Metabolic engineering of *Caldicellulosiruptor bescii* yields increased hydrogen production from lignocellulosic biomass

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Abstract

**Background:** Members of the anaerobic thermophilic bacterial genus *Caldicellulosiruptor* are emerging candidates for consolidated bioprocessing (CBP) because they are capable of efficiently growing on biomass without conventional pretreatment. *C. bescii* produces primarily lactate, acetate and hydrogen as fermentation products, and while some *Caldicellulosiruptor* strains produce small amounts of ethanol *C. bescii* does not, making it an attractive background to examine the effects of metabolic engineering. The recent development of methods for genetic manipulation has set the stage for rational engineering of this genus for improved biofuel production. Here, we report the first targeted gene deletion, the gene encoding lactate dehydrogenase (*ldh*), for metabolic engineering of a member of this genus.

**Results:** A deletion of the *C. bescii* L-lactate dehydrogenase gene (*ldh*) was constructed on a non-replicating plasmid and introduced into the *C. bescii* chromosome by marker replacement. The resulting strain failed to produce detectable levels of lactate from cellobiose and maltose, instead increasing production of acetate and H₂ by 21-34% relative to the wild type and ΔpyrFA parent strains. The same phenotype was observed on a real-world substrate—switchgrass (*Panicum virgatum*). Furthermore, the *ldh* deletion strain grew to a higher maximum optical density than the wild type on maltose and cellobiose, consistent with the prediction that the mutant would gain additional ATP with increased acetate production.

**Conclusions:** Deletion of *ldh* in *C. bescii* is the first use of recently developed genetic methods for metabolic engineering of these bacteria. This deletion resulted in a redirection of electron flow from production of lactate to acetate and hydrogen. New capabilities in metabolic engineering combined with intrinsic utilization of lignocellulosic materials position these organisms to provide a new paradigm for consolidated bioprocessing of fuels and other products from biomass.

**Keywords:** *ldh*, Metabolic engineering, Switchgrass, Biohydrogen, *Caldicellulosiruptor*

Background

Fuel production from plant biomass offers the opportunity to generate energy from a sustainable feedstock, reduce dependence on petroleum, and reduce the negative environmental impact of increased CO₂ emissions. The major obstacle in the use of lignocellulosic feedstocks is the recalcitrance of the biomass itself. Plants have evolved to resist deconstruction by microbes, and plant cell wall components such as cellulose, hemicellulose, and lignin play a major role in recalcitrance [1-3]. Industrial conversion of plant biomass to fuels currently relies on thermal and chemical treatment of biomass to remove hemicellulose and lignin, followed by enzymatic hydrolysis to solubilize the plant cell walls to generate a fermentable substrate for fuel-producing organisms [4-6]. However, these methods add cost, produce hydrolysates that are toxic to microorganisms [7] and are destructive to the sugars in the biomass [8]. An alternative approach is to use consolidated bioprocessing (CBP), in which the fermentative organism is also responsible for production of the biomass-solubilizing...
Thermococcales morphiles such as can produce similar amounts [17-19], some hyperthermophiles such as ethanol or butanol production to reoxidize electrons being routed to lactate or H₂. Thus, production of acetate is obligately coupled to H₂ production to allow reoxidation of NADH and ferredoxin. While enteric bacteria such as Enterobacter aerogenes, Enterobacter cloacae and Escherichia coli produce 1 ~ 2 moles of H₂ per mole of glucose [15,16], and Clostridium spp. can produce similar amounts [17-19], some hyperthermophiles such as Thermococcales spp., Pyrococcus furiosus, Thermotogales spp., and Caldicellulosiruptor spp. produce about 3 ~ 4 moles of H₂ per mole of glucose [20-25]. Enterics typically utilize a formate-H₂ lyase and Clostridium spp. use a ferredoxin-dependent hydrogenase to avoid the thermodynamically unfavorable formation of H₂ from NADH, instead using other pathways such as ethanol or butanol production to reoxidize NADH. Thermotoga (and likely all the above-mentioned hyperthermophiles), on the other hand, use an electron bifurcating hydrogenase [26] to simultaneously oxidize NADH and ferredoxin to produce H₂, using the thermodynamically favorable oxidation of ferredoxin to drive the unfavorable oxidation of NADH. This bifurcating hydrogenase allows a theoretical maximum yield of 4 moles of H₂ per mole of glucose, whereas wild type E. coli has a theoretical maximum of 2 moles of H₂ per mole of glucose. C. bescii encodes a putative bifurcating hydrogenase (Cbes1295-1299). This, combined with the native ability of C. bescii to catabolize plant biomass and the newly developed genetic transformation system, makes C. bescii a compelling platform to engineer high yield H₂ production directly from plant biomass without conventional pretreatment.

We hypothesized that by developing the necessary tools to delete genes from the C. bescii chromosome [14,27,28], we would enable metabolic engineering to increase H₂ production. Here, we demonstrate the utility of gene deletion in the pyruvate metabolic pathway for rational strain engineering of C. bescii while simultaneously creating a platform for further strain modification for advanced production of fuels and chemicals from renewable plant feedstocks.

Results
Deletion of lactate dehydrogenase (ldh) from the C. bescii chromosome
We recently reported a method for DNA transformation and marker replacement in Caldicellulosiruptor bescii based on uracil prototrophic selection [14,27,28]. C. bescii strain JWCB005 (ΔpyrFA, ura⁻/5-FOA⁶) contains a deletion of the pyrFA locus making the strain a uracil auxotroph resistant to 5-fluoroorotic acid (5-FOA) [27], allowing the use of pyrF as both a selectable and counter-selectable marker (Figure 2A). A deletion of the L-lactate dehydrogenase gene (Cbes1918) was constructed by fusing the 5' and 3' flanking regions of the ldh

![Figure 1](https://example.com/figure1.png)  
*Figure 1* A simplified version of predicted metabolic pathways for fermentation, glycolysis and electron transfer in *Caldicellulosiruptor bescii*. (1) L-Lactate dehydrogenase; (2) Pyruvate-ferredoxin oxidoreductase; (3) Phosphotransacetylase; (4) Acetate kinase; (5) Bifurcating (reduced ferredoxin: NADH-dependent) hydrogenase. The thick grey arrows represent potential heterologous pathways that do not exist in *Caldicellulosiruptor* species but could be used for renewable fuel and chemical production.
gene and cloning the fused product into a non-replicating plasmid vector, resulting in plasmid pDCW121. This vector also contains the wild type pyrF allele under the transcriptional control of a ribosomal protein gene promoter (Cbes2105, 30S ribosomal protein S30EA), allowing both positive (uracil prototrophy) and negative (5-FOA sensitivity) selection. Plasmid pDCW121 was transformed into C. bescii JWCB005 selecting uracil prototrophy resulting from plasmid recombination into the targeted region, followed by counter-selecting 5-FOA resistance (resulting from plasmid excision). The resulting strain, JWCB017, contained a deletion of the ldh wild type gene in the chromosome. To confirm the ldh deletion in JWCB017, the region of the ldh locus was amplified by PCR using primers outside of the plasmid regions of homology used to construct the deletion (Figure 2B). The wild type and the ΔpyrFA strain (JWCB005) gave the same expected 3.0 kb bands, while PCR from JWCB017 resulted in the smaller 2.0 kb band, as predicted. The PCR product was also sequenced to verify that the deletion in the chromosome was the same as that constructed on the plasmid.

Deletion of ldh eliminates lactate production and increases acetate and H₂ production

Cbes1918 is the only predicted lactate dehydrogenase gene encoded in the C. bescii genome. To confirm that this gene is solely responsible for the production of lactate in C. bescii, wild type, JWCB005 and JWCB017 were grown on 0.5% maltose, and fermentation products were analyzed by high-performance liquid chromatography (HPLC) (Figure 3AB) and nuclear magnetic resonance (NMR) analysis (Figure 3C). No lactate was detected in the mutant by either method, as compared to approximately 5.0 mM lactate from the wild-type and parental strains.

To compare the production of lactate, acetate and hydrogen, C. bescii wild-type and mutant strains were grown in LOD medium [29] with soluble celloolodextrins (cellulbiose) or plant biomass (switchgrass) as carbon source. When grown on 0.5% cellobiose for 30 hours, JWCB017 showed 29% and 21% more acetate production and 37% and 34% more hydrogen production than wild type and parent strains, respectively (Figure 3D). Cells grown for 120 hours on LOD medium supplemented with 0.5% switchgrass as the sole carbon source showed a similar profile to that on cellulbiose, with the Δldh strain producing 38% and 40% more acetate and 55% and 70% more hydrogen than wild-type and parent strains (Figure 3E).

Growth yield increases upon deletion of ldh

Growth of JWCB017 was compared to the wild type and parental strains in defined media [29] supplemented with either 0.5% maltose or 0.5% cellulbiose. While growth of the ΔpyrFA parent strain on both maltose (Figure 4A) and cellulbiose (Figure 4B) was indistinguishable from the wild type [29], the JWCB017 mutant strain...
reached a 34-53\% higher final optical density than the wild type and parent. Interestingly, while the growth rate was comparable, the exponential growth phase of JWCB017 was extended resulting in higher cell densities.

**Discussion**

We have built upon recent advances in the genetic manipulation of *Caldicellulosiruptor* [14,27,28] to delete the gene encoding lactate dehydrogenase. While the wild type strain produced roughly equimolar amounts of acetate and lactate, the JWCB017 mutant strain no longer produced lactate, instead rerouting carbon and electron flux to acetate and $\text{H}_2$, respectively. JWCB001 ($\sim 1.8$ mol/mol of glucose) and JWCB005 ($\sim 1.7$ mol/mol of glucose) appeared a bit lower in hydrogen yield than reported values for *C. saccharolyticus* ($\sim 2.5$ mol/mol of glucose) [22]). However, in this report, *C. saccharolyticus* was grown in culture media with added yeast extract, which improves yields [22]. JWCB017 ($\sim 3.4$ mol/mol of glucose) was more than that reported for *C. saccharolyticus*. Yield and titer of acetate and $\text{H}_2$ were increased in the *C. bescii ldh* deletion strain from both model soluble

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**Figure 3 Fermentation products by *C. bescii* mutant strains.** (A) Lactic acid and (B) acetic acid production were measured during growth on 0.5\% maltose by the parent strain (JWCB005 ΔpyrFA; filled circles) and JWCB017 (ΔpyrFA Δldh; open circles). (C) Production of lactic and acetic acids by wild type and JWCB017 were further measured by NMR analysis after 48 hours incubation. (D) End products of *C. bescii* wild-type and mutant strains were measured by HPLC on cellobiose after 30 hours incubation, and (E) switchgrass after 120 hours incubation. Acetate, Black; Lactate, Light gray; Hydrogen, Dark gray.
substrates and real-world plant biomass. A similar approach has been applied to other thermophilic biomass-degrading bacteria, including xylanolytic *Thermoanaerobacterium saccharolyticum* and cellulolytic *Clostridium thermocellum* [30-32], with the goal of increasing ethanol production.

Members of the genus *Caldicellulosiruptor* offer special advantages for biomass conversion to products of interest in that they are hyperthermophiles with optimal growth temperatures between 70°C ~ 80°C and they are capable of using biomass without conventional pretreatment. Interestingly, deletion of *ldh* resulted in a higher cell yield and longer exponential growth phase relative to the wild type. The increase in cell density is likely caused by an increase in acetate production, which should increase ATP production per glucose via acetate kinase providing more energy for biosynthesis and growth. The evolutionary pressures that selected for maintenance of *ldh* are not clear, though it may be related to the partial pressure of H2 found in the environment. Further, the molecular mechanism by which *C. bescii* switches from production of acetate + H2 to lactate is unknown. *C. thermocellum* encodes a lactate dehydrogenase that is allosterically activated by fructose-1,6-bisphosphate [33], such that lactate is only produced when the rate of substrate uptake exceeds glycolytic flux. It would be interesting to examine whether *C. bescii* utilizes a similar mechanism for flux control at the pyruvate node of glycolysis. Independent of mechanism, the fact that *C. bescii* JWCB017 grows to a higher density without an obvious effect on growth rate suggests that further engineered strains may be able to compete well with the wild type strain and thrive in an industrial setting.

Recent progress in genetic tool development opened the possibility of more advanced metabolic engineering strategies to increase the utility of *C. bescii* for industrial applications. In addition to the construction of gene deletions, this will enable gene insertion into the chromosome (so called gene knock-ins), simplifying the process of heterologous gene expression by eliminating the need for plasmid maintenance and increasing the number of genes that can be stably expressed. Thus, we have created a new platform for rational strain design for lignocellulosic bioconversion, enabling future efforts to increase the titer of H2, express heterologous pathways for production of liquid fuels and chemicals, increase robustness, and improve upon the native ability of *Caldicellulosiruptor* species to deconstruct and convert biomass without conventional pretreatment.

Characterization of the JWCB017 mutant also sheds light on the basic physiology of *C. bescii*, which will inform future metabolic modeling and engineering efforts. For instance, this strain has now been engineered to produce only acetate and H2 from sugars, providing further evidence that *C. bescii* uses a bifurcating hydrogenase to funnel all the electrons to H2. Examination of the *C. bescii* genome sequence reveals that glycolysis likely yields NADH from glyceraldehyde-3-phosphate oxidation, based on the presence of glyceraldehyde-3-phosphate dehydrogenase (Cbes1406) and lack of glyceraldehyde-3-phosphate: ferredoxin oxidoreductase. Conversion of pyruvate to acetyl-CoA, on the other hand, presumably reduces ferredoxin, based on the presence of pyruvate: ferredoxin oxidoreductase (Cbes0874-0877) and the lack of pyruvate dehydrogenase and pyruvate-formate lyase. Because H2 evolution from NADH is thermodynamically unfavorable except in extremely low H2 partial pressures [34], this implies that the favorable production of H2 from ferredoxin drives the unfavorable NADH-dependent H2 production. Further genetic modification will increase our understanding of metabolic flux in *C. bescii*, allowing better metabolic models and further informing metabolic engineering efforts.

**Conclusions**

Here we show the first application of recently developed genetic methods for metabolic engineering of a member
of the genus *Caldicellulosiruptor*. The method for creating a deletion of the *ldh* gene in the *C. bescii* chromosome was efficient enough to allow targeted marker replacement using non-replicating plasmids. The resulting mutant grew to a higher cell density and produced more hydrogen than the wild-type strain. Using the tools developed here, *C. bescii* JWCB017 will serve as a platform for additional rational strain engineering for production of fuels and chemicals from lignocellulosic feedstocks.

### Methods

#### Strains, growth conditions and molecular techniques

A spontaneous mutant containing a deletion within the *pyrFA* locus of *C. bescii*, JWCB005 [27,28], was used in this study to select transformants. *C. bescii* strains were grown in modified DSMZ516 medium or LOD (low osmolality defined growth medium) [29] containing 0.5% maltose as carbon source, final pH 7.0. Liquid cultures were grown from a 0.5% inoculum or a single colony and incubated at 75°C in anaerobic culture bottles degassed with five cycles of vacuum and argon. A solid medium was prepared by mixing an equal volume of liquid medium at a 2× concentration with the same volume of (wt/vol) agar, 3.6% (Difco, Sparks, MD) that had been previously autoclaved. Both solutions were kept at 70°C and poured into petri dishes immediately after mixing. A series of dilutions of this culture were mixed with 4 ml of soft top agar (1.5% of agar) and poured across the top of the solid agar medium. The plates were degassed with five cycles of vacuum and argon and incubated at 75°C for 4 days in anaerobic jars.

*E. coli* DH5α was used to prepare plasmid DNA. Cells were grown in LB broth supplemented with apramycin (50 µg/ml) and a mixture of 19 amino acids (5% inoculum, v/v) [29]. The 500 ml culture was incubated at 75°C for 5 hours and cooled to room temperature for 1 hr. Cells were harvested by centrifugation (6000 × g, 20 min) at 25°C and washed three times with 50 ml of pre-chilled 10% sucrose. After the third wash, the cell pellet was resuspended in 50 µl of pre-chilled 10% sucrose in a pre-chilled 1 mm cuvette using a Bio-Rad gene Pulser (Bio-Rad, Hercules, CA). Transformants were selected on LB solid medium containing apramycin (50 µg/ml final).

#### Competent cells, transformation and mutant selection in *C. bescii*

To prepare competent cells, a 50 ml culture of JWCB005 was grown in LOD minimal medium at 75°C for 18 hours (to mid exponential phase) and 25 ml of the culture was used to inoculate a 500 ml culture of LOD (low osmolality defined growth medium) supplemented with 40 µM uracil and a mixture of 19 amino acids (5% inoculum, v/v) [29]. The 500 ml culture was incubated at 75°C for 5 hours and cooled to room temperature for 1 hr. Cells were harvested by centrifugation (6000 × g, 20 min) at 25°C and washed three times with 50 ml of pre-chilled 10% sucrose. After the third wash, the cell pellet was resuspended in 50 µl of pre-chilled 10% sucrose in a microcentrifuge tube and stored at −80°C until needed. Before transformation, plasmids from *E. coli* cells were methylated *in vitro* with *C. bescii* methyltransferase (M.CbeI, [14]) and methylated plasmid DNAs (0.5-1.0 µg) were added to the competent cells, gently mixed and incubated for 10 minutes in ice.

### Table 1 Plasmids and *C. bescii* strains (JWCB) used in this study

| Strains and Plasmids | Description and/or relevant characteristics | Source or reference |
|----------------------|---------------------------------------------|---------------------|
| pDCW88               | Non-replicating plasmid in *C. bescii*      | [28]                |
| pDCW121              | *ldh* knock-out plasmid                     |                     |
| JWCB001              | *C. bescii* wild-type DSM 6725              | [35]                |
| JWCB005              | DSM 6725 Δ*pyrFA*  | [27]                |
| JWCB017              | DSM 6725 Δ*pyrFA*Δ*ldh*                     | This study          |

### Table 2 Oligonucleotides used in this study

| Primer | Sequence 5’→3’ |
|--------|----------------|
| DC081  | TCCATGTGAGTTAGGCTGT |
| DC348  | GAATCTCTGGCGCTACGTGGAAGAA |
| DC349  | GAAACAAATGCGCTGAGGAGGCAGGCTGT |
| DC350  | TGCCAGCTTGGAGGATAGGAGGCAGGCTG |
| DC351  | TGCCAGATAGGAAAGGAGGCAG |
| DC356  | CGTCCATCATGCTGACATGACAGTATTAA |

Fragment containing a KpnI site upstream of the *ldh* gene was amplified using primers DC348 and DC349. A 1,011 bp fragment containing an EcoRI site downstream of *ldh*, was amplified using primers DC350 and DC351. The two fragments were joined by overlapping PCR using primers DC348 and DC351 to generate a 2,020 bp product that was cloned into pDCW88 [28] using the KpnI and EcoRI sites. The resulting plasmid, pDCW121, was transformed into *E. coli* DH5α by an electrotransformation via a single electric pulse (1.8 kV, 25 µF and 200 Ω) in a pre-chilled 1 mm cuvette using a Bio-Rad gene Pulser (Bio-Rad, Hercules, CA). Transformants were selected on LB solid medium containing apramycin (50 µg/ml final).
Electrotransformation of the cell/DNA mixture was performed via single electric pulse (1.8 kV, 25 μF and 350 Ω) in a pre-chilled 1 mm cuvette using a Bio-Rad gene Pulser (Bio-Rad, Hercules, CA). After pulsing, cells were inoculated into 10 ml of LOC medium (low osmolarity complex growth medium, [29]) and incubated for 4 hours at 75°C. 100 μl of the culture was transferred into 20 ml of defined medium without uracil. After 18 hours incubation at 75°C, cells were harvested by centrifugation (at 6000 × g for 20 min) and resuspended in 1 ml of 1× basal salts. 100 microliters of the cell suspension was plated onto solid defined media with 40 μM uracil and 8 mM 5-FOA (5-fluoroorotic acid monohydrate).

Analytical techniques for determining fermentation end products
Batch fermentations were conducted in stoppered 125 ml serum bottles containing 50 ml LOD medium with 5 g/l maltose, cellobiose or switchgrass. Cultures of JWCB005 and JWCB017 were supplemented with 40 μM uracil. Triplicate bottles were inoculated with a fresh 2% (v/v) inoculum and incubated at 75°C without shaking. Total cell dry weight (CDW) was determined by concentrating 25 ml of each culture on dried, preweighed 47 mm Supor membrane filters (0.45, Pall Corp., Ann Arbor, MI) and washed with 10 ml of ddH2O. Cell retentates were dried for 16 hours at 85°C and weighed on an analytical balance. Culture supernatants were analyzed via HPLC using a Waters Breeze 2 software and compared against peak areas and retention times of known standards for each analyte of interest. H2 was measured using an Agilent Technologies 6850 Series II Gas Chromatograph equipped with a thermal conductivity detector at 190°C with a N2 reference flow and a HP-PLOT U Column (30 m * 0.32 mm). To measure organic acid production, Nuclear magnetic resonance (NMR) analysis was performed. One-dimensional 1H-NMR spectra were recorded at 298 K with a Varian Inova-NMR operating at 600 MHz for 1H and equipped with a 5-mm NMR cold probe. Samples (500 μL) of cell free culture media were mixed with 150 μL of D2O as internal lock and immediately analyzed. 128 scans were recorded for each sample using a pre-saturation method to suppress the water resonance. The amounts of the most abundant components in the samples were calculated by integration of the proton signals in the spectra. The data were normalized to the amount of acetic acid in each sample.

Biomass preparation
Air-dried switchgrass (Panicum virgatum, Alamo variety) was reduced to 60 mesh using a Wiley Mini-Mill (Thomas Scientific, Swedesboro, NJ, USA). The ground switchgrass was subjected to a hot water treatment similar to that described by Yang et al. [12] however the biomass was boiled in distilled H2O (2% w/v) for 1 hour rather than treating overnight at 75°C. The switchgrass was then washed and dried overnight at 50°C before dispensing into serum bottles as previously described [12].

Abbreviations
lacH: L-lactate dehydrogenase; CBP: Consolidated bioprocessing; 5-FOA: 5-Fluoroorotic acid; HPLC: High-performance liquid chromatography; NMR: Nuclear magnetic resonance; LOD: Low osmolarity defined growth medium; LOC: Low osmolarity complex growth medium; CDW: Cell dry weight; RI: Refractive index; GC: Gas chromatography.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
MC and DC designed and carried out the genetic and growth experiments, analyzed results and participated in the writing of the manuscript. JGE and AWG designed and carried out the analysis of fermentation products and participated in writing the manuscript. JW participated in design of the study, coordination of the work, and writing of the manuscript. All authors read and approved the final manuscript.

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