J Ind Microbiol Biotechnol
DOI 10.1007/s10295-017-1982-4

GENETICS AND MOLECULAR BIOLOGY OF INDUSTRIAL ORGANISMS - ORIGINAL PAPER

Heterologous expression of a β-D-glucosidase in *Caldicellulosiruptor bescii* has a surprisingly modest effect on the activity of the exoproteome and growth on crystalline cellulose

Sun-Ki Kim[1,3] · Daehwan Chung[2,3] · Michael E. Himmel[2,3] · Yannick J. Bomble[2,3] · Janet Westpheling[1,3]

Received: 16 May 2017 / Accepted: 14 September 2017

Abstract Members of the genus *Caldicellulosiruptor* are the most thermophilic cellulolytic bacteria so far described and are capable of efficiently utilizing complex lignocellulosic biomass without conventional pretreatment. Previous studies have shown that accumulation of high concentrations of cellobiose and, to a lesser extent, cellotriose, inhibits cellulase activity both in vivo and in vitro and high concentrations of cellobiose are present in *C. bescii* fermentations after 90 h of incubation. For some cellulolytic microorganisms, β-D-glucosidase is essential for the efficient utilization of cellobiose as a carbon source and is an essential enzyme in commercial preparations for efficient deconstruction of plant biomass. In spite of its ability to grow efficiently on crystalline cellulose, no extracellular β-D-glucosidase or its GH1 catalytic domain could be identified in the *C. bescii* genome. To investigate whether the addition of a secreted β-D-glucosidase would improve growth and cellulose utilization by *C. bescii*, we cloned and expressed a thermostable β-D-glucosidase from *Acidothermus cellulolyticus* (Acel_0133) in *C. bescii* using the CelA signal sequence for protein export. The effect of this addition was modest, suggesting that β-D-glucosidase is not rate limiting for cellulose deconstruction and utilization by *C. bescii.*

Keywords Consolidated bioprocessing · Biomass deconstruction · β-D-Glucosidase · *Caldicellulosiruptor*

Electronic supplementary material The online version of this article (doi:10.1007/s10295-017-1982-4) contains supplementary material, which is available to authorized users.

✉ Janet Westpheling
janwest@uga.edu

Sun-Ki Kim
skkim@uga.edu

Daehwan Chung
Daehwan.Chung@nrel.gov

Michael E. Himmel
Mike.Himmel@nrel.gov

Yannick J. Bomble
yannick.bomble@nrel.gov

[1] Department of Genetics, University of Georgia, Athens, GA 30602, USA

[2] Biosciences Center, National Renewable Energy Laboratory, Golden, CO, USA

[3] The BioEnergy Science Center, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA

Introduction

Caldicellulosiruptor bescii is the most thermophilic cellulolytic bacterium so far described. It can efficiently utilize complex plant biomass without pretreatment, producing more than 50 glycoside hydrolases, many of which consist of multi-domain enzymes that contain more than one catalytic domain. CelA, the most abundant enzyme in the *C. bescii* exoproteome, has been shown to be important for both complex biomass deconstruction as well as growth on crystalline cellulose [29]. A major product of the GH48 exoglucanase domain of CelA is cellobiose [28]. Previous studies have shown that accumulation of high concentrations of cellobiose and, to a lesser extent, cellotriose, is present in *C. bescii* fermentations after 90 h of incubation [27]. For some cellulolytic microorganisms, β-D-glucosidase is essential for the efficient utilization of cellobiose as a carbon source. In fact, β-glucosidases are essential enzymes in commercial preparations for efficient deconstruction of plant biomass as the activity of cellulases in these mixtures has been shown to be inhibited by cellobiose. Inhibition of cellulase action by

Published online: 23 September 2017

the accumulation of cellobiose would be expected to limit the effectiveness of cellulose deconstruction by *C. bescii*. In a previous study, it was shown that the addition of exogenous β-D-glucosidase purified from *Thermotoga maritima* substantially improved the performance of CelA on Avicel in vitro [4]. In spite of its ability to grow efficiently on crystalline cellulose, no extracellular β-D-glucosidase or its GH1 catalytic domain could be identified in the *C. bescii* genome [9, 12]. To investigate whether or not the addition of a secreted β-D-glucosidase could improve growth and cellulose utilization by *C. bescii*, we cloned and expressed a thermally stable β-D-glucosidase from *Acidothermus cellulolyticus* in *C. bescii* using the CelA signal sequence for protein export. We then examined the effect of this addition on the activity of the exoproteome as well as its growth on Avicel. The effect in both cases was modest, suggesting that β-D-glucosidase is not rate limiting for cellulose deconstruction and utilization by *C. bescii.*

Materials and methods

Strains, media, and culture conditions

E. coli and *C. bescii* strains used in this study are listed in Table 1. All *C. bescii* strains were grown under anaerobic conditions at 65 °C on solid or in liquid low osmolarity defined (LOD) medium [15], as previously described, with 5 g/L maltose or cellobiose as the sole carbon source for routine growth and transformation experiments [11]. For growth of uracil auxotrophs, the defined medium contained 40 μM uracil. *E. coli* DH5α was used as a host for plasmid DNA construction and preparation using standard techniques. *E. coli* cells were cultured in LB broth containing apramycin (50 μg/mL). Plasmid DNA was isolated using a Qiagen Miniprep Kit (Qiagen, Valencia, CA, USA). Chromosomal DNA from *C. bescii* strains was extracted using the Quick-gDNA MiniPrep (Zymo, Irving, CA) as previously described [10].

Construction and transformation of β-D-glucosidase expression vectors

Q5 High-Fidelity DNA polymerase (New England BioLabs, Ipswich, MA, USA) was used for all PCRs, and restriction enzymes (New England BioLabs, Ipwich, MA, USA) and the Fast-link DNA ligase kit (Epicentre Biotechnologies, Madison, WI, USA) were used for plasmid construction according to the manufacturer's instruction. Plasmid pSKW12 (Fig. 1a) was constructed in two cloning steps. First, a 1.4-kb DNA fragment containing the coding sequence of Acel_0133 without the signal sequence was amplified using primers SK015 (with an ApaLI site) and SK016 (with an AvrII site) using *A. cellulolyticus* 11B gDNA as template. The 5.9-kb DNA fragment containing the regulatory region of Cbes2303 (S-layer protein), the CelA signal sequence, a *C*-terminal 6X histidine-tag, and a Rho-independent transcription terminator was amplified by PCR with primers DC699 (with ApaLI site) and DC700 (with AvrII site) using pDCW212 [19] as template. These two linear DNA fragments were digested with ApaLI and AvrII, and ligated to construct a

Table 1 Strains and plasmids used in this study

Name	Description	References
E. coli		
JW522	DH5α containing pSKW12 (Apramycin^R)	This study
JW527	DH5α containing pSKW18 (Apramycin^R)	This study
C. bescii		
JWCB18	Δ*pyrFA ldh::ISCbe4* Δ*cbe1*(*ura*$^-$/5-FOA^R)	[11]
JWCB52	Δ*pyrFA ldh::ISCbe4* Δ*cbe1::P$_{S\text{-}layer}$acel0614(E1)* (*ura*$^-$/5-FOA^R)	[9]
JWCB21	JWCB18 containing pDCW89 (*ura*$^+$/5-FOA^S)	[5]
JWCB73	JWCB52 containing pJGW07 (*ura*$^+$/5-FOA^S)	[19]
JWCB76	JWCB52 containing pSKW12 (*ura*$^+$/5-FOA^S)	This study
JWCB77	JWCB52 containing pSKW18 (*ura*$^+$/5-FOA^S)	This study
Plasmids		
pDCW89	*E. coli*/*C. bescii* shuttle vector containing the *C. bescii pyrF* gene (Apramycin^R)	[5]
pJGW07	*E. coli*/*C. bescii* shuttle vector containing the *C. thermocellum pyrF* gene (Apramycin^R)	[16]
pDCW173	Intermediate vector 1 (Apramycin^R)	[8]
pDCW212	Intermediate vector 2 (Apramycin^R)	[19]
pDCW218	Source of Acel_0133 open reading frame (Apramycin^R)	This study
pSKW12	Expression vector for extracellular Acel_0133 (Apramycin^R)	This study
pSKW18	Expression vector for intracellular Acel_0133 (Apramycin^R)	This study

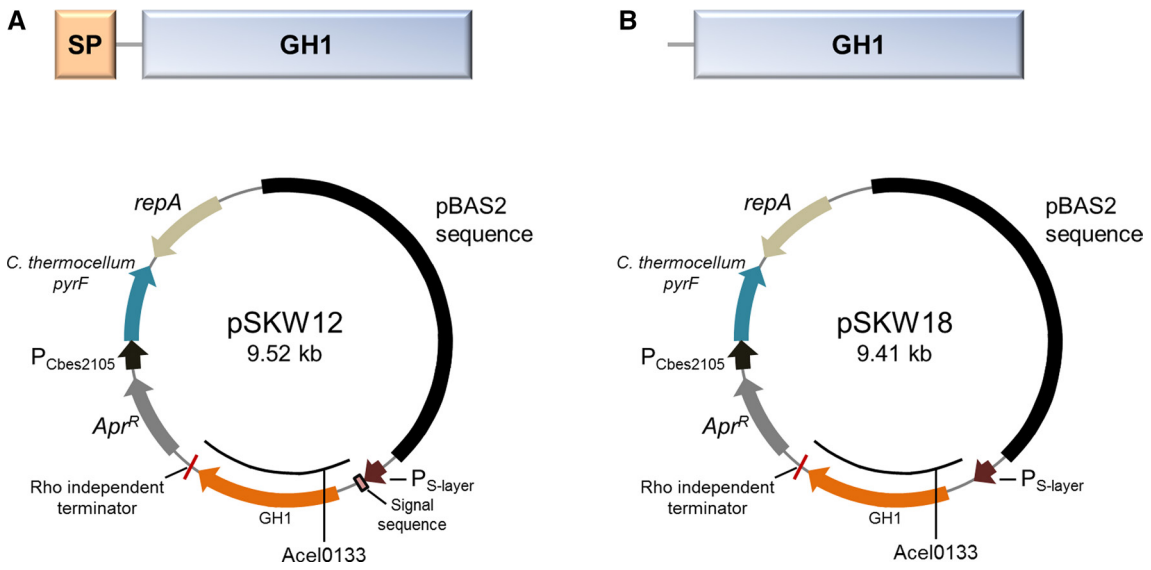

Fig. 1 Construction of vectors for the expression of β-D-glucosidase from *A. cellulolyticus* (Acel_0133) in *C. bescii*. Schematic diagrams and maps of expression vectors for extracellular (**a**) and intracellular (**b**) expression of Acel_0133. Genes were expressed under the transcriptional control of the *C. bescii* S-layer promoter. The expression vectors contain a CelA signal sequence, a *C*-terminal 6X His-tag, a Rho-independent terminator, the *pyrF* (from *C. thermocellum*) cassette for selection, and pBAS2 sequences for replication in *C. bescii.* SP, signal peptide; GH1, a family 1 glycoside hydrolase

7.3 kb intermediate vector, pDCW218. In a second step, the 1.8 kb Acel_0133 expression cassette, containing the regulatory region of Cbes2303 (S-layer protein), the CelA signal sequence, a *C*-terminal 6X histidine-tag, and a Rho-independent transcription terminator, was amplified by PCR with primers SK043 (with SphI site) and DC461 (with NotI site) using pDCW218 as template. A 7.7 kb DNA fragment containing the pSC101 replication origin for *E. coli*, a *C. bescii* replication origin from pBAS2, an apramycin resistance gene cassette (Apr^R), and a *Clostridium thermocellum pyrF* expression cassette was amplified with primers SK044 (with SphI site) and DC482 (with NotI site) using pDCW173 as template. These two linear DNA fragments were digested with Sph1 and Not1 and ligated to construct pSKW12 (Fig. 1a). Plasmid pSKW18 is identical to pSKW12 except that it does not contain the CelA signal sequence (Fig. 1). To make this change, a 9.4-kb DNA fragment without the CelA signal sequence was amplified with primers SK051 (with ApaI site) and SK052 (with ApaI site) using pSKW12 as template. This linear DNA fragment was digested with ApaI and ligated to construct pSKW18 (Fig. 1b). These plasmids were introduced into *E. coli* DH5α by electroporation in a 1-mm-gap cuvette at 1.8 kV and transformants were selected for apramycin resistance. All plasmids were sequenced by automatic sequencing (Genewiz, South Plainfield, NJ, USA). Electrotransformation of *C. bescii* cells was performed as previously described [16]. After being electro-pulsed with plasmid DNA (~0.5 µg), the cultures were recovered in low osmolarity complex (LOC) medium [15] at 65 °C. Recovery cultures were transferred to liquid LOD medium [15] without uracil to allow selection of uracil prototrophs. Cultures were plated on solid LOD media to obtain isolated colonies, and total DNA was extracted. Taq polymerase (Sigma, St. Louis, MO, USA) was used for PCRs to confirm the presence of the plasmid. PCR amplification with primers (DC460 and DC228) outside of the gene cassette on the plasmid was used to confirm the presence of the plasmid with the Acel_0133 gene. Primers used for plasmid constructions and confirmations are listed in Table S1.

Preparation of protein and zymogram assays

To collect cell-free extracts (CFE), *C. bescii* cells were grown in 500 mL of LOD medium with 40 mM MOPS buffer in closed bottles at 65 °C with shaking at 100 rpm to an OD_{680} of 0.25, harvested by centrifugation at 6000×*g* at 4 °C for 10 min, suspended in CelLytic B cell lysis reagent (Sigma, USA), and lysed by a combination of 4× freeze–thawing and sonication (three times for 15 s at 40 amps with 1-min rests on ice). Samples were centrifuged to separate protein lysate from cell debris and the supernatant was used as CFE. To collect the extracellular protein (ECP) fraction, *C. bescii* cells were grown in 2 L of LOD medium with 40 mM MOPS buffer in closed bottles at 65 °C with shaking at 100 rpm to an OD_{680} of 0.25 to 0.3. Culture broth was centrifuged (6000×*g* at 4 °C for 15 min), filtered (glass fiber, 0.7 µm), to separate out cells, and concentrated with a 3 kDa molecular weight cut-off column.

The concentrated ECP was exchanged with 20 mM MES buffer/2 mM β-mercaptoethanol (pH 5.5), and protein concentrations were determined using the Bio-Rad protein assay kit with bovine serum albumin (BSA) as the standard. CFE (50 µg) and ECP (20 µg) samples were electrophoresed in 4 to 20% gradient Mini-Protein TGX gels (BIO-RAD) and protein bands were visualized by staining with Coomassie Brilliant Blue G-250. For the zymogram analysis, the gel was soaked for 1 h in 2.5% (v/v) Triton X-100 solution to remove the SDS and washed in distilled water. After incubating the gel at 75 °C for 30 min in reaction buffer containing 5 mM 4-methylumbelliferyl β-D-glucopyranoside (MUG), 20 mM MES buffer (pH 5.5), 1 mM dithiothreitol (DTT), 1 mM $CaCl_2$, and 1 mM $MgCl_2$, the presence of fluorescent reaction product was visualized under UV light. The quantification of band intensity was carried out using the densitometry software (Total Lab 1.01, Nonlinear Dynamics Ltd.).

Enzyme activity assays

The reaction mixture for β-D-glucosidase activity contained 750 µL of distilled water, 100 µL of 200 mM MES buffer (pH 5.5), 10 µL of 100 mM dithiothreitol (DTT), 10 µL of 100 mM $CaCl_2$, 10 µL of 100 mM $MgCl_2$, and 20 µL of the crude enzyme solution, and was preheated at 75 °C for 10 min. The absorbance change at 75 °C and 405 nm wavelength was monitored using a Jenway Genova spectrophotometer after adding 100 µL of 50 mM *p*-nitrophenyl β-D-glucopyranoside (*p*-NPG, Sigma, USA). One unit (U) of β-D-glucosidase activity was defined as the amount of enzyme able to release 1 µmol *p*-NP (*p*-nitrophenol) from *p*-NPG per min. Specific enzyme activity (U/mg protein) was estimated by dividing the enzyme activity by the total protein concentration of the enzyme solution. Protein concentrations were determined using the Bio-Rad protein assay kit. Cellulolytic activity was measured using 10 g/L of either CMC or Avicel in MES reaction buffer (pH 5.5) as previously described [18]. Cells were grown in 2 L of LOD medium with 40 mM MOPS buffer and cellobiose as carbon source. Twenty-five µg/mL of the extracellular protein fraction was added to each reaction and incubated at 75 °C (1 h for CMC and 24 h for Avicel). Reducing sugars in the supernatant were measured using dinitrosalicylic (DNS) acid. Samples and standards (glucose) were mixed 1:1 with DNS reaction solution, boiled for 2 min and measured at OD_{575}. Activity was reported as mg/mL of sugar released.

Growth of recombinant strains on cellobiose and Avicel

To measure growth on cellobiose, cells were subcultured twice in LOD medium with 5 g/L maltose as the sole carbon source and this culture was used to inoculate media with 5 g/L cellobiose (1% total volume for all experiments) as the sole carbon source in 50 mL of LOD medium with 40 mM MOPS buffer, and incubated at 65 °C with shaking at 150 rpm. Cell growth on cellobiose was measured by optical density (OD) at 680 nm using a spectrophotometer. To measure growth on Avicel, cells were subcultured in LOD medium with 5 g/L maltose first and then 5 g/L Avicel. Colony-forming units (CFU) were measured by plating cells on LOC medium.

Results and discussion

Intracellular and extracellular expression of a β-D-glucosidase from *A. cellulolyticus* 11B in *C. bescii*

To construct a plasmid for extracellular expression of Acel_0133 from *A. cellulolyticus* (Fig. 1) in *C. bescii*, the gene was amplified from *A. cellulolyticus* gDNA and cloned into a shuttle vector pSKW12 (Fig. 1a) under the transcriptional control of the *C. bescii* S-layer promoter [6, 8]. The CelA signal sequence was selected for β-D-glucosidase secretion because CelA is the most abundant extracellular enzyme produced by *C. bescii* [20, 27] and we recently reported the use of this signal peptide for the secretion of xylanase proteins from *A. cellulolyticus* in *C. bescii* [9, 19]. For intracellular expression of Acel_0133, pSKW18 (Fig. 1b) was identical to the vector used for extracellular expression (pSKW12), except that it had no signal sequence. These plasmids were introduced into a *C. bescii* background strain JWCB52 that contains a deletion of *pyrF* for selection of plasmid transformants. This strain also expresses the *A. cellulolyticus* E1 protein, a thermostable, endo-1,4-β-glucanase (GH5) with a family 2 carbohydrate-binding module [9]. Transformants were selected for uracil prototrophy as the plasmids contained a wild-type *pyrF* allele, to generate JWCB76 (containing pSKW12) and JWCB77 (containing pSKW18). The resulting strains were grown at 65 °C to accommodate the expression of *C. thermocellum pyrF* gene used for complementation and plasmid selection. To verify the presence of the plasmids in the host strains, primers (DC460 and DC228) were used not only to amplify the portion of the plasmid containing the open reading frame of the targeted proteins, but also annealing to regions of the plasmid outside the gene to avoid amplification of sequences residing on the chromosome (Fig. S1A). Total DNA from JWCB76 and JWCB77 strains was also back-transformed into *E. coli*, and two different restriction endonuclease digests performed on plasmid DNA purified from three independent back-transformants resulted in identical digestion patterns to the original plasmid (Fig. S1). These results

indicated that the plasmids were successfully transformed into *C. bescii* and structurally stable during transformation and replication in *C. bescii* and back-transformation into *E. coli.*

To verify expression of the Acel_0133 β-D-glucosidase in *C. bescii*, strains JWCB76 and JWCB77 (Table 1) were grown in low osmolarity defined (LOD) medium with 40 mM MOPS buffer [7]. Intracellular proteins in the CFE and extracellular proteins in the ECP were separated by SDS-PAGE (Fig. 2a). The gel was then submerged in a 2.5% Triton X-100 solution to remove the SDS, allowing the proteins to re-nature in the gel. β-D-Glucosidase activity was detected by submerging the gel in a buffered solution of MUG, which permitted enzyme activity to be detected by visual inspection under UV light. Although β-D-glucosidases were difficult to detect in gels using Coomassie blue staining (Fig. 2a), zones of reactivity using the MUG substrate were clearly visible (Fig. 2b), indicating the presence of active proteins.

As shown in Fig. 2b, several proteins exhibiting β-D-glucosidase activity were detected in the intracellular and extracellular protein fractions. *C bescii* contains no annotated extracellular β-D-glucosidases and only two proteins annotated as intracellular β-D-glucosidases: a native GH3 enzyme (Cbes_2354) that has a predicted molecular weight of 87 kDa and a native GH1 enzyme that has a predicted molecular weight of 53 kDa [12]. Protein bands consistent with a molecular weight of ~115 and 110 kDa were detected and we speculate that these species represent dimers of the native 87 kDa enzyme and the heterologous 55 kDa protein (the predicted molecular weight of Acel_0133). The predicted dimer of the *A. cellulolyticus* β-D-glucosidase was not present in the parental strain of *C. bescii* and only detected in strains containing the cloned gene. In the case of a denaturing gel, the β-D-glucosidase from *Pyrococcus furiosus* (BGLPf) appears to form a dimer that is stable in the presence of sodium dodecyl sulfate and this dimer migrated in reducing SDS-PAGE even after incubation at 95 °C [17].

In SDS-PAGE of the extracellular protein fraction, a protein band estimated to be 55 kDa, corresponding to the *A. cellulolyticus* β-D-glucosidase, was absent in the parental strain culture, but clearly detected in strains containing the cloned gene. Additionally, an 87-kDa enzyme, corresponding to the native *C. bescii* enzyme, as well as the predicted dimer of the native 87-kDa enzyme, was detected and we speculate that these β-D-glucosidase activities in the extracellular fraction result from cell lysis. This result is consistent with previous studies showing the presence of cytoplasmic proteins without signal sequences in cell supernatants due to cell lysis [1, 14]. In the ECP fraction, activity of a β-D-glucosidase protein of 55 kDa was observed from both JWCB76 (with signal sequence) and JWCB77 (without the signal sequence) (Fig. 2b). Comparison of the band intensities from the ECP fraction showed that the β-D-glucosidase activity in the band of Acel_0133 in JWCB76 was 2.1 times greater than that in JWCB77, suggesting that attachment of the CelA signal sequence at the *N*-terminal end of Acel_0133 increased secretion efficiency through the SecB-dependent secretion pathway [8, 26]. We point out that the intracellular accumulation of Acel_0133 with a signal sequence may be a consequence of the limited secretion capacity of the *C. bescii* transport apparatus [24].

Expression of the β-D-glucosidase from *A. cellulolyticus* 11B in *C. bescii* results in a significant but modest increase in extracellular β-D-glucosidase activity

To test intracellular and extracellular β-D-glucosidase activity of Acel_0133 from the *C. bescii* host strains, cells were grown at 65 °C and total proteins were collected from the

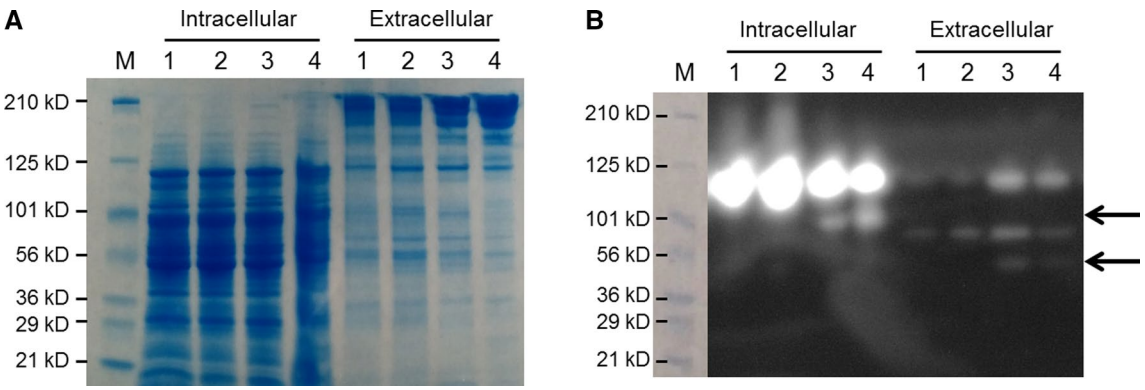

Fig. 2 Confirmation of β-D-glucosidase expression and activity in *C. bescii*. Concentrated intracellular (50 µg) and extracellular (20 µg) proteins were electrophoresed either for SDS-PAGE analysis (**a**) or for zymogram analysis (**b**) using 5 mM MUG as a substrate. *M* pre-stained SDS-PAGE standards, Broad range (Bio-Rad Laboratories); *1* JWCB21 (parental strain); *2* JWCB73 (E1-expressing strain); *3* JWCB76 (E1 + extracellular Acel_0133-expressing strain); *4* JWCB77 (E1 + intracellular Acel_0133-expressing strain)

supernatant (the extracellular fraction), as well as from cell extracts (the intracellular fraction). These fractions were then assayed at 75 °C. Although the optimal temperature for growth of *A. cellulolyticus* is 55 °C [23], enzymes from *A. cellulolyticus* are reported to be optimally active above 55 °C (80 °C for E1 and 90 °C for the Acel_0372 xylanase) [2, 3]. The intracellular and extracellular β-D-glucosidase activities of the parental strain (JWCB21) and the E1-expressing background strain (JWCB73) were indistinguishable (Fig. 3a). Somewhat surprisingly, for the strain expressing β-D-glucosidase without a signal sequence, there was virtually no difference in intracellular activity compared to the parental strain and a slight but detectable increase in extracellular activity. For the strain expressing β-D-glucosidase with the CelA signal sequence there was, as expected, an increase in extracellular activity.

The intracellular activity of JWCB21 and JWCB73 was 35- and 32-fold ($P_{values} < 0.001$) higher, respectively, than the extracellular activity (Fig. 3a). This result may indicate that *C. bescii* strains prefer to hydrolyze cellobiose intracellularly after importing cellobiose with cellodextrin transporters [12]. Cellobiose is a known inhibitor of cellulase activity, and studies have shown that overexpression of β-D-glucosidase in *Trichoderma reesei* and *C. thermocellum* strains results in higher cellulolytic activity [13, 22]. Moreover, the exogenous addition of β-D-glucosidase purified from *Thermotoga maritima* was reported to markedly improve the performance of CelA, the most abundant extracellular protein produced by *C. bescii*, on Avicel [4]. Carboxymethylcellulose (CMC) and Avicel are traditionally used as an assay for endoglucanase and exoglucanase activities, respectively. As previously reported [9], the exoproteome from an E1-expressing strain (JWCB73) of *C. bescii* showed 10.6% ($P_{value} = 0.008$) higher activity on CMC and 20.3% ($P_{value} = 0.016$) higher activity on Avicel compared to the parental strain (Fig. 3b). Increased activity of the concentrated culture supernatants from the Acel_0133-expressing strains (JWCB76 and 77) was observed for both CMC and Avicel substrates compared to the parental strain (JWCB21). Whereas the activity of the exoproteome from JWCB76 (Acel_0133 with signal sequence) on CMC and Avicel increased 17.6% ($P_{value} = 0.008$) and 32.8% ($P_{value} = 0.002$), the activity from JWCB77 (Acel_0133 without signal sequence) increased 24.0% ($P_{value} = 0.001$) and 48.9% ($P_{value} = 0.002$), respectively (Fig. 3b). The fact that there is a larger increase in exoglucanase activity (on Avicel) in the Acel_0133-expressing strains than endoglucanase activity (on CMC) is consistent with the observation that cellobiose is a stronger inhibitor of exoglucanases than endoglucanases [25]. Interestingly, although the exoproteome of JWCB76 (extracellular expression of Acel_0133) showed a 56.5% ($P_{value} = 0.003$) higher β-D-glucosidase activity than JWCB77 (intracellular expression of Acel_0133) (Fig. 3a), a higher increase of activity when acting on CMC and Avicel was observed in JWCB77. Similar results were also reported by Dashtban et al. [13], in which the endoglucanase and exoglucanase activities of *T. reesei* strains with high β-D-glucosidase activity were lower than that of the *T. reesei* strains with low β-D-glucosidase activity. One possible explanation for this result is that high expression levels of β-D-glucosidases in the exoproteome might reduce expression or secretion of other enzymes, including endoglucanases and exoglucanases. Moreover, although there was a significant increase in extracellular β-D-glucosidase

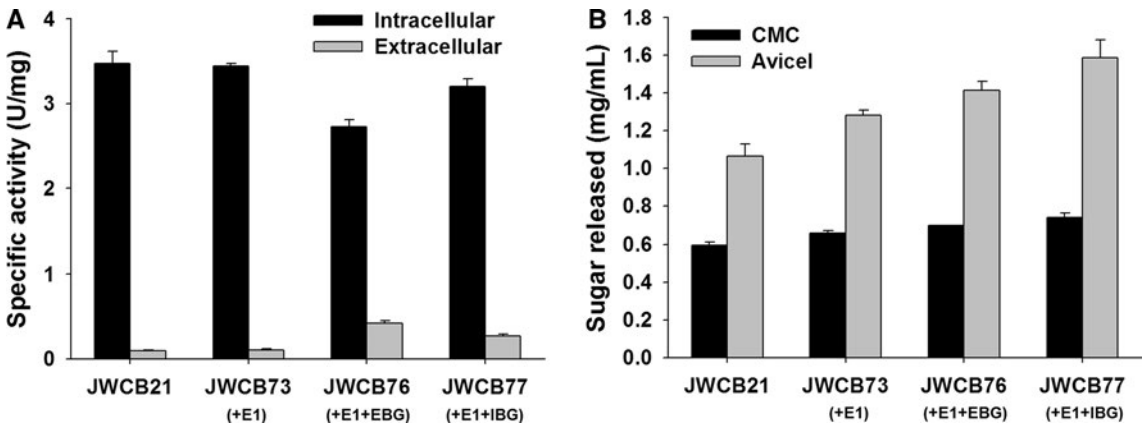

Fig. 3 Intracellular and extracellular β-D-glucosidase (BG) activity of *C. bescii* strains on CMC and Avicel. **a** The enzyme was incubated at 75 °C for 10 min in reaction buffer containing 5 mM *p*-nitrophenyl β-D-glucopyranoside (*p*-NPG), 20 mM MES buffer (pH 5.5), 1 mM dithiothreitol (DTT), 1 mM $CaCl_2$, and 1 mM $MgCl_2$. **b** Activity of extracellular protein (25 µg/mL concentrated protein) on carboxymethylcellulose (CMC) and Avicel was measured in triplicate. JWCB21, the parent strain used in these experiments (see Table 1 for genotype details); JWCB73, the E1-expressing strain; JWCB76, the E1-expressing strain containing Acel_0133 with a signal sequence; JWCB77, the E1-expressing strain containing Acel_0133 without a signal sequence; Eβ-D-glucosidase (EBG), extracellular β-D-glucosidase; Iβ-D-glucosidase (IBG), intracellular β-D-glucosidase

activity in the strains producing Ace_0133, relatively little extracellular β-D-glucosidase activity was detected—a 4.2-fold ($P_{value} = 0.002$) increase from JWCB76 (Ace_0133 with the signal sequence) and a 2.7-fold ($P_{value} = 0.002$) increase from JWCB77 (Ace_0133 without the signal sequence). This outcome may be the result of the accumulation of cellobiose in the supernatant that inhibits further cellulose hydrolysis. This effect was reported by a previous study, showing the accumulation of high concentrations of cellobiose and, to a lesser extent, cellotriose, after a 90-h incubation inhibited growth of *C. bescii* on crystalline cellulose [27]. Increasing the extracellular β-D-glucosidase activity might be expected to increase activity on CMC and/or Avicel. To test this hypothesis, the β-D-glucosidase activity of the Acel_0133-expressing strains was compared to the parental and background strains on CMC and Avicel (Fig. 3b). There was virtually no difference in activity on CMC and a small but significant increase on Avicel, suggesting that increasing the extracellular β-D-glucosidase does, in fact, increase activity on cellulose hydrolysis.

Expression of the β-D-glucosidase from *A. cellulolyticus* 11B in *C. bescii* results in an increase in the growth of *C. bescii* on crystalline cellulose

To investigate whether or not the expression of the *A. cellulolyticus* β-D-glucosidase in *C. bescii* would improve its ability to grow on crystalline cellulose, growth of the strains on Avicel was compared. Growth of the parent strain (JWCB21), the E1-expressing strain (JWCB73), and the strains containing the *A. cellulolyticus* β-D-glucosidase with and without the signal sequence (JWCB76 and JWC77) was first examined on cellobiose. Note that the presence of the β-D-glucosidase should not make a dramatic difference to growth on cellobiose, as cellobiose is a poor substrate for this enzyme. As shown in Fig. 4a, growth of these strains on cellobiose was virtually identical. This result also suggests that there is no obvious detrimental effect on growth of *C. bescii* expressing this heterologous protein. Furthermore, after 24-h cultivation on Avicel, the E1-expressing strain (JWCB73) showed 3.4-fold ($P_{value} = 0.040$) increase in growth (1.3×10^6 CFU/mL) compared to the parental strain JWCB21 (Fig. 4b). There was no significant difference in cell growth on Avicel between the E1-expressing strain and the β-D-glucosidase-expressing strain without a signal sequence (JWCB77). There was, however, a small but significant increase in growth of JWC76. Similar results were also reported by Maki et al. [22] in which an increase in total cellulase activity of *C. thermocellum* by β-D-glucosidase overexpression did not lead to a similar increase in ethanol production during batch fermentation. We suggest that this result could be due to a metabolic burden in these anaerobic bacteria caused by β-D-glucosidase overexpression. β-D-Glucosidases hydrolyze cellobiose producing two glucose molecules that must be phosphorylated to be utilized metabolically, a process that is not energetically favorable. Cellobiose phosphorylase converts cellobiose to glucose and glucose-1-phosphate without depleting ATP levels, so phosphorolytic pathways may be preferred by these bacteria. The scarcity of ATP under anaerobic conditions may also be a crucial factor in the preponderance of the phosphorolytic pathway in obligate anaerobic bacteria [21, 30]. In fact, in *C. bescii*, putative cellobiose/cellodextrin phosphorylases (Cbes_0459 and Cbes_0460) are detected in much higher amounts than β-D-glucosidase [12]. The fact that the addition of an extracellular β-glucosidase did not have a dramatic effect on cellulose utilization may suggest that the metabolic burden of cellulose hydrolysis is more complicated than the simple expression and activity of these enzymes.

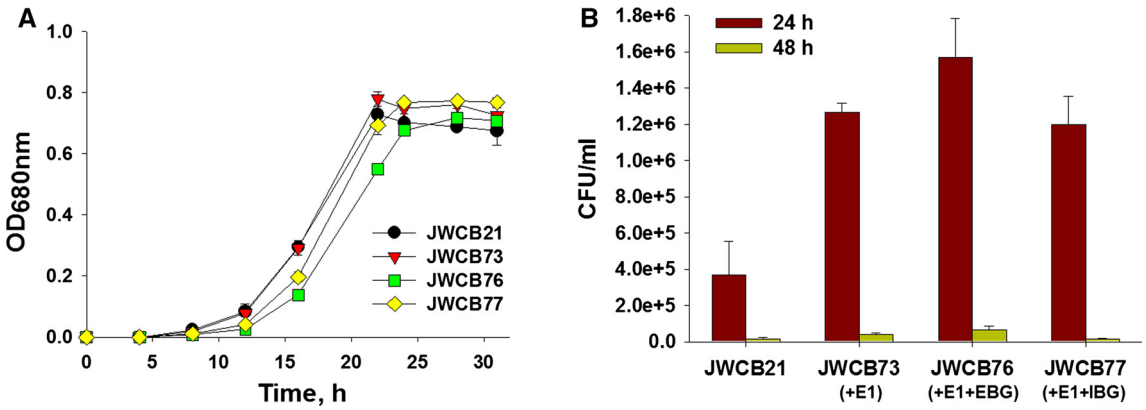

Fig. 4 Growth of *C. bescii* strains on cellobiose (**a**) or Avicel (**b**). JWCB21, the parent strain used in these experiments (see Table 1 for genotype details); JWCB73, the E1-expressing strain; JWCB76, the E1-expressing strain containing Acel_0133 with signal sequence; JWCB77, the E1-expressing strain containing Acel_0133 without signal sequence; Eβ-D-glucosidase, extracellular β-D-glucosidase (EBG); Iβ-D-glucosidase (IBG), intracellular β-D-glucosidase

Acknowledgements We thank Shreena Patel for technical assistance and Joseph Groom for critical review of the manuscript. This work was supported by the BioEnergy Science Center, US DOE Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science. Oak Ridge National Laboratory is managed by UT-Battelle, LLC, for the US DOE under contract DE-AC05-00OR22725. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

1. Antelmann H, van Dijl JM, Bron S, Hecker M (2005) Proteomic survey through secretome of *Bacillus subtilis*. In: microbial proteomics. Wiley, New York, pp 179–208. doi:10.1002/0471973165.ch12
2. Baker JO, Adney WS, Nieves RA, Thomas SR, Wilson DB, Himmel ME (1994) A new thermostable endoglucanase, *Acidothermus cellulolyticus* E1—synergism with *Trichoderma reesei* CBH-I and comparison to *Thermomonospora fusca* E_5. Appl Biochem Biotechnol 45:245–256. doi:10.1007/Bf02941803
3. Barabote RD, Parales JV, Guo YY, Labavitch JM, Parales RE, Berry AM (2010) Xyn10A, a thermostable endoxylanase from *Acidothermus cellulolyticus* 11B. Appl Environ Microbiol 76:7363–7366. doi:10.1128/Aem.01326-10
4. Brunecky R, Alahuhta M, Xu Q, Donohoe BS, Crowley MF, Kataeva IA, Yang SJ, Resch MG, Adams MWW, Lunin VV, Himmel ME, Bomble YJ (2013) Revealing nature's cellulase diversity: the digestion mechanism of *Caldicellulosiruptor bescii* CelA. Science 342:1513–1516. doi:10.1126/science.1244273
5. Chung D, Cha M, Farkas J, Westpheling J (2013) Construction of a stable replicating shuttle vector for *Caldicellulosiruptor* species: use for extending genetic methodologies to other members of this genus. PLoS One 8:e62881. doi:10.1371/journal.pone.0062881
6. Chung D, Cha M, Guss AM, Westpheling J (2014) Direct conversion of plant biomass to ethanol by engineered *Caldicellulosiruptor bescii*. Proc Natl Acad Sci USA 111:8931–8936. doi:10.1073/pnas.1402210111
7. Chung D, Cha M, Snyder EN, Elkins JG, Guss AM, Westpheling J (2015) Cellulosic ethanol production via consolidated bioprocessing at 75 °C by engineered *Caldicellulosiruptor bescii*. Biotechnol Biofuels. doi:10.1186/S13068-015-0346-4
8. Chung D, Young J, Bomble YJ, Vander Wall TA, Groom J, Himmel ME, Westpheling J (2015) Homologous expression of the *Caldicellulosiruptor bescii* CelA reveals that the extracellular protein is glycosylated. PLoS One. doi:10.1371/journal.pone.0119508
9. Chung D, Young J, Cha M, Brunecky R, Bomble YJ, Himmel ME, Westpheling J (2015) Expression of the *Acidothermus cellulolyticus* E1 endoglucanase in *Caldicellulosiruptor bescii* enhances its ability to deconstruct crystalline cellulose. Biotechnol Biofuels. doi:10.1186/S13068-015-0296-X
10. Chung D, Huddleston JR, Farkas J, Westpheling J (2011) Identification and characterization of CbeI, a novel thermostable restriction enzyme from *Caldicellulosiruptor bescii* DSM 6725 and a member of a new subfamily of HaeIII-like enzymes. J Ind Microbiol Biotechnol 38:1867–1877. doi:10.1007/s10295-011-0976-x
11. Chung D, Farkas J, Westpheling J (2013) Overcoming restriction as a barrier to DNA transformation in *Caldicellulosiruptor* species results in efficient marker replacement. Biotechnol Biofuels. doi:10.1186/1754-6834-6-82
12. Dam P, Kataeva I, Yang SJ, Zhou FF, Yin YB, Chou WC, Poole FL, Westpheling J, Hettich R, Giannone R, Lewis DL, Kelly R, Gilbert HJ, Henrissat B, Xu Y, Adams MWW (2011) Insights into plant biomass conversion from the genome of the anaerobic thermophilic bacterium *Caldicellulosiruptor bescii* DSM 6725. Nucleic Acids Res 39:3240–3254. doi:10.1093/nar/gkq1281
13. Dashtban M, Qin WS (2012) Overexpression of an exotic thermotolerant β-glucosidase in *Trichoderma reesei* and its significant increase in cellulolytic activity and saccharification of barley straw. Microb Cell Fact. doi:10.1186/1475-2859-11-63
14. Durmaz E, Hu Y, Aroian RV, Klaenhammer TR (2016) Intracellular and extracellular expression of *Bacillus thuringiensis* crystal protein Cry5B in *Lactococcus lactis* for use as an anthelminthic. Appl Environ Microbiol 82:1286–1294. doi:10.1128/Aem.02365-15
15. Farkas J, Chung D, Cha M, Copeland J, Grayeski P, Westpheling J (2013) Improved growth media and culture techniques for genetic analysis and assessment of biomass utilization by *Caldicellulosiruptor bescii*. J Ind Microbiol Biotechnol 40:41–49. doi:10.1007/s10295-012-1202-1
16. Groom J, Chung D, Young J, Westpheling J (2014) Heterologous complementation of a *pyrF* deletion in *Caldicellulosiruptor hydrothermalis* generates a new host for the analysis of biomass deconstruction. Biotechnol Biofuels. doi:10.1186/S13068-014-0132-8
17. Kado Y, Inoue T, Ishikawa K (2011) Structure of hyperthermophilic β-glucosidase from *Pyrococcus furiosus*. Acta Crystallogr Sect F Struct Biol Cryst Commun 67:1473–1479. doi:10.1107/S1744309111035238
18. Kanafusa-Shinkai S, Wakayama J, Tsukamoto K, Hayashi N, Miyazaki Y, Ohmori H, Tajima K, Yokoyama H (2013) Degradation of microcrystalline cellulose and non-pretreated plant biomass by a cell-free extracellular cellulase/hemicellulase system from the extreme thermophilic bacterium *Caldicellulosiruptor bescii*. J Biosci Bioeng 115:64–70. doi:10.1016/j.jbiosc.2012.07.019
19. Kim SK, Chung D, Himmel ME, Bomble YJ, Westpheling J (2016) Heterologous expression of family 10 xylanases from *Acidothermus cellulolyticus* enhances the exoproteome of *Caldicellulosiruptor bescii* and growth on xylan substrates. Biotechnol Biofuels. doi:10.1186/s13068-016-0588-9
20. Lochner A, Giannone RJ, Rodriguez M Jr, Shah MB, Mielenz JR, Keller M, Antranikian G, Graham DE, Hettich RL (2011) Use of label-free quantitative proteomics to distinguish the secreted cellulolytic systems of *Caldicellulosiruptor bescii* and *Caldicellulosiruptor obsidiansis*. Appl Environ Microbiol 77:4042–4054. doi:10.1128/AEM.02811-10
21. Lou J, Dawson KA, Strobel HJ (1996) Role of phosphorolytic cleavage in cellobiose and cellodextrin metabolism by the ruminal bacterium *Prevotella ruminicola*. Appl Environ Microbiol 62:1770–1773
22. Maki ML, Armstrong L, Leung KT, Qin WS (2013) Increased expression of β-glucosidase A in *Clostridium thermocellum* 27405 significantly increases cellulase activity. Bioengineered 4:15–20. doi:10.4161/bioe.21951
23. Mohagheghi A, Grohmann K, Himmel M, Leighton L, Updegraff DM (1986) Isolation and characterization of *Acidothermus cellulolyticus* gen. nov., sp. nov., a new genus of thermophilic, acidophilic, cellulolytic bacteria. Int J Syst Bacteriol 36:435–443. doi:10.1099/00207713-36-3-435
24. Rosenberg HF (1998) Isolation of recombinant secretory proteins by limited induction and quantitative harvest. Biotechniques 24:188–190
25. Teugjas H, Valjamae P (2013) Product inhibition of cellulases studied with ^{14}C-labeled cellulose substrates. Biotechnol Biofuels. doi:10.1186/1754-6834-6-104
26. Tjalsma H, Bolhuis A, Jongbloed JDH, Bron S, van Dijl JM (2000) Signal peptide-dependent protein transport in *Bacillus subtilis*: a genome-based survey of the secretome. Microbiol Mol Biol Rev 64:515–547. doi:10.1128/Mmbr.64.3.515-547.2000

27. Yang SJ, Kataeva I, Hamilton-Brehm SD, Engle NL, Tschaplinski TJ, Doeppke C, Davis M, Westpheling J, Adams MW (2009) Efficient degradation of lignocellulosic plant biomass, without pretreatment, by the thermophilic anaerobe "*Anaerocellum thermophilum*" DSM 6725. Appl Environ Microbiol 75:4762–4769. doi:10.1128/AEM.00236-09
28. Yi Z, Su X, Revindran V, Mackie RI, Cann I (2013) Molecular and biochemical analyses of CbCel9A/Cel48A, a highly secreted multi-modular cellulase by *Caldicellulosiruptor bescii* during growth on crystalline cellulose. PLoS One 8:e84172. doi:10.1371/journal.pone.0084172
29. Young J, Chung D, Bomble YJ, Himmel ME, Westpheling J (2014) Deletion of *Caldicellulosiruptor bescii* CelA reveals its crucial role in the deconstruction of lignocellulosic biomass. Biotechnol Biofuels. doi:10.1186/s13068-014-0142-6
30. Zhang YHP, Lynd LR (2005) Cellulose utilization by *Clostridium thermocellum*: bioenergetics and hydrolysis product assimilation. Proc Natl Acad Sci USA 102:7321–7325. doi:10.1073/pnas.0408734102