As was also noted in previous studies (Atsumi et al., 2008a), we found this particular substitution to provide inferior results, as butanol production decreased by 55% compared with strain EB1.0. The utility of other non-Clostridial source enzyme homologs also provided unsubstantial improvements to the maximum butanol titer. In contrast to previously published work (Atsumi et al., 2008a), the replacement of *thil* with *atoB* from *E. coli* in strain EB3.0, led to only a modest improvement in titer, to 220 mg/L butanol. The replacement of *C. acetobutylicum adhE1* in strain EB3.0 with *adhE2* led to only 230 mg/L butanol (strain EB4.0; Figure 2). Although it was reported that *adhE2* was the most highly active of these homologs in alcohologenic cultures of *C. acetobutylicum* (Fontaine et al., 2002), little difference between these two homologs was observed in our system.

Expression of NADH-regenerating and glycolytic flux-enhancing enzymes

In the butanol biosynthetic pathway, four moles of NADH are consumed (by oxidation to NAD+) per each mole of butanol produced from acetyl-CoA (Figure 1). In an effort to improve butanol synthesis by strain EB4.0, we explored the effects of introducing formate dehydrogenase (*fdh1*) from *Saccharomyces cerevisiae*. Yeast formate dehydrogenase catalyzes the conversion of formate to CO_2 while producing one molecule of NADH (Figure 3). While formate dehydrogenase also exists in *E. coli*, the bacterial enzyme catabolizes formate to CO_2 and H_2 without generation of NAD(P)H. For this reason, yeast formate dehydrogenase has been exploited in a variety biocatalytic applications requiring ample NADH molecules for product formation (Berrios-Rivera et al., 2002; Harris et al., 2000; Kaup et al., 2004; Sanchez et al., 2005; Tishkov and Popov,

2004). Expression of *fdh1* in strain EB4.0 resulted in the generation of strain EB4.F. As shown in Figure 4, the optimum biobutanol synthesis from strain EB4.F was achieved after 48 h, similar to that of strain EB4.0. Expression of the yeast *fdh1* also resulted in ~74% butanol production improvement over EB4.0, reaching as high as 400 mg/L. Supplementation of the media with 1 g/L formate, increased maximum butanol titers up to 520 mg/L with EB4.F, whereas no difference was observed with EB4.0 (Figure 2). In *E. coli*, glyceraldehyde 3-phosphate dehydrogenase mediates the conversion of glyceraldehyde 3-phosphate to 1,3-diphosphateglycerate in the glycolytic pathway (Figure 3). Thus, overexpression of glyceraldehyde 3-phosphate dehydrogenase (*gapA*) should promote higher rates of substrate flux through the glycolytic pathway. Expression of *gapA* in strain EB4.0 resulted in the generation of strain EB4.G. Again, the maximum biobutanol titer from strain EB4.G was achieved after 48 h, and reached a butanol titer of 580 mg/L, demonstrating that *gapA* overexpression resulted in ~150% greater butanol production (relative to EB4.0). The effects of simultaneously expressing both *gapA* and *fdh1* was tested in strain EB4.GF. Interestingly, however, butanol synthesis from strain EB4.GF was only up to 320 mg/L, which was lower than either EB4.G or EB4.F (Figures 2 and 4). It is possible that simultaneous overexpression of *gapA* and *fdh1* along with the Clostridial enzymes negatively impacted host fitness.

Characterization of EB4.0 fermentation byproducts revealed that after 48 h, succinate, lactate, and ethanol constituted the majority of the end-products, at concentrations of 1400 mg/L, 1700 mg/L, and 1600 mg/L, respectively (Figure 5). Overexpression of *gapA* in EB4.G not only resulted in butanol production increase, but also increased ethanol production by ~18%, likely as a result of increased availability of

384 the precursor acetyl-CoA. The expression of *fdh1* in strain EB4.F resulted in a ~42%
385 decrease of formate accumulation compared to EB4.0, down to 250 mg/L. In addition to
386 an increase in butanol production, *fdh1* co-expression in strain EB4.F also caused ethanol
387 production to increase by ~12% (Figure 5). In fact, with the exception of lactate, flux
388 through each of the natural NADH-consuming fermentative pathways of *E. coli* (i.e.,
389 ethanol and succinate) was enhanced when *fdh1* was co-expressed. Deletions of *adhE*
390 and/or *ldhA* did not improve carbon flux and/or NADH availability, instead resulting in a
391 decrease in growth rate and butanol production in all strains tested (data not shown).
392
393 **Engineering *P. putida* and *B. subtilis* for Butanol Biosynthesis**
394 The previously observed sensitivity of *E. coli* to elevated butanol concentrations
395 motivated the engineering of alternative butanol production platforms in more tolerant,
396 yet still well-characterized, strains. *P. putida* strains PS1.0 and PS2.0 were obtained by
397 co-transformation with either prBCS and pmTA1 or prBCS and pmTBA1, respectively.
398 These strains were cultured in TB medium supplemented with 0.5% glucose or glycerol,
399 however only aerobic conditions could be studied as *P. putida* is an obligately aerobic
400 organism. As seen in Table 3, PS1.0 and PS2.0 achieved initial butanol titers of 44 ± 6
401 and 50 ± 6 mg/L with glucose, respectively, and 122 ± 12 and 112 ± 14 mg/L with
402 glycerol, respectively. As with *E. coli*, butanol production was highest when using the
403 more reduced substrate, glycerol. In this case butanol production by PS1.0 and PS2.0 was
404 ~260% (3.6-fold) and ~240% (3.4-fold) greater than their respective *E. coli* counterparts
405 (ED1.0 and ED2.0, respectively; Figure 2). Again, the co-expression of *bdhB* was found
406 to yield an insignificant effect on butanol production. The capacity to produce such

butanol titers under aerobic conditions in engineered strains of *P. putida* is of particular phenomenological interest because it questions the longstanding and generally accepted position that the *bcd-etfAB* complex of *C. acetobutylicum* is inactive in the presence of oxygen. For instance, a recent report has shown that the activity of *C. kluyveri* butyryl-CoA dehydrogenase could in fact be demonstrated *in vitro*, but only when the enzymes were prepared under a strict oxygen-free environment, and in the presence of FAD (Li et al., 2008). The presence of an acyl-CoA dehydrogenase (*acd*) in *P. putida* KT2440 with catalytic activity on crotonyl-CoA/butyryl-CoA under aerobic conditions has recently been reported (McMahon et al., 2005). To determine if the heterologous *bcd-etfAB* complex was responsible for providing butyryl-CoA dehydrogenase activity in butanol-producing strains of *P. putida*, or whether this activity was perhaps alternatively derived from an endogenous *acd* or other homologous protein, we constructed a *bcd*- and *etfB*-deficient derivative of the BCS operon which was expressed from pRK415 as prCCS (strains PS1.A, PS2.A). After 72 h of culture, no butanol production was detected in the culture medium with either strain PS1.A or PS2.A (Figure 2). In a complementary experiment, expression of *acd* from *P. putida* instead of *bcd-etfAB* in *E. coli* (strain EB2.A) abolished butanol biosynthesis. These two experiments provide strong evidence that the *bcd-etfAB* complex is in fact functional under aerobic conditions.

Reconstruction of the butanol biosynthetic pathway in *B. subtilis* was achieved by serial transformation with each of plasmids pdBCS, pjT, and ppA2, producing strain BK1.0. In this case, the expression of the polycistronic BCS operon was performed using a single, strong (*hyper-spank*) promoter. Although it had demonstrated no distinct benefits in strain EB4.0, *adhE2* was chosen among aldehyde/alcohol dehydrogenase

homologs based on its previously demonstrated activity in *C. acetobutylicum* (Fontaine et al., 2002). *bdhB*, on the other hand, was excluded from this design because it demonstrated no beneficial effects in either *E. coli* or *P. putida*. BK1.0 was cultured in TB medium supplemented with 0.5% glucose or glycerol, under both aerobic and anaerobic conditions. As seen in Table 3, no butanol production was detected under aerobic conditions. However, after 72 h under anaerobic conditions, BK1.0 produced a maximum of 23 ± 4 and 24 ± 4 mg/L butanol with glucose and glycerol supplementation, respectively. Therefore, despite sharing greater phylogentic similarity with *Clostridium* than both *E. coli* and *P. putida*, expression of Clostridial genes in *B. subtilis* did not improve the apparent activity of this heterologous pathway.

Assessing the butanol tolerance of *E. coli*, *P. putida*, and *B. subtilis*

To assess the production potential of the various butanol-producing strains constructed, we studied the inhibitory effects of butanol on the dynamic and steady-state growth phenotypes of *E. coli*, *P. putida*, and *B. subtilis*. As seen in Figure 6, the addition of at least 0.5% (wt./vol.) butanol to cultures of *E. coli* and *P. putida*, or at least 1.0% (wt./vol.) butanol to *B. subtilis* cultures, resulted in rapid and markedly negative effects on both the growth rate and biomass yield. Addition of 2.0% (wt./vol.) butanol was completely lethal to all cultures tested within about 30 min. The addition of butanol at 0.5% (wt./vol.) or higher to *E. coli* cultures caused a rapid decrease (within 30 minutes) in growth rate and OD_{600} relative to the control culture. Viability, however, was only observed to decrease significantly in the presence of at least 1.0% (wt./vol.) butanol over the same time period. Adaptation to the solvent stresses was observed for *E. coli* cultures

with between 0.5 and 0.75% (wt./vol.) butanol, as shown by increases in viable cell concentration after approximately 100 minutes. *P. putida* and *B. subtilis* also demonstrated similar behaviors, though with respect to different characteristic butanol concentrations. A pseudo-steady state at which there was nearly no net change in OD_{600} or culture viability relative to that at the time of butanol addition occurred after addition of 0.75, 1.0, and 1.25% (wt./vol.) butanol to cultures of *P. putida*, *E. coli*, and *B. subtilis*, respectively. Above these respective concentrations, however, decreases in both measurements were observed.

Discussion

Although the bacterium *E. coli* is a well-characterized microorganism from both a genetic and metabolic perspective and boasts a vast availability of genetic tools for its engineering, the susceptibility of *E. coli* to high butanol concentrations complicates its development as a butanol production strain. Nevertheless, we selected *E. coli* as our first prototype strain for the development of an alternative butanol producer in order to provide a baseline for comparison with our other non-native hosts, as well as with other works recently reported in the literature. Enhanced expression of the butanol pathway genes was achieved via replacement of the polycistronic BCS operon to provide expression using individual promoter-RBS sequences and resulted in nearly a six-fold improvement in product titer. It has recently been shown that the expression levels of several butanol biosynthetic genes were significantly higher in a butanol hyper-producing mutant of *C. beijerinckii* than that of the wild-type strain (Chen and Blaschek, 1999).

475 Recruitment of enzymes with homologous function from alternative genetic sources had
476 limited results, which is consistent with previous reports (Atsumi et al., 2008a).

477 Co-expression of *fdh1* from yeast to provide cofactor regeneration in *E. coli*
478 resulted in further improvement of butanol titers up to 400 mg/L in shake flask cultures.
479 However, it was expected that this strategy could provide only limited improvements as
480 endogenous formate levels were quite low (Figure 5). By provision of exogenous
481 formate, flux through the butanol pathway was increased, presumably as a result of
482 elevated rates of NADH regeneration. Although supplementation of the culture media
483 with formate does not represent a sustainable practice for butanol fermentations, it does
484 suggest that an insufficient supply of intracellular NADH may limit the activity of the
485 heterologous butanol pathway in engineered *E. coli*. Meanwhile, overexpression of *gapA*
486 from *E. coli* to promote greater glycolytic flux and increase the acetyl-CoA pool resulted
487 in final butanol titers of 580 mg/L. This titer is comparable to the maximum reported by
488 Atsumi et al. (2008) and did not require the deletion of endogenous genes. As shown in
489 Figure 5, the co-expression of both *gapA* and *fdh1* resulted in a notable increase in
490 succinate production, a phenomenon that can be explained two-fold. Firstly, the
491 overexpression of *gapA* increases the flux through phosphoenolpyruvate carboxylase
492 towards the synthesis of oxaloacetate, a precursor of succinate (Causey et al., 2004).
493 Since the overexpression of *gapA* was also likely to result in increased accumulation of
494 acetyl-CoA (as indicated by observed increases in ethanol accumulation; Figure 5), a
495 substrate of malate synthase, this would ultimately lead to increased production of malate
496 and succinate. Secondly, the NADH regenerated via *fdh1* co-expression can also serve as
497 an electron donor for both malate dehydrogenase and fumarate reductase (Causey et al.,

2004), two enzymes involved in succinate biosynthesis. Overall, the elevation of fermentative byproducts reflects an increase in the intracellular NADH/NAD+ ratio (de Graef et al., 1999).

One means of reducing carbon flux towards fermentative byproducts and affecting the co-factor balance could involve deletions of *adhE* and *ldhA*, the primary enzymes responsible for the production of ethanol and lactate, respectively. In this study, deletion of either or both of these genes led to decreases in butanol productivity. These two deletions were previously found to improve butanol titers from glucose in engineered *E. coli* when combined with additional gene deletions (Atsumi et al., 2008a). Similarly, glucose flux to pyruvate was significantly enhanced when *adhE* and *ldhA* deletions were included among several genetic modifications (Causey et al., 2004). Thus, it may be that additional mutations (e.g., *frd* deletion) are required to observe the positive effect associated with *adhE* and/or *ldhA* deletions. 1,2-Propanediol productivity was previously improved in an *ldh* mutant of *E. coli*, but only with glucose as a substrate (Berrios-Rivera et al., 2003). Considering this observation, our use of the more reduced glycerol as a substrate may also have impacted these results. Finally, it should be noted that our experiments were performed with BL21(DE3), a B strain, while each of the previous studies utilized K-12 strains. The reported differences in metabolic activity between these strains (Phue et al., 2005; Phue and Shiloach, 2004) may have also contributed to the observed lack of effect of *adhE* and *ldhA* in this study.

Ultimately, the availability of excess reducing equivalents and/or enhanced metabolite flux could only provide limited improvements towards butanol production due likely to the slow enzyme kinetics of the engineered butanol pathway. Our results

support those of previous studies (Li et al., 2008) in suggesting that the slow turn-over rate of the *Clostridium* butyryl-CoA dehydrogenase complex likely limited the capacity of the engineered butanol pathway. In *C. kluyveri*, activity of the enzyme encoded by the *bcd-etf* complex has recently been found to be rate-limiting in butyraldehyde synthesis. It was postulated that the slow kinetics demonstrated by this enzyme complex may result from complexities associated with the endergonic reduction of ferredoxin with NADH and the exergonic reduction of crotonyl-CoA with NADH (Li et al., 2008). *In vitro* activity of *bcd-etfAB* could not be detected from any of the strains constructed in this study. Previous works have also highlighted the difficulty associated with confirming the *in vitro* activity of *bcd-etfAB* as expressed in recombinant *E. coli* (Boynton et al., 1996) or from *C. acetobutylicum* itself (Hartmanis and Gatenbeck, 1984), often citing the possible sensitivity of this enzyme complex to oxygen. Although product titers were quite low, functional expression of the butanol pathway has previously been achieved in *E. coli* under aerobic conditions, despite the inability to assay *bcd-etfAB* activity *in vitro* (Atsumi et al., 2008a). Compared to that work, butanol titers obtained under aerobic conditions in this study were improved by nearly 15-fold using *P. putida* as the host organism (122 mg/L vs. ~8 mg/L). If *C. acetobutylicum bcd-etfAB* did in fact suffer from decreased activity in the presence of oxygen, then the functional pathway construction in *P. putida* under aerobic conditions could have been aided by the activity of native isozymes catalyzing the same reaction of crotonyl-CoA to butyryl-CoA. However, as we have demonstrated, butanol production was dependent upon the presence of *bcd-etfAB* and expression of *acd* did not complement butanol biosynthesis in our engineered strains of *E. coli*, indicating that background enzymatic activity alone was insufficient for

544 catalyzing this reaction under the culture conditions studied. These results support the
545 notion that it may be the method of analysis, and not the sensitivity of this enzyme to
546 dissolved oxygen, that is in fact responsible for the inability to assay this step of the
547 butanol biosynthetic pathway.

548 The susceptibility of *E. coli* to the toxic effects of butanol prompted our interest in
549 engineering of butanol-producing strains of both *P. putida* and *B. subtilis*. In this case,
550 we found that *B. subtilis* displayed elevated butanol tolerance, when compared with *P.*
551 *putida* and *E. coli*. The polycistronic expression of *C. acetobutylicum* genes in *B. subtilis*
552 resulted in butanol synthesis at similar titers to engineered *E. coli* ED strains. This result
553 represents a step toward the generation of an alternative butanol production platform with
554 improved solvent tolerant characteristics. As with *E. coli*, we anticipate the butanol titers
555 from *P. putida* and *B. subtilis* could now be substantially improved through host-specific
556 strategies. Significant improvements of butanol biosynthesis were achieved in *E. coli* as
557 a result of an improved gene expression strategy that involved individual promoter and
558 RBS sequences associated with each pathway enzyme, as well as through the
559 overexpression of enzymes to increase glycolytic flux or facilitate cofactor regeneration.
560 Furthermore, as has been previously demonstrated in *E. coli*, *in vivo* evolution of
561 heterologous pathway elements can also lead to improved productivities in a non-native
562 host (Meynial Salles et al., 2007). In *P. putida* strains, improved expression could also be
563 achieved through the use of a *Pseudomonas*-derived promoter as opposed to the *E. coli*-
564 derived *lac* promoter employed in this study. Furthermore, the lack of extensive natural
565 product biosynthesis in *Pseudomonas* sp. also reduces the potential for molecular cross-
566 talk, contamination, and competition with native pathways in heterologous production

efforts (Zhang et al., 2008a). The functional expression of the butanol biosynthesis pathway in *P. putida* and *B. subtilis* further illustrates the potential of these under-utilized, yet industrially relevant, strains as production hosts.

In addition to n-butanol, *E. coli* has also recently been engineered for the production of other potential biofuels consisting of higher alcohols such as iso-butanol (Atsumi et al., 2008b), 2-methyl-1-butanol (Cann and Liao, 2008), 3-methyl-1-butanol (Connor and Liao, 2008), as well as n-pentanol, 3-methyl-1-pentanol, and n-hexanol (Zhang et al., 2008b). Despite their favorable thermodynamic properties, it has been thoroughly demonstrated that the cytotoxicity of an alcohol is elevated with an increasing carbon chain length (Heipieper and Debont, 1994; Osborne et al., 1990a; Vermue et al., 1993). Thus, the problems associated with the cytotoxicity of both conventional and second-generation biofuels will remain apparent, and represent an increasing requirement for robust biocatalyst platforms.

The pseudo-steady state behavior observed with respect to butanol inhibition represents a critical condition above which growth and viability became most severely inhibited, and below which cultures remained prosperous. This state was found to be a distinguishing feature of each organism, however the characteristic range of critical butanol concentrations was found to be seemingly narrow (0.75 to 1.25%). This finding is consistent with a previous study which found that a selection of Gram-positive and Gram-negative bacteria, including *Arthrobacter*, *Norcadia*, *Acinetobacter*, and *Pseudomonas* sp., each displayed very similar tolerance to a series of n-alkanol solvents, including butanol (Vermue et al., 1993). More recently, the inhibitory effects of butanol on the growth of 24 different microorganisms, including several species of bacteria and

590 yeast, was investigated (Knoshaug and Zhang, 2008). Although *Pseudomonas* and
591 *Bacillus* were excluded from that study, those findings also confirmed the existence of a
592 narrow range of toxic thresholds (between 1 and 2% (wt./vol.)) for most strains. Two
593 strains of *Lactobacillus*, however, were found to capable of maintaining growth in
594 butanol concentrations as high as 3%. Meanwhile, additional strains of *Lactobacillus* and
595 the phylogenetically related species *Enterococcus* have also been reported as capable of
596 tolerating up to 2.5% (wt./vol.) butanol on solid media (Bramucci et al., 2007; Bramucci
597 et al., 2008). Interspecies similarity of butanol toxic threshold concentrations is likely a
598 result of high homology between the cytoplasmic membrane structures of the studied
599 organisms particularly when it is considered that the inhibitory mechanism involves
600 membrane accumulation leading to structural distortion. Although the specific strains of
601 *P. putida* investigated here showed a relatively low sensitivity threshold to butanol,
602 evolved strains of *P. putida* that can grow in the presence of up to 6% (wt./vol.) butanol
603 have recently been isolated (Ruhl et al., 2009). Since the *P. putida* pathway
604 reconstruction strategy outlined here would be compatible with these novel organisms,
605 these hosts would make excellent candidates as alternative butanol production strains.
606 Solvent tolerance can be further engineered, for example, as it was in *Clostridium*
607 through the overexpression of stress (heat shock) proteins (Tomas et al., 2003). Other
608 widely employed approaches towards enhancing the desired phenotypes of industrial
609 biocatalysts most frequently rely upon mutagenic techniques. However, the identification
610 of enhanced phenotypes obtained via stochastic mutation procedures typically requires
611 laborious screening and selection processes. Such techniques can also elicit an
612 unforeseen impact on host fitness and decrease its overall industrial utility (Bonomo et al.,

2006). Furthermore, since complex phenotypes such as solvent tolerance are not monogenic (Alper et al., 2006), several distinct mutation events would be required to be performed in highly specific combinations. In contrast, broader mechanisms utilizing global transcription machinery engineering (gTME) have been successfully demonstrated for the elevation of ethanol tolerance in yeast (Alper et al., 2006) and *E. coli* (Alper and Stephanopoulos, 2007). The use of naturally solvent tolerant microorganisms as host productions strains does not negate the relevance or applicability of previously developed molecular techniques. Rather, the selection of an appropriate host as a starting point is a critical challenge for the for the engineering of solvent tolerant phenotypes via such combinatorial procedures (Fischer et al., 2008). The outlined approach thus remains compatible with such emerging techniques while providing an elevated baseline of natural solvent tolerance from which next generation butanol producing microorganisms can be engineered.

Conclusion

Although *Clostridia* are the traditional organisms employed in biobutanol production, a significant and growing amount of research is centered on the engineering of more robust strains capable of elevated production. Because systematic approaches to improve butanol production traits of *Clostridium* are currently impeded by a lack of characterization and genetic tools, this work has focused on the generation of a variety of tractable strains which allow for versatile manipulations with the objective of improving butanol fermentation. Functional butanol pathways were successfully constructed in *E. coli*, *P. putida*, and *B. subtilis*. Experimental titers were highest in *E. coli* and benefited

from optimized expression strategies. Although *B. subtilis* displayed the most solvent tolerant phenotype among the studied strains, thus providing it with the greatest production potential, it was found to be the poorest producing strain. Preliminary titers obtained in engineered strains of *P. putida* were superior to those obtained by *E. coli* under aerobic conditions in previous studies, and titers from both *P. putida* and *B. subtilis* were notably better than those recently achieved in yeast (Steen et al., 2008). Although the specific strain studied displayed sensitivity to butanol, with concurrent work on the evolution of more solvent tolerant strains of *P. putida*, pseudomonads may constitute an effective butanol production host in the future. Overall, this work has demonstrated the engineering of butanol biosynthesis in heterologous, solvent-tolerant microorganisms.

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References

Alexeyev, M. F., et al., 1995. Improved Antibiotic-Resistance Gene Cassettes and Omega-Elements for Escherichia-Coli Vector Construction and in-Vitro Deletion Insertion Mutagenesis. Gene. 160**,** 63-67.

660 Alper, H., et al., 2006. Engineering yeast transcription machinery for improved ethanol
661 tolerance and production. Science. 314**,** 1565-1568.
662 Alper, H., Stephanopoulos, G., 2007. Global transcription machinery engineering: A new
663 approach for improving cellular phenotype. Metabolic Engineering. 9**,** 258-267.
664 Antoni, D., et al., 2007. Biofuels from microbes. Appl Microbiol Biotechnol. 77**,** 23-35.
665 Atsumi, S., et al., 2008a. Metabolic engineering of Escherichia coli for 1-butanol
666 production. Metab Eng. 10**,** 305-311.
667 Atsumi, S., et al., 2008b. Non-fermentative pathways for synthesis of branched-chain
668 higher alcohols as biofuels. Nature. 451**,** 86-9.
669 Berrios-Rivera, S. J., et al., 2002. Metabolic engineering of Escherichia coli: Increase of
670 NADH availability by overexpressing an NAD(+)-dependent formate
671 dehydrogenase. Metabolic Engineering. 4**,** 217-229.
672 Berrios-Rivera, S. J., et al., 2003. The effect of carbon sources and lactate dehydrogenase
673 deletion on 1,2-propanediol production in Escherichia coli. J Ind Microbiol
674 Biotechnol. 30**,** 34-40.
675 Bonomo, J., et al., 2006. A comparative study of metabolic engineering anti-metabolite
676 tolerance in Escherichia coli. Metabolic Engineering. 8**,** 227-239.
677 Borden, J. R., Papoutsakis, E. T., 2007. Dynamics of genomic-library enrichment and
678 identification of solvent tolerance genes for *Clostridium acetobutylicum*. Applied
679 and Environmental Microbiology. 73**,** 3061-3068.
680 Bowles, L. K., Ellefson, W. L., 1985. Effects of butanol on *Clostridium acetobutylicum*.
681 Applied and Environmental Microbiology. 50**,** 1165-1170.
682 Boynton, Z. L., et al., 1996. Cloning, sequencing, and expression of clustered genes
683 encoding beta-hydroxybutyryl-coenzyme A (CoA) dehydrogenase, crotonase, and
684 butyryl-CoA dehydrogenase from Clostridium acetobutylicum ATCC 824. J
685 Bacteriol. 178**,** 3015-24.
686 Bramucci, M., et al., Solvent tolerant microorganisms and methods of isolation. Vol. US
687 Patent 20070259411. EI DuPont de Nemours and Co., 2007.
688 Bramucci, M., et al., Solvent tolerant microorganisms and methods of isolation. Vol. US
689 Patent 20080124774. EI DuPont de Nemours and Co., 2008.
690 Britton, R. A., et al., 2002. Genome-wide analysis of the stationary-phase sigma factor
691 (sigma-H) regulon of Bacillus subtilis. J Bacteriol. 184**,** 4881-90.
692 Buday, Z., et al., 1990. Improved acetone-butanol fermentation analysis using
693 Subambient HPLC column temperature. Enzyme and Microbial Technology. 12**,**
694 24-27.
695 Cann, A. F., Liao, J. C., 2008. Production of 2-methyl-1-butanol in engineered
696 Escherichia coli. Appl Microbiol Biotechnol. 81**,** 89-98.
697 Cascone, R., 2008. Biobutanol - A Replacement for Bioethanol? Chem Eng Progr. 104**,**
698 S4-S9.
699 Causey, T. B., et al., 2004. Engineering Escherichia coli for efficient conversion of
700 glucose to pyruvate. Proc Natl Acad Sci U S A. 101**,** 2235-40.
701 Chen, C. K., Blaschek, H. P., 1999. Examination of physiological and molecular factors
702 involved in enhanced solvent production by clostridium beijerinckii BA101. Appl
703 Environ Microbiol. 65**,** 2269-71.
704 Connor, M. R., Liao, J. C., 2008. Engineering of an Escherichia coli strain for the
705 production of 3-methyl-1-butanol. Appl Environ Microbiol. 74**,** 5769-75.

706 Datsenko, K. A., Wanner, B. L., 2000. One-step inactivation of chromosomal genes in
707 Escherichia coli K-12 using PCR products. Proceedings of the National Academy
708 of Sciences of the United States of America. 97**,** 6640-6645.
709 de Bont, J. A. M., 1998. Solvent-tolerant bacteria in biocatalysis. Trends in
710 Biotechnology. 16**,** 493-499.
711 de Carvalho, C. C., et al., 2004. Mycobacterium sp., Rhodococcus erythropolis, and
712 Pseudomonas putida behavior in the presence of organic solvents. Microsc Res
713 Tech. 64**,** 215-22.
714 de Graef, M. R., et al., 1999. The steady-state internal redox state (NADH/NAD) reflects
715 the external redox state and is correlated with catabolic adaptation in Escherichia
716 coli. Journal of Bacteriology. 181**,** 2351-2357.
717 Errington, J., Mandelstam, J., 1983. Variety of Sporulation Phenotypes Resulting from
718 Mutations in a Single Regulatory Locus, Spoiia, in Bacillus-Subtilis. Journal of
719 General Microbiology. 129**,** 2091-2101.
720 Ezeji, T. C., et al., 2007. Bioproduction of butanol from biomass: from genes to
721 bioreactors. Curr Opin Biotechnol. 18**,** 220-7.
722 Fischer, C. R., et al., 2008. Selection and optimization of microbial hosts for biofuels
723 production. Metabolic Engineering. 10**,** 295-304.
724 Fontaine, L., et al., 2002. Molecular characterization and transcriptional analysis of
725 adhE2, the gene encoding the NADH-dependent aldehyde/alcohol dehydrogenase
726 responsible for butanol production in alcohologenic cultures of Clostridium
727 acetobutylicum ATCC 824. J Bacteriol. 184**,** 821-30.
728 Guerout-Fleury, A. M., et al., 1996. Plasmids for ectopic integration in Bacillus subtilis.
729 Gene. 180**,** 57-61.
730 Harris, L. M., et al., 2000. Characterization of recombinant strains of the *Clostridium*
731 *acetobutylicum* butyrate kinase inactivation mutant: need for new
732 phenomenological models for solventogenesis and butanol inhibition? Biotechnol
733 Bioeng. 67**,** 1-11.
734 Hartmanis, M. G., Gatenbeck, S., 1984. Intermediary Metabolism in Clostridium
735 acetobutylicum: Levels of Enzymes Involved in the Formation of Acetate and
736 Butyrate. Appl Environ Microbiol. 47**,** 1277-1283.
737 Heipieper, H. J., Debont, J. A. M., 1994. Adaptation of Pseudomonas-Putida S12 to
738 Ethanol and Toluene at the Level of Fatty-Acid Composition of Membranes.
739 Applied and Environmental Microbiology. 60**,** 4440-4444.
740 Hwang, E. I., et al., 2003. Production of plant-specific flavanones by Escherichia coli
741 containing an artificial gene cluster. Appl Environ Microbiol. 69**,** 2699-706.
742 Inui, M., et al., 2008. Expression of Clostridium acetobutylicum butanol synthetic genes
743 in Escherichia coli. Appl Microbiol Biotechnol. 77**,** 1305-16.
744 Isken, S., de Bont, J. A. M., 1998. Bacteria tolerant to organic solvents. Extremophiles. 2**,**
745 229-238.
746 Jones, D. T., Woods, D. R., 1986. Acetone-butanol fermentation revisited.
747 Microbiological Reviews. 50**,** 484-524.
748 Kang, H. J., et al., 2007. Functional characterization of Hsp33 protein from Bacillus
749 psychrosaccharolyticus; additional function of HSP33 on resistance to solvent
750 stress. Biochem Biophys Res Commun. 358**,** 743-50.

751 Kaup, B., et al., 2004. Metabolic engineering of Escherichia coli: construction of an
752 efficient biocatalyst for D-mannitol formation in a whole-cell biotransformation.
753 Appl Microbiol Biotechnol. 64**,** 333-9.
754 Keen, N. T., et al., 1988a. Improved Broad-Host-Range Plasmids for DNA Cloning in
755 Gram-Negative Bacteria. Gene. 70**,** 191-197.
756 Keen, N. T., et al., 1988b. Improved broad-host-range plasmids for DNA cloning in
757 gram-negative bacteria. Gene. 70**,** 191-7.
758 Knoshaug, E. P., Zhang, M., 2008. Butanol Tolerance in a Selection of Microorganisms.
759 Appl Biochem Biotechnol. DOI: 10.1007/s12010-008-8460-4.
760 Li, F., et al., 2008. Coupled ferredoxin and crotonyl coenzyme A (CoA) reduction with
761 NADH catalyzed by the butyryl-CoA dehydrogenase/Etf complex from
762 Clostridium kluyveri. J Bacteriol. 190**,** 843-50.
763 McMahon, B., et al., 2005. The protein coded by the PP2216 gene of Pseudomonas
764 putida KT2440 is an acyl-CoA dehydrogenase that oxidises only short-chain
765 aliphatic substrates. Fems Microbiology Letters. 250**,** 121-127.
766 Mermelstein, L. D., et al., 1994. Genetic and metabolic engineering of Clostridium
767 acetobutylicum ATCC 824. Ann N Y Acad Sci. 721**,** 54-68.
768 Meynial Salles, I., et al., 2007. Evolution of a Saccharomyces cerevisiae metabolic
769 pathway in Escherichia coli. Metab Eng. 9**,** 152-9.
770 Middleton, R., Hofmeister, A., 2004. New shuttle vectors for ectopic insertion of genes
771 into Bacillus subtilis. Plasmid. 51**,** 238-45.
772 Morales, V. M., et al., 1991. A Series of Wide-Host-Range Low-Copy-Number Vectors
773 That Allow Direct Screening for Recombinants. Gene. 97**,** 39-47.
774 Nijkamp, K., et al., 2005. The solvent-tolerant Pseudomonas putida S12 as host for the
775 production of cinnamic acid from glucose. Applied Microbiology and
776 Biotechnology. 69**,** 170-177.
777 Osborne, S. J., et al., 1990a. Correlation of biocatalytic activity in an organic-aqueous
778 two-liquid phase system with solvent concentration in the cell membrane.
779 Enzyme Microb Technol. 12**,** 281-91.
780 Osborne, S. J., et al., 1990b. Correlation of biocatalytic activity in an organic aqueous 2-
781 liquid phase system with solvent concentration in the cell-membrane. Enzyme and
782 Microbial Technology. 12**,** 281-291.
783 Phue, J. N., et al., 2005. Glucose metabolism at high density growth of E. coli B and E.
784 coli K: differences in metabolic pathways are responsible for efficient glucose
785 utilization in E. coli B as determined by microarrays and Northern blot analyses.
786 Biotechnol Bioeng. 90**,** 805-20.
787 Phue, J. N., Shiloach, J., 2004. Transcription levels of key metabolic genes are the cause
788 for different glucose utilization pathways in E. coli B (BL21) and E. coli K
789 (JM109). J Biotechnol. 109**,** 21-30.
790 Ruhl, J., et al., 2009. Selected Pseudomonas putida strains can grow in the presence of
791 high butanol concentrations. Appl Environ Microbiol. DOI:10.1128/AEM.00225-
792 09.
793 Sanchez, A. M., et al., 2005. Effect of different levels of NADH availability on metabolic
794 fluxes of Escherichia coli chemostat cultures in defined medium. J Biotechnol.
795 117**,** 395-405.

Sardessai, Y., Bhosle, S., 2002. Organic solvent-tolerant bacteria in mangrove ecosystem. Current Science. 82**,** 622-623.

Schmid, A., et al., 2001. Industrial biocatalysis today and tomorrow. Nature. 409**,** 258-68.

Shen, C. R., Liao, J. C., 2008. Metabolic engineering of Escherichia coli for 1-butanol and 1-propanol production via the keto-acid pathways. Metabolic Engineering. 10**,** 312-320.

Sillers, R., et al., 2008. Metabolic engineering of the non-sporulating, non-solventogenic Clostridium acetobutylicum strain M5 to produce butanol without acetone demonstrate the robustness of the acid-formation pathways and the importance of the electron balance. Metab Eng. 10**,** 321-32.

Steen, E. J., et al., 2008. Metabolic engineering of Saccharomyces cerevisiae for the production of n-butanol. Microbial Cell Factories. 7**,** -.

Tishkov, V. I., Popov, V. O., 2004. Catalytic mechanism and application of formate dehydrogenase. Biochemistry (Mosc). 69**,** 1252-67.

Tomas, C. A., et al., 2003. Overexpression of groESL in Clostridium acetobutylicum results in increased solvent production and tolerance, prolonged metabolism, and changes in the cell's transcriptional program. Appl Environ Microbiol. 69**,** 4951-65.

Vermue, M., et al., 1993. Toxicity of homologous series of organic-solvents for the Gram-positive bacteria *Arthrobacter* and *Nocardia* Sp and the Gram-negative bacteria *Acinetobacter* and *Pseudomonas* Sp. Biotechnology and Bioengineering. 42**,** 747-758.

Wallace, K. K., et al., 1995. Purification of crotonyl-CoA reductase from Streptomyces collinus and cloning, sequencing and expression of the corresponding gene in Escherichia coli. Eur J Biochem. 233**,** 954-62.

Welch, R. W., et al., 1989. Purification and characterization of the NADH-dependent butanol dehydrogenase from Clostridium acetobutylicum (ATCC 824). Arch Biochem Biophys. 273**,** 309-18.

Wierckx, N. J. P., et al., 2005. Engineering of solvent-tolerant Pseudomonas putida S12 for bioproduction of phenol from glucose. Applied and Environmental Microbiology. 71**,** 8221-8227.

Zhang, H., et al., 2008a. Bacterial hosts for natural product production. Mol Pharm. 5**,** 212-25.

Zhang, K., et al., 2008b. Expanding metabolism for biosynthesis of nonnatural alcohols. Proc Natl Acad Sci U S A. 105**,** 20653-8.

LIST OF TABLES

Table 1. Plasmids used or constructed in this study.

Plasmid	Description	Source
pRK415	Tet^r, *lac*	(Keen et al., 1988b)
pMMB206	Cm^r, *lacI, taclac*	(Morales et al., 1991)
pMMB206G	Gm^r, *lacI, taclac*	This study
pETDuet-1	Ap^r, *lacI,* T7*lac*	Novagen
pCDFDuet-1	Sm^r, *lacI,* T7*lac*	Novagen
pCOLADuet-1	Km^r, *lacI,* T7*lac*	Novagen
pACYCDuet-1	Cm^r, *lacI,* T7*lac*	Novagen
pDG1664	Ap^r (*E. coli*), Em^r (*B. subtilis*), *thrC* locus	(Guerout-Fleury et al., 1996)
pPyr-Kan	Ap^r Km^r (*E. coli*), Km^r (*B. subtilis*), *pyrD* locus	(Middleton and Hofmeister, 2004)
pDR111	Ap^r (*E. coli*), Sp^r (*B. subtilis*), *lac I, hyper-spank, amyE* locus	(Britton et al., 2002)
pJBN1	Ap^r (*E. coli*), Em^r (*B. subtilis*), *lac I, hyper-spank, thrC* locus	This study
pDRPyr-Kan	Ap^r Km^r (*E. coli*), Km^r (*B. subtilis*), *lac I, hyper-spank, pyrD* locus	This study
prBCS	Tet^r, *lac:* BCS (*crt, bcd, etfAB, hbd*)	This study
prCCS	Tet^r, *lac:* BCS (*crt, etfA, hbd*)	This study
pmTA1	Gm^r, *lacI, taclac: thil, adhE1*	This study
pmTBA1	Gm^r, *lacI, taclac: thil, bdhB, adhE1*	This study
peT	Ap^r, *lacI,* T7*lac: thil*	This study
peA	Ap^r, *lacI,* T7*lac: atoB*	This study
pcHC	Sm^r, *lacI,* T7*lac: hbd,* T7*lac: crt*	This study
pkBE	Km^r, *lacI,* T7*lac: bcd,* T7*lac: etfAB*	This study
pkA	Km^r, *lacI,* T7*lac: acd*	This study
pkC	Km^r, *lacI,* T7*lac: Synccr*	This study
paA1	Cm^r, *lacI,* T7*lac: adhE1*	This study
paA2	Cm^r, *lacI,* T7*lac: adhE2*	This study
peAF	Ap^r, *lacI,* T7*lac: atoB,* T7*lac: fdh1*	This study
peAG	Ap^r, *lacI,* T7*lac: atoB,* T7*lac: gapA*	This study
peAGF	Ap^r, *lacI,* T7*lac: atoB,* T7*lac: gapA,* T7*lac: fdh1*	This study
pdBCS	Ap^r (*E. coli*), Sp^r (*B. subtilis*), *lac I, hyper-spank:* BCS	This study
pjT	Ap^r (*E. coli*), Em^r (*B. subtilis*), *lac I, hyper-spank: thil*	This study
ppA2	Ap^r Km^r (*E. coli*), Km^r (*B. subtilis*), *lac I, hyper-spank: adhE2*	This study

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Table 2. Strains of *E. coli*, *P. putida*, and *B. subtilis* engineered for this study.

Strain	Genotype or plasmid inserted	Source
E. coli strains		
DH10B	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lac*ZΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara leu*)7697 *galU galK rpsL endA1 nupG*	Invitrogen
ED1.0	prBCS, pmTA1	This study
ED2.0	prBCS, pmTBA1	This study
BL21Star(DE3)	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)	Invitrogen
EB1.0	peT, pcHC, pkBE, paA1	This study
EB2.A	peT, pcHC, pkA, paA1	This study
EB2.C	peT, pcHC, pkC, paA1	This study
EB3.0	peA, pcHC, pkBE, paA1	This study
EB4.0	peA, pcHC, pkBE, paA2	This study
EB4.F	peAF, pcHC, pkBE, paA2	This study
EB4.G	peAG, pcHC, pkBE, paA2	This study
EB4.GF	peAGF, pcHC, pkBE, paA2	This study
P. putida strains		
S12	Wild type	ATCC
PS1.0	prBCS, pmTA1	This study
PS1.A	prCCS, pmTA1	This study
PS2.0	prBCS, pmTBA1	This study
PS2.A	prCCS, pmTBA1	This study
B. subtilis strains		
KS438	*spoIIAI SPβ^o*	(Errington and Mandelstam, 1983)
BK1.0	Δ*amyE::*pdBCS, Δ*thrC ::*pjT, Δ*pyrD::*ppA2	This study

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Table 3. Comparing butanol production (mg/L) by engineered strains of *P. putida* S12 (PS) and *B. subtilis* KS438 (BK) in different media, and under different growth conditions.

	Aerobic, TB media with 0.5% (wt./vol.)		Anaerobic, TB media with 0.5% (wt./vol.)	
P. putida strains	Glucose	Glycerol	Glucose	Glycerol
PS1.0	44 ± 6	122 ± 12	n.a. [1]	n.a. [1]
PS2.0	50 ± 6	112 ± 14	n.a. [1]	n.a. [1]
B. subtilis strains				
BK1.0	n.d.	n.d.	23 ± 4	24 ± 4

n.a., not applicable
n.d., not detected
[1] note that *P. putida* is obligately aerobic

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FIGURE CAPTIONS

Figure 1. The Acetone-Butanol-Ethanol (ABE) fermentation pathway of *C. acetobutylicum.* Enzymatic steps used to reconstruct the biobutanol pathway are shown in bold. Relevant *C. acetobutylicum* genes are also indicated, while those genes encoding enzymes of homologous function from alternative genetic sources are shown in parentheses.

Figure 2. A comparison of the maximum butanol titers for all strains constructed in this study, including those which utilize polycistronic gene expression (A) or individual gene expression (B). All strains cultured in TB media with 0.5% (wt./vol.) glycerol with (diagonal) or without (hashed and gray) supplementation with 1 g/L formate. Error bars shown at one standard deviation.

Figure 3. Engineering the central metabolic pathway of *E. coli* to increase glycolytic flux and promote NADH regeneration in support of butanol synthesis.

Figure 4. Effects of co-expression of *gapA* and/or *fdh1* on butanol production by strains EB4.0 (control, solid squares), EB4.G (*gap*A$^+$, open circles), EB4.F (*fdh*1$^+$, solid circles), and EB4.GF (*gap*A$^+$ *fdh*1$^+$, half-filled circles) as a function of time. Error bars shown at one standard deviation.

Figure 5. Metabolite byproduct formation by strains EB4.0 (black), EB4.G (dark gray), EB4.F (light gray), and EB4.GF (white). Error bars shown at one standard deviation.

Figure 6. Effect of butanol addition on growing cultures of *E. coli* BL21 (DE3), *P. putida* S12, and *B. subtilis* KS438 as determined by viable cell concentration and optical density. Butanol was added to mid-exponential stage cultures at final aqueous concentration (% wt./vol.) of: 0 (solid diamonds), 0.25 (open squares), 0.5 (solid triangles), 0.75 (open circles), 1.0 (solid circles), 1.25 (open triangles), 1.5 (solid squares), and 2.0 (open diamonds). Note that series data were excluded when zero viable cells were obtained, as was observed after 1.5 and/or 2% (wt./vol.) butanol addition. Error bars shown at one standard deviation.

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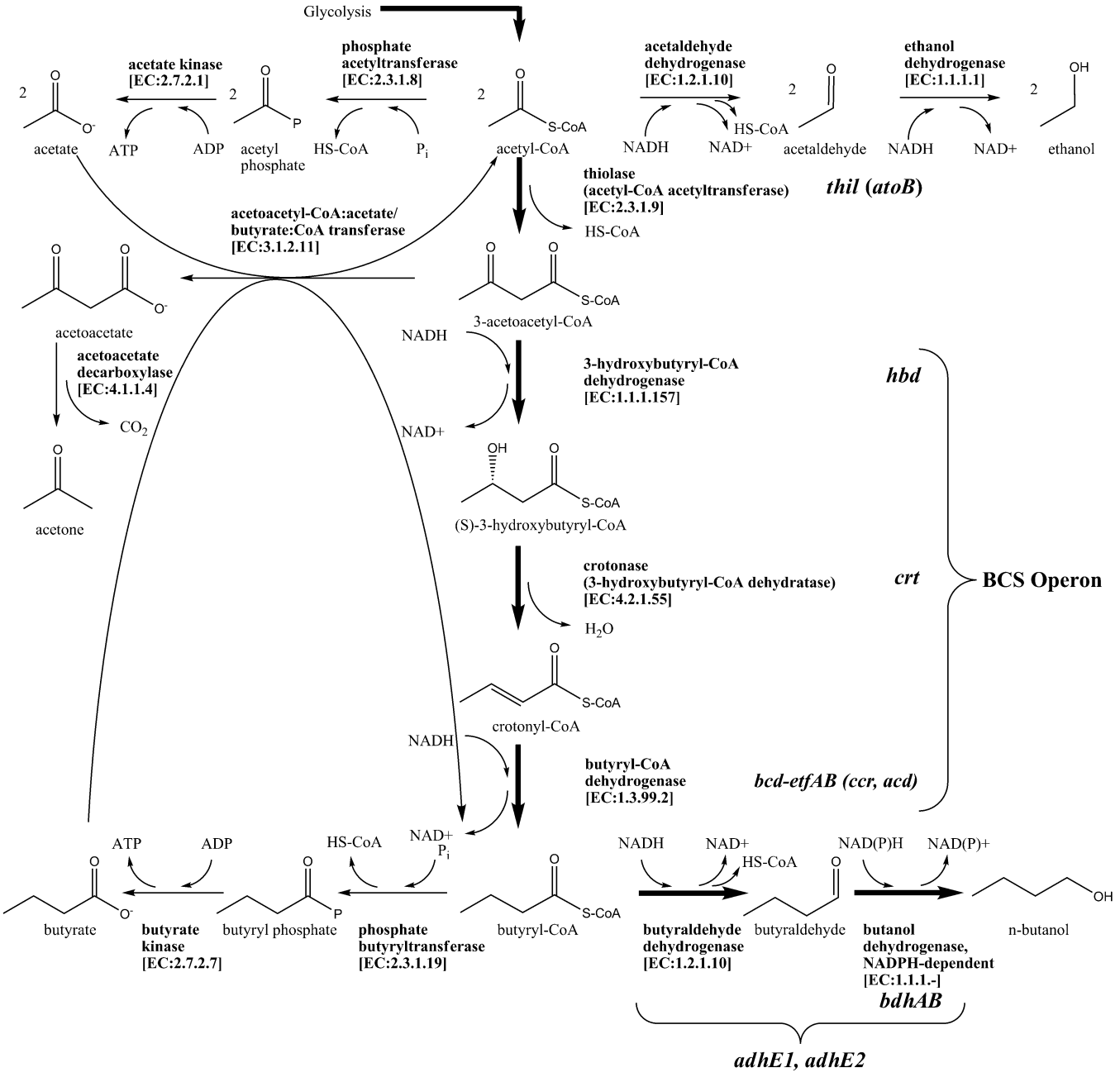

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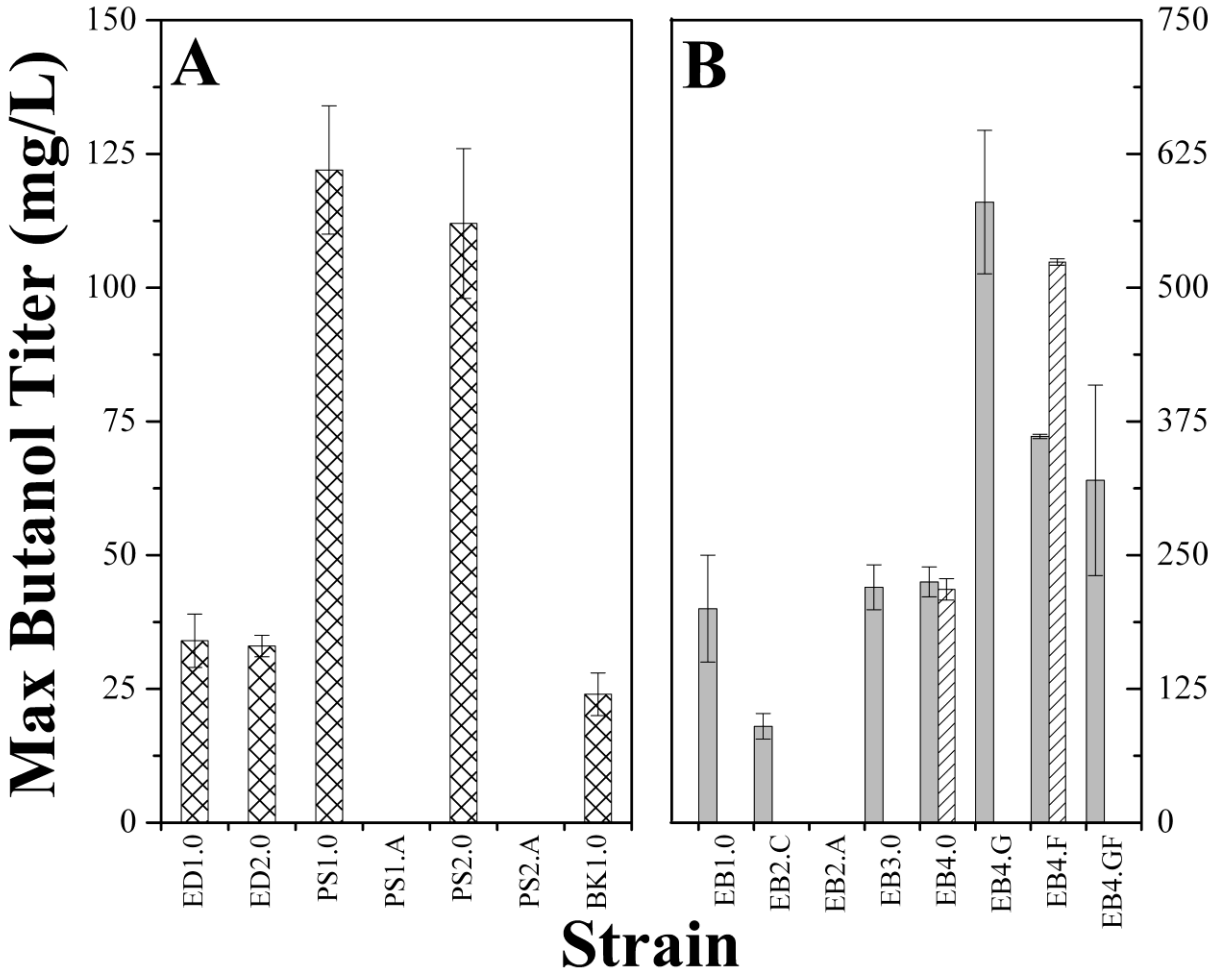

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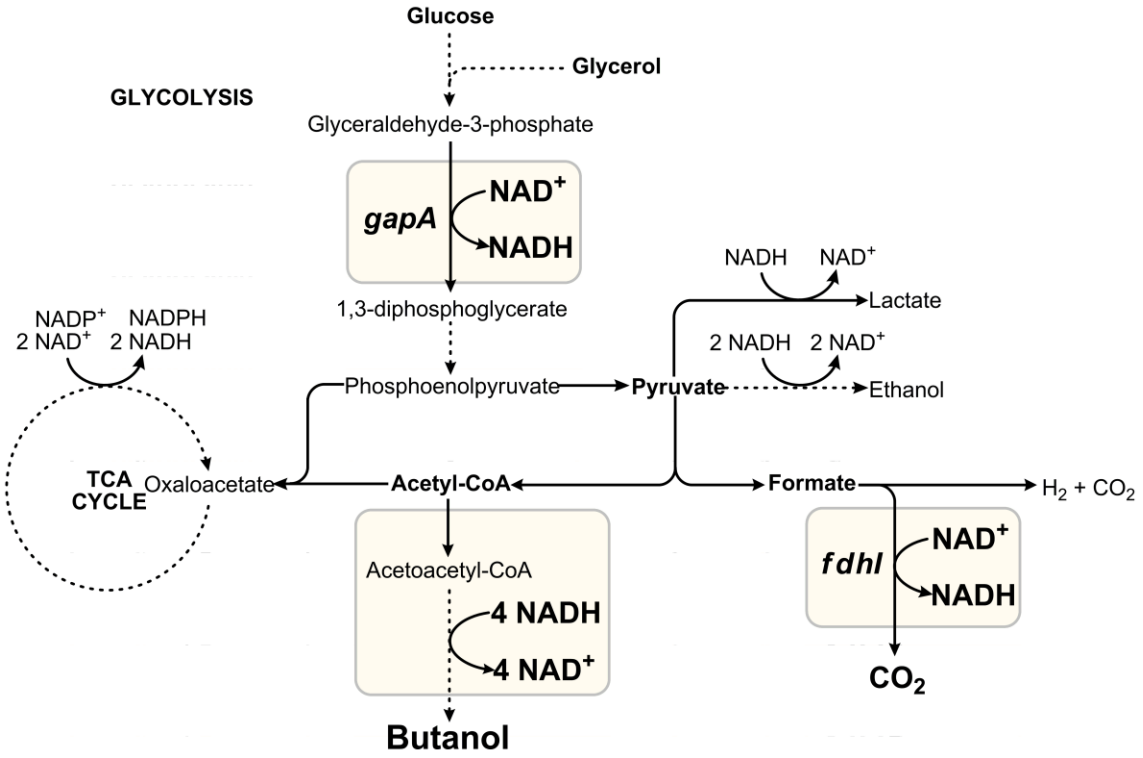

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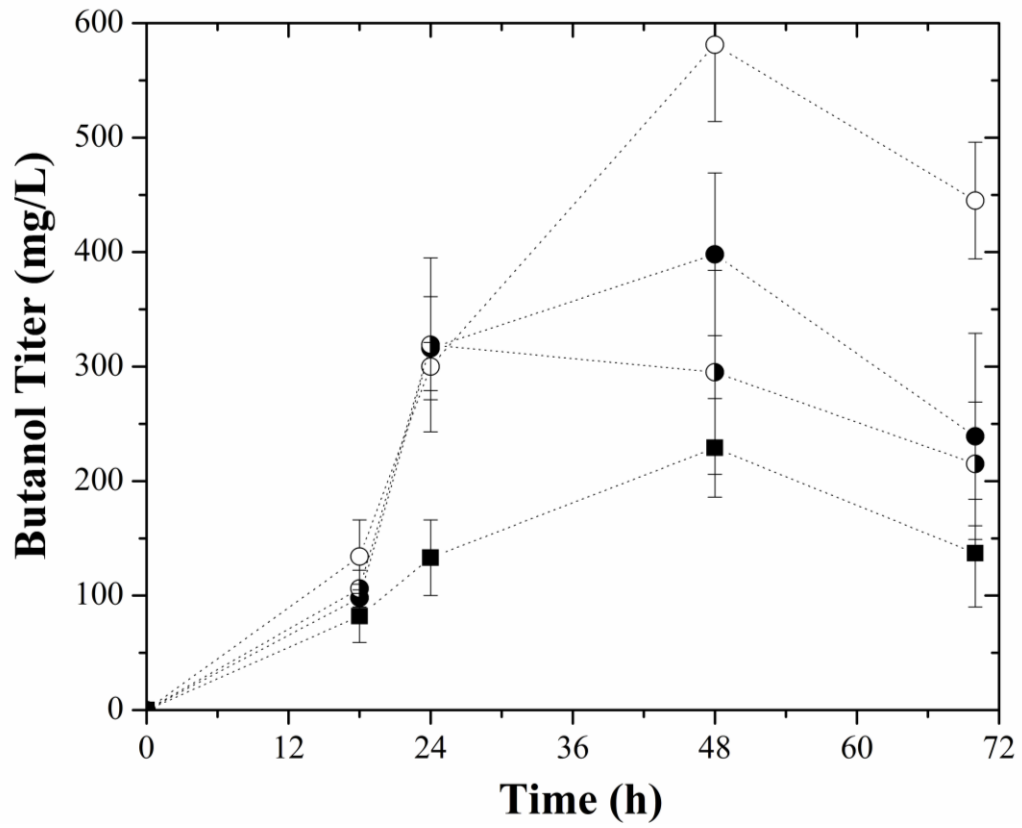

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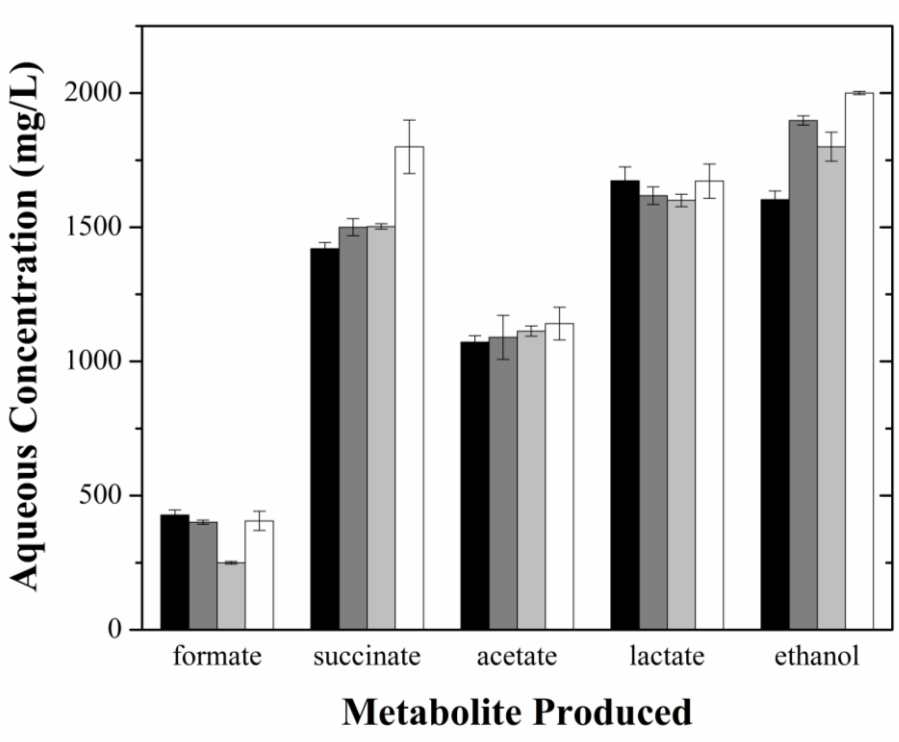

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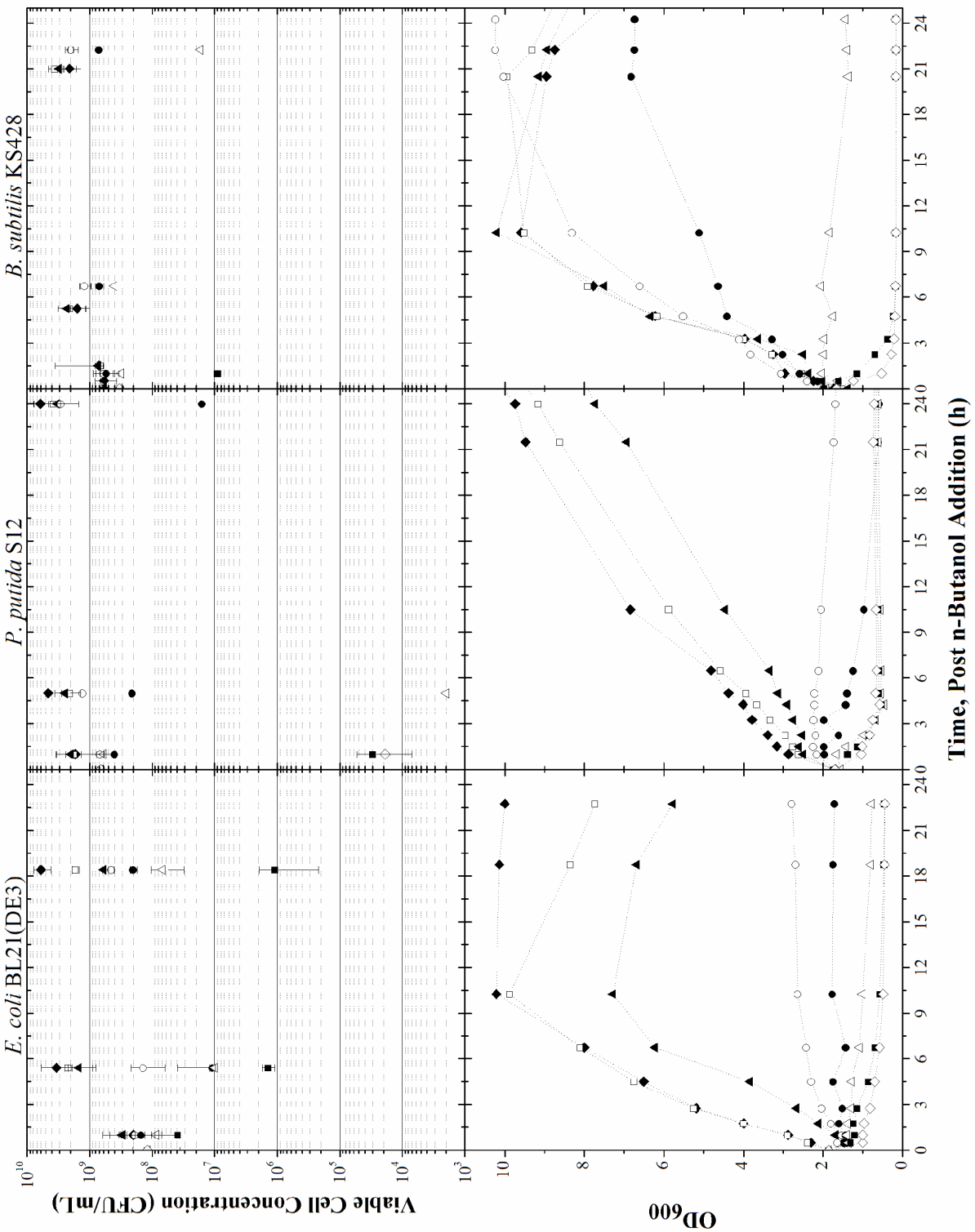

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