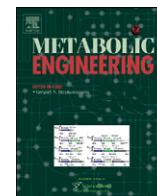




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Engineering the robustness of *Clostridium acetobutylicum* by introducing glutathione biosynthetic capability

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ABSTRACT

To improve the aero- and solvent tolerance of the solvent-producing *Clostridium acetobutylicum*, glutathione biosynthetic capability was introduced into *C. acetobutylicum* DSM1731 by cloning and over-expressing the *gshAB* genes from *Escherichia coli*. Strain DSM1731(pITAB) produces glutathione, and shows a significantly improved survival upon aeration and butanol challenge, as compared with the control. In addition, strain DSM1731(pITAB) exhibited an improved butanol tolerance and an increased butanol production capability, as compared with the recombinant strains with only *gshA* or *gshB* gene. These results illustrated that introducing glutathione biosynthetic pathway, which is redundant for the metabolism of *C. acetobutylicum*, can increase the robustness of the host to achieve a better solvent production.

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1. Introduction

Global environmental problems and fuel crises have been promoting public attentions on the development of biotechnological process and alternative sources for the production of fuels and chemicals. Currently, a wide variety of chemicals, materials, and biofuels can be produced by microorganisms from renewable biomass. This includes ethanol, higher alcohols (Atsumi et al., 2008b; Zeng and Biebl, 2002; Zhang et al., 2006), biodiesel (Kalscheuer et al., 2006), and alkanes (Schirmer et al., 2010). However, these metabolites are generally toxic to microbial cells, leading to a low final fermentation titer (Nicolaou et al., 2010; Papoutsakis, 2008). For example, wild type solventogenic *Clostridium acetobutylicum* can rarely produce *n*-butanol higher than 13 g/L due to butanol toxicity (Jones and Woods, 1986), while the butanol produced by genetically engineered or chemically mutated strains of *Clostridium* rarely exceeded 20 g/L (Ezeji et al., 2010; Papoutsakis, 2008; Sillers et al., 2008). *n*-Butanol can also be produced by engineered non-solventogenic bacteria, for instance by engineered *Escherichia coli* (Atsumi et al., 2008a), *Pseudomonas putida*, and *Bacillus*

subtilis (Nielsen et al., 2009), but the final titer of butanol has not exceeded 1 g/L. Moreover, several chemical stressants always co-exist during fermentation, resulting in overlapping stresses to cells (Nicolaou et al., 2010). Therefore, the robustness of *C. acetobutylicum* against multiple stresses needs to be improved.

Robustness refers to the ability to maintain phenotypic stability when facing diverse perturbations (Kitano, 2004). Microbial robustness coping with high osmolarity, salinity, and temperature can be improved through overexpression of heat shock proteins, leading to significantly improved cryo-, thermo-, and solvent tolerance in *Lactobacillus* (Desmond et al., 2004; Fiocco et al., 2007). Our previous studies have shown that introducing glutathione (GSH) into *Lactococcus lactis* can improve the cellular resistance to oxidative, acid, and osmotic stresses (Li et al., 2003; Zhang et al., 2007; Zhang et al., 2010). Since GSH is also involved in protein stabilization, antioxidation, and detoxification (Lu, 2009), we therefore wonder if introducing GSH biosynthetic capability into *C. acetobutylicum* can improve its aero-tolerance and butanol tolerance, thus improving butanol production. To test this hypothesis, we engineered *C. acetobutylicum* for GSH production, and investigated the effect of the produced GSH on cellular physiology.

2. Materials and methods

2.1. Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used are listed in Table 1. *E. coli* strains were grown aerobically in Luria–Bertani (LB)

Abbreviations: γ -GC, γ -glutamyl-cysteine; γ -GCS, γ -glutamylcysteine synthetase; CFE, cell free extracts; CFU, colony forming unit; CGM, clostridium glucose medium; Cys, cysteine; Cys-Gly, cysteinyl-glycine; GS, glutathione synthetase; GSH, glutathione; LB, Luria–Bertani; mBB, monobromobimane; RBS, ribosomal binding site; RCM, reinforced clostridial medium; SDS-PAGE, sulfate polyacrylamide gel electrophoresis

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Table 1
Strains and plasmids used in this study.

Strains and plasmids	Relevant characteristics	Reference or source
<i>Strains</i>		
<i>C. acetobutylicum</i> DSM1731	Wild type, the parent strain in this study, highly similar to <i>C. acetobutylicum</i> ATCC824 according to our genome sequencing results (unpublished)	DSMZ
<i>C. acetobutylicum</i> DSM1731(pIMP1)	The plasmid control strain of DSM1731, harboring pIMP1	This study
<i>C. acetobutylicum</i> DSM1731(pITA)	The <i>gshA</i> expressing recombinant, harboring pITA	This study
<i>C. acetobutylicum</i> DSM1731(pITB)	The <i>gshB</i> expressing recombinant, harboring pITB	This study
<i>C. acetobutylicum</i> DSM1731(pITAB)	The <i>gshA</i> and <i>gshB</i> expressing recombinant, harboring pITAB, producing GSH	This study
<i>E. coli</i> JM109	<i>recA1 mcrB^r hsdR17</i>	Lab storage
<i>E. coli</i> TOP10(pNA1)	<i>mcrA Δ(mrr-hsdRMS-mcrBC) recA1</i> , applied for plasmid methylation before transformed into <i>C. acetobutylicum</i>	Invitrogen, (Mermelstein et al., 1992)
<i>Plasmids</i>		
pAN1	Φ3TI, <i>p15a ori</i> , Cm ^r	Mermelstein et al. (1992)
pIMP1	The control plasmid without any promoters, MLS ^r Amp ^r , shuttle vector of <i>E. coli-C. acetobutylicum</i>	Mermelstein et al. (1992)
pITF	MLS ^r Amp ^r , pIMP1 derivative for <i>fdh</i> expression under control of PthI	Dong et al. (2010)
pITA	pITF harboring <i>gshA</i> gene under PthI promoter, replaced the original <i>fdh</i> gene, MLS ^r , Amp ^r	This study
pITB	pITF harboring <i>gshB</i> gene under PthI promoter, replaced the original <i>fdh</i> gene, MLS ^r , Amp ^r	This study
pITAB	pITF harboring <i>gshA</i> and <i>gshB</i> gene under PthI promoter, replaced the original <i>fdh</i> gene, MLS ^r , Amp ^r	This study

Abbreviations: Amp^r, ampicillin resistance; Cm^r, chloramphenicol resistance; MLS^r, macrolide, lincosamide, and streptogramin B resistance; Φ3TI, Φ3TI methyltransferase gene of *Bacillus subtilis* phage Φ3TI. PthI, the promoter of thiolase gene in *C. acetobutylicum*; DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany.

medium supplemented with, when necessary, ampicillin (100 μg/mL) or chloramphenicol (35 μg/mL). *C. acetobutylicum* strains were grown in an anaerobic chamber at 37 °C. Liquid cultures were grown in reinforced clostridial medium (RCM) for routine growth, and mRCM medium (RCM containing 20 g/L glucose as the sole carbohydrate) for preparing competent cells (Dong et al., 2010). *C. acetobutylicum* strains harboring different plasmids were grown in RCM supplemented with erythromycin (25 μg/mL). *E. coli* strains were stored at –80 °C in LB medium with 10% glycerol. *C. acetobutylicum* strains were frozen at –80 °C in RCM with 15% glycerol.

2.2. DNA isolation, manipulation, and transformation

Plasmids in *E. coli* were isolated using E.Z.N.A Plasmid Extraction Kit, while genomic DNA of *C. acetobutylicum* and *E. coli* were isolated using E.Z.N.A Bacterial DNA Isolation Kit (Omega Biotek Inc., Guangzhou, China). DNA restriction and cloning were performed according to standard procedures (Sambrook and Russell, 2001). Electrotransformation of *C. acetobutylicum* was carried out as previously described (Dong et al., 2010).

2.3. Plasmids construction

Plasmid pITF that was derived from pIMP1 with PthI promoter and *fdh* gene (Dong et al., 2010) was used as a parent vector for cloning. *E. coli* JM109 was used for vector construction. The *gshA* gene of *E. coli* (encoding γ-glutamylcysteine synthetase, γ-GCS) was amplified with the primers gA-F: 5'-TTCAGAGGATCCA-TCCCGGACGTATCACAGGCGC-3' and gA-R: 5'-CTGCTGGCGCCT-CAGGCGTGTITTTCCAGCCACAC (the bases underlined are the recognition site of restriction enzymes). The resulted 1580-bp PCR product was cloned into the *Bam*HI and *Bbe*I sites of plasmid pITF to replace the *fdh* gene, yielding *gshA* expression plasmid pITA (Fig. 1). The *gshB* gene (encoding glutathione synthetase, GS) was amplified from genomic DNA of *E. coli* with the primers gB-F: 5'-TTCAGAGGATCCATGATCAAGCTCGGCATCGTG-3' and gB-R: 5'-AAGCGGAATTCCTACTGCTGCTGTAACGTGCTTC-3'. The 974-bp PCR product was cloned into the *Bam*HI and *Eco*RI sites of plasmid pITF to replace the *fdh* gene, yielding *gshB* expression plasmid pITB (Fig. 1). The *gshAB* operon was created by fusion PCR using purified PCR products of *gshA* and *gshB* as templates. The primers used were gA-F, gAB-L: GCGGTGTGGCTGGAAAAACA-CGCTAATTTAAGGAGGTTAAGAGGATGATCAAGCTCGGCATCGT-GATGG, and gB-R. The bases underlined in primer gAB-L are the ribosomal binding site (RBS) sequence. The 2549-bp PCR product was cloned into the *Bam*HI and *Bbe*I sites of plasmid pITF to replace the *fdh* gene, yielding *gshAB* expression plasmid pITAB (Fig. 1). Plasmids pITA, pITB, or pITAB were constructed in *E. coli* JM109, methylated in *E. coli* TOP10(pAN1), and then transformed into *C. acetobutylicum* DSM1731. Plasmid pIMP1 that does not contain any promoters was used as the empty vector control, and was also transformed into strain DSM1731.

2.4. Preparation of cell free extracts and protein expression analysis

Ten milliliters fresh cultures were harvested by centrifugation (10,000g for 10 min at 4 °C). Cell pellets were washed twice with pre-cooled saline (0.85% NaCl, w/v) and re-suspended in 1 mL of 200 mM phosphate buffer (pH 7.0) containing 2 mM EDTA. The cells were sonicated on ice for 8 min using a Sonifier S-450D (Branson Ultrasonics Corp., Danbury, CT, USA) with the following protocol: 4-s sonication with 6-s interval, set at 50% duty cycle. Cell debris was removed by centrifugation (12,000g for 10 min at 4 °C), resulting in cell free extracts (CFE). Total protein concentrations of CFE were determined using an RC DC protein assay kit (Bio-Rad) with bovine serum albumin as a standard (the standard error was less than 10%). For protein expression analysis, CFE was mixed with an equal amount of 2-fold concentrated loading buffer (10 mmol/L Tris-HCl (pH6.8), 4% (w/v) sodium dodecyl sulfate, 20% (v/v) glycine, 0.2% bromophenol blue, 2% (v/v) 2-mercaptoethanol). After boiling for 10 min, 10 μl of each sample was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The target bands in gel were excised and subjected to in-gel-digestion and MALDI-TOF MS analysis according to our previous report (Mao et al., 2010).

2.5. Thiol assay

The monobromobimane (mBBr) fluorescent labeling and HPLC methods were used to determine the intracellular and extracellular thiol (cysteine (Cys), γ-glutamyl-cysteine (γ-GC), and glutathione) concentrations according to the method described previously (Li et al., 2003). Ten millimolar stock solutions of GSH, γ-GC, cysteinyl-glycine (Cys-Gly), and Cys in 0.01 mol/L HCl were prepared, aliquoted, and stored at –20 °C (to be used within

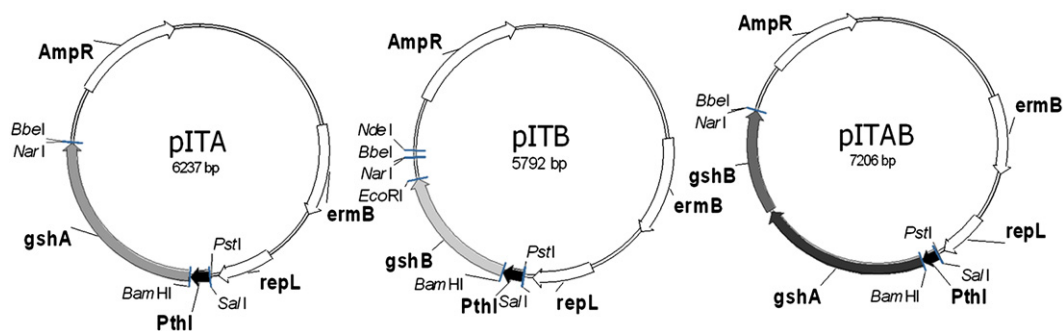


Fig. 1. Structure of plasmids pITA, pITB, and pITAB. Three recombinant plasmids were derived from the expression vector pITF (Dong et al., 2010), which contained PthI promoter and an *fdh* gene. By replacing the *fdh* gene, target gene(s) (*gshA*, *gshB*, or *gshA* and *gshB*) were inserted under PthI promoter, resulting in pITA, pITB, and pITAB, respectively.

one month). Working standard solutions were freshly prepared from each stock solution by dilution in 0.01 mol/L HCl. Fifty micromolar Cys-Gly was added to each sample as an internal standard. Five microliter mBBR-labeled samples were injected into a ZORBAX Eclipse XDB C18 column (4.6 mm × 250 mm) packed with 5 μm reversed-phase material (Agilent Technologies, Beijing, China) and with a C18 guard column (4 mm × 20 mm, Agilent Technologies), using an Agilent Technologies HPLC 1200 Series equipped a fluorescence detector (Agilent Technologies, Beijing, China). The mBBR labeling procedure and HPLC analysis conditions were the same as described previously (Li et al., 2003).

2.6. Butanol challenge experiments

For butanol challenge experiments, RCM containing 19 g/L butanol were prepared and filter-sterilized (0.2 μm). Ten milliliter fresh culture (grown for 20 h until early-stationary-phase) was centrifuged (5000g for 5 min). Cell pellets were re-suspended in 10 mL fresh RCM contained 19 g/L butanol. The serially diluted cultures were plated onto the RCM agar (pH 5.8) and the colony forming unit (CFU) was determined after incubating anaerobically at 37 °C for 48 h. Each experiment was carried out in triplicate.

For butanol challenge growth experiment, RCM containing different concentrations of butanol (0–18 g/L) were prepared and filter-sterilized (0.2 μm). 0.2 mL fresh culture ($OD_{600}=1.0$) of *C. acetobutylicum* DSM1731 or its derivatives were inoculated into 10 mL RCM containing different concentrations of butanol. The cultures were incubated anaerobically at 37 °C for 52 h. Cell concentration was determined by measuring the turbidity at 600 nm using a microtitre plate reader (SPECTRAMax PLUS 384; Molecular Devices, Sunnyvale, Calif.). Each experiment was carried out in triplicate.

2.7. Aeration challenge experiments

To determine if GSH can improve the aero-tolerance of *C. acetobutylicum*, the effect of GSH addition in media on survival of DSM1731 against aeration challenge was detected. Additionally, the aero-tolerance of strain DSM1731 and its derivatives was compared. One milliliter fresh cultures ($OD_{600}=1.0$) of *C. acetobutylicum* DSM1731 and its derivatives harboring different plasmids were harvested by centrifugation (5000g for 5 min) under anaerobic conditions. Cell pellets were washed with pre-cooled saline to remove the residual medium. Cells were centrifuged again and re-suspended in 10 mL fresh RCM (adding different concentrations of GSH or antibiotics, when necessary) in 100 mL flasks. The culture was shaken at 200 rpm, 37 °C. Survival rate of *C. acetobutylicum* cells was calculated by dividing the CFU at a sampling time against the initial cell number. The serially diluted cultures were

plated onto the RCM Agar (pH 5.8); CFU was determined after incubating anaerobically at 37 °C for 48 h. The CFU per mL for each sample were calculated. Each experiment was carried out in triplicate.

2.8. Assay of fermentation phenotype

Metabolic phenotype of *C. acetobutylicum* was assayed in pH-controlled BioFlo 110 bioreactors (New Brunswick Scientific, Edison, N.J.) with 4.0-L initial working volume as previously reported (Mao et al., 2010). Clostridium glucose medium (CGM) (Wiesenborn et al., 1988) was used as fermentation medium and supplemented with 25 μg/mL of erythromycin and 0.15% anti-foam C (Sigma Chemical Co., St. Louis, Mo.). The pH value (≥ 5.0) was automatically controlled by the addition of 6 M ammonium hydroxide. Nitrogen was introduced to maintain the anaerobic environments. The serially diluted cultures were plated on RCM plates with and without erythromycin to ensure the presence of the plasmid. Cell growth (OD_{600}) was determined with a UNICO UV/vis-2802PC Spectrophotometer (UNICO Instruments Co., Ltd, Shanghai, China). The concentrations of acetate, butyrate, acetone, butanol, ethanol, and glucose were analyzed by an Agilent Technologies high-pressure liquid chromatography system 1200 Series (Agilent Technologies, Beijing, China). A Bio-Rad (Bio-Rad Laboratories, Inc., Hercules, Calif.) Aminex[®] HPX-87H ion exchange column (7.8 mm × 300 mm) with a Cation H guard column (4.6 mm × 30 mm) was used with a mobile phase of 0.05 mM sulfuric acid flowing at 0.50 mL/min at 15 °C. A refractive index (RI) detector (Agilent) was used for signal detection at 30 °C. The data displayed represent the mean value of two independent experiments.

3. Results

3.1. Addition of GSH improves the aero-tolerance of *C. acetobutylicum*

Aeration is quite severe for the survival of *C. acetobutylicum*. When DSM1731 cells were incubated aerobically, cell growth immediately ceased. A 5 log decrease in cell number was observed in the first 3 h of aeration, while no viable cells could be detected after 5 h of aeration (Fig. 2). Survival of *C. acetobutylicum* cells could be improved when GSH was added to the medium in the beginning of the culture (Fig. 2). Exogenous addition of Cys also showed protective effect, but less effective as compared to GSH. Fig. 2 shows that addition of 3.2 mM Cys is equivalent to addition of 0.8 mM GSH, while addition of 19.2 mM Cys is equivalent to the addition of 3.2 mM GSH. This suggests that addition of GSH is about four to six times more effective as

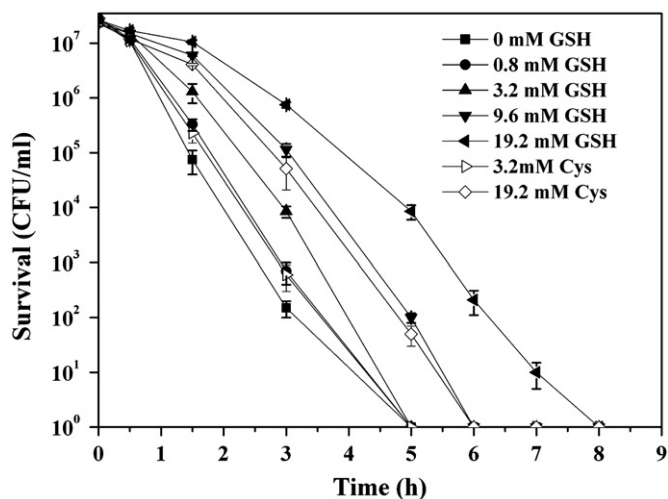


Fig. 2. Survival of aeration-challenged *C. acetobutylicum* DSM1731 when different concentration of GSH and Cys were added to the medium. One milliliter fresh culture ($OD_{600}=1.0$) of *C. acetobutylicum* DSM1731 was harvested, washed, and transferred to 10 mL fresh RCM containing different concentrations of GSH and Cys, followed by incubating aerobically at 37 °C and 200 rpm on a rotary shaker. CFU on the RCM agar plate after incubating anaerobically for 48 h was counted to determine the survival. The GSH and Cys concentrations are shown in the graph.

compared to the addition of Cys. We also determined the intracellular thiol concentration in *C. acetobutylicum* upon addition of exogenous Cys or GSH (cultivated for 12 h). When 19.2 mM Cys or 19.2 mM GSH was individually added to the medium, the intracellular Cys or GSH concentration reached 10.5 ± 1.2 nmol/mg and 5.6 ± 0.7 nmol/mg protein, respectively (GSH and Cys can be stably maintained in the medium and inside the cells at least for 28 h, data not shown). On one hand, this shows that *C. acetobutylicum* is able to accumulate more Cys; on the other hand, it provides further evidence showing that GSH is more effective in *C. acetobutylicum* than Cys, as GSH functions much greater even in a lower intracellular concentration.

3.2. Biosynthesis of GSH in *C. acetobutylicum* improves the aero-tolerance of the host

GSH is not present in most Gram-positive bacteria (Fahey et al., 1978) and is not detected in *C. acetobutylicum* DSM1731. The expression of target proteins of strain DSM1731 and its derivatives were examined by SDS-PAGE. Fig. 3A shows that γ -GCS, GS, γ -GCS, and GS were overexpressed in DSM1731(pITA), DSM1731(pITB), and DSM1731(pITAB), respectively. The relevant bands were excised and subjected to mass spectrometry identification for validation. The band excised from the position of about 58 kDa was identified to be a protein mixture that contains γ -GCS from *E. coli* and ATP synthase F1(alpha subunit) from *C. acetobutylicum* according to peptide mass fingerprint (Fig. 3B). The band excised from the position of about 36 kDa was identified to be GS from *E. coli* (Fig. 3C).

Introducing pITA into strain DSM1731 resulted in the production of significant amount of γ -GC, and introducing pITAB resulted in the production of GSH and a minor amount of γ -GC (Fig. 4A). Introducing pITB into strain DSM1731 did not change the thiol spectrum (Fig. 4A), suggesting that *C. acetobutylicum* DSM1731 lacks the activity of γ -GCS, although a homolog encoding a putative γ -GCS can be found in the *C. acetobutylicum* genome (CAC1539). Notably, although the expression level of GS in DSM1731(pITAB) (Fig. 3A, lane 5) was much lower than that in DSM1731(pITB) (Fig. 3A, lane 4), it did not affect the biosynthesis of GSH. Interestingly, strain DSM1731(pITAB) was able to secrete GSH to the medium (Fig. 4B).

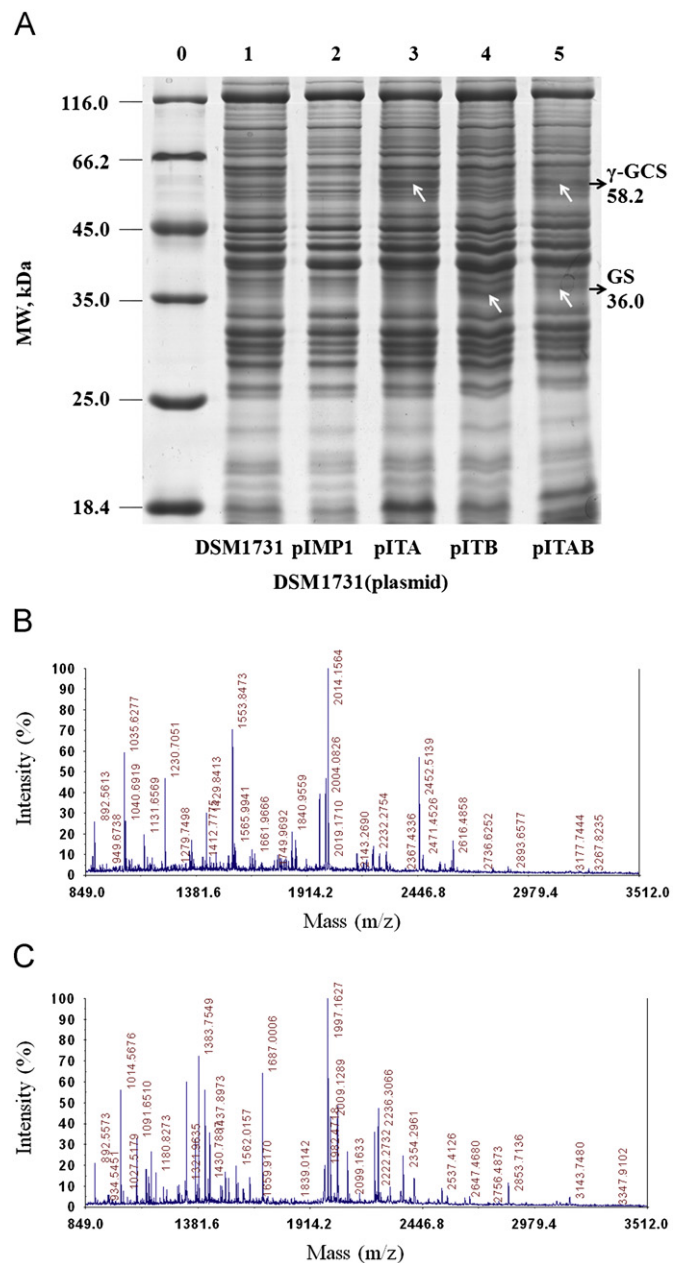


Fig. 3. Analysis of gene expression in strain DSM1731 and its derivatives. (A) SDS-PAGE analysis of CFE of *C. acetobutylicum* DSM1731 and its derivatives at OD_{600} of 1.0. Lane 1–5: wild type strain DSM1731 and strain DSM1731 harboring empty plasmid pIMP1, pITA, pITB and pITAB, respectively; Lane 0: protein marker. (B) The peptide mass fingerprint of bands in the size of about 58 kDa. The bands were identified as γ -GCS from *E. coli* and ATP synthase F1(alpha subunit) from *C. acetobutylicum*. (C) The peptide mass fingerprint of bands in the size of about 36 kDa. The bands were identified as GS from *E. coli*. Mascot score greater than 53 (the default MASCOT threshold for such searches) was accepted as significant (p value < 0.05).

When subjected to aeration, strain DSM1731(pITAB) exhibited significantly improved survival rate, as compared with strain DSM1731 harboring pIMP1, pITA, and pITB. Ten thousand fold difference in survival was observed between DSM1731(pITAB) and its empty vector control DSM1731(pIMP1) after 3 h of aeration (Fig. 5). Strain DSM1731(pITA) that was able to produce γ -GC also showed an improved aero-tolerance, but to a less pronounced level as compared to strain DSM1731(pITAB) (Fig. 5). It suggests that γ -GC is also functional against oxidative stress, but less effective as compared to GSH.

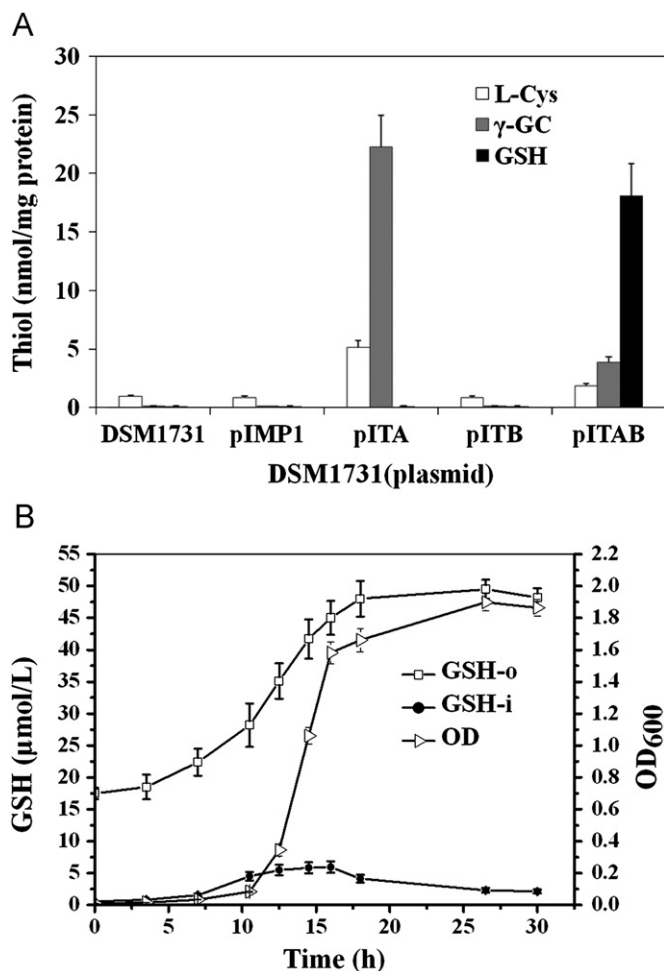


Fig. 4. The thiols concentration detected by HPLC. (A) Intracellular thiols concentrations of the five strains when they were grown to OD₆₀₀ of 1.0 in RCM medium. Cys: cysteine; γ-GC: γ-glutamylcysteine, GSH: glutathione. (B) Extracellular and intracellular GSH concentration profiles of strain DSM1731(pITAB) grown in RCM medium. GSH-o: extracellular GSH concentration; GSH-i: intracellular GSH concentration (the unit of which was converted from nmol/mg protein to μmol/L by multiplying the cell concentration).

3.3. Biosynthesis of GSH in *C. acetobutylicum* improves butanol tolerance and decreases growth inhibition by butanol

To test if GSH can protect *C. acetobutylicum* against butanol stress, cells of DSM1731(pIMP1/pITA/pITB/pITAB) were grown to early-stationary-phase, harvested, and exposed to 19 g/L butanol challenge (Fig. 6). No viable cells of strains DSM1731(pIMP1/pITA/pITB) could be detected after challenged with 19 g/L butanol for 180 min, while the viable cells of strain DSM1731(pITAB) remained 10³ CFU/mL. This indicates that GSH can protect the host against lethal butanol challenge.

We also tested if GSH can help cells grow better in the presence of butanol. Cells of DSM1731 wild type and DSM1731(pIMP1/pITA/pITB/pITAB) were grown in RCM containing different levels of butanol (up to 18 g/L). No growth differences were found in RCM free of additional butanol (Fig. 7A) or addition of 10 g/L butanol (Fig. 7B). As shown in Fig. 7C and D, when challenged with high concentrations of butanol (14.5 or 16.5 g/L), strain DSM1731(pITAB) and its plasmid control strain DSM1731(pIMP1) obviously grew better than the wild type DSM1731 and the single gene expression strains DSM1731(pITA) and DSM1731(pITB). Notably, strain DSM1731, DSM1731(pITA), and DSM1731(pITB) could hardly grow in the presence of 16.5 g/L of butanol; while strain DSM1731(pITAB) could grow, and grew slightly

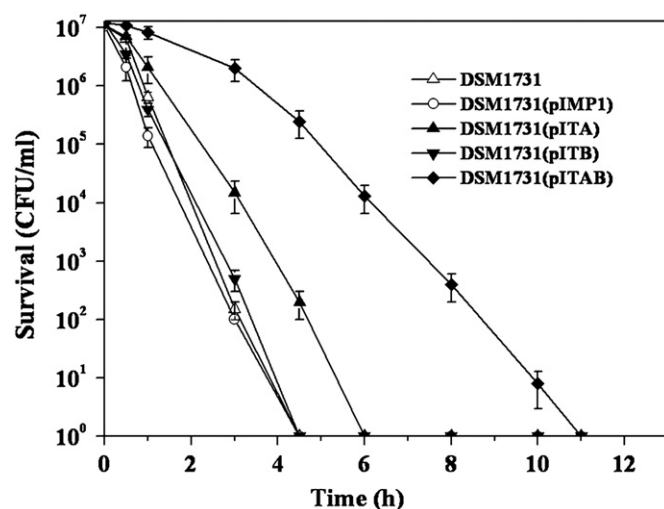


Fig. 5. Survival of *C. acetobutylicum* DSM1731 harboring different plasmids under aeration conditions. After anaerobically cultivated to an OD₆₀₀ of 1.0, cells of different strains were individually transferred to fresh RCM, and incubated aerobically on a rotary shaker (200 rpm) at 37 °C. Survival was determined after incubating anaerobically for 48 h.

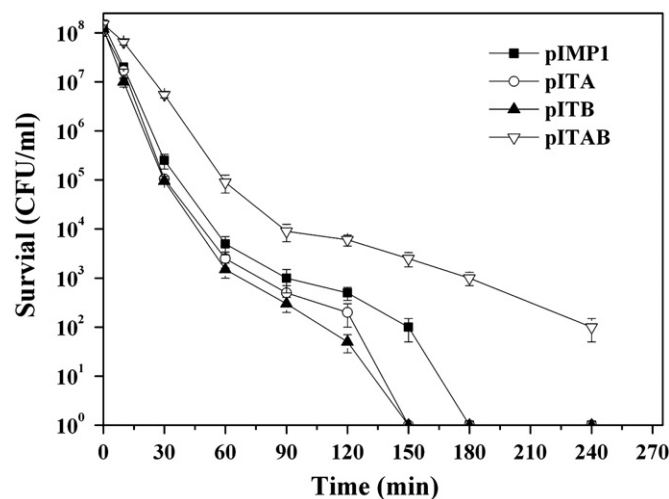


Fig. 6. Survival of *C. acetobutylicum* DSM1731 harboring different plasmids under 19 g/L butanol stress. After anaerobically cultivated to early-stationary-phase (20 h), cells of different strains were individually transferred to fresh RCM contained 19 g/L butanol, and incubated anaerobically at 37 °C. CFU was determined after incubating anaerobically on RCM agar for 48 h.

better than DSM1731(pIMP1) (Fig. 7D). At 18 g/L of butanol challenge, none of the strains could grow (data not shown). The phenomenon that *C. acetobutylicum* strain harboring the empty vector pIMP1 exhibited better growth ability under butanol stress has been reported, which was ascribed to the interaction between host and plasmid (Tomas et al., 2003; Walter et al., 1994). Strain DSM1731(pITAB) exhibited improved butanol resistance as compared to its control strain DSM1731(pITA) and DSM1731(pITB), suggesting that GSH synthesis can improve the butanol tolerance of the host.

3.4. Biosynthesis of GSH in *C. acetobutylicum* improves butanol producing capability

During pH-controlled batch fermentations, target plasmids could be stably maintained within the cell (data not shown). According to the profiles of cell growth and major metabolites (Fig. 8), strain DSM1731(pITAB) produced 14.8 g/L butanol, 3.7 g/L

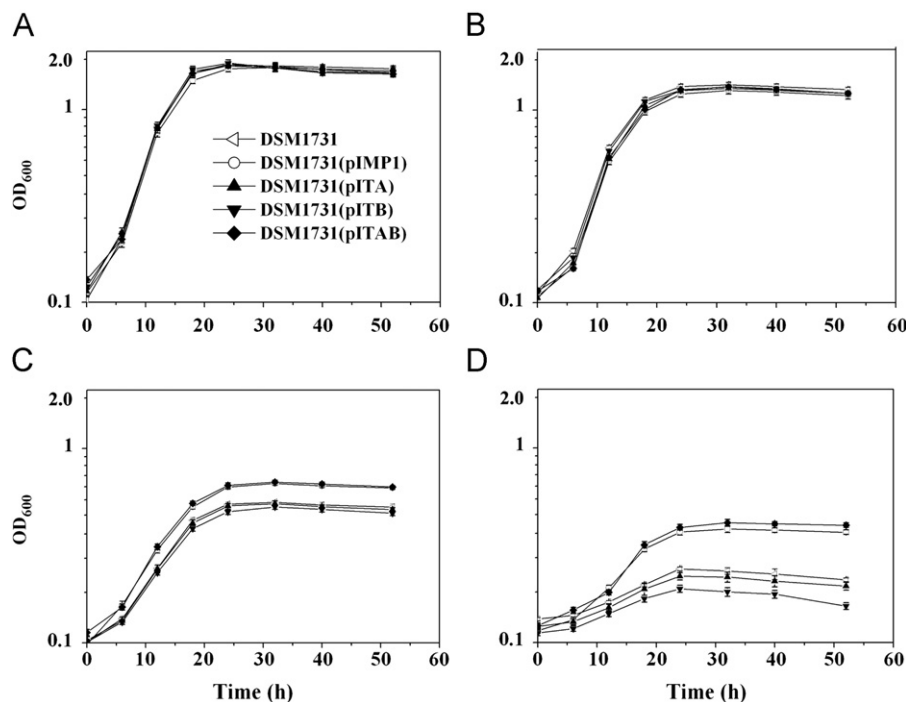


Fig. 7. Growth profile of strain DSM1731 and its derivatives under butanol-stressed conditions. After anaerobically cultivated to an OD_{600} of 1.0, cells of strain DSM1731 and its derivatives were inoculated into fresh RCM containing different concentrations of butanol ((A) 0 g/L; (B) 10 g/L; (C) 14.5 g/L; (D) 16.5 g/L). Static cultures were performed anaerobically at 37 °C and cell concentrations were determined by measuring the turbidity at 600 nm. Data represent the mean value of three independent experiments.

acetone, and 1.2 g/L ethanol (total solvents 19.7 g/L with a ratio of 75:19:6) after 64 h of fermentation (Table 2). The butanol produced by strain DSM1731(pITAB) is 66% and 37% higher than its control strain DSM1731(pITB) and the wild-type strain DSM1731 free of plasmid, respectively (Table 2). Although the individual solvent produced by strain DSM1731(pITAB) was only slightly higher than that of strain DSM1731(pIMP1) (total solvents 18.6 g/L with a ratio of 72:19:9), solvent ratio was altered towards the direction of producing more butanol in the presence of GSH. The final concentration of acetate and butyrate did not show significant difference during the fermentation of the four strains.

4. Discussion

Glutathione is the most prevalent non-protein thiol compound in living organisms that performs many diverse functions in metabolism (Lu, 2009; Meister, 1988) and stress resistance (Masip et al., 2006). However, most Gram-positive bacteria do not synthesize GSH, except for *Streptococcus agalactiae*, *Streptococcus pyrogens*, *Enterococcus faecalis*, and *Listeria monocytogenes* (Gopal et al., 2005; Janowiak and Griffith, 2005; Sherrill and Fahey, 1998). Some Gram-positive bacteria, including *Leuconostoc kimchi* and *Leuconostoc mesenteroides*, alternatively accumulate γ -GC rather than GSH (Kim et al., 2008). In *E. coli*, GSH was synthesized through two ATP-dependent reactions, which were catalyzed by the enzymes of γ -GCS and GS in turn. The *E. coli* γ -GCS is a monomer of 58.3 kDa (Huang et al., 1988) and GS is tetramer with four identical subunits of 35.6 kDa (Yamaguchi et al., 1993), the size of which is almost equal to that of the expressed recombinant proteins shown in Fig. 3A. Previously, we have introduced the GSH biosynthesis pathway into *L. lactis* subsp. *cremoris* NZ9000, resulted in an improved resistance to oxidative stress (Fu et al., 2006) and acid stress (Zhang et al., 2007) of the host. Although the protein encoded by CAC1539 in

the genome of *C. acetobutylicum* is annotated as γ -GCS; the fact that no γ -GC was detected in *C. acetobutylicum* cells suggested this gene is not functional. No gene homologous to *gshB* was found in the genome of *C. acetobutylicum*, this is consistent with the fact that introducing *gshA* into strain DSM1731 only resulted in the production of γ -GC but not GSH.

Introducing *gshAB* into strain DSM1731 resulted in the production of GSH, demonstrating for the first time that, GSH can be produced by *C. acetobutylicum* and plays physiological roles. Although *C. acetobutylicum* is a typical obligatory anaerobe, it can grow under continuous microoxic conditions (5% O_2) (Kawasaki et al., 2004). In *C. acetobutylicum*, only when dissolved oxygen accumulates so much that central metabolic enzymes like pyruvate-ferredoxin oxidoreductase (PFOR) are destructed, metabolism is halted and viability decreases (Hillmann et al., 2009a). While GSH could protect some central metabolic enzymes like glyceraldehyde-3-phosphate dehydrogenase (GapA) in *E. coli* (Cotgreave et al., 2002; Leichert et al., 2008) and up-regulated synthesis of several glycolytic enzymes in *L. lactis* (Zhang et al., 2010), we postulate that GSH might also protect the central metabolism of *C. acetobutylicum*.

C. acetobutylicum possesses several pathways for detoxification of reactive oxygen species (ROS), including superoxide reductase (SOR) (Riebe et al., 2007), reverse rubrerythrin (revRbr) (Kawasaki et al., 2004), NADH rubredoxin oxidoreductase, and some glutathione peroxidase-like proteins (Kawasaki et al., 2005). Deletion of a peroxide repressor PerR-homologous protein resulted in a prolonged aero-tolerance, limited growth under aerobic conditions, and rapid consumption of oxygen from an aerobic environment (Hillmann et al., 2008), demonstrating that PerR acts as a switch for aero-tolerance in *C. acetobutylicum*. Although naturally *C. acetobutylicum* does not synthesize GSH, the aero-tolerance of this strict anaerobe could be improved by adding external GSH or assembling a synthetic pathway for GSH biosynthesis. This shows that the aero-tolerance or even the ability to grow in the presence

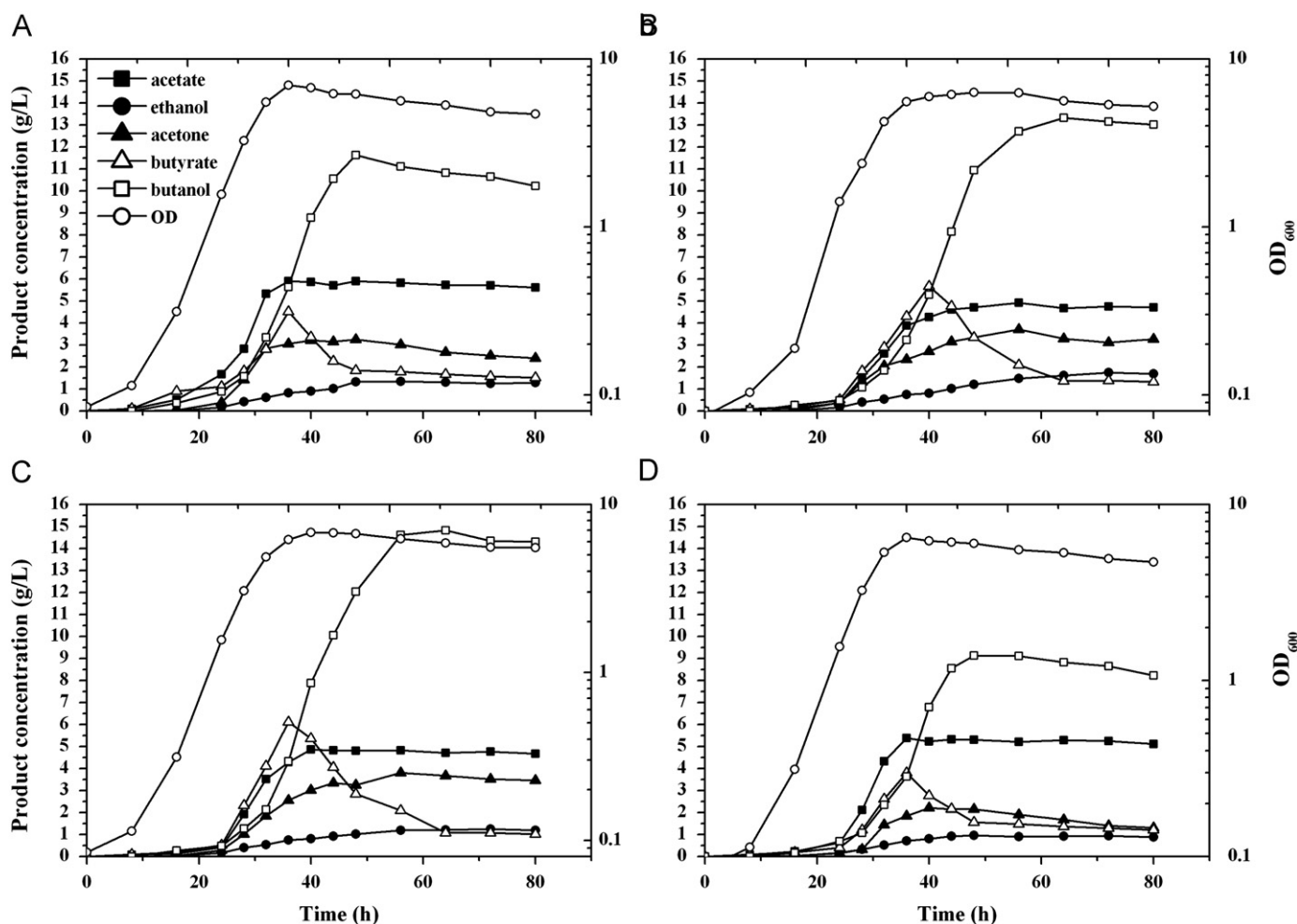


Fig. 8. Profiles of cell growth and metabolites production for pH-controlled ($\text{pH} \geq 5.0$) anaerobic batch fermentations of DSM1731 and its derivatives. Strains DSM1731 (A), DSM1731(pIMP1) (B), DSM1731(pITAB) (C), and DSM1731(pITB) (D) were cultivated anaerobically in CGM. Metabolites concentrations were determined by HPLC and cell concentrations were determined by measuring the turbidity at 600 nm. Data shown represent the averages of two independent fermentations.

Table 2

Major product concentrations in pH-controlled ($\text{pH} \geq 5.0$) anaerobic batch fermentations of DSM1731 and its derivatives^a.

	Acetate (g/L)	Ethanol (g/L)	Acetone (g/L)	Butyrate (g/L)	Butanol (g/L)	Residual glucose (g/L)
DSM1731	5.7 ± 0.2	1.3 ± 0.1	2.8 ± 0.1	1.7 ± 0.2	10.8 ± 0.3	6.6 ± 0.3
DSM1731(pIMP1)	4.7 ± 0.2	1.6 ± 0.1	3.6 ± 0.2	1.3 ± 0.2	13.4 ± 0.4	4.3 ± 0.4
DSM1731(pITB)	5.3 ± 0.3	0.9 ± 0.1	1.8 ± 0.2	1.4 ± 0.2	8.9 ± 0.3	12.6 ± 0.8
DSM1731(pITAB)	4.7 ± 0.2	1.2 ± 0.1	3.7 ± 0.1	1.1 ± 0.1	14.8 ± 0.3	3.5 ± 0.3

^a Data shown were from 64 h fermentation. The initial glucose concentration was 71.5 ± 1.3 g/L.

of oxygen, two desirable biotechnological traits of anaerobes, can be improved by natural/external/synthetic approaches.

The H_2O_2 and O_2 detoxification pathway involves rubredoxin (Rd) and NADH: rubredoxin oxidoreductase (NROR) as electron transport intermediaries and two revRbrs as the terminal component of an NADH peroxidase and NADH oxidase, which catalyze the four-electron reduction of molecular oxygen to water (Hillmann et al., 2009b; Riebe et al., 2009). Glutathione peroxidase in *C. acetobutylicum* has not yet been characterized in detoxification of ROS, but the expressions of glutathione peroxidase, thioredoxin reductase, and thiol peroxidase, were up-regulated quickly upon flushing with 5% O_2 (Kawasaki et al., 2005). The synthesized GSH in *C. acetobutylicum* may be involved in scavenging ROS and maintaining cellular redox balance through these peroxidases and reductases, resulting in a significantly enhanced aero-tolerance of strain DSM1731(pITAB). As it is

very hard to maintain strictly anaerobic conditions on an industrial scale, an improved aero-tolerance of *C. acetobutylicum* is of industrial significance.

Introducing GSH biosynthetic ability into *C. acetobutylicum* also resulted in a less growth inhibition when challenged with butanol of higher than 14.5 g/L. Butanol accumulation may disrupt many physiological functions in cell membrane, including transmembrane ΔpH and $\Delta\psi$, substance transportation, and membrane-bound ATPase (Bowles and Ellefson, 1985; Huang et al., 1985; Terracciano and Kashket, 1986) and induce autolysin production (Ezeji et al., 2010). These stresses may induce cellular general stress response including up-regulation of all major chaperones to stabilize proteins (Alsaker and Papoutsakis, 2005; Schaffer et al., 2002), up-regulation of glycerol metabolism genes *glpA* and *glpF* as conserved mechanism for solvent tolerance (Alsaker et al., 2010), and significant oxidative stress response

to consume increased ROS (Rutherford et al., 2010). In bacteria, GSH can protect the Gram-negative *E. coli* (Csonka, 1989; Smirnova et al., 2001) and the Gram-positive *L. lactis* (Zhang et al., 2007; Zhang et al., 2010) against osmotic stress and acid stress. The role of GSH in solvent stress resistance might be similar to that in osmotic stress and acid stress, as these stresses primarily disrupt function of cell membrane. Solvent, acid, and osmotic stresses may interfere with proteins involved in respiratory functions and oxidation–reduction reactions, whose inactivation consequentially accumulates ROS (Rutherford et al., 2010; Smirnova et al., 2000). Thus, scavenging of ROS in the presence of GSH may help cells to survive the solvent, acid, and osmotic stresses longer. Obviously, the increased butanol production could be partially ascribed to the increased resistance to butanol stress.

Toxicity of inhibitory substrates and metabolites are often encountered in bioprocesses to produce fuels and chemicals from renewable resources. Although the knowledge on how cells respond to these chemicals stresses (such as solvent and carboxylic-acid stress) is accumulating, developing a stress-tolerant robust strains is still a difficult task because stress response is complex and often overlapped (Nicolaou et al., 2010). Genome-scale approaches have been developed to engineering cellular tolerance in *C. acetobutylicum*, such as butanol tolerance (Borden and Papoutsakis, 2007) and acid tolerance (Borden et al., 2010). Lacking the native GSH biosynthetic ability in *C. acetobutylicum* suggests that GSH is not essential for this bacterium. In this sense, the GSH biosynthetic pathway is redundant for the metabolism of *C. acetobutylicum*. Our results demonstrated that introducing this metabolic redundancy can improve the robustness of the host, one of the most important physiological functionalities for efficient production of fuels and chemicals (Zhang et al., 2009). Therefore, strategies of exploiting and engineering the functional metabolic redundancy may also help microbes to better adapt themselves to the industrial environments.

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