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Use of Proteomic Analysis To Elucidate the Role of Calcium in Acetone-Butanol-Ethanol Fermentation by *Clostridium beijerinckii* NCIMB 8052

Bei Han,[a,c] Victor Ujor,[a] Lien B. Lai,[b] Venkat Gopalan,[b] Thaddeus Chukwuemeka Ezeji[a]

Department of Animal Sciences, The Ohio State University, and Ohio State Agricultural Research and Development Center (OARDC), Wooster, Ohio, USA[a]; Department of Chemistry and Biochemistry, and Center for RNA Biology, The Ohio State University, Columbus, Ohio, USA[b]; Department of Public Health, School of Medicine, Xi'an Jiaotong University, Xi'an, China[c]

Calcium carbonate increases growth, substrate utilization, and acetone-butanol-ethanol (ABE) fermentation by *Clostridium beijerinckii* NCIMB 8052. Toward an understanding of the basis for these pleiotropic effects, we profiled changes in the *C. beijerinckii* NCIMB 8052 proteome that occur in response to the addition of $CaCO_3$. We observed increases in the levels of different heat shock proteins (GrpE and DnaK), sugar transporters, and proteins involved in DNA synthesis, repair, recombination, and replication. We also noted significant decreases in the levels of proteins involved in metabolism, nucleic acid stabilization, sporulation, oxidative and antibiotic stress responses, and signal transduction. We determined that $CaCO_3$ enhances ABE fermentation due to both its buffering effects and its ability to influence key cellular processes, such as sugar transport, butanol tolerance, and solventogenesis. Moreover, activity assays *in vitro* for select solventogenic enzymes revealed that part of the underpinning for the $CaCO_3$-mediated increase in the level of ABE fermentation stems from the enhanced activity of these catalysts in the presence of Ca^{2+}. Collectively, these proteomic and biochemical studies provide new insights into the multifactorial basis for the stimulation of ABE fermentation and butanol tolerance in the presence of $CaCO_3$.

Growing concerns over increased emissions of greenhouse gases from the combustion of fossil fuels and the global energy crisis have recently spawned extensive research into renewable energy. As a result, there is a resurgent interest in butanol as an alternative fuel, due mainly to its higher energy content than ethanol and its compatibility with gasoline, with the latter trait making it more compatible with existing pipelines for distribution (1, 2). However, the cost of butanol production, which currently relies on petroleum feedstock, is not favorable compared to gasoline (3). Although acetone-butanol-ethanol (ABE) fermentation with solventogenic *Clostridium* species holds promise as a potentially cheaper means of butanol production, low yields and productivity due to butanol toxicity to the fermenting cells have hampered the commercialization of biobutanol (4, 5).

To increase yield and productivity, fermentation broth additives such as acetate (6, 7) and calcium carbonate (3, 8, 9) have been successfully utilized. During ABE fermentation by solventogenic *Clostridium* species, $CaCO_3$ has been shown to stimulate sugar utilization, butanol production, and butanol tolerance (3, 8, 9). For example, during *Clostridium acetobutylicum* fermentation, the addition of 8 g/liter butanol (to mimic solvent intolerance) limited xylose utilization to 30 g/liter (from a starting concentration of 60 g/liter); however, upon the addition of $CaCO_3$ (10 g/liter), xylose utilization increased to 43 g/liter (8). Similarly, when ABE fermentation was conducted in an iron-deficient medium, which modifies carbon and electron flow to favor early butanol accumulation, the xylose utilization by *C. acetobutylicum* was drastically inhibited; this effect was significantly reversed by the addition of 10 g of $CaCO_3$/liter (8). In both cases, better sugar utilization resulted in increased butanol production.

To engender these physiological changes, Ca^{2+} must exert a wide range of effects on the cellular machinery of solventogenic bacteria. Toward uncovering such mechanisms, we first undertook a characterization of the proteome of *Clostridium beijerinckii* NCIMB 8052 (referred to here as *C. beijerinckii*) without and with exogenous $CaCO_3$. We used two-dimensional (2-D) gel electrophoresis and mass spectrometry to profile and identify differentially expressed proteins; as support for the proteomic data, changes in the mRNA levels of some of these candidates were validated by real-time quantitative PCR (qRT-PCR). We discuss changes in levels of proteins that could potentially impact the biosynthetic machinery, butanol stress tolerance, cell division, and glucose utilization. Second, to assess the extent to which the buffering capacity of $CaCO_3$ contributes to its stimulatory effects on ABE fermentation, we evaluated the effects of a range of carbonates on butanol production and sugar utilization by *C. beijerinckii*. Our results indicate that the pronounced pleiotropic effects of $CaCO_3$ exceed its buffering capacity and likely reflect its ability to mediate cellular signaling events. Finally, by measuring the activity of key solventogenic pathway enzymes in the absence and presence of Ca^{2+}, we found that Ca^{2+} also modestly enhances their activities *in vitro*, and this might provide an unexpected indirect mechanism *in vivo* to alter the pH of the medium. We integrate these results to highlight the multifactorial basis for the Ca^{2+}-induced increase in ABE fermentation.

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Address correspondence to Thaddeus Chukwuemeka Ezeji, ezeji.1@osu.edu.
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MATERIALS AND METHODS

Fermentation and culture conditions. *C. beijerinckii* NCIMB 8052 (ATCC 51743) was obtained from the American Type Culture Collection, Manassas, VA. Laboratory stocks were routinely maintained as spore suspensions in sterile, double-distilled water at 4°C. Spores (200 μl) were heat shocked for 10 min at 75°C and cooled on ice prior to inoculation into 10 ml of anoxic presterilized tryptone-glucose-yeast extract (TGY) medium. To create anaerobic conditions and generate anoxic TGY medium, loosely capped bottles with sterilized TGY medium were kept for 24 h in an anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, MI) with a modified atmosphere of 82% N_2, 15% CO_2, and 3% H_2. Cultures were incubated for 12 to 14 h at 35°C ± 1°C under anaerobic conditions for inoculum buildup, as described elsewhere previously (4, 5). This was followed by the transfer of 8 ml of the actively growing inoculum into 92 ml anoxic presterilized TGY medium. The culture was incubated until it reached an optical density at 600 nm (OD_{600}) of 0.9 to 1.1 (4 to 5 h).

To determine the optimal $CaCO_3$ concentration for ABE production and for subsequent proteomic studies, fermentations were conducted for 72 h in semidefined P2 medium (4, 5) supplemented with 2, 4, 6, 8, or 10 g/liter of $CaCO_3$. Precultures grown in TGY medium (6%) were transferred into loosely capped 250-ml Pyrex medium bottles containing P2 medium plus $CaCO_3$ (2, 4, 6, 8, or 10 g/liter). P2 medium without $CaCO_3$ was used as the negative control. Unless otherwise stated, all fermentations were conducted in triplicate, and the temperature was maintained at 35°C ± 1°C, without agitation or pH control. The pH profile was monitored with a Beckman Φ500 pH meter (Beckman Coulter Inc., Brea, CA). The growth of *C. beijerinckii* was estimated by using a DU800 spectrophotometer (Beckman Coulter Inc., Brea, CA) to measure the OD_{600}, which was converted to cell dry weight by using a predetermined correlation (4). The concentration of glucose was analyzed by using a hexokinase- and glucose-6-phosphate dehydrogenase-coupled enzymatic assay, as described previously (5). The concentrations of the fermentation products acetate, butyrate, acetone, butanol, and ethanol were measured by using an Agilent Technologies 7890A gas chromatograph (Agilent Technologies Inc., Wilmington, DE) equipped with a flame ionization detector (FID) and a 30 μm (length) by 320 μm (internal diameter) by 0.50 μm (HP-Innowax film) J x W 19091N-213 capillary column, as described previously (10).

Protein extraction. To investigate the effect of $CaCO_3$ on the proteome of *C. beijerinckii*, precultures grown in TGY medium (6%) were transferred, as described above, into $CaCO_3$-supplemented (4 g/liter) (treatment) and unsupplemented P2 medium in triplicate. Samples for protein extraction were taken from the control (P2 medium) and treatment (P2 medium plus 4 g/liter $CaCO_3$) cultures in the early solventogenic phase, when cultures were producing the same amount of butanol (~5.5 g/liter for 12 h and 24 h for treatment and control cultures, respectively). Sampling at this concentration ensured that cells for protein extraction were actively growing and in the solventogenic phase (when solventogenic enzymes are fully expressed). Cells were harvested from 300-ml cultures by centrifugation at 10,000 × *g* at 4°C for 15 min, washed twice with sterile distilled water, and stored overnight at −70°C. Cell pellets were then sent to Kendricks Laboratory Inc., Madison, WI, where two-dimensional (2-D) polyacrylamide gel electrophoresis, image analysis, and mass spectrometry were conducted.

Cells were disrupted by vigorous vortexing with intermittent freezing in 600 μl of osmotic lysis buffer (9.5 M urea, 2% [wt/vol] nonyl phenoxypolyethoxylethanol [NP-40], 2% ampholines [pH 4 to 9], 5% [vol/vol] β-mercaptoethanol) containing 1% (vol/vol) protease inhibitor cocktail {20 mM AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride], 1 mg/ml leupeptin, 0.36 mg/ml E-64 cysteine protease inhibitor, 500 mM EDTA, and 5.6 mg/ml benzamidine}, nucleases to final concentrations of 50 μg/ml (RNase) and 100 μg/ml (DNase) in 10 mM Tris-HCl (pH 7) and 5 mM $MgCl_2$, a phosphatase inhibitor (EMD Biosciences, Darmstadt, Germany), and 100 mg of washed glass beads (with a mesh size of 425 to 6,000 μm; catalog number G9268; Sigma). This was followed by the addition of 300 μl of sodium dodecyl sulfate (SDS) boiling buffer (62.5 mM Tris [pH 6.8], 10% [vol/vol] glycerol, 2.3% [wt/vol] SDS, 50 mM dithiothreitol). The samples were then heated in a boiling water bath for 5 min before the protein concentration was determined by using a bicinchoninic acid (BCA) assay kit (Pierce Chemical Co., Rockford, IL) (11). The samples were then diluted to 6 mg/ml in a 1:1 solution of SDS boiling buffer-lysis (sample) buffer, both of which are described above.

Proteomics. Six hundred micrograms of each crude protein extract was resolved in the first dimension (isoelectric focusing [IEF]) on a thin-tube gel with an inner diameter of 3.3 mm containing 2% ampholines with a pH range of 4 to 9 (Serva, Heidelberg, Germany) at 1,000 V for 20 h, according to methods described previously by O'Farrell (12) and Burgess-Cassler et al. (13). In the second dimension, samples were separated and visualized on 18.5-cm-wide dried-slab gels by using Coomassie blue R-350. Tropomyosin (1 μg) was added to each sample as an IEF internal standard prior to loading. Duplicate gels were run and analyzed for each protein sample from the treated and control cultures. Gel images were captured with a quantitative densitometer (GE Imagescanner III; GE Healthcare, Piscataway, NJ) and analyzed by using Progenesis Same Spots software (version 4.0; Nonlinear Dynamics, Durham, NC) and Progenesis PG240 software (version 2006; Nonlinear Dynamics, Durham, NC), such that all spots of interest and all varying spots were outlined, quantified, and matched on all the gels. The densitometer was tested for linearity prior to scanning with a calibrated neutral-density filter set (Melles Griot, Irvine, CA). The relative amount of each spot was computed by the software as a function of the intensity of each individual spot in comparison to the intensity of the entire gel. Spots of interest were sequenced by matrix-assisted laser desorption ionization–time of flight tandem mass spectrometry (MALDI-TOF-MS/MS). Jvirgel 2.0 (14) (http://www.jvirgel.de/) was used to identify the pI of proteins, followed by validation with the EXPASY Bioinformatics Resource Portal (http://expasy.org/).

qRT-PCR. Following protein profiling, four proteins (Cbei_0830, Cbei_1049, Cbei_0829, and Cbei_2831) that showed increased levels and five proteins (Cbei_2110, Cbei_1273, Cbei_0476, Cbei_0422, and Cbei_2146) that were detected in smaller amounts in response to $CaCO_3$ treatment were selected for validation by qRT-PCR. Most of these proteins were selected largely due to previous reports on their putative involvement in solventogenesis and/or the response to butanol stress; however, we stress that their role in Ca^{2+}-mediated signaling has not been explored. Culture samples for RNA isolation were taken as described above for protein extraction. RNA was isolated by using the Qiagen RNA isolation kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. The RNA content was determined spectrophotometrically by the use of a NanoDrop 3300 spectrophotometer (Thermo Scientific, Wilmington, DE). For qRT-PCR, primers specific for the Cbei_0830, Cbei_1049, Cbei_0829, Cbei_2831, Cbei_2110, Cbei_1273, Cbei_0476, Cbei_0422, Cbei_2146, and 16S rRNA genes were designed (Table 1). These gene-specific primers were synthesized by Eurofins MWG Operon (Huntsville, AL).

The stable 16S rRNA gene was used as a reference for comparing transcript levels of *C. beijerinckii* grown in the presence (treatment) and the absence (control) of $CaCO_3$ at similar phases in their growth. We are aware that there might be variations in 16S rRNA levels at different stages in a bacterial growth curve, although this phenomenon did not occur in our case. The 16S rRNA qRT-PCRs were performed as internal controls to normalize the quantity of RNA inputs. Total RNAs (2 μg) were reverse transcribed to cDNA with SuperScript III reverse transcriptase (Invitrogen Corporation, Carlsbad, CA), according to the supplier's protocol. The resulting cDNA was used in qRT-PCRs using the DyNAmo HS SYBR green quantitative PCR (qPCR) kit (New England BioLabs Inc., Ipswich, MA). Quantitative real-time PCR (RT-PCR) analysis was conducted in triplicate by using the Bio-Rad iCycler continuous fluorescence detection system (Bio-Rad, Hercules, CA). The RT-PCR conditions were as follows: 15 min at 95°C and then 40 cycles of 10 s at 95°C and 30 s at 55°C, followed by heating from 55°C to 95°C with a ramp speed of 1°C per 10 s. This resulted in melting curves. The expression levels of all the tested genes

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TABLE 1 Sequences of oligonucleotides used for amplification of target genes

Gene (protein)[a]	Primer (sequence)
Cbei_R0001 (16S rRNA)	16S-F (5′-GAAGAATACCAGTGGCGAAGGC-3′) 16S-R (5′-ATTCATCGTTTACGGCGTGGAC-3′)
Cbei_2110 (ArsA)	ArsA-F (5′-GGGCTTGAAAACGTAAGAGC-3′) ArsA-R (5′-CTCCGCCTTTACCCATTGTA-3′)
Cbei_1273 (hypothetical protein)	hp1-F (5′-ATTAGAGCAGGCGTTTTGGA-3′) hp2-R (5′-AACCTTCTTTCAGCCGCATA-3′)
Cbei_0476 (hypothetical protein)	hp2-F (5′-GCAAGCAGGCTTCTAGCATT-3′) hp2-R (5′-CAGAAATGCAAATTGCTCCA-3′)
Cbei_0422 (SpoIID)	spoIID-F (5′-TGATACAATTACTGCCAAG-3′) spoIID-R (5′-GTTTTTCCAGAGCTGGTTGC-3′)
Cbei_2146 (PdaA)	pdaA-F (5′-CATGAGAAGGATTGGCATCA-3′) pdaA-R (5′-TGGTAAAGCCTTCGCTGTTT-3′)
Cbei_0830 (DnaK)	dnak-F (5′-ACTAAGGATGCAGGTAAG-3′) dnaK-R (5′-CACCACCTAAGTCATAAA-3′)
Cbei_1049 (ESBP)	espb-F (5′-TTGGGAGTAAGCTTGGTTGC-3′) esbp-R (5′-TGGAACTATTTCCCCGTTCTC-3′)
Cbei_0829 (GrpE)	grpE-F (5′-GCTGTTGCAGCTGATGGTAG-3′) grpE-R (5′-CCCTTTTGGAAAACTTCTGC-3′)
Cbei_2831 (CBP)	cbp-F (5′-GCAGCAACAACAAAAGCAGA-3′) cbp-R (5′-GGGTTATATGGTGCTGTTCC-3′)

[a] ESBP, extracellular solute-binding protein.

were standardized against the expression level of the internal control gene (16S rRNA) (10).

Effect of carbonates on *C. beijerinckii* ABE production. To evaluate effects of different carbonates on ABE production by *C. beijerinckii*, batch fermentations were conducted in 250-ml Pyrex screw-cap medium bottles, as previously described (4, 5, 15), with minor modifications. Pyrex screw-cap medium bottles containing 150 ml P2 medium were supplemented with 2 g/liter $CaCO_3$, $(NH_4)_2CO_3$, NH_4HCO_3, K_2CO_3, $NaHCO_3$, and Na_2CO_3. These carbonates were added to a final concentration of 2 g/liter, because some of the tested compounds were toxic above this concentration. Unless otherwise stated, all fermentations were conducted in triplicate or more, the temperature was maintained at 35°C ± 1°C, and no agitation or pH control was used. Samples (5 ml) were withdrawn at 12-h intervals to measure the cell concentration, pH, glucose concentration, and ABE concentration.

Effect of Ca^{2+} on *C. beijerinckii* NCIMB 8052 growth and ABE production. To test whether the stimulatory effects associated with $CaCO_3$ treatment could in part be ascribed to Ca^{2+}, ABE production by *C. beijerinckii* was measured in cultures supplemented with $CaCO_3$ and $CaCl_2$ (0.5 g/liter each) in 250-ml Pyrex screw-cap bottles. Fermentation was carried out as described above. Samples (5 ml) were collected at 12-h intervals to measure the cell concentration, ABE concentration, glucose concentration, pH, and acid levels. The growth of *C. beijerinckii* was estimated by using a DU800 spectrophotometer (Beckman Coulter Inc., Brea, CA) to measure the OD_{600}, which was converted to cell dry weight by using a predetermined correlation (4). The ABE and acid (acetic and butyric) concentrations were measured by using an Agilent Technologies 7890A gas chromatograph, as previously described (10). The yield was defined as total grams of ABE produced per total grams of glucose utilized.

Effect of calcium on EDTA-treated *C. beijerinckii* fermentations. To further assess the potency of calcium in enhancing ABE fermentation in *C. beijerinckii*, a TGY-grown preculture was subcultured in 1 mM EDTA-containing P2 medium. After 4 h of incubation, the following additions were made in triplicate: solution X (0.5 g/liter $CaCl_2$), solution Y (0.23 g/liter total of $FeSO_4$-$MnSO_4$-$MgSO_4$ at a ratio of 2:3:40), and solution Z (0.5 g/liter $CaCl_2$ plus 0.23 g/liter total of $FeSO_4$-$MnSO_4$-$MgSO_4$ at a ratio of 2:3:40). Cultures in P2 medium without the addition of EDTA or metal ion and those containing 1 mM EDTA were used as controls. To further probe whether the effects of $CaCO_3$ on ABE fermentation resulted from its buffering capacity, we supplemented EDTA-treated cultures with non-buffering $CaCl_2$ (in place of $CaCO_3$ as a source of Ca^{2+}).

Determination of intracellular Ca^{2+} concentrations in *C. beijerinckii*. In alkaline buffer, Ca^{2+} reacts in a concentration-dependent fashion with *o*-cresolphthalein complexone to form a purple color that absorbs at 575 nm, and this observation has been employed extensively for the quantification of Ca^{2+} (16, 17). Fifty milliliters of *C. beijerinckii* was harvested at 12-h intervals during the course of a 36-h batch fermentation in P2 medium supplemented with 0.5 g/liter Ca^{2+}. P2 medium without $CaCO_3$ was used as a control. Cells were collected by centrifugation at 10,000 × *g* for 5 min, followed by washing twice with double-distilled H_2O and drying at 60°C for 2 to 3 days. Dried *C. beijerinckii* cells (20 to 40 mg) were suspended in 1 ml of 10% (vol/vol) HCl and incubated overnight at 30°C to break the cells. This was followed by centrifugation at 8,000 × *g* for 15 min at 4°C. The resulting supernatant was used to determine the intracellular Ca^{2+} concentration in *C. beijerinckii* cells according to the method of Dorey and Draves (17).

Enzyme activity assays. Cell extracts of *C. beijerinckii* were assayed for solventogenic enzyme (coenzyme A transferase [CoAT], acetate kinase [AK], acetoacetate decarboxylase [ACDC], butyraldehyde dehydrogenase [BDDH], and butanol dehydrogenase [BDH]) activities. *C. beijerinckii* cells were harvested at 24 h of batch fermentation in semidefined P2 medium and centrifuged at 8,000 × *g* for 5 min at 4°C. These pellets were suspended in their respective buffers [50 mM MOPS (morpholinepropanesulfonic acid) (pH 7), 500 mM $(NH_4)_2SO_4$, and 20% (vol/vol) glycerol for CoAT; 100 mM Tris-HCl (pH 7.6) for AK, BDDH, and BDH; and 50 mM sodium acetate (pH 5) for ACDC]. Aliquots (~2 to 3 ml; 1.8 mg [dry weight]) of *C. beijerinckii* cells were frozen at −70°C and thawed on ice, followed by the addition of 2 mg/ml lysozyme and incubation at 37°C for 1 h. Complete *C. beijerinckii* lysis was accomplished by passing the resulting suspension through a TissueLyser LT instrument (Qiagen Inc., Valencia, CA) for 8 min at a setting of 5,000. The mixture was clarified by centrifugation at 14,000 × *g* for 5 min at 4°C. Cell extracts were transferred into fresh sterile tubes and used immediately for enzyme assays. The protein concentration was estimated by using Bradford reagent according to the manufacturer's protocol (Amresco, Solon, OH).

To determine possible effects of Ca^{2+} on the activities of the selected enzymes, assays were conducted with the addition of $CaCl_2$ to a final concentration of 2 mM to the assay mixture. The CoAT assay was carried out according to previously described methods based on the extinction coefficient of acetoacetyl-CoA (8 mM^{-1} cm^{-1}) at 310 nm (6, 18). One unit of CoAT activity was defined as the amount required for the disappearance of 1 μmol acetoacetyl-CoA per min per mg protein. The AK activity assay was conducted by using a combination of the hydroxamate methods described previously by Rose (19) and Chen and Blaschek (6), at an end product molar extinction coefficient of 0.691 mM^{-1} cm^{-1} at 540 nm. ACDC activity was determined as described previously (20). One unit of ACDC activity was defined as the amount of CO_2 (in microliters) produced per minute per mg protein. BDDH and BDH activities were assayed at 340 nm, as described previously (21, 22). One unit was defined as the amount of enzyme required to oxidize 1 μmol of NADH per min with butyryl-CoA or butyraldehyde as the substrate.

We assayed 6-phospho-β-glucosidase as described previously by Setlow et al. (23). *C. beijerinckii* cells grown with and without 4 g/liter $CaCO_3$, as described above for the proteomic study, were harvested from 1 ml of culture by centrifugation and resuspended in 200 μl of phosphate-buffered saline. A clear cell lysate was generated by bead beating (100 μl of 0.1-mm zirconia/silica beads; Biospec Products, Bartlesville, OK) and centrifugation at 18,000 × *g* for 5 min. Glycerol was then added to a final concentration of 10% (vol/vol). The 6-phospho-β-glucosidase in these

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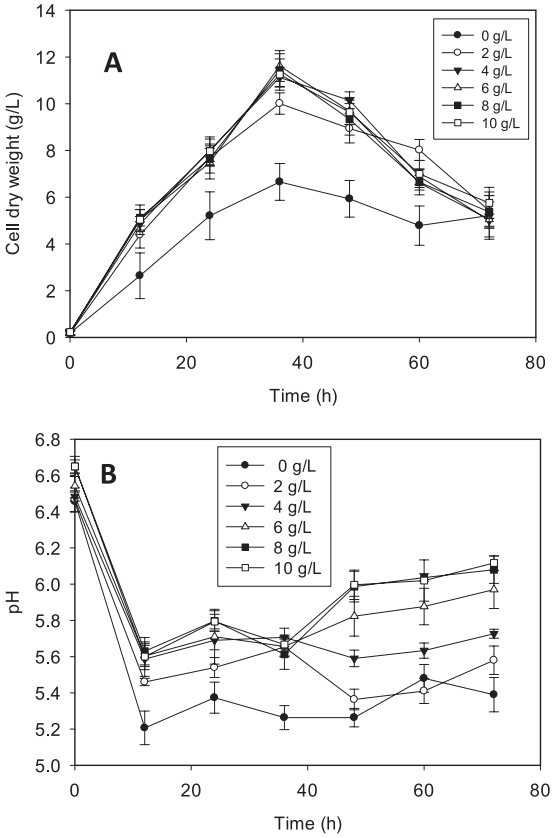

FIG 1 Comparative cell dry weight (A) and pH (B) profiles of *C. beijerinckii* ABE fermentation in P2 medium supplemented with the indicated concentrations of $CaCO_3$.

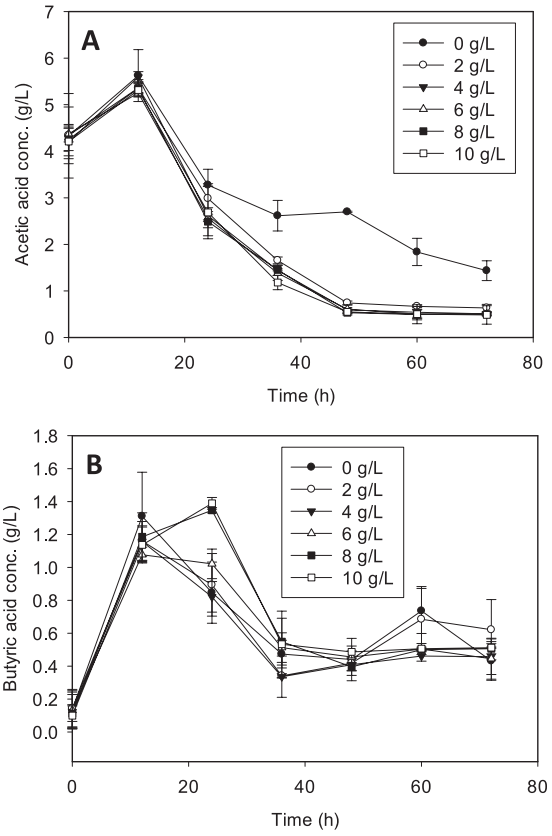

FIG 2 Acetic (A) and butyric (B) acid concentrations in the fermentation medium of *C. beijerinckii* grown in P2 medium and P2 medium supplemented with the indicated concentrations of $CaCO_3$.

cell extracts was assayed at 37°C with the substrate 4-methylumbelliferyl-β-D-glucopyranoside-6-phosphate (MUG-P), a generous gift from Barbara and Peter Setlow (University of Connecticut Health Center, Farmington, CT). Time course reactions (50 μl per reaction mixture containing 50 mM potassium phosphate [pH 7.4] and 1 mM MUG-P) were initiated by the addition of crude extracts. Reactions were quenched with 50 μl of 1.4 M K_2CO_3. The fluorophore 4-methyl-umbelliferone (4-MU) (excitation/emission at 365 nm/455 nm) produced in these assays was measured with an Infinite M1000Pro instrument (Tecan Austria GmbH). The product formed in these assays was calculated by using a 4-MU standard curve as a reference and was used to determine initial velocities. Specific activities were calculated based on the total protein in the crude extract, which was determined by using the Bradford protein assay (24) with bovine serum albumin as the standard.

Statistical analyses. All fermentation experiments were performed under anaerobic conditions at least in triplicate ($n \geq 3$). Multiple one-way analyses of variance (ANOVAs) were conducted to evaluate the effects of different concentrations of $CaCO_3$ (2 to 10 g/liter) (independent variable) in P2 medium on the growth of and sugar utilization, ABE production, acid production, and assimilation (dependent variable) by *C. beijerinckii*. All statistical analyses were performed by using the general linear model (GLM) procedure of SAS statistical software, version 9.1.3 (SAS Institute Inc., Cary, NC). Differences among means were separated by using the Student-Newman-Keuls test procedure ($P \leq 0.05$).

RESULTS

$CaCO_3$-induced modulation of *C. beijerinckii* ABE fermentation: effects on medium pH, cell density, glucose utilization, and ABE production. To determine the dose-dependent effects of $CaCO_3$ on *C. beijerinckii* ABE fermentation and to determine the optimum $CaCO_3$ concentration for proteomic studies, we supplemented P2 medium with 2, 4, 6, 8, or 10 g/liter of $CaCO_3$ and measured changes in pH, cell density, glucose utilization, and ABE production relative to values for cultures grown in P2 medium without $CaCO_3$. The addition of $CaCO_3$ led to the rapid growth of *C. beijerinckii* cells, resulting in at least a 50% increase in cell dry weight (Fig. 1A). At >2 g/liter $CaCO_3$, the cell densities observed for the treated cultures were nearly indistinguishable. We observed a dose-dependent modulation of the pH of the medium by $CaCO_3$. With the exception of cultures supplemented with 2 g/liter $CaCO_3$, the pH of $CaCO_3$-treated cultures was consistently higher than ~5.6 for the entire duration of fermentation, whereas the pH of the control cultures ranged from 5.2 to 5.5, following the initial pH decline 12 h after inoculation (Fig. 1B). The total acid (acetic and butyric) levels detected in both treated and untreated cultures were reflective of the respective pH profiles observed. While no significant difference in butyric acid concentrations was observed between treated and untreated cultures, acetic acid levels decreased significantly with treatment (Fig. 2). In fact, the incorporation of $CaCO_3$ led to 2-fold (2 g/liter $CaCO_3$) and 3-fold (4, 6, 8, and 10 g/liter $CaCO_3$) decreases in acetic acid concentrations compared to concentrations in control cultures, thus providing a possible basis for the increased pH which we observed.

As with cell growth, glucose utilization by *C. beijerinckii* was rapid during growth in P2 medium supplemented with $CaCO_3$. As

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TABLE 2 Complete list of proteins whose levels showed significant increases and partial list of proteins with lower levels when *C. beijerinckii* cells were grown in P2 medium supplemented with $CaCO_3$[a]

Protein	Function or description	Fold change	*P* value (*t* test)
YP_001307969.1	Heat shock protein GrpE	28.1	0.001
YP_001307970.1	DnaK/molecular chaperone (Hsp)	11.7	0.015
YP_001308102.1	Sigma 54 factor interaction domain protein	7.7	0.029
YP_001309935.1	Carbohydrate-binding protein	7.6	0.017
YP_001307580.1	Integral membrane histidine kinase	4.4	0.004
YP_001307648.1	Conserved hypothetical protein	4.2	0.006
YP_001308374.1	Glycoside hydrolase	3.9	0.001
YP_001308398.1	NNDP[b]	2.6	0.012
YP_001307436.1	Hypothetical protein	2.5	0.027
YP_001310550.1	Histidine kinase/DNA gyrase/Hsp90	2.4	0.005
YP_001307689.1	Putative transketolase	2.2	0.020
YP_001308203.1	Peptidase	1.9	0.006
YP_001311102.1	6-Phospho-β-glucosidase	1.8	0.016
YP_001307790.1	Thiamine biosynthesis protein	1.8	0.003
YP_001310387.1	Hypothetical protein	−11.5	0.034
YP_001307566.1	Stage II sporulation protein D	−6.7	0.001
YP_001311201.1	Pullulanase/glycoside hydrolase	−6.1	0.003
YP_001309441.1	Rubrerythrin/rubredoxin	−4.9	0.007
YP_001311728.1	Polysaccharide deacetylase	−4.7	0.003
YP_001309233.1	Hypothetical protein[c]	−4.3	0.032
YP_001309233.1	Arsenite-activated ATPase	−4.2	0.004
YP_001310303.1	Chloramphenicol *O*-acetyltransferase	−4.1	0.005
YP_001307470.1	Putative 8-oxoguanine DNA glycosylase	−3.2	0.020
YP_001308366.1	LacI; regulatory protein	−2.6	0.034

[a] See Table S3 in the supplemental material for a complete list of proteins whose levels decreased when the culture was grown in the presence of $CaCO_3$.
[b] NNDP, nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase.
[c] Nucleoside triphosphate hydrolase conserved domain.

a result, the addition of ≥4 g/liter $CaCO_3$ to the fermentation medium resulted in maximum glucose utilization within a shorter period by *C. beijerinckii*. In effect, glucose was used up by cultures grown in the presence of ≥4 g/liter $CaCO_3$ (4, 6, 8, and 10 g/liter) in 60 h, whereas 17.8 g/liter residual glucose was detected in the control experiments 72 h after inoculation. Additionally, no significant difference in glucose utilization was observed among *C. beijerinckii* cells grown in P2 medium containing ≥4 g/liter $CaCO_3$ (data not shown). The increased glucose utilization with the addition of $CaCO_3$ translated into higher ABE yields and productivity. A yield of 0.36 g of ABE/g of glucose was determined for the untreated cultures. Conversely, the addition of $CaCO_3$ resulted in ABE yields of 0.43, 0.44, 0.42, 0.41, and 0.41 g/g with 2, 4, 6, 8, and 10 g/liter of $CaCO_3$, respectively. The increased yield was attributable to a relatively shorter fermentation time, thereby minimizing ABE losses and facilitating better acid uptake and conversion to ABE. Similarly, while the ABE productivity level in the control experiments was 0.22 g/liter/h, the average productivity level in cultures grown in the presence of $CaCO_3$ was 0.42 g/liter/h, a 2-fold increase compared with the level in control cultures.

Comparative proteomic analysis of Ca^{2+}-treated *C. beijerinckii*. We profiled the global response of *C. beijerinckii* to $CaCO_3$ (4 g/liter) at the proteome level using 2-D gel electrophoresis. The choice of 4 g/liter was dictated by findings from our dose-dependent studies of $CaCO_3$ establishing the threshold for the maximal increase in cell growth (Fig. 1), superior glucose utilization, enhanced assimilation of acetic acid (Fig. 2), and improved ABE yields and productivity (see Table S1 in the supplemental material). Since 87% of proteins of known function and 93% of solventogenic enzymes in *C. beijerinckii* NCIMB 8052 have pIs of between 4 and 9 (see Tables S2A and S2B in the supplemental material), we focused on this pH range for isoelectric focusing. A total of 540 protein spots were resolved from pH 4 to 9, of which 96 proteins displayed differences in levels in the Ca^{2+}-treated cultures compared to the controls (fold change ≥1.7; $P < 0.05$). While one-fifth (of these 96 proteins) showed an increase, the remainder was detected in smaller amounts.

Proteins which showed increased levels are involved predominantly in the heat shock response (13%) (DnaK and GrpE); DNA synthesis, transcription, and repair (27%) (GHKL [gyrase B/topoisomerase VI, Hsp90, histidine kinases, and MutL], nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase, and transketolase); and carbohydrate metabolism/transport (20%) (carbohydrate-binding protein [CBP] with a glycoside hydrolase conserved domain, a glycoside hydrolase, and 6-phospho-β-glucosidase) (Table 2 and Fig. 3B). Proteins with the GHKL (DNA mismatch repair proteins) conserved domain

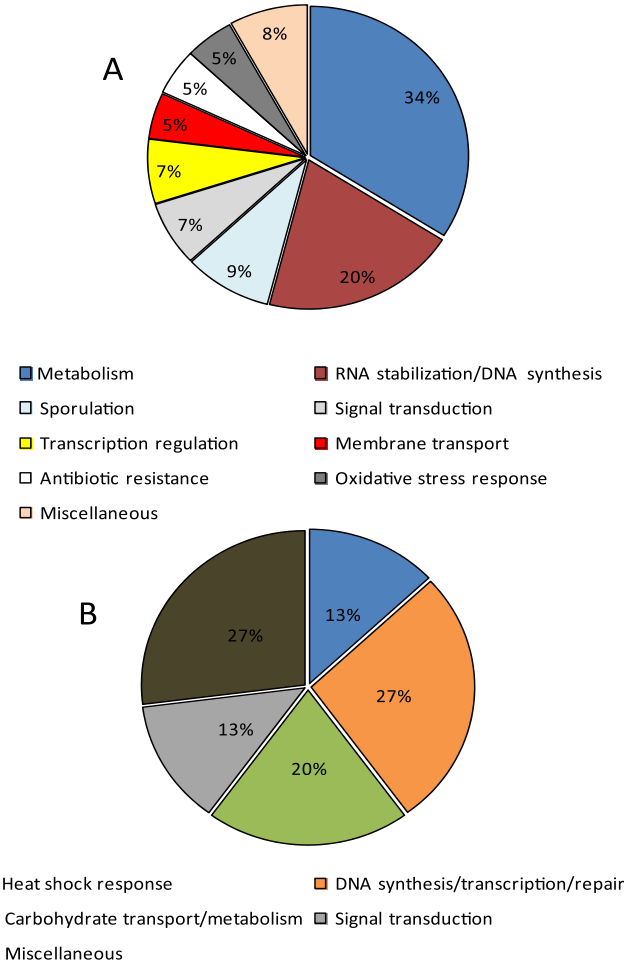

FIG 3 Functional classification of proteins whose levels decreased (A) or increased (B) in *C. beijerinckii* NCIMB 8052 cells grown in Ca^{2+}-supplemented P2 medium relative to growth in P2 medium.

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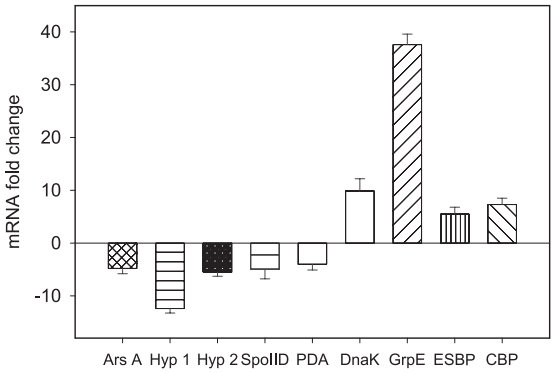

FIG 4 qRT-PCR analysis to illustrate changes in the expression levels of selected genes in Ca^{2+}-treated *C. beijerinckii* NCIMB 8052 cells at 12 h of fermentation relative to levels in untreated cells at 24 h. Relative expression levels confirmed the downregulation of genes encoding an arsenite-activated ATPase (ArsA), two hypothetical proteins (Hyp 1 and Hyp 2), stage II sporulation protein D (SpoIID), and polysaccharide deacetylase (PDA). The genes coding for DnaK, GrpE, an extracellular solute-binding protein (ESBP), and a carbohydrate-binding protein (CBP) also mirrored the increases in levels observed for their respective proteins.

typically comprise ATPases which perform at least one of the highlighted functions.

Figure 3A shows the functional classification of proteins whose levels decreased with $CaCO_3$ treatment, most of which are involved in carbohydrate metabolism (34%); the disruption of RNA secondary structures (which normally accumulate under stress conditions); DNA repair (20%); sporulation (9%); signal transduction (7%); transcription regulation (7%); antibiotic (5%) and oxidative stress (5%) responses; and membrane transport (5%) (see Table S3 in the supplemental material for a full list).

Independent confirmation of the findings from proteomic studies. While we realize that posttranscriptional regulation can often complicate correlations between mRNA and protein levels, we undertook quantitative RT-PCR analysis of nine selected genes to determine if we could confirm mRNA level changes for nine of the differentially expressed proteins. Samples for proteomic and mRNA analyses were taken at the same butanol concentration (~5.5 g/liter), which corresponds to 12 h for the $CaCO_3$ treatment and 24 h for the control. There was a striking agreement between the changes at the mRNA and protein levels, corroborating the patterns of protein expression (Fig. 4 and Table 2). For example, the levels of carbohydrate-binding protein, *dnaK*, and *grpE* mRNAs increased 7-, ~10-, and 38-fold, respectively, in the $CaCO_3$-supplemented cultures relative to the controls (Fig. 4); this mirrors the ~8-, 12-, and 28-fold changes in their respective protein amounts. Additionally, the mRNA levels of polysaccharide deacetylase and stage II sporulation protein D decreased by 4- and 5-fold, respectively, upon $CaCO_3$ treatment; these changes are consistent with the 5- and 7-fold decreases established for the respective proteins in our proteomic studies.

To independently verify the proteomic changes using an enzyme assay, we focused on the 1.8-fold change in the 6-phospho-β-D-glucosidase level (Table 2). In many bacteria (e.g., *Bacillus subtilis*), cellobiose and aryl-β-D-glucosides are taken up by the phosphotransferase systems, with the subsequent intracellular accumulation of either cellobiose 6-phosphate or an aryl-phospho-β-D-glucoside (23). For these glucosides to be subjected to glycolysis, these phosphoglucosides need to be hydrolyzed by a phospho-β-D-glucosidase. We observed in our proteomic studies that Ca^{2+} increased the levels of a phospho-β-glucosidase by 1.8-fold. Our enzyme assays using 4-methylumbelliferyl-β-D-glucopyranoside-6-phosphate as the substrate indeed confirmed that Ca^{2+} elicits a 1.6-fold increase in the level of phospho-β-glucosidase activity; the specific activities from two independent experiments (each involving initial velocity determinations in triplicate) were 2.15 ± 0.23 and 1.34 ± 0.08 nmol/min/mg with and without Ca^{2+}, respectively.

Delineating the dual contribution of $CaCO_3$ to ABE fermentation by *C. beijerinckii*. Recognizing that the effects of $CaCO_3$ on ABE fermentation could be due to its buffering capacity and/or other cellular effects mediated by Ca^{2+} signaling, we sought to delineate this dual contribution even though we realized that this objective is complicated by the peculiarity of solventogenic clostridia (see below). To hone in on only the contribution of buffering, we first compared the effects of different carbonates. Subsequently, we compared $CaCO_3$ and $CaCl_2$, with the expectation that $CaCl_2$, which is incapable of buffering, would only be able to mimic the signaling effects mediated by Ca^{2+}.

To determine whether the effects of $CaCO_3$ on ABE fermentation were due solely to its buffering capacity, *C. beijerinckii* cells were grown for 72 h individually with each carbonate [$(NH_4)_2CO_3$, NH_4HCO_3, K_2CO_3, $NaHCO_3$, Na_2CO_3, and $CaCO_3$]. While total ABE levels increased by 1.6-fold with $CaCO_3$, the other carbonates enhanced ABE levels by only 1.3-fold (Table 3); in fact, $CaCO_3$ elicited a 2.5-fold increase in the acetone level, while the highest change elicited by any other carbonate was 1.6-fold ($NaHCO_3$). These results attest to the favorable effect on ABE conferred by the buffering capacities of different carbonates.

The addition of $CaCl_2$ to the medium used for *C. beijerinckii* ABE fermentation clearly demonstrated that the effects of $CaCO_3$ do not stem solely from its buffering effect. In fact, the addition of 0.5 g/liter $CaCl_2$ to the medium also resulted in increased total ABE production (approximately 17 g/liter) (see Fig. S2 in the supplemental material). Similarly, the total solvent in the cultures treated with 0.5 g/liter $CaCO_3$ was approximately 15 g/liter, whereas the unsupplemented cultures produced less than 14 g/liter total ABE (see Fig S2 in the supplemental material). The moderately more favorable effects on ABE generation by 0.5 g/liter $CaCl_2$ than by $CaCO_3$ are due largely to the former's higher cellular uptake (see below and Fig. 6). However, when the $CaCO_3$ concentration was increased to 4 g/liter, 25.5 g/liter total ABE was produced (see Table S1 in the supplemental material). We could not test the effect of 4 g/liter $CaCl_2$ on ABE fermentation because

TABLE 3 List of carbonates tested on *C. beijerinckii* and their corresponding effects on ABE production

Treatment (2 g/liter)	Mean product concn (g/liter) ± SD[a]			
	Acetone	Ethanol	Butanol	Total ABE
Control	$3.01^{A} \pm 0.92$	$0.12^{A} \pm 0.08$	$11.47^{A} \pm 0.96$	$14.6^{A} \pm 1.1$
Na_2CO_3	$3.91^{AB} \pm 0.28$	$0.16^{A} \pm 0.05$	$12.03^{A} \pm 0.38$	$16.1^{B} \pm 0.49$
K_2CO_3	$3.4^{A} \pm 0.19$	$0.18^{A} \pm 0.03$	$12.48^{AB} \pm 0.09$	$16.06^{B} \pm 0.3$
$(NH_4)_2CO_3$	$4.75^{B} \pm 0.22$	$0.31^{B} \pm 0.11$	$13.43^{BC} \pm 0.26$	$18.49^{C} \pm 0.52$
$NaHCO_3$	$4.76^{B} \pm 0.38$	$0.29^{B} \pm 0.01$	$13.8^{C} \pm 0.22$	$18.85^{C} \pm 0.61$
NH_4HCO_3	$4.58^{B} \pm 0.31$	$0.29^{B} \pm 0.09$	$13.34^{C} \pm 0.37$	$18.21^{C} \pm 0.66$
$CaCO_3$	$7.66^{C} \pm 0.81$	$0.33^{B} \pm 0.07$	$15.5^{D} \pm 0.83$	$23.49^{D} \pm 1.2$

[a] Means with same superscript letters are not significantly different.

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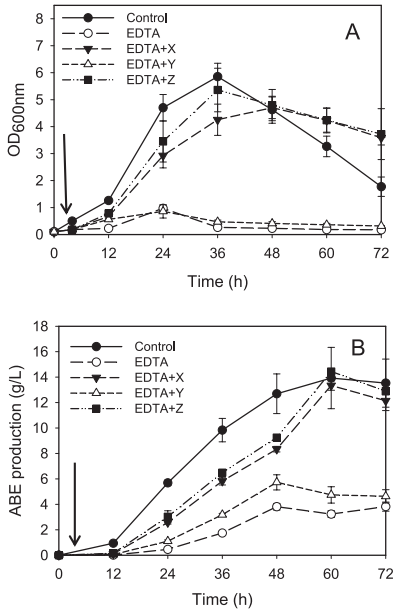

FIG 5 Biomass (A) and total ABE (B) profiles of *C. beijerinckii* fermentation in EDTA-supplemented P2 medium following the addition of solutions X (0.5 g/liter Ca^{2+}), Y (0.23 g/liter total Fe^{2+}, Mn^{2+}, and Mg^{2+} at a ratio of 2:3:40), and Z (0.5 g/liter Ca^{2+} plus 0.23 g/liter total Fe^{2+}, Mn^{2+}, and Mg^{2+} at a ratio of 2:3:40). Arrows indicate the addition of solutions X, Y, and Z at 4 h of fermentation.

concentrations above 0.5 g/liter were found to be toxic to *C. beijerinckii* NCIMB 8052 due to its bacteriostatic and bactericidal effects on the microorganism (data not shown).

Calcium-mediated recovery of fermentation in EDTA-treated *C. beijerinckii*. The assumption that Ca^{2+} elicits key mechanisms which favorably affect ABE fermentation in *C. beijerinckii* was demonstrated by the patterns of growth and solvent production in EDTA-treated cultures to which either Ca^{2+}; Fe^{2+}, Mg^{2+}, and Mn^{2+}; or both were subsequently added, relative to those of the untreated cultures. Fe^{2+}, Mn^{2+}, and Mg^{2+} are known to be involved in glycolysis and ABE synthesis. EDTA (1 mM) inhibited *C. beijerinckii* growth and solventogenesis (Fig. 5A). The addition of Fe^{2+}, Mg^{2+}, and Mn^{2+} to a total concentration of 0.23 g/liter was unable to alleviate this EDTA-induced inhibition; higher concentrations of Fe^{2+}, Mg^{2+}, and Mn^{2+} are toxic. However, the addition of 0.5 g/liter Ca^{2+} increased both cell growth and solventogenesis to the levels observed for the untreated cultures (approximately 14 g/liter ABE) (Fig. 5B). Of further interest is the observation that the addition of Ca^{2+} alone, i.e., without Fe^{2+}, Mg^{2+}, and Mn^{2+}, abolished (within 1 h) the EDTA-induced inhibition of growth and solventogenesis, suggesting that recovery following the addition of both $Ca^{2+}$and Fe^{2+}, Mg^{2+}, and Mn^{2+} is mediated largely by Ca^{2+}.

Calcium uptake by *C. beijerinckii*. Using a fluorescence assay, we investigated if there is an increase in intracellular Ca^{2+} levels in *C. beijerinckii* when it is grown in Ca^{2+}-supplemented medium compared to regular P2 medium. Indeed, Ca^{2+}-treated cells had a 3-fold-higher intracellular Ca^{2+} concentration than the untreated cells at 36 h of fermentation (6.5 mg versus 2 mg Ca^{2+}/g cell dry weight) (Fig. 6). The much higher intracellular Ca^{2+} concentrations in the $CaCl_2$- and $CaCO_3$-treated cells strongly suggest that Ca^{2+} plays specific physiological/cellular roles during ABE fermentation, which may account for the stimulatory effects on solventogenesis that were previously ascribed to the buffering capacity of $CaCO_3$.

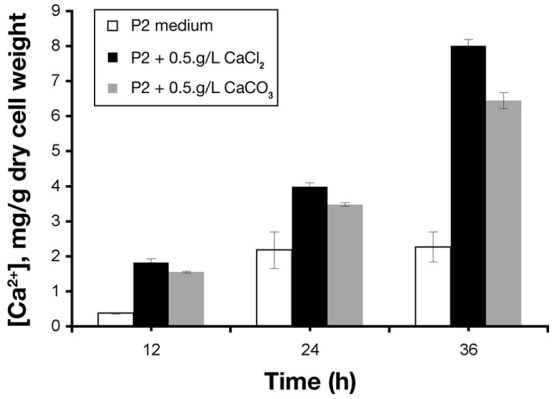

FIG 6 Intracellular Ca^{2+} concentrations in *C. beijerinckii* cells during ABE fermentation in P2 medium without or with supplementation of either $CaCl_2$ or $CaCO_3$.

Ca^{2+} increases activities of selected solventogenic enzymes of *C. beijerinckii*. To test the hypothesis that Ca^{2+} enhances the activities of important enzymes involved in ABE production, aliquots of cell extracts of *C. beijerinckii* were assayed for CoAT, AK, ACDC, BDDH, and BDH activities with or without 2 mM Ca^{2+}. We observed 2.3-, 1.2-, and 1.4-fold increases in activity for CoAT, AK, and ACDC, respectively, when 2 mM Ca^{2+} was included in the assay mixtures. A negligible difference in activity was observed for BDH and BDDH upon Ca^{2+} supplementation in their respective assay mixtures (Fig. 7).

DISCUSSION

While the role of Ca^{2+} as a universal messenger involved in signaling and regulating a range of cellular processes in eukaryotes is well appreciated (25–27), the emerging picture in bacteria reveals some similarities but also unanticipated roles (28–33). There is a growing body of evidence implicating Ca^{2+} in cell division, spo-

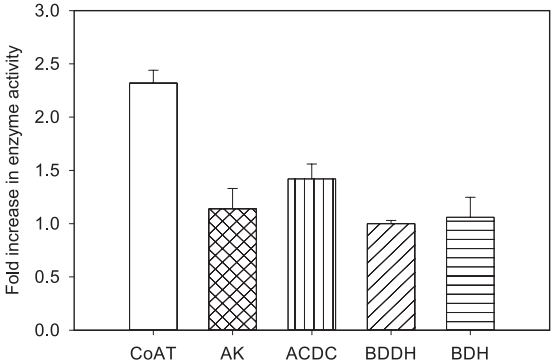

FIG 7 Ca^{2+}-induced increases in the activities of coenzyme A transferase (CoAT), acetate kinase (AK), and acetoacetate decarboxylase (ACDC) in cell extracts of *C. beijerinckii*. No changes in activity were observed for butyraldehyde dehydrogenase (BDDH) or butanol dehydrogenase (BDH). Increases in activity are expressed as fold differences between the Ca^{2+}-supplemented cell extracts and the unsupplemented extracts. The basal activities measured in the unsupplemented cell extracts (without Ca^{2+}) were 0.22 (CoAT), 0.07 (AK), 4.56 (ACDC), 0.01 (BDDH), and 0.91 (BDH) U/mg protein/min.

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rulation, the maintenance of cell structure, chemotaxis, and DNA replication in bacteria (29, 31, 34). In this study, we investigated how $CaCO_3$ increases butanol production, sugar utilization, and butanol tolerance by solventogenic *Clostridium* species (3, 8, 9). We examined whether these multifaceted effects of Ca^{2+} on solventogenesis are mediated through changes in gene expression levels and/or by other means (e.g., direct changes in the activities of enzymes). The findings from the present study are grouped under different attributes.

Solventogenesis. Given the favorable effects of $CaCO_3$ on ABE fermentation, it was hypothesized that Ca^{2+} induced the expressions of key enzymes in the clostridial solventogenic pathway. Although this premise is not supported by our proteomic data, we uncovered, somewhat unexpectedly, that Ca^{2+} increases the activities of CoAT, AK, and ACDC by 2.3-, 1.2-, and 1.4-fold, respectively (Fig. 7). Since CoAT is central to the uptake of acetic and butyric acids (which in turn is needed for subsequent conversion to butanol), and it is also the rate-limiting enzyme for acetone production (35), we have now identified a novel basis for the favorable solventogenesis upon the uptake of Ca^{2+} present in the medium. The increase in the CoAT activity is consistent with the marked decrease in acetic acid (and increased pH) levels in the Ca^{2+}-treated cultures (Fig. 2A). We postulate that the activities of a broader range of enzymes/proteins (other than those involved in solventogenesis) are also likely to be positively influenced by Ca^{2+} *in vivo*.

Variations in pH exert a major effect on the outcome of ABE fermentation (1, 36, 37). This is ascribed primarily to the severe toxicity of acids produced during acidogenesis (38); as a result, during the exponential phase, it is critical to maintain cell growth within a favorable pH range to achieve significant cell density and considerable acid accumulation for optimal solvent production in the latter stages (36, 37). By using a two-stage controlled-pH strategy, where fermentation was maintained at pH 5.5 during acidogenesis, Guo et al. (37) previously achieved a ~40% (20.3 g/liter) increase in ABE production when using *C. acetobutylicum* XY16. Likewise, Bryant and Blaschek (36) previously reported significant increases in cell growth and ABE production (10 g/liter) by incorporating acetate-, carbonate-, citrate-, and phosphate-based buffers in P2 medium when using *C. acetobutylicum*.

In the present study, the addition of ≥4 g/liter $CaCO_3$ to the fermentation medium resulted in pH values typically >pH 5.6 (Fig. 1B). In parallel, we observed significant increases in cell growth (Fig. 1A), glucose utilization, and ABE production (25.5 g/liter) (see Table S1 in the supplemental material). We infer that the pH profile resulting from treatment with ≥4 g/liter $CaCO_3$ (pH-buffering effect), which may have been influenced in part by the interplay between Ca^{2+} and CoAT activities, leading to increased acetic acid assimilation (and, consequently, increases in pH) and contributing to the positive effects of $CaCO_3$. This observation echoes previous reports which documented how the efficient buffering of ABE fermentation modulates the equilibrium between dissociated and undissociated acids by favoring the accumulation of the former, which are less toxic to solventogenic clostridia and are more readily utilized for solvent production (1, 36).

Moreover, $CaCO_3$ dissolves sparingly in water and in a pH-dependent manner (39), an attribute that likely favors ABE fermentation. For instance, at pH 6, the solubility of $CaCO_3$ in water is roughly 2 g/liter; however, a decrease in pH promotes the dissolution of $CaCO_3$ with concomitant proton consumption and, thus, alkalinization, a process thought to be critical for natural buffering in coral reefs (39). Hence, the addition of $CaCO_3$ in excess of 2 g/liter can serve as a buffer reservoir, which responds to the characteristic decreases in pH during ABE fermentation. This is underscored by the slightly reduced cell density (an average of ~11% in the earlier stages of growth) (Fig. 1A), decreased assimilation of acetic acid (~20% on average) (Fig. 2A), and reduced pH-buffering capacity (Fig. 1B) observed with 2 g/liter $CaCO_3$ relative to ≥4 g/liter $CaCO_3$, where the observed positive effects on *C. beijerinckii* ABE fermentation appeared to plateau above 4 g/liter.

Although we chose to compare $CaCO_3$ and $CaCl_2$ under the assumption that the latter lacks the natural buffering capacity of the former, we believe that this is an oversimplification, since the biological effects of Ca^{2+}-mediated signaling also encompass pH changes in the medium. Calcium chloride (like $CaCO_3$) will stimulate CoAT activity; in fact, this is likely to be partly responsible for the increased ABE fermentation supported by 0.5 g/liter $CaCl_2$. As a consequence, there will be increased acetic acid assimilation, which in turn will increase the pH of the medium. Taking into consideration the idiosyncratic switch of clostridial growth from acidogenic to solventogenic phases, our findings suggest that explaining pH changes purely on the merits of the additive's buffering capacity alone might underestimate ripple effects from changes in the activities of solventogenic enzymes, which in turn have implications for the pH of the medium.

Butanol toxicity and glucose utilization. The chaotropic effect of butanol on the cell membrane (and the attendant effects on the pH gradient) is largely the underlying basis for its toxicity. Previous studies, which showed that Ca^{2+} promotes glucose/xylose utilization while lowering butanol toxicity, speculated that Ca^{2+} might have stabilizing effects on membrane proteins, particularly xylose and glucose permeases, which are inhibited by 8 and 12 g/liter butanol, respectively (8, 40). Although this is certainly a possibility, we stress that the P2 medium used for ABE fermentation contains Fe^{2+}, Mn^{2+}, and Mg^{2+}, and these cations are unable to confer any Ca^{2+}-like protective traits during solventogenesis (Fig. 5). Either Fe^{2+}, Mn^{2+}, and Mg^{2+} are unable to mimic Ca^{2+} in exerting a stabilizing effect on the membrane/membrane proteins, or, in addition to its stabilizing effect on membranes and/or membrane proteins, the ability of Ca^{2+} to trigger a network of cellular events is the underpinning for its distinctive ability to alleviate butanol toxicity. Although we favor the latter idea, we first consider the former.

The binding of Ca^{2+} and Mg^{2+} to the negatively charged surface lipopolysaccharides (LPS) in Gram-negative bacteria generates ionic cross bridges, which exert a stabilizing effect on the outer cell membrane (41–44). Notwithstanding variations in the cell wall/membrane makeup and structure between Gram-positive and Gram-negative bacteria (45), we considered whether the formation of Ca^{2+}-mediated cross bridges in the Gram-positive bacterium *C. beijerinckii* might partly contribute to the effects of Ca^{2+} on ABE fermentation. However, such a premise is not supported by our observation that Fe^{2+}, Mn^{2+}, and Mg^{2+} are poor mimics of Ca^{2+} in enhancing ABE fermentation (Fig. 5).

Although *C. beijerinckii* cells grown in the presence of $CaCO_3$ showed elevated levels of growth and glucose utilization (see Table S1 in the supplemental material), glycolytic proteins were not detected in larger amounts upon Ca^{2+} treatment, suggesting that the barrier to glucose utilization is not its ca-

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tabolism and that a roadblock elsewhere is somehow being circumvented in the presence of Ca^{2+}. Most likely, impaired sugar transport is the limiting factor for glucose utilization, especially during growth in the presence of butanol. In this regard, we stress that levels of a carbohydrate-binding protein (YP_001309935.1) with a glycoside hydrolase conserved domain, a glycoside hydrolase (YP_001308374.1), and 6-phospho-β-glucosidase (YP_001311102.1) (Table 2), all involved in carbohydrate metabolism and transport, increased significantly (Table 2; see also Fig. S1 in the supplemental material). In fact, since 6-phospho-β-glucosidase is involved in cellobiose metabolism, and our growth substrate in the medium was glucose, we were surprised by this ~2-fold increase and thus confirmed the same through enzyme activity assays. These data also furnish the first experimental evidence for the presence of this enzyme in *C. beijerinckii* NCIMB 8052. Broadly, these results suggest that Ca^{2+} (preemptively) sets the stage for metabolic fine-tuning that is not necessarily based on the growth substrate; in fact, this inference has already inspired us to examine the likely favorable effects of Ca^{2+} on cellobiose utilization by *C. beijerinckii* NCIMB 8052.

Butanol tolerance. The most important conclusions from the proteomic study are the Ca^{2+}-induced 12- and 28-fold increases in the levels of DnaK and GrpE, respectively (Table 2); while the former is a molecular chaperone and heat shock protein (HSP) (member of the Hsp70 family), the latter is a nucleotide exchange factor which facilitates the efficient release of DnaK-bound nonnative protein substrates and aids in the recycling of DnaK. Modest increases in butanol concentrations induce the heat shock response (46), and Hsp70 chaperones have been shown to participate in the repair of aberrant proteins and the prevention of stress-mediated damage to proteins (47); hence, DnaK and GrpE will be crucial when butanol accumulates to high concentrations in the fermentation broth.

Since samples for protein extraction were taken at the same butanol concentration (~5.5 g/liter) for both $CaCO_3$-treated and untreated cultures, we contend that the pronounced increases in the amounts of DnaK and GrpE are not due to butanol stress but rather to a Ca^{2+}-mediated response, albeit as a secondary effect. The addition of ≥4 g/liter $CaCO_3$ to the *C. beijerinckii* fermentation medium evoked rapid growth, leading to at least a 1.7-fold increase in cell density (Fig. 1A) compared to that of the control; in fact, similar effects were reported previously for Ca^{2+} in *Escherichia coli* (48, 49) and *Rhizobium fredii* (50). Increased rates of cell replication are accompanied by increases in levels of DNA and protein synthesis (51), which amplify flux through the biosynthesis machinery, often associated with the upregulation of chaperones to prevent protein misfolding or to enhance the refolding of misfolded proteins, among other functions (51–53). Taken together, we conclude that Ca^{2+} elicits rapid cell replication in *C. beijerinckii*, thereby triggering attendant increases in the levels of proteins involved in DNA and protein processing and stabilization. Therefore, the Ca^{2+}-induced increase in DnaK/GrpE levels upon an increase in cell replication and the associated stabilization of the biosynthesis and growth machinery might account for the robust growth of *C. beijerinckii* NCIMB 8052 in the presence of $CaCO_3$; such an expectation is further supported by the finding that DnaK plays a key role in DNA replication and cell division (54). Additionally, increased levels of HSPs have been shown to improve butanol tolerance and/or production in *E. coli* (2, 55), *Lactobacillus plantarum* (56), and *C. acetobutylicum* (57, 58), often with improved substrate utilization.

In the aerobic Gram-positive bacteria *Bacillus subtilis* and *Streptococcus pneumoniae*, Ca^{2+} in fact represses the expression of the *dnaK* and *groE* operons by increasing the levels of the class I HSP repressor *hrcA*, which in turn binds to the conserved *cis*-active CIRCE (controlling inverted repeat of expression) element required for the expression of class I HSPs (59, 60). Although *hrcA* lies upstream of the *hsp70* gene cluster on the *C. beijerinckii* genome (*hrcA-grpE-dnaK-dnaJ*), a search of the promoter regions of the upregulated *grpE* and *dnaK* genes with the CIRCE element sequence (TTAGCACTC-N_9-GAGTGCTAA) (57) did not produce a significant match. Most likely, the expression of the class I HSPs in *C. beijerinckii* follows a different regulatory path in comparison to its aerobic relatives. An in-depth understanding of the molecular mechanisms involved in the increased gene expression levels of *dnaK* and *grpE* in Ca^{2+}-treated *C. beijerinckii* NCIMB 8052 cells will prove instructive. Also of further interest is the increased expression level of an ATPase (histidine kinase/DNA gyrase/Hsp90) with the GHKL conserved domain (Table 2), found in DNA gyrase B, topoisomerase VI, Hsp90, histidine kinases, and MutL (61). Proteins carrying the GHKL conserved domain, which have also been associated with increased rates of cell replication (62), have been implicated in the refolding of damaged polypeptides; signal transduction; DNA replication, repair, transcription, and recombination; and genome stabilization (61, 63, 64), which would also be crucial during growth under conditions of butanol stress.

Several other findings from our proteomic study suggest that Ca^{2+} mitigates stress through unknown mechanisms. In the presence of Ca^{2+}, we found decreases in the levels of DNA/RNA helicases (2-fold for YP_001307495.1 and 3-fold for YP_001309141.1) (see Table S3 in the supplemental material) and rubrerythrin/rubredoxin (5-fold for YP_001309441.1) (see Fig. S1 in the supplemental material), which is involved in the anaerobic oxidative stress response. Under conditions of abiotic stress, helicases function as nucleic acid chaperones by disrupting stable misfolded RNA structures, thereby restoring their normal function (65, 66). They are also central to the DNA repair machinery, especially during heat shock or oxidative stresses (66, 67), which often phenocopy butanol stress (2). Similarly, rubrerythrin has been shown to participate in the detoxification of reactive oxygen species in anaerobic bacteria (68) and exhibits superoxide dismutase activity *in vitro* (69). In *E. coli* (2) and *C. acetobutylicum* (70), the upregulation of the oxidative stress response machinery is among the strongest effects during butanol exposure, perhaps because both oxidative and alcohol stresses primarily cause the membrane potential to collapse (2, 57, 71).

Additional lines of evidence support our premise that Ca^{2+} alleviates stress caused by solventogenesis. First, the observed protein profile of *C. beijerinckii* in the presence of Ca^{2+} agrees with the transcription profile of *C. acetobutylicum* overexpressing GroESL (a heat shock protein), wherein stress- and metabolism-related genes were downregulated, with concomitant increases in butanol production, sugar utilization, and cell growth (57). Also, a recent study which compared the proteomes of wild-type *C. acetobutylicum* and an Rh8 mutant strain which has higher butanol tolerance and yields showed that chaperones and solventogenesis enzymes were upregulated in the mutant compared to the wild type (72). While the nature of the mutation in strain Rh8 is unknown, we

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highlight a thematic parallel with our Ca^{2+}-induced changes and the shared outcomes. Second, sporulation is an independent stress indicator during the onset of solventogenesis and the subsequent accumulation of butanol in *C. beijerinckii* and *C. acetobutylicum*; sporulation is initiated upon the expression/activation of a transcription factor encoded by the *spo0A* gene (73–75). We observed decreases in levels of sporulation-related proteins (YP_001307345.1, YP_001307566.1, and YP_001307539.1) (Table 2; see also Fig. S1 and Table S3 in the supplemental material) in *C. beijerinckii* NCIMB 8052 cells grown in the presence of $CaCO_3$, even though samples for protein extraction were taken at the same butanol concentration for both treated and control cultures. Finally, while several genetic and biochemical factors dictate biofilm formation, solvent stress is a positive effector (76); in this regard, it is notable that *C. beijerinckii* cells grown in the presence of $CaCO_3$ showed 5- and 2-fold decreases in levels of polysaccharide deacetylase and diguanylate cyclase, respectively (see Fig. S1 and Table S3 in the supplemental material), two enzymes that facilitate biofilm formation due to their roles in exopolysaccharide deacetylation and the generation of the bis-(3′-5′)-cyclic dimeric GMP (c-di-GMP) biofilm-signaling molecule, respectively (77). Collectively, these data, which indicate the absence of stress-related sequelae, support the idea that Ca^{2+} treatment eases the stress on *C. beijerinckii* during solventogenesis.

In conclusion, while pH-buffering effects may contribute to the stimulatory effects of $CaCO_3$ on ABE fermentation, Ca^{2+} has broader effects at the cellular and protein levels, which directly (through the enhancement of CoAT activity) and indirectly (signaling) amplify the growth of *C. beijerinckii* and promote acid assimilation and ABE production. Collectively, the stabilizing mechanisms of HSPs, increased DNA synthesis and replication, and the direct enhancement of the activities of key solventogenic enzymes in the presence of Ca^{2+} contribute to robust cell replication and solventogenesis during ABE fermentation. However, specific analyses of the various aspects of this complex global response to Ca^{2+} are required to facilitate a rational reconstruction of *C. beijerinckii* ABE fermentation for improved butanol production.

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