Supporting Information

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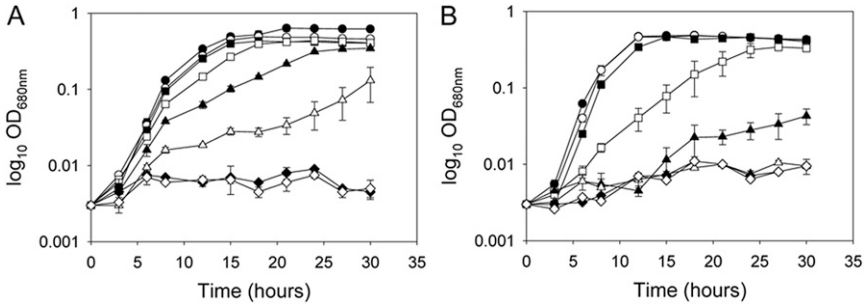

Fig. S1. Analysis of ethanol tolerance of the *Caldicellulosiruptor bescii* wild-type DSM 6725. Growth of *C. bescii* on 1% cellobiose as the carbon source with different amounts of ethanol at 65 °C (*A*) and 75 °C (*B*) monitored by measuring culture turbidity ($\log_{10}$ OD_{680nm}). ●, no ethanol; ○, 200 mM; –■, 300 mM; □, 400 mM; ▲, 450 mM; △, 500 mM; ◆, 600 mM; ◇, 700 mM. Error bars based on two biologically independent experiments.

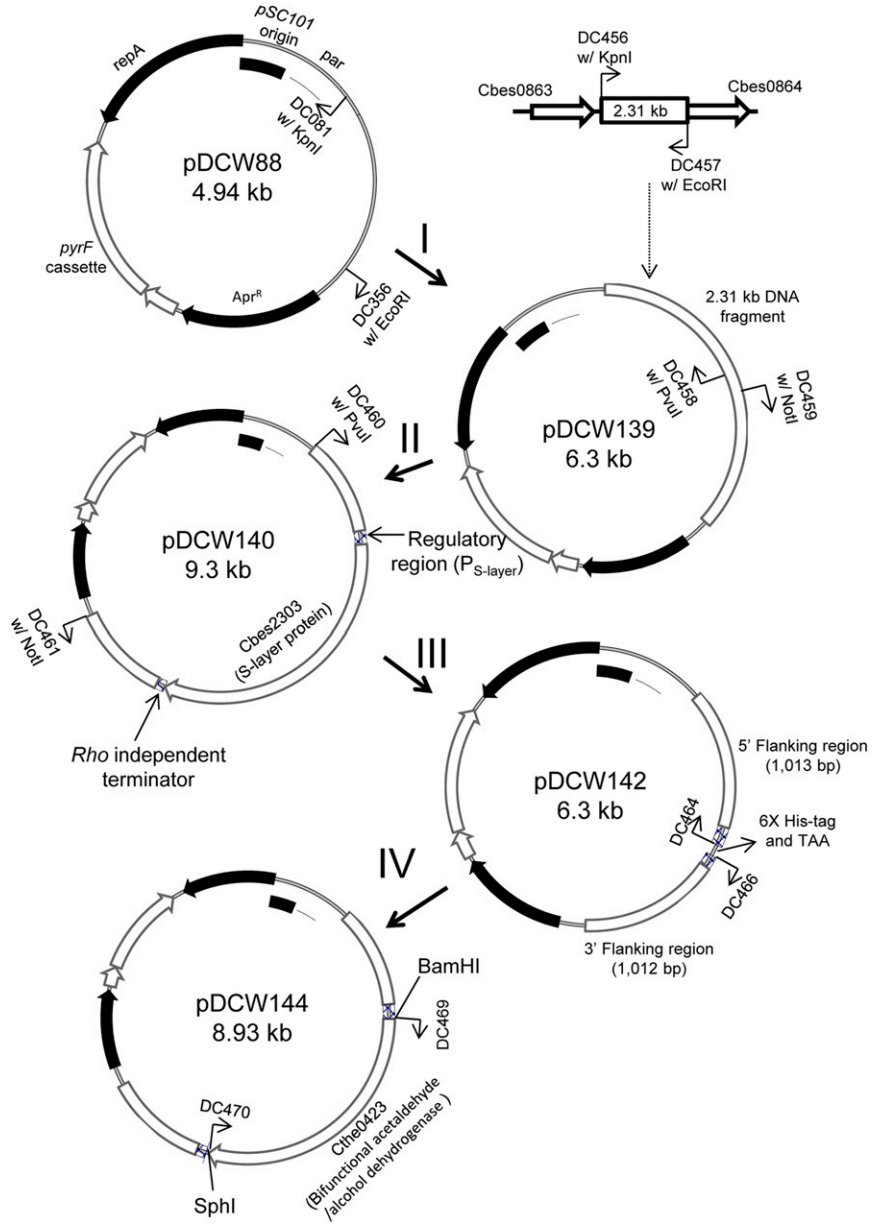

Fig. S2. Construction of knock-in vector pDCW144. Plasmid pDCW144 was constructed in four cloning steps. ORFs from *C. bescii* and *Clostridium thermocellum* are indicated as empty arrows. ORFs from *Escherichia coli* indicated as black arrows. Apr^R, the apramycin-resistant gene cassette; *pSC101,* low-copy replication origin in *E. coli*; *repA*, a plasmid-encoded gene required for *pSC101* replication; *par*, partition locus; *pyrF* cassette; 5′ and 3′ franking sequences of the targeted insertion site in *C. bescii* chromosome; regulatory and *rho* independent terminator sequences surrounding Cbes2303 (marked as a cross-hatched box); C-terminal 6x Histidine-tag in front of stop codon are indicated. All primers and two restriction sites (BamHI and SphI) used in this construction are also indicated.

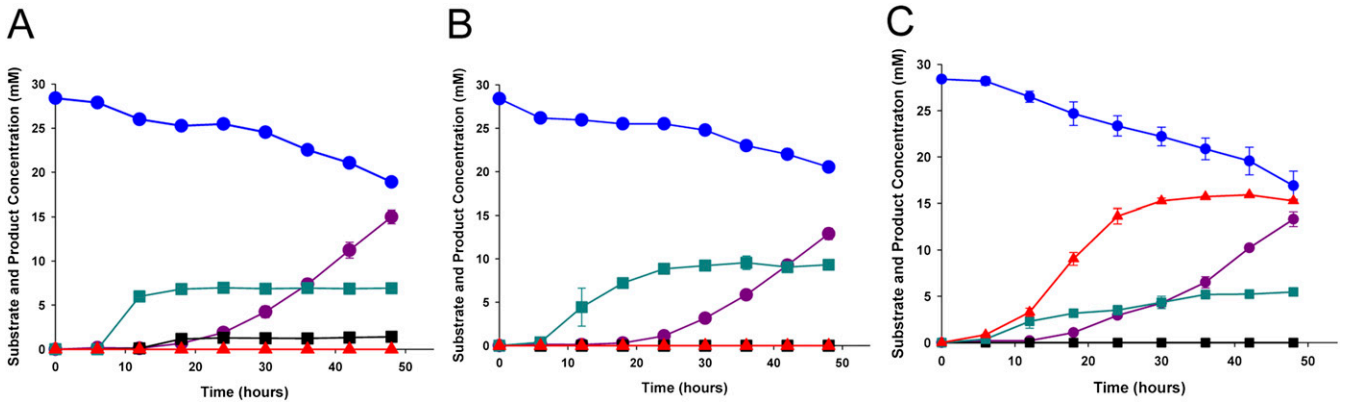

Fig. S3. Fermentation of 1% cellobiose at 65 °C. (*A*) *C. bescii* JWCB001, (*B*) *C. bescii* JWCB018, and (*C*) *C. bescii* JWCB032. Cellobiose (filled blue circles), glucose (filled purple circles), lactate (filled black squares), acetate (filled green squares), and ethanol (filled red triangles) were determined by HPLC analysis as indicated in *Methods*. Error bars based on two biologically independent experiments.

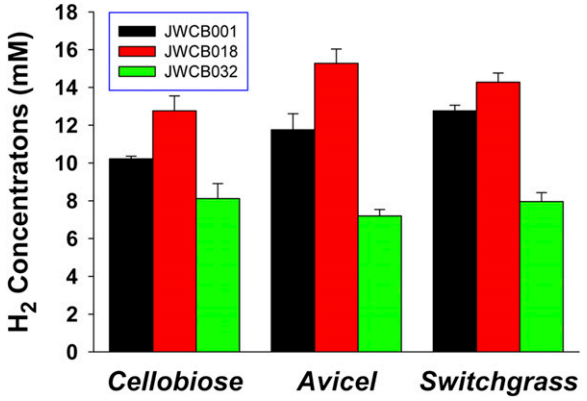

Fig. S4. The final H_2 concentrations produced by *C. bescii* wild-type and mutant strains grown on 1% cellobiose, 2% (wt/vol) Avicel, and 2% (wt/vol) switchgrass. The hydrogen concentrations were measured by a gas chromatography (GC) after 48 h fermentation at 65 °C. The GC analysis was performed as indicated in *Methods*. Error bars based on two biologically independent experiments.

Table S1. Effect of ethanol addition on growth rates of *C. bescii* wild-type at 65 °C and 75 °C

Ethanol concentrations (mM)	Growth rate at 65 °C (Doubling time, h)	Growth rate at 75 °C (Doubling time, h)
0	4.0	2.7
200	3.9	2.7
300	4.3	3.0
400	4.5	5.2
450	6.1	ND
500	9.7	ND
600	ND	ND
700	ND	ND

The growth rates were presented as the average of two biologically independent experiments. ND, not detected.

Table S2. Growth rates and cell yields of *C. bescii* wild-type and mutant strains

	Grown at 65 °C		Grown at 75 °C	
Strain	Doubling time (h)	Maximum cell density (cells/mL)	Doubling time (h)	Maximum cell density (cells/mL)
JWCB001	4.0	6.40E+08	2.7	4.90E+08
JWCB018	4.1	4.70E+08	3.0	4.10E+08
JWCB032	3.2	5.50E+08	2.7	4.20E+08

The values were presented as the average of two biologically independent experiments.

Table S3. Data supporting values for carbon balance at 48-h fermentation

	Concentrations of residual compounds (at 48 h) (mM)									Product yields (mol/mol)			
Strains	Cellobiose *initial*	Cellobiose *final*	Glucose	Cellobiose *catabolized*	Lactate	Acetate	Ethanol	Hydrogen	CR (%)	$Y_{L/C}$	$Y_{A/C}$	$Y_{E/C}$	$Y_{H/C}$
JWCB001	28.4 ± 0.11	18.9 ± 0.42	15.0 ± 0.78	2.0 ± 0.28	1.3 ± 0.07	6.9 ± 0.23	0.0	11.8 ± 0.21	103	0.65	3.45	0.0	5.90
JWCB018	28.4 ± 0.36	19.7 ± 0.27	12.9 ± 0.67	2.3 ± 0.22	0.0	9.3 ± 0.56	0.0	14.5 ± 0.53	103	0.0	4.05	0.0	6.30
JWCB032	28.4 ± 0.25	16.9 ± 1.57	13.4 ± 0.81	4.8 ± 1.71	0.0	5.5 ± 0.30	15.3 ± 0.19	9.8 ± 0.64	107	0.0	1.14	3.19	2.04

Substrate and product concentration of JWCB001, 018, and 032 are reported in millimolarity, with average percent carbon recovery (CR) and average yields in mol/mol. The fermentation conditions were as described in *Methods*. Product yields are calculated as: $Y_{L/C}$, lactate yield per mole cellobiose; $Y_{A/C}$, acetate yield per mole cellobiose; $Y_{E/C}$, ethanol yield per mole cellobiose; $Y_{H/C}$, hydrogen yield per mole cellobiose. SDs based on two biologically independent experiments.

Table S4. Primers used in this study

Primers	Sequences (5′ to 3′)	Description
DC081	TCCAATGATCGAAGTTAGGCTGGT	To construct pDCW139
DC356	TCTGAATTCTCTGACGCTCAGTGGAACGAA	To construct pDCW139
DC456	AGAGGTACCTGTGAGGGCATGTCAATTTACGA	To construct pDCW139
DC457	AGAGAATTCTCTTTTCGATGGAATCTTCTTCGGA	To construct pDCW139
DC458	AGAGAGCGATCGTCTATTGTAACTTTCACTTCAGTGCA	To construct pDCW140
DC459	AGAAGAAGGCGGCCGCTGGAAGAACTTGAAAGCAGGCT	To construct pDCW140
DC460	AGAGAGCGATCGACAGTTTGATTACAGTTTAGTCAGAGCT	To construct pDCW140
DC461	AGAAGAAGGCGGCCGCTTGGTTCCTTAAATCTAAGAGGTATGA	To construct pDCW140
DC462	TGCTGGCAGAGAAGAGCGAAA	Sequencing primer for pDCW140
DC463	TCTTCATCCCAATCTTCAACTTC	Sequencing primer for pDCW140
DC464	ACTGGATCCCTCACCAAACCTCCTTGTATGAT	To construct pDCW142
DC466	AGAGCATGCCATCACCATCACCATCACTAATAATAAAGCTGAAATAAAAGAGGGTGAGA	To construct pDCW142
DC469	ACTGGATCCATGACGAAAATAGCGAATAAATACGAAGT	To construct pDCW144 and 145
DC470	AGAGCATGCTTTCTTCGCACCTCCGTAATAAGCGTTCAGA	To construct pDCW144 and 145
DC471	TGGTAATGAGAGAAGCAGATG	Sequencing primer for pDCW144 and 145
DC472	TGATAAAAAGCACCCAGTTTGT	Sequencing primer for pDCW144 and 145
DC477	TGGTTGACCAGGAGAATTTTACACA	Sequencing primer to verify the insertion
DC478	AGCAACAATCCTGCATTTGTAAG	Sequencing primer to verify the insertion