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Transcriptional analysis of degenerate strain *Clostridium beijerinckii* DG-8052 reveals a pleiotropic response to CaCO₃-associated recovery of solvent production

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Degenerate *Clostridium beijerinckii* strain (DG-8052) can be partially recovered by supplementing CaCO₃ to fermentation media. Genome resequencing of DG-8052 showed no general regulator mutated. This study focused on transcriptional analysis of DG-8052 and its response to CaCO₃ treatment via microarray. The expressions of 5168 genes capturing 98.6% of *C. beijerinckii* NCIMB 8052 genome were examed. The results revealed that with addition of CaCO₃ 565 and 916 genes were significantly up-regulated, and 704 and 1044 genes significantly down-regulated at acidogenic and solventogenic phase of DG-8052, respectively. These genes are primarily responsible for glycolysis to solvent/acid production (*poR, pfo*), solventogensis (*buk, ctf, aldh, adh, bcd*) and sporulation (*spo0A, sigE, sigma-70, bofA*), cell motility and division (*ftsA, ftsK, ftsY, ftsH, ftsE, mreB, mreC, mreD, rodA*), and molecular chaperones (*grpE, dnaK, dnaJ, hsp20, hsp90*), etc. The functions of some altered genes in DG-8052, totalling 5.7% at acidogenisis and 8.0% at sovlentogenisis, remain unknown. The response of the degenerate strain to CaCO₃ was suggested significantly pleiotropic. This study reveals the multitude of regulatory function that CaCO₃ has in clostridia and provides detailed insights into degeneration mechanisms at gene regulation level. It also enables us to develop effective strategies to prevent strain degeneration in future.

Solventogenic *Clostridium* species are unique natural occurring microorganisms capable of performing acetone-butanol-ethanol (ABE) fermentation with butanol as the major metabolite. One challenge with industrial ABE fermentation by solventogenic *Clostridia* is strain degeneration due to repeated subculture or continuous fermeantion, which often leads to significantly reduced fermentation efficiency and even predetermined fermentation[1,2]. Another cause of strain degeneration is bacteriophage infection due to which many industrial ABE fermentation processes were compromised in early 20th century[3]. Over the past three decades, extensive research were carried out to find solutions for overcoming *Clostridia* strain degeneration. It was reported that addition of sodium acetate to MP2 medium could prevent degeneration in *Clostridium beijerinckii* NCIMB 8052 and BA101, a solvent-hyperproducing mutant derived from 8052[4]; Recently, in hyper-butanol producing *C. ace-tobutylicum* JB200, the integrated gas stripping to remove product during fermentation was found to increase butanol productivity without culture degeneration[5,6].

Degenerate solventogenic *Clostridium* strains are known to partially or completely lose their capability to catabolize acetic acid and butyric acid generated at acidogenic phase for ABE production at the following solventogenic phase. The resulting acid accumulation exerts a strong stress to degenerate strains, eventually leading to

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cell death/lysis and cease of solvent production[7,8]. The degeneration mechanism varies among *Clostridium* strains. *C. acetobutylicum* (ATCC 824) is degenerated as a result of the loss of the mega-plasmid pSOL1 which harbours *sol* operon (*aad-ctfA-ctfB*) expressing alcohol/aldehyde dehydrogenase and CoA transferase responsible for the acid uptake[9]. For *C. saccharoperbutylacetonicum*, a degenerate strain is identified with a defect in genes encoding for enzymes responsible for the NADH formation from the catabolism of pyruvate[10]. *C. beijerinckii* NCIMB 8052 has all the genes responsible for ABE fermentation in the genome, and more degenerate variants of *C. acetobutylicum* was found than *C. acetobutylicum* (ATCC 824)[4]. During ABE fermentation, $CaCO_3$ has been shown to stimulate sugar utilization, butanol production, and butanol tolerance[11]. We reported previously that $CaCO_3$ could enhance ABE fermentation in both the wild type *C. beijerinckii* strain (NCIMB 8052) and degenerate one. The proteomic analysis results showed that key cellular processes, such as sugar transport, butanol tolerance, and solventogenesis were influenced by the addition of $CaCO_3$[12,13].

The objective of this study is to elucidate the regulation mechanism of calcium on the degenerate strain of *C. beijerinckii* NCIMB8052 by transcriptional analysis. We first compared the differences between the whole genomes of DG-8052 and WT-8052 using a genome resequencing strategy, and then compared the abundance of transcription of genes in $CaCO_3$ treated and untreated DG-8052, especially in ABE fermentation, cell division and spore forming. This study is expected to provide possible strategies in engineering *C. beijerinckii* NCIMB 8052 to prevent strain degeneration and improve ABE fermentation.

Results and Discussion

Effect of $CaCO_3$ on cell growth of DG-8052. In solventogenic *Clostridium* species, the spore development (sporulation) is usually accompanied with solvent production. Usually, during the acidogenic phase, cells in most cultures consist of straight, short or long rods with round ends (Fig. 1A). Towards the end of exponential growth the rod-shaped cells typically begin to accumulate granulose, assuming a swollen cigar-shaped clostridia form, and produce extracellular slime or capsules (Fig. 1B)[14,15]. In contrast, DG-8052 showed a large proportion of non-split and elongated straight rod cells ($8-10\,\mu m \times 0.8\,\mu m$ at acidogenic phase, $8-12\,\mu m \times 0.8\,\mu m$ at solventogenic phase) (Fig. 1C and D). DG-8052 only retained little capability of solvent production, having very small amounts of solvents produced (0.10 g/l acetone, 0.19 g/l ethanol and 0.58 g/l butanol) during 48 hours or longer culture period[13]. Similar phenomena were observed with a degenerate strain *C. acetobutylicum* M5 which lost the capability of sporulation and solvent production[8]. Our previous study indicated that $CaCO_3$ played an important role in improving the performance of DG-8052 on cell growth, glucose utilization, and solventogenesis. With the addition of 4 g/l $CaCO_3$, the solventogenesis was partially recovered, reaching 1.94 g/l acetone, 0.25 g/l ethanol and 5.44 g/l butanol throughout the fermentation period of 60 h[13]. In addition, the DG-8052 growing in the media supplement with $CaCO_3$ appeared to have short rod-like morphology ($2-6\,\mu m \times 0.8\,\mu m$) (Fig. 1E and F), resembling those of the wide type one much, especially at the exponential phase. The morphological change along with partially restored solventogenic capability indicated that $CaCO_3$ was beneficial to *C. beijerinckii* DG-8052.

Overall gene transcription dynamics. Transcriptome profiles of DG-8052 treated with and without $CaCO_3$ were compared by microarray analysis. Since ABE fermentation is a biphasic process, gene expressions were compared at acidogenic phase and solventogenic phase, respectively. Total 5168 genes capturing 98.6% of the *C. beijerinckii* NCIMB 8052 genome were examed (Fig. 2). With the addition of $CaCO_3$, DG-8052 had 565 and 916 genes significantly up-regulated at acidogenic phase and solventogenic phase, respectively (Additional file Table S3A, S3C). According to the enrichment analysis of GO terms and KEGG pathway, these genes were significantly overexpressed in terms of cellular functions such as amino acid transport and metabolism, organic acid biosynthetic process, and bacteria chemotaxis (Table 1, Fig. 3). Significantly down-regulated 704 and 1044 genes at acidogenic phase and solventogenic phase, respectively (Additional file Table S3B, S3D) had primary functions in ion transmembrane transport, ATP synthesis, and oxidative phosphorylation (Table 1, Fig. 3). The COG (Clusters of Orthologous Groups) distribution for these up- and down-regulated genes in the transcriptome was determined (Additional file Table S2). Not all of the regulated genes had known function, and COG analysis revealed that 292 and 425 regulated genes at acidogenisis and solventogensis, respectively, had unspecified functions. The differentially expressed genes are described in detail in the latter sections.

At proteomic level, it was found that 3% detected proteins changed in DG-8052, resulting in corresponding morphological and physiological changes; moreover, the addition of $CaCO_3$ partially recovered the metabolism, including solvent production, spore formation, and carbon utilization, and there had only about 0.7% detected proteins changed[12]. Transcriptomic data will help to reveal the responses of this bacterium to $CaCO_3$, and expatiate on the mechanisms that $CaCO_3$ contributed to the up-regulation of ABE fermentation in degenerate *Clostridium* strain.

Solvent production. $CaCO_3$ can improve solvent production in both WT-8052 and DG-8052 strains with higher transcription and expression of solvent producing pathway-related enzymes[12,13]. At the acidogenic phase, the addition of $CaCO_3$ increased the expression level of genes encoding acids producing enzymes (butyrate kinase Cbei_4609) expressed (+6.05) in the DG-8052 culture (Fig. 3A, Additional file Table S5A). Enough amounts of acetic acid and butyric acid accumulated, then they were re-assimilated and converted into acetyl-CoA and bytyryl-CoA, respectively, by CoA-transferase[16]. The corresponding ORFs of CoA-transferase (Cbei_2040, Cbei_3278, Cbei_3833 and Cbei_3834) showed different transcription. When $CaCO_3$ added, Cbei_3833 and Cbei_3834 showed no obviously expressing change at both acidogenisis and solventogensis, while Cbei_2040 and Cbei_3278 showed up-regulated fold change from +3.40, +3.99 (acidogenisis) to +4.69, +12.99 (solventogensis), separately. The regulation mechanism of sharp increased expression of CoA-transferase in DG-8052 with $CaCO_3$ is still unknown. It may be because of the effects of Ca^{2+} and the buffering capacity of $CaCO_3$ to the fermentation media[12,13]. The acetyl-CoA acetyltransferase (Cbei_3630) showed increased transcription while Cbei_0411
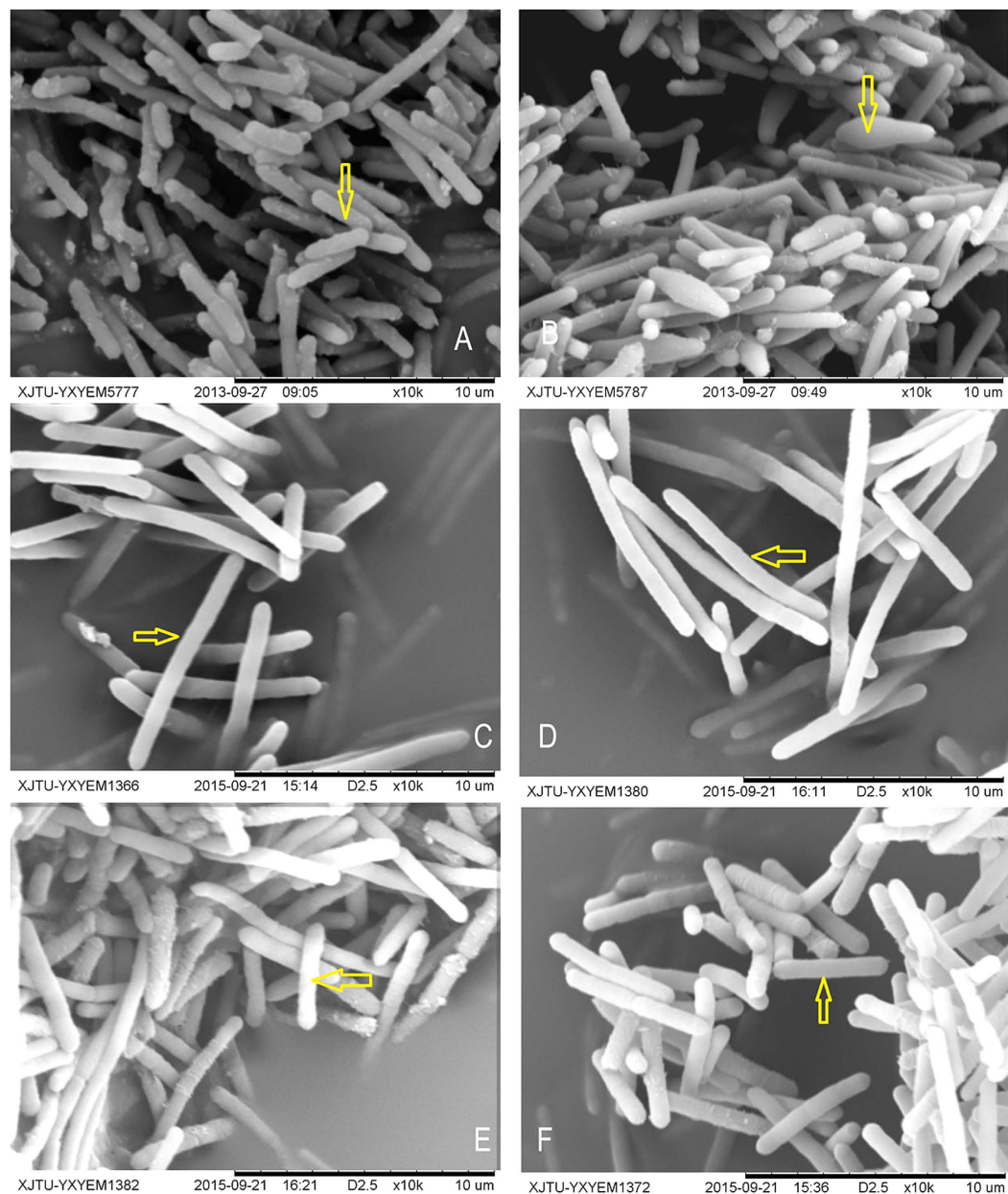
Figure 1. Electron micrograph of *C. beijerinckii* cells grown on P2 medium with and without CaCO₃. WT-8052 strain cultured at 12 h (**A**) and 24 h (**B**); DG-8052 strain at 12 h (**C**) and 24 h (**D**); DG-8052 cells strain cultured with 4 g/L CaCO₃ at 12 h (**E**) and 24 h (**F**). The typical cells were indicated by yellow arrows.

showed decreased transcription, and there had 86% similarity in their amino acid sequences, which may cause the different sensitivity to CaCO₃. The transcription of 3-hydroxybutyryl-CoA dehydrogenase (Cbei_0325), 3-hydroxybutyryl-CoA (Cbei_0324) and butyryl-CoA dehydrogenase (Cbei_0322) was not changed significantly by the addition of CaCO₃ to the DG-8052 culture.

It is reported that phosphoenolpyruvate (PEP), pyruvate, and acetyl-CoA form three main key nodes in the flux distribution[17]. In solvetogenic *clostridia*, glucose is converted into pyruvate via glycolysis accompanied with 2 ATP and 2 NADH, and the produced pyruvate is further converted into acetyl-CoA for the production of acetic and butyric acid at acidogenic phase. It had no significant difference in transcription of enzymes responsible for acid production and subsequent conversion to acetyl-CoA and butyryl-CoA between the DG-8052 cells with and without CaCO₃. Thus, the increased production of solvent may be attributed to either the up-regulated aldehyde dehydrogenase and alcohol dehydrogenase, or the high amount of reaction substrate (acetyl-CoA). We found in DG-8052 supplied with CaCO₃, pyruvate synthase (Cbei_2063), puryvate/ferredoxin oxidoreductase (Cbei_1853, Cbei_1458, Cbei_4042) were all up-regulated at acidogenisis and solventogensis, and Cbei_1458 had a maximum fold change of 41.18 at solventogenisis (Figs 4 and 5A, Additional file Table S5A), which may result in high concentration of acetyl-CoA. Energy and reducing power (NADH/NADPH) has been reported to have a significant
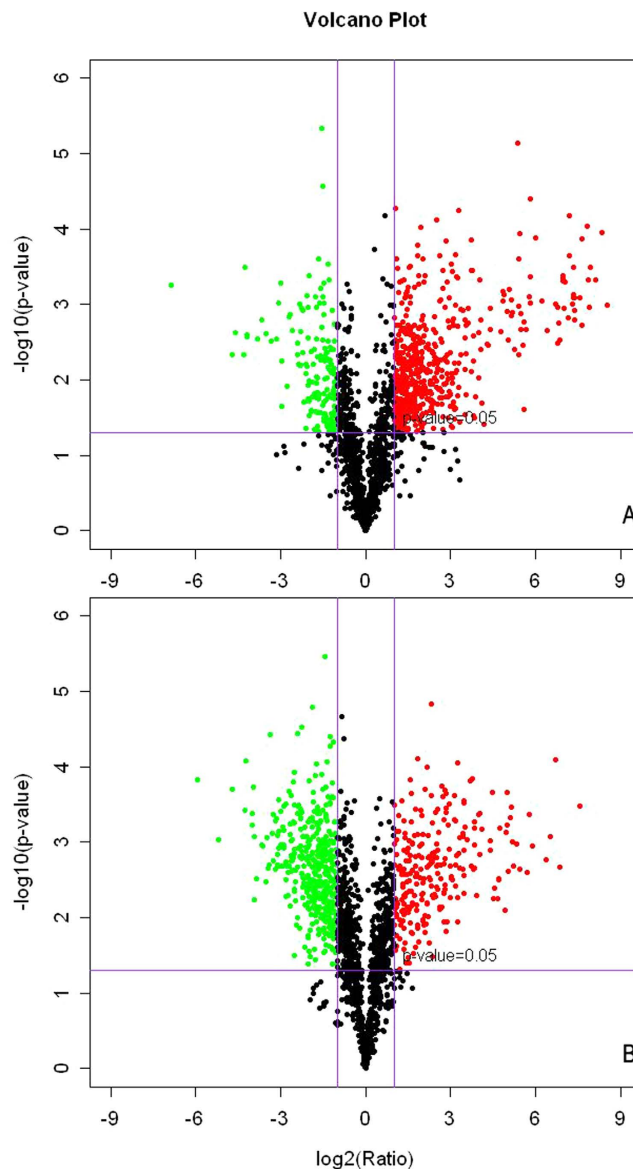
Volcano Plot

Figure 2. Comparison of gene expression in DG-8052 in P2 medium with CaCO$_3$ vs DG-8052 in P2 medium. Cells from both strains were taken at 12 h and 24 h to compare transcriptome profiles at acidogenic (**A**) and solventogenic (**B**) phases, respectively. Genes with significantly differential expression (fold change >2 and p-value < 0.05) between the two strains were shown in green (down-regulated) and red (up-regulated) while the others with non-significantly differential expression were shown in black.

influence on cell growth, metabolism and solvent production[14]. The problem with formation of NADH from pyruvate[10] was report to lead to degeneration of *C. saccharoperbutylacetonicum*. CaCO$_3$ appeared to increase high level of NADH accompanied with increased producing of Acetyl-CoA from pyruvate, which could contribute to some restored functions of DG-8052. Further study is needed to verify the positive effects of CaCO$_3$ on the formation of NADH.

Aldehyde dehydrogenase was reported to play key roles in primary alcohol production of *C. beijerinckii* 8052[18,19]. Cbei_1722 and Cbei_2181 in DG-8052 with 94–99% amino acid sequence identical to that of *adhA* and *adhB*, showed no change in their expression in response to CaCO$_3$. The aldehyde dehydrogenase (Cbei_2518, Cbei_0305 and Cbei_4936) showed up-regulation folds from +4.33, +3.36, +164.7 (acidogenisis) to +4.83, +4.01, +39.86 (solventogensis), separately. Alcohol dehydrogenase (Cbei_4552, Cbei_4354 and Cbei_1937) were also all up-transcripted from +3.42, +8.44, +159.77 (acidogenisis) to +5.31, +3.0 +11.52 (solventogensis), separately. There were 12 aldehyde dehydrogenase encoded genes and 25 alcohol dehydrogenase encoded genes in *C. beijerinckii* NCIMB 8052, suggesting the great potential for the solvent production in this bacterium. Most of these genes were induced at the early acidogenic phase and highly expressed in the solventogenic stage (Additional file Table S5A). Our previous report revealed the broader effects of calcium at the cellular and protein levels on carbohydrate utilization, acids uptake, butanol production and tolerance in ABE production by

culture	KEGG ID	Pathway definition	p value	Benjamini[*]	Count[**]
Up-regulated					
12 h	cbe02010	ABC transporters	1.8E-3	1.0E-1	30
	cbe00562	Inositol phosphate metabolism	2.4E-3	7.2E-2	5
	cbe00400	Phenylalanine, tyrosine and tryptophan biosynthesis	2.5E-3	5.0E-2	9
	cbe02020	Two-component system	4.2E-2	6.2E-2	16
	cbe00620	Pyruvate metabolism	4.0E-2	3.9E-2	10
24 h	cbe00040	Pentose and glucuronate interconversions	9.4E-8	6.3E-6	20
	cbe02020	Two-component system	5.6E-4	1.9E-2	24
	cbe00400	Phenylalanine, tyrosine and tryptophan biosynthesis	1.0E-3	2.3E-2	12
	cbe00030	Pentose phosphate pathway	8.1E-3	1.3E-1	16
	cbe00450	Selenoamino acid metabolism	9.1E-3	1.1E-1	8
	cbe02010	ABC transporters	1.0E-2	1.1E-1	40
	cbe00562	Inositol phosphate metabolism	1.5E-2	1.3E-1	5
	cbe00290	Valine, Leucine and isoleucine biosynthesis	3.4E-2	2.5E-1	9
	cbe00480	Glutathione metabolism	6.6E-2	3.1E-1	5
	cbe00620	Pyruvate metabolism	6.6E-2	3.7E-1	13
	cbe00020	Citrate cycle (TCA cycle)	7.6E-2	3.8E-1	5
Down-regulated					
12 h	cbe00190	Oxidative phosphorylation	5.2E-4	2.8E-2	10
	cbe00330	Arginine and proline metabolism	7.5E-4	2.0E-2	11
	cbe00440	Phosphonate and phosphinate metabolism	1.1E-2	1.8E-1	4
	cbe00250	Alanine, aspartate and glutamate metabolism	6.5E-2	6.0E-1	7
	cbe00230	Purine metabolism	9.0E-2	6.5E-1	11
24 h	cbe00440	Phosphonate and phosphinate metabolism	4.0E-3	1.3E-1	5
	cbe00730	Thiamine metabolism	1.7E-3	1.1E-1	9
	cbe00860	Porphyrin and chlorophyll metabolism	6.3E-3	1.4E-1	11
	cbe00330	Arginine and proline metabolism	2.6E-2	3.7E-1	11
	cbe00190	Oxidative phosphorylation	4.5E-2	4.7E-1	9

Table 1. KEGG pathway showing significant changes in *C. beijerinckii* DG-8052 cultures with addition of 4 g/L CaCO$_3$. [*]Significant groups were selected based upon Benjamini (<0.05). [**]Number of genes within the given KEGG ID showing a significant change in their expression level.
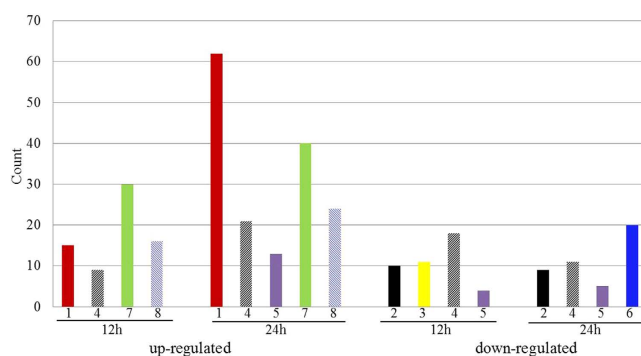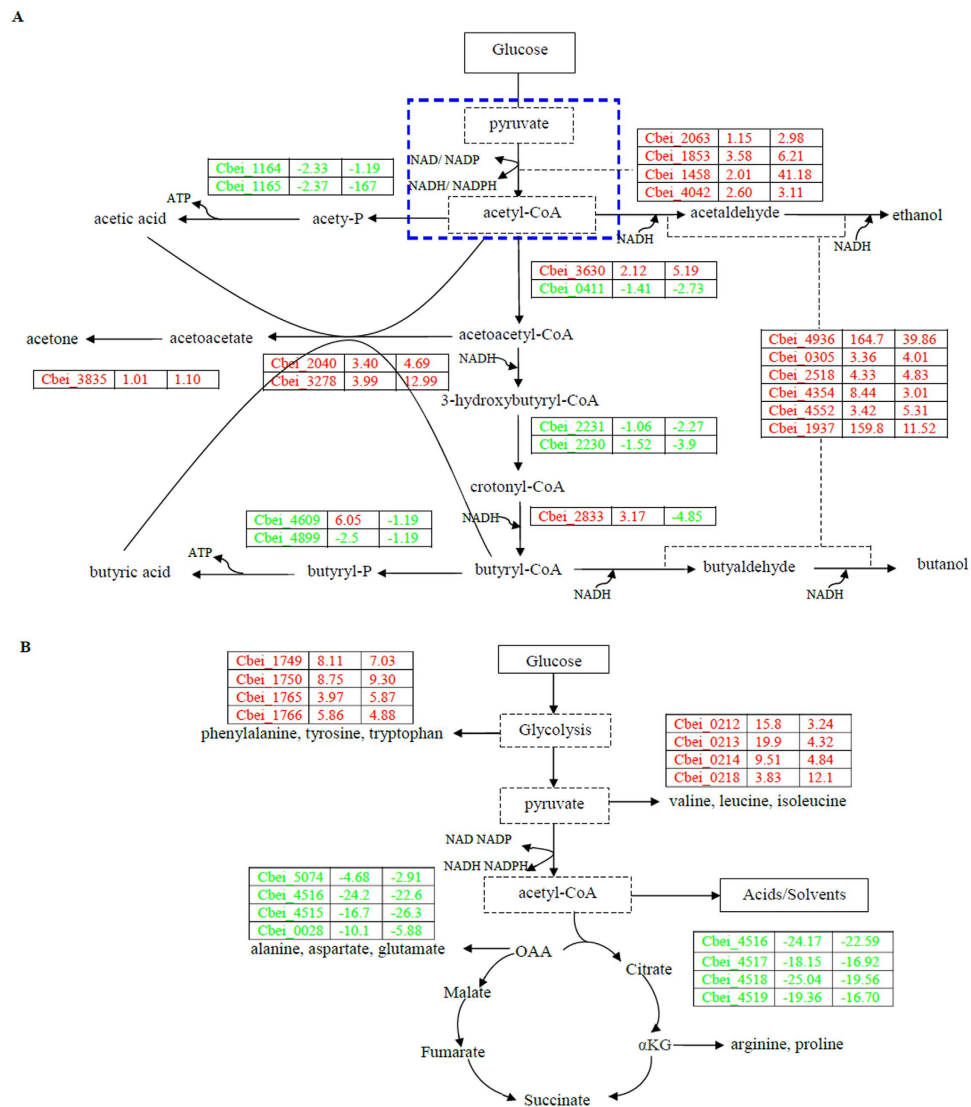

Figure 3. Counts of differentially expressed genes by KEGG pathway categories. 1, carbohydrate metabolism; 2, energy metabolism; 3, nucleotide metabolism; 4, amino acid metabolism; 5, metabolism of other amino acid; 6, metabolism of co-factors and vitamins; 7, membrane transporter; 8, signal transduction.

C. beijerinckii NCIMB 8052[12]. The metal-driven stimulatory effects on ABE production were somehow multifactorial and multifunctional due to the complex metabolic network of *clostridia*[20]. The specific functions of different

Figure 4. Comparative expression patterns for genes related to (**A**) glycolysis, acidogensis and solventogensis, (**B**) sigma factor and sporulation, (**C**) Cell division and cytoskeleton, (**D**) Sugar transporter/metabolism, (**E**) amino acid biosynthesis/metabolism, (**F**) Signal transduction. Left column is the acidogenisis, and right column is the solventogenisis.

Figure 5. Schematic diagram showing the primary steps in conversion of glucose into fermentation products in *C. beijerinckii* DG-8052. Acids/solvents producing pathway (**A**), amino acids producing pathway (**B**). The fold change (FC) value is indicated directly beside the gene ID of 12 h and 24 h respectively. Text in red and green represents the up-regulated and down-regulated genes, respectively. The blue dash box indicated the synthesis of acetyl-CoA from pyruvate. Detailed enzymes and their associated gene ID can be found in Supplementary Data Table S4A, 4C.

aldehyde dehydrogenase and alcohol dehydrogenase genes in *C. beijerinckii* NCIMB 8052 and different transcription in DG-8052 required further investigation.

Sugar transporter and metabolism. Our previous report indicated that the DG-8052 could metabolite more glucose when supplied with $CaCO_3$ during the batch fermentation. The phosphoenolpyruvate-dependent phosphotransferase system (PTS) is the predominant sugar uptake pathway in *C. beijerinckii*[21]. In *C. beijerinckii* 8052, EI (enzyme I, Cbei_0196) and HPr (heat stable, histidine-phosphorylatable protein, Cbei_1219), phosphorylate the sugar in the cytoplasm, which were all down-regulated in DG-8052 when supplied with $CaCO_3$. Except this, other sugar PTS transporters were all not affected, which may point out that in DG-8052, the genes related to carbohydrate metabolism pathways were not impacted by $CaCO_3$, or the addition of $CaCO_3$ did not affect PTS sugar transportation system (Fig. 4A,B, Additional file Table S5B). In *Cyanobacterium synechocystis* sp. PCC 6803, the sugar catabolic pathways were positive regulated by SigE[22]. Our results showed than compared with DG-8052 without $CaCO_3$, SigE (Cbei_1120) got down-regulated by 2.76 and 4.79 folds in DG-8052 with $CaCO_3$ (Additional file Table S5D). And the down-regulated SigE may repress the sugar metabolism related genes.

However, not all carbohydrates are accumulated in PTS system, and there were a non-PTS system probably energized by the transmembrane proton gradient in *C. beijerinckii*[23]. In DG-8052 supplied with $CaCO_3$, the increased transport of glucose accompanied with increased production of acetic and butyric acids, while those acids were converted into dissociated form and increased the transmembrane proton gradient. In *C. beijercinkii*

CC101, which derived from NCIMB8052, the addition of citrate could inhibit cell growth by reducing the cells internal pH and proton motive force, and changing cell membrane permeability[24]. In our research, the supplied $CaCO_3$ could convert the undissociated acids into dissociated form which reduced the acids stress to cell, while, the produced butyrate and acetate may contribute like citrate did in CC101, which may explain the partial recovery of ABE producing ability in DG-8052.

And it showed the non-PTS transport system contributed significantly to glucose uptake at solventogenic phase but not acidogenic phase[23]. In the present study we found in DG-8052, the putative ABC transport system including phosphate binding protein (Cbei_1127), phosphate ABC transporter permease (Cbei_1128, Cbei_1129), phosphate ABC transporter ATPase (Cbei_1130) were down-regulated at acidogenisis, but up-regulated at solventogenisis when supplied with $CaCO_3$ (Fig. 4B, Additional file Table S5B). It may contribute to the higher transport of sugar in DG-8052 with presence of $CaCO_3$.

Generally, glycolysis genes encoding enzymes for conversion of glucose to pyruvate were expressed at high levels to provide enough ATP for cell growth. At the generic level, expression of glycolysis pathway enzymes in DG-8052 cell were down regulated by adding $CaCO_3$, except ROK family protein (Cbei_4565, Cbei_3517) and fructose-bisphosphate aldolase (Cbei_4551) (Fig. 4A). Compared with the proteomic data, the translation of those down-transcript genes had no significant change in DG-8052 with and without $CaCO_3$[13]. It may indicate the sugar could be transported into the cell via non-PTS system (ABC transporter), and metabolized by glycolysis.

Amino acid biosynthesis/metabolism. Amino acids are key metabolites that reflect the intercellular status[17,25]. In general, biosynthesis of phenylalanine, tyrosine and tryptophan (cbe00400), valine, leucine and isoleucine (cbe00290) was apparently up-regulated (Fig. 3, Table 1, Additional file Table S5C). Genes involved in phenylalanine, tyrosine, tryptophan biosynthesis (Cbei_1749, Cbei_1750, Cbei_1752, Cbei_1753, Cbei_1754, Cbei_1755) were up-regulated about 10-fold at solventogenisis. Genes involved in valine, leucine and isoleucine biosynthesis (Cbei_0212, Cbei_0213, Cbei_0217) were up-regulated about 20-fold at acidogenesis, and 3–18 fold at solventogenisis, along with their transporter genes (ABC transporter genes, Cbei_1762, Cbei_1763, Cbei_1764, Cbei_1766, Cbei_1767, 4–6 fold increased), which are the side pathways from glycolysis and pyruvate, separately.

The biosynthesis of arginine and proline (cbe00330), alanine, aspartate and glutamate (cbe00250) was down-regulated. Genes involved in alanine, aspartate and glutamate (Cbei_4516, Cbei_4515, Cbei_5074) were down-regulated about 16 to 26-fold both at acidogensis and solventogenisis, which is the by-pathway of TCA. In *C. acetobutylicum*, arginine biosynthesis genes were often found to be strongly up-regulated under butyric acid stress and down-regulated under butanol stress, and it was suggested that arginine expression played an important role in metabolite stress[26,27]. In both WT-8052 and DG-8052 strains supplied with $CaCO_3$, the produced butyric acid was neutralized to form butyrate by $CaCO_3$, resulting in a balance in butyric acid and butyrate and decreased acid stress as well as improved butanol production[12,13]. It could be explained that biosynthesis of arginine and proline was down-regulated during the whole fermentation in DG-8052 when supplied with $CaCO_3$. Additionally, the prevalence of this pathway suggests that wide type *C. beijerinckii* NCIMB 8052 was accumulating proline intracellularly to act as an osmoregulator, as was shown to occur previously in other bacteria, particularly gram-positive strains[27]. However, In DG-8052, the addition of $CaCO_3$ broke down this osmoregulating balance, and the genes related in proline metabolism (cbe00330) were down-regulated.

With addition of $CaCO_3$, DG-8052 could move towards solventogenisis, and begin to form spores, during which cells required more ATP, NADH and intermediate products. The biosynthesis of amino acid is the most energetically expensive pathway. On the other hand, reduced availability of metabolic energy might be involved in the reduced supply of the 'expensive' amino acids[28]. Therefore, DG-8052 cells metabolized glucose into pyruvate, which was in turn converted into acetyl-CoA, followed by entering acids/solvents pathway instead of TCA cycle, which may cause the up-regulation of phenylalanine, tyrosine, tryptophan, valine, leucine and isoleucine, and down-regulation of arginine, proline, alanine, aspartate and glutamate (Fig. 5B).

Sporulation. The initiation of sporulation in *C. beijerinckii* 8052 was concurrent with the onset of solventogenisis. Sporulation is generally regulated by the transcription factor Spo0A both in bacilli and clostridia, which act as a switch from vegetative growth to sporulation[29,30]. Our results revealed the expression of *spo0A* (Cbei_1712) at acidogenic phase was down-regulated by 1.5 folds and further repressed at solventogenic phase 2.31 folds (Fig. 4F). Phosphorylated *spo0A* has been reported to activate transcription of some genes and represses transcription of other genes, which include the soloventogenic operon and multiple sporulation sigma factor genes in *C. acetobutylicum*[31]. Spo0A represses *abrB* gene, a repressor of sporulation genes, and initiates transcription of sporulation genes in *Bacillus sublitis* and *C. acetobutylicum*[32]. In DG-8052, there were 6 ORFs annotated as genes encoding AbrB family transcript regulator (Cbei_0088, Cbei_1757, Cbei_2219, Cbei_2270, Cbei_3375, Cbei_4885). When supplied with $CaCO_3$, Cbei_2219 had a 2.24 folds increase at acidogenic phase, while Cbei_0088 had a 3.06 folds increase at solventogenic phase, and Cbei_2270 had a 5.07 folds decrease at solventogenic phase. Although DG-8052 cells supplied with $CaCO_3$ advanced its growth to solventogenisis, no complete spores were formed. We speculate that the incomplete sporulation was caused by down-regulation of *spo0A* and different regulation of AbrB family transcript regulators.

Sigma factor SigH, encoded by *spo0H*, is the earliest acting sporulation associated sigma factor in *B. subtilis* that controls genes in the transition phase[33], and SigH regulates the expression of *spo0A*[34]. In this study, the expression of sigma-70 (Cbei_0135, Cbei_3569, Cbei_3576, Cbei_3675) were significant down-regulated in DG-8052 supplied with $CaCO_3$ at acidogenisis and at solventogenisis, where Cbei_3569 and Cbei_3576 was highly repressed by -14.28, -14.65 at 12 h and -7.33, -7.60 at solventogenisis (Additional file Table S5D), which may result from the repressed expression of SigH.

As we reported before, when supplying $CaCO_3$ to WT- 8052, the proteomic analysis showed a great increase in the expression of heat shock proteins (GrpE, $+28.1$; DnaK, $+11.7$) at early solventogensis stage[12]. A similar

increase in the transcription and expression of heat shock proteins *grpE-dnaK-dnaJ* operon (Cbei_0829, + 2.67; Cbei_0830, +4.98; Cbei_0831, +3.52) was observed at solventogensis stage of DG-8052 supplied with CaCO₃DG-8052. However, there was a down-regulation for this operon at acidogenisis stage (Cbei_0829, −2.10; Cbei_0830, −2.48; Cbei_0831, −1.56). Higher transcription and expression of *grpE-dnaK-dnaJ* operon specific at solventogenesis/sporulation may be due to the fact that CaCO₃ acts as solventogenesis-inducing substances like a quorum sensing signal and thus contributes to the spore formation[2].

Cell division and cytoskeleton. The addition of CaCO₃ could improve the sugar metabolism, growth and solvent producing to DG-8052, but not full recovery was observed with ABE fermentation by DG-8052. Although the morphology of DG-8052 cells supplied with CaCO₃ changed from non-split and elongated straight rod shape to short rod cells at acidogenic phase (Fig. 1E and F), it was not same as that of wild-type 8052 Figure (Fig. 1A and B). Incomplete recovery of DG-8052 at the presence of CaCO₃ treatment was verified by the cell division related genes expression (Fig. 4C,E, Additional file Table S5E). There was a great increase in the expression of cell division related protein FtsH (Cbei_3037), with 13.9 folds at 12 h, 16.6 folds at 24 h. In *B. subtilis*, FtsH accumulates in the mid-cell septum during vegetative cell division and at the onset of sporulation at positions near the cell poles that appear to coincide with future division sites[35]. Cell division protein FtsZ is the first protein to move to the division sites, and is essential for recruiting other proteins that produce a new cell wall between the dividing cells, FtsA and FtsK are large integral membrane proteins that coordinate chromosome segregation and cell division[36]. Our results showed at solventogenesis, FtsZ (Cbei_1118) in CaCO₃ treated DG-8052 cell was not different from untreated cell, while FtsA (Cbei_1117), FtsK (Cbei_1213), FtsY (Cbei_1173) were all down-regulated for 3.12, −3.88 and −3.01 folds, respectively. All these findings may partially explain the morphology change in CaCO₃ treated DG-8052 cells.

In gram-positive bacteria, the members of the morphogenetic system include MreB (or Mbl), MreC, MreD, PBP2 and RodA. PBP2 particularly determines rod shape, and RodA is required for the maintenance of the rod cell shape and is essential for the elongation of the lateral wall of the cell[37]. Our results showed MreB (Cbei_0491), MreC (Cbei_0492), MreD (Cbei_0494), PBP2 (Cbei_0491), and RodA (Cbei_3365) were all in down-regulation with fold change of −5.28, −2.1, −2.14, −4.42 and −3.66. respectively, at solventogenic (Additional file Table S4E). In DG-8052 supplied with CaCO₃, cells were still in rod shape at acidogenic phase, but shorten than DG-8052 cells without CaCO₃; while at 24 h, DG-8052 cells grew into solventogenic stage and began to from spore by the induction of CaCO₃ and the rod-shape-determining proteins got repressed.

Cell motility. The cell motility-related genes encoded products responsible for chemotactic responses and flagellar assembly[38]. Motility-related genes in clostridia and bacilli are usually down-regulated in sporulation cells[31]. In this study, the flagellar/chemotaxis multi-gene cluster (Cbei 4312–4302) showed no significant difference between DG-8052 cells treated with and without CaCO₃, except the increasing of Cbei_4311 (+2.51) and Cbei_4312 (+2.69). Other genes annotated as motility-related, among them, Cbei_0755 (*cheW*) was down-regulated by 3.31-fold, Cbei_4018 (*cheA*) and Cbei_4019 (*cheW*) were up-regulated by 5.80- and 2.86-fold at 24 h (Fig. 4C). The flagellar motility related genes *flgB* (Cbei_4271), *flgC* (Cbei_4270) were down-regulated at 24 h. And it was reported in *B. subtilis* the expression of the flagellar/chemotaxis operon is negatively regulated by Spo0A[39]. Spo0A (Cbei_1712) got down-regulated both at 12 h (−1.5 fold) and 24 h (−2.13 fold), and it seems the regulation effects of Spo0A to the flagellar/chemotaxis genes in *C. beijerinckii* degenerate strain were different from that of *B. subtilis*.

Validation of gene expression data from microarray analysis by Q-RT-PCR. To validate results of gene expressions obtained from microarray analysis, q-RT-PCR was applied to quantify gene expression levels in cultures of DG-8052 supplied with and without CaCO₃ at the same condition. There were 10 genes at acidogenic phase (12 h) and 10 genes at solventogenic phase (24 h) evaluated. Genes for validation were selected from three blocks: up-regulation (gene expression ratio of DG-8052 with CaCO₃ to DG-8052 > 2 folds), down-regulation (the gene expression ratio of DG-8052 with CaCO₃ to DG-8052 < 2 folds) and no remarkable regulation (the gene expression ratio of DG-8052 with CaCO₃ to DG-8052 between −2 and 2 folds). The results from two expression assays, microarray and Q-RT-PCR, showed that gene expressions in DG-8052 with CaCO₃ had a high degree of correlation at both acidogenic (R = 0.96) and solventogenic phases (R = 0.91) (Fig. 6, Additional file Figure S1, Table S1). Those genes displayed similar trends and expression levels in the microarray and q-RT-PCR analysis.

DG-8052 genome resequencing. In order to distinguish the transcriptional modifications associated to the genetic mutations from those associated to the addition of CaCO₃ during fermentation processes, the DG-8052 was genome resequenced and its results were compared to that of WT-8052. A total of 20 SNPs (17 non-synonymous, 2 premature_stop, 1 intergenic) and 16 InDels (10 coding and 6 non-coding) were found in DG-8052 genome (Table 2). The mutated genes in DG-8052 (microarray data: GSE63671) regulated by CaCO₃ were Cbei_0769 (extracellular solute-binding protein), Cbei_2569 (DNA mismatch repair protein MutS), Cbei_2653 (3-oxoacid CoA-transferase subunit B), Cbei_1662 (hypothetical protein) and Cbei_2885 (L-lactate transport). It is worth exploring those mutated genes further as they were sensitive to CaCO₃. While among those genes, there were no general or specific regulators, which may indicate the strain degeneration was not caused by the mutation of them. And the transcriptional changes of DG-8052 were affected by the addition of CaCO₃ during fermentation.

Summary. During the growth of *C. beijerinckii* DG-8052, the accumulation of acetic and butyric acid led to the decreasing of pH (<5.5), and provided stress to the cell, which may cause the cell to die rapidly. With the addition of CaCO₃, not only the produced acetic and butyric acid was neutralized from undissociated form into dissociated form, but the medium had an increased buffering capacity, which improved the viability of DG-8052
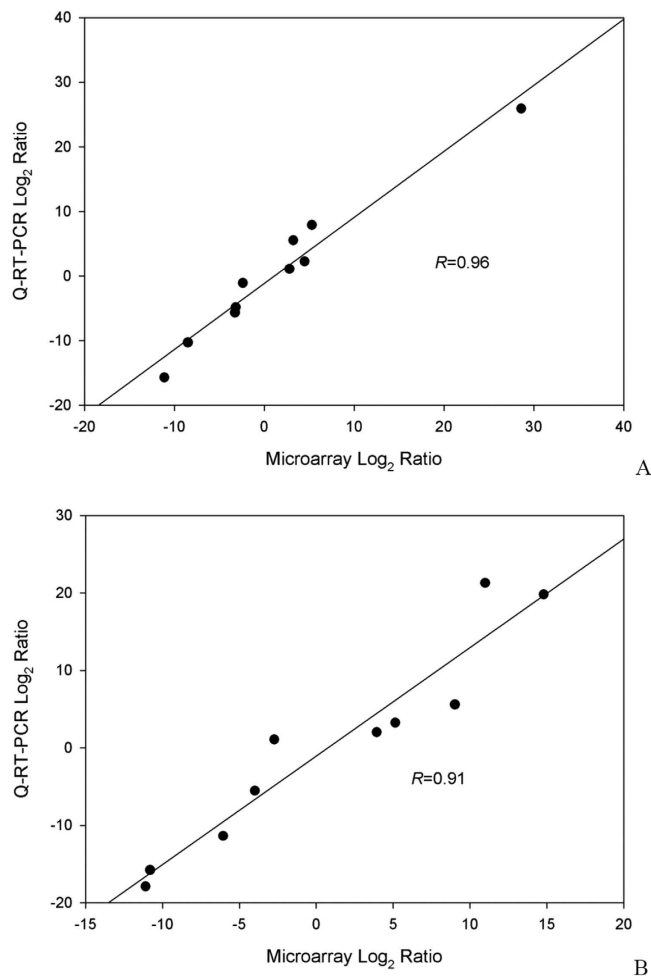
Figure 6. Validation of microarray data from *C. beijerinckii* DG-8052 at two growth stages using Q-RT-PCR. (**A**) acidogenic phase; (**B**) solventogenic phase.

cells. At the same time, the soluble Ca^{2+} maybe transmembrane and affect to the enzymes activities of some key cellular processes, such as sugar transport, butanol tolerance and solventogenesis. The transcriptional analysis showed multiple genes prominently responded to the supplementary $CaCO_3$. These genes are primarily responsible for glycolysis to solvent/acid production, solventogensis and sporulation, cell motility and division, molecular chaperones, where the specific gene target need more experiment verification. And there had 5–8% altered genes with unknown functions. Genome re-sequencing result showed that had no general or specific regulator mutated. It suggests the $CaCO_3$ associated response for intracellular metabolisms be significantly pleiotropic. This study enables us to deeply understand the multitude of regulatory function $CaCO_3$ has in clostridia, and provides valuable targets for engineered strain development of *Clostridium* species to degeneration prevention.

Methods

Bacterial strains. *Clostridium beijerinckii* NCIMB 8052 is a wild-type strain purchased from ATCC (ATCC 51743, WT-8052), and *C. beijerinckii* DG-8052, a degenerate strain of wild type NCIMB 8052, was generated as described previously[13].

Growth and fermentation. The stock culture of DG-8052 was maintained at $-75\,°C$, and revived by sequential culture in anoxic sterile tryptone glucose yeast extract (TGY) medium. Seeds cultures were inoculated into sterile TGY medium and incubated for 12 to 14 h at $35 \pm 1\,°C$ in anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, Michigan) with a modified atmosphere of 82% N_2, 15% CO_2, and 3% H_2. For batch fermentation, DG-8052 precultures in TGY medium (6%) were transferred to loosely capped 250 ml Pyrex medium bottles containing semi-defined P2 medium plus 4 g/l $CaCO_3$. The DG-8052 culture without $CaCO_3$ was used as the control. Unless otherwise stated, all fermentations were conducted in triplicate at $35 \pm 1\,°C$ for 72 h without agitation or pH control. The pH profile was monitored with a Beckman Φ500 pH meter (Beckman Coulter Inc., Brea, CA). Growth of *C. beijerinckii* was estimated using the F-7000 spectrophotometer (Hitachi High-Tech Inc., JP) to measure OD_{600}. The concentrations of fermentation products (acetate, butyrate, acetone, butanol, ethanol), were measured using a 7890 A Agilent Technologies gas chromatograph (Agilent Technologies Inc., Wilmington,

CDS region variants								
					Fold change of genes by microarray analysis			
					DG-8052 vs WT-8052		DG-8052 (CaCO$_3$+) vs DG-8052	
Gene ID	Product	Variant type	Coden	Protein change	12 h	24 h	12 h	24 h
Cbei_0769	extracellular solute-binding protein	C 935449 T	CAA → TAA	Q → stop	+2.18	+3.28	NC	−3.56
Cbei_1046	hypothetical protein	T 1255087 C	GTA → GCA	V → A	NC	NC	NC	+8.25
Cbei_1854	peptidase S8/S53 subtilisin kexin sedolisin	T 2149286 C	GTT → GCT	V → A	NC	−4.35	ND	ND
Cbei_2569	DNA mismatch repair protein MutS	C 2979111 A	GCT → GAT	A → D	NC	−2.11	NC	+5.51
Cbei_2653	3-oxoacid CoA-transferase subunit B	C 3079373 T	GCT → GTT	A → V	NC	−36.19	−3.11	ND
Cbei_2769	extracellular ligand-binding receptor	G 3218907 A	GTG → ATG	V → M	NC	NC	+368.18	ND
Cbei_3134	aldo/keto reductase	C 3665903 T	CCT → CTT	P → L	NC	NC	−2.35	NC
Cbei_4046	ethanolamine utilization protein EutJ family protein	A 4651590 G	ACT → GCT	T → A	ND	ND	+2.39	+4.25
Cbei_4761	cell wall binding repeat-containing protein	T 5556196 C	GTT → GCT	V → A	NC	NC	−2.20	NC
Cbei_0144	DNA-directed RNA polymerase subunit beta	A 182836 G	ACT → GCT	T → A	NC	NC	NC	NC
Cbei_1069	2-nitropropane dioxygenase	T 1282245 C	GTT → GCT	V → A	NC	NC	NC	NC
Cbei_1685	hypothetical protein	G 1960764 T	AAG → AAT	K → N	ND	ND	ND	ND
Cbei_1933	glucose-inhibited division protein A	A 2232660 G	AAT → GAT	N → D	NC	NC	NC	NC
Cbei_1935	PucR family transcriptional regulator	C 2234607 A	CAT → AAT	H → N	NC	NC	ND	ND
Cbei_1975	adenine deaminase	G 2295776 T	GCT → TCT	A → S	NC	ND	ND	ND
Cbei_3078	PAS/PAC sensor hybrid histidine kinase	G 3591468 T	GAA → TAA	E → stop	NC	NC	NC	NC
Cbei_4400	Serine/threonine protein phosphatase-likeprotein	G 5075403 T	CGG → CTG	R → L	NC	NC	NC	ND
Cbei_4500	esterase	A 5205979 T	AAT → AAA	N → K	NC	NC	NC	ND
Cbei_4905	glucose-1-phosphate adenylyltransferase	T 5755002 A	TTG → ATG	L → M	NC	NC	NC	NC
Cbei_1662	hypothetical protein	Ins ATCCGG after 1942328	frameshift		−5.28	−3.31	−2.78	−3.34
Cbei_4308	chemotaxis protein CheR	Del C after 4962014	frameshift		NC	NC	+2.80	NC
Cbei_2885	lactate permease	Ins T after 3369627	frameshift		+5.21	+3.71	NC	−2.74
Cbei_2830	glycoside hydrolase	Del CTACA after 3296490	frameshift		−9.94	−16.81	NC	NC
Cbei_0234	LacI family transcriptional regulator	Del AATAC after 266219	frameshift		NC	NC	+3.53	+2.50
Cbei_1790	single-stranded DNA-binding protein	Del A after 2074768	frameshift		−2.38	−2.30	NC	NC
Cbei_2386	fructose-6-phosphate aldolase	Del GAAGA after 2754381	frameshift		NC	NC	NC	+6.87
Cbei_4998	ATPase	Ins A after 5883107	frameshift		NC	NC	NC	NC
Cbei_0751	hypothetical protein	Ins T after 907959	frameshift		NC	NC	NC	NC
Cbei_2303	DNA-directed RNA polymerase sigma-70 factor	Del T after 2664570	frameshift		NC	NC	ND	ND
Intergenic region								
C 2611044 T	Intergenic of Cbei_2247 and Cbei_2248			No future found				
Ins AG after 432413	Intergenic of Cbei_0356 and Cbei_0357			No future found				
Del AA after 929926	Intergenic of Cbei_R0070 and Cbei_0765			No future found				
Del TT after 1702123	Intergenic of Cbei_1445 and Cbei_1446			No future found				
Ins TG after 2399098	Intergenic of Cbei_2060 and Cbei_2061			No future found				
Del TTT after 3043073	Intergenic of Cbei_2618 and Cbei_2619			No future found				
Del TATCTC after 5382086	Intergenic of Cbei_4644 and Cbei_4645			No future found				

Table 2. Variants identified in *Clostridium beijerinckii* DG-8052[a] and their transcriptional change during fermentation. [a]single nucleotide variations (SNVs) and InDel were identified by aligning the sequenced data with the reference genome of *C. beijerinckii* NCIMB8052. *ND, not detected, NC, no changed.

DE) equipped with a flame ionization detector (FID) and 30 m (length) x 320 m (internal diameter) x 0.50 m (HP-Innowax film) J x W 19091N-213 capillary column as described previously[12].

The cells were collected at 12 h and 24 h for the morphological observation under scanning electron microscope (Hitachi TM-1000, JP). And at the same time, cell pellets were used to purify total RNA for microarray analysis.

Total RNA purification. Cellular biomass of DG-8052 in P2 medium and P2 medium plus 4 g/l $CaCO_3$ were collected at 12 h (acidogenic phase) and 24 h (early solventogenic phase) of fermentation. Total cellular RNA was extracted from homogenized cells using RiboPure™ RNA Purification Kit, bacteria (Ambion®, Life Technologies, Inc., US). RNA concentration was measured by NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE, US). RNA quality was analyzed by 1.2% denatured formaldehyde gel electrophoresis. RNA samples for microarray hybridization had 23 S:16 S rRNA $\geq$ 2:1 and A260/A280 $\geq$ 1.80.

Comparative microarray hybridization. Generation of complementary DNA (cDNA) and its subsequent amino-allyl labelling was performed as described. Using Crystal Core® cDNA amplified RNA labelling kit (CapitalBio, Beijing, China), 1 μg of total RNA was processed to generate cDNA and labelled using 100 μM each dATP, dTTP, dGTP and 25 μM Cy3-label or Cy5-label dCTP. For two–colour microarray hybridization, Cy5 labelled DG-8052 (P2 with $CaCO_3$) cDNA and Cy3 labelled DG-8052 (P2) cDNA from the samples collected at the same time point were pooled resulting to six samples for hybridization (triplicate 12-h samples in triplicate and 24-h samples in triplicate). The microarray probes were designed with Agilent eArray software (https://earray.chem.agilent.com/earray/) based on the genomic sequence of *C. beijerinckii* NCIMB 8052. Out of 5243 annotated genes, 5168 genes were examined capturing 98.6% of the genome. The hybridization was performed by CapitalBio and Agilent Technologies (CapitalBio, Beijing, China) using a custom-made Agilent chip (15000 probes/array). To reduce technique variation, the triplicates of each *C. beijerinckii* 8052 probe sequence (60mer) together with 60 probes of negative and positive control were fabricated on each array. The samples were hybridized to six arrays and the hybridized slides were scanned using Agilent G2565CA Microarray Scanner, and scanned images were extracted and analyzed with GenePix® Pro 7 Microarray Acquisition and Analysis Software (Molecular Devices, Sunnyvale, US).

Microarray data analysis. Data generated from microarray experiments were processed using Molecular Annotation System V4.0 (CapitalBio, Beijing, China). Firstly, LOWESS (Locally Weighted Scatterplot Smoothing) intensity-based normalization was applied. For each array, gene expression ratio was calculated as dividing the Cy5 intensity (signal of DG-8052 fermented in P2 medium with $CaCO_3$) by the Cy3 intensity (signal of DG-8052 fermented in P2 medium). Expression ratio for each gene was the average ratio of replicate. To facilitate a fair comparison of up- and down-regulated genes, fold change was calculated as follows: for genes with an expression ratio $\geq$1, the fold change is the same as the expression ratio, whereas folds change of genes whose ratio is <1 equals the reciprocal of the expression ratio multiplied by −1.

The data were analyzed by pairwise and point-by-point comparison between the gene expression of DG-8052 in P2 medium with and without $CaCO_3$ using SAM (Significant Analysis of Microarrays, version 2.23b). Genes exhibiting expression changes of >2-fold and a p-value that was <0.05 were considered to be significantly altered. Enrichment analysis of Gene Ontology terms (GO), including biological process, cellular component and molecular function, and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment pathway analysis were performed using DAVID Functional Annotation Bioinformatics Microarray Analysis (DAVID Bioinformatics Resources 6.7) to identify statistically overrepresented biological terms[18,40,41].

Microarray data accession number. All protocols related to this microarray platform, which include information on probe sequences and synthesis, labelling, hybridization and scan protocols, and microarray data have been submitted to NCBI's Gene Expression Omnibus database at http://www.ncbi.nlm.nih.gov/geo/ with GEO accession number of GSE81808.

Real-time quantitative PCR (qRT-PCR). Following microarray analysis results, 10 up-regulated genes (Cbei_0311, Cbei_2826, Cbei_0795, Cbei_0870, Cbei_1113, Cbei_3472, Cbei_1108, Cbei_1255, Cbei_4644, Cbei_1130) and 10 down-regulated genes (Cbei_4123, Cbei_0331, Cbei_4871, Cbei_1221, Cbei_4669, Cbei_0411, Cbei_2261, Cbei_1227, Cbei_4406, Cbei_4491) were selected to validate microarray by q-RT-PCR. Culture samples for RNA isolation were taken as described before. RNA was isolated and RNA content was determined as described above. For qRT-PCR, primers specific to the 20 genes were designed (Additional file Table S1). These gene-specific primers were synthesized by Sangon Biotech (Shanghai, China). The 16 S rRNA of *C. beijerinckii* 8052 was selected as amplified internal standard for the qRT-PCR analysis. Prior to selection of 16 S rRNA as an internal standard, the expression of 16 s rRNA of $CaCO_3$ treated and untreated *C. beijerinckii* DG-8052 cultures was analyzed and confirmed for constant expression under the reaction condition of the study. Total RNAs (2 μg) were reverse transcribed to cDNA with the SuperScript™ III reverse transcriptase (Invitrogen corporation, Carlsbad, CA). The mRNA levels of genes of interest (Additional Table S1) were quantified by subjecting cDNA to Q-RT-PCR analysis in triplicate using a Bio-Rad iCycler continuous fluorescence detection system (Bio-Rad, Hercules, CA). The conditions were as follows: step 1, 95 °C for 2 min, step 2, 95 °C for 15 sec, step 3, 55 °C for 30 sec, 40 cycles of step 2 and 3, step 4, 95 °C for 1 min, step 5, 55 °C for 1 min, and step 6, heat from 65 °C to 95 °C with a ramp speed of 1 °C per 10 sec, resulting in melting curves. Expression levels of *C. beijerinckii* 8052 genes were quantified by the comparative C_T method as previously described[12].

Genome resequencing. DG-8052 genomic DNA was extracted using Bacterial Genomic DNa Isolation kit (B518225, Sangon Biotech, Shanghai, China) from 50 ml 12 h culture grown in P2 medium. Genomic DNA

was firstly fragmented, then adapters were ligated to the ends of fragments, and adapter-modified DNA fragments were enriched by PCR and sequenced by Illumina Hiseq 2500. Finally the filtered reads were aligned to the *Clostridium beijerinckii* NCIMB 8052 reference sequence (GenBank: NC_009617.1). This genomic DNA sequencing was analyzed by the commercial sequencing company RealGene Bio-Tec (Shanghai, China), and the sequences obtained in this study have been submitted to the Sequence Read Archive (SRA) database with accession numbers of SRP082285.

Statistical analysis. The data were analyzed by ANOVA, and $p < 0.05$ was selected prior to the experiments to reflect statistical significance. Unless otherwise stated, all results are expressed as the mean $\pm$ SD ($n \geq 3$). All the analyses were conducted using the General Linear Model (GLM) procedure of SAS Version 9.1.3 (SAS Institute Inc., Cary, NC, USA).

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S.J., J.L. and R.D. conducted the work presented here and performed data analysis; Y.Z., R.Z. and B.H. drafted the manuscript. Y.Z., C.W. and B.H. revised the overall paper; B.H. supervised the work. All authors Contributed to data interpretation, wrote and revised various parts of the paper.

Additional Information

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