Native xylose-inducible promoter expands the genetic tools for the biomass-degrading, extremely thermophilic bacterium *Caldicellulosiruptor bescii*

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Received: 4 December 2017 / Accepted: 2 April 2018
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Abstract
Regulated control of both homologous and heterologous gene expression is essential for precise genetic manipulation and metabolic engineering of target microorganisms. However, there are often no options available for inducible promoters when working with non-model microorganisms. These include extremely thermophilic, cellulo-lytic bacteria that are of interest for renewable lignocellulosic conversion to biofuels and chemicals. In fact, improvements to the genetic systems in these organisms often cease once transformation is achieved. This present study expands the tools available for genetically engineering *Caldicellulosiruptor bescii*, the most thermophilic cellulose-degrader known growing up to 90 °C on unpretreated plant biomass. A native xylose-inducible (Pₓᵢ) promoter was utilized to control the expression of the reporter gene (ldh) encoding lactate dehydrogenase. The Pₓᵢ-ldh construct resulted in a both increased ldh expression (20-fold higher) and lactate dehydrogenase activity (32-fold higher) in the presence of xylose compared to when glucose was used as a substrate. Finally, lactate production during growth of the recombinant *C. bescii* strain was proportional to the initial xylose concentration, showing that tunable expression of genes is now possible using this xylose-inducible system. This study represents a major step in the use of *C. bescii* as a potential platform microorganism for biotechnological applications using renewable biomass.

Keywords Anaerobes · Biodegradation of cellulosic · Biotechnology of thermophiles · Genetics · Molecular biology · Genetics of extremophiles

Abbreviations
5-FOA 5-Fluoroorotic acid
CBP Consolidated bioprocessing
LDH Lactate dehydrogenase
XI Xylose isomerase
PCR Polymerase chain reaction

Introduction
*Caldicellulosiruptor bescii* is the most thermophilic cellulose-degrading bacterium known, growing optimally at 78 °C. It is capable of utilizing plant biomass as the sole carbon source without prior pretreatment (Yang et al. 2009). These attributes make *C. bescii* a candidate organism for lignocellulosic-based biotechnology via consolidated bioprocessing (CBP) (Blumer-Schuette et al. 2014; Zeldes et al. 2015). CBP is an economically attractive alternative for generating bio-based fuels and chemicals from renewable plant biomass without the need for expensive exogenous cellulase enzymes (Lynd et al. 2005; Olson et al. 2012). However, metabolic engineering of *C. bescii* is required since the organism does not naturally produce industrial products (Yang et al. 2010). Thus, a robust and stable genetic system is required if *C. bescii* is to become a platform organism for CBP (Chung et al. 2012, 2013).

The first genetic background of *C. bescii* was based on the uracil auxotrophic strain, JWCB005, which was selected
by growth on 5-fluoroorotic acid (5-FOA) (Chung et al. 2012). 5-FOA is toxic to uracil prototrophic strains and, thus, can be used for counter selection for loss of plasmid backbones when making chromosomal manipulations in C. bescii (Chung et al. 2012). The majority of C. bescii strains have been developed in this background strain leading to increased understanding of plant biomass deconstruction, native metabolism and metabolic engineering (Cha et al. 2013a, 2016; Chung et al. 2014a, b, 2015a, b, c, d; Conway et al. 2016, 2017; Scott et al. 2015). A second uracil auxotrophic strain was developed demonstrating the effectiveness of a kanamycin resistance strategy, adding antibiotic resistance to the genetic tools available in C. bescii (Lipscomb et al. 2016). It was later shown that an active insertion element, ISCbed4, is found in a lower copy number in the MACB1018 lineage (Williams-Rhaesa et al. 2017).

While major improvements have been made in the C. bescii genetic system, genetic tools are still limited, especially for controlling both homologous and heterologous gene expression in this organism. To date, only two promoters have been deployed and both are high expression constitutive promoters in C. bescii that are responsible for the expression of genes encoding the S-layer protein and the ribosomal protein S30EA (Lipscomb et al. 2016). While these are useful in some cases, they do not allow for targeted control of gene expression or decreased expression of essential genes within C. bescii. To address this, we describe here the use of a xylose-inducible promoter in C. bescii based on the gene encoding lactate dehydrogenase (LDH) (Athe_1918, ldh) as a reporter. In the recombinant strain, the ldh mRNA transcript level, LDH activity and lactate production were all increased in xylose-containing media compared to glucose-containing media. Additionally, the amount of lactate produced was directly dependent on the initial xylose concentration in the medium, demonstrating titratable expression using this inducible promoter system.

Materials and methods

Growth of C. bescii

Strains were grown under anaerobic conditions at 75 °C. All strains used in this study are described in Table 1. For genetic manipulation, C. bescii competent cells were grown in the low osmolarity defined medium, containing amino acids as described (Farkas et al. 2013) with high purity argon in the headspace. Recovery, selection, and growth experiments were performed in modified DSMZ 516 medium having the following composition per liter: 1× salt solution, 1× vitamin solution, 1× trace element solution, 0.16 μM sodium tungstate, 0.25 mg resazurin, 5 g glucose (or 5 g xylose where described), 0.5 g yeast extract, 1 g cysteine hydrochloride, 1 g sodium bicarbonate and 1 mM potassium phosphate buffer, pH 7.2. The 50× stock salt solution contained the following per liter: 16.5 g NH₄Cl, 16.5 g KCl, 16.5 g MgCl₂·6H₂O and 7 g CaCl₂·2H₂O. Stock solutions of 200× vitamins and 1000× trace elements were prepared, as previously described (Yang et al. 2010). All media were filter sterilized using a Millipore 0.22 μm filter. The complex medium containing glucose as the carbon source will be referred to as CG516 and the same medium containing xylose as the carbon source will be referred to as CX516. Both CG516 and CX516 use 20% carbon dioxide and 80% nitrogen in the culture headspace. For cultures with varying initial xylose concentration below 33.3 mM, 27.8 mM glucose was used as a supplemental carbon source, except when 16.6 mM xylose was used which had 13.9 mM glucose. All uracil auxotrophic strains were grown with 20 μM uracil in the growth medium. Kanamycin and 5-fluoroorotic acid (5-FOA) were used at concentrations of 50 μg/mL and 4 mM, respectively, when appropriate for selection (Lipscomb et al. 2016).

The solid medium for genetic manipulation was prepared by mixing equal volumes of pre-heated 2X CG516 medium with 6% (wt/vol) agar. One hundred microliters (100 μL) of culture dilution were pipetted into an empty petri dish and the mixed medium was poured directly into the plate while swirling to distribute cells. Plates were allowed to solidify by cooling at room temperature for 20 min, inverted and placed into anaerobic canisters, as described (Lipscomb et al. 2016), and flushed with 20% carbon dioxide and 80% nitrogen gas prior to incubation at 70 °C for 2 days. Colonies were picked using toothpicks pressed into the agar and inoculated into 4–5 mL medium in screw-cap Hungate tubes.

Vector construction

The plasmid, pAR012 (Fig. S1), was constructed for insertion of the lactate dehydrogenase gene (Athe_1918, ldh) under the control of the xylose isomerase promoter (Pxi promoter Fig. 1). The promoter regulatory binding site was

<table>
<thead>
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<th>Strain</th>
<th>Genotype</th>
<th>Parent strain</th>
<th>References</th>
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<td>MACB1018</td>
<td>ΔpyrE</td>
<td>C. bescii DSM 6725</td>
<td>(Lipscomb et al. 2016)</td>
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<td>ΔpyrE, Δldh, Pxi-ldh</td>
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<td>This study</td>
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determined through use of DNA motif recognition software Virtual Footprint version 3.0 with the highest scoring pattern belonging to the XylR binding site of *Bacillus subtilis* (Schmiedel and Hillen 1996). The plasmid was assembled from polymerase chain reaction (PCR) products using NEBuilder® HiFi DNA assembly (New England Biosciences). Primers used in this study can be found in Table 2.

The 200 bp promoter region (note that *genes* are designated *Athe*), as well as the 1.2 kb upstream and 1.1 kb downstream flanking regions were amplified from *C. bescii* strain DSMZ 6725 genomic DNA. The plasmid backbone was generated by amplifying the colE1 origin of replication, the *P*<sub>530</sub> promoter, *Cbhtk* and *pyrE* regions from pGL100 (Lipscomb et al. 2016). All PCR products were generated using Takara PrimeSTAR Max polymerase, following manufacturer protocols. The plasmid was sequence verified and found to have a silent mutation in the *Cbhtk* gene after passage through *E. coli* changing the codon for T44 from ACA to ACG; however, this did not affect kanamycin resistance.

**Table 2 Primers used in this study**

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in *C. bescii* strains transformed with this plasmid (data not shown).

### Plasmid DNA methylation

Plasmid was methylated with recombinant M. CbeI as described (Chung et al. 2012). The *E. coli* strain, JW284, contains the M. CbeI expression plasmid, pDCW73, used for heterologous expression of M. CbeI that can be purified from cell lysate using the His-Spin Protein Miniprep kit (Zymo Research) (Chung et al. 2012). The Plasmid was methylated with recombinant M. CbeI as described (Chung et al. 2012). The Plasmid DNA methylation was performed using the Brilliant III Ultra-Fast SYBR® Green QRT-PCR Master Mix run on an Agilent MX3000p qPCR instrument and analysis was performed using the MxPro software (Agilent). Controls not subjected to RT were verified to have no amplification prior to use of cDNA for experimental quantification of mRNA. Biological duplicates were tested and all samples were analyzed in technical duplicates. Expression was determined relative to *gapdh* which has been shown previously to be expressed at constitutively high levels on both glucose and xylose (Scott et al. 2015). Expression and standard deviation calculations were performed according to the “Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR” (Applied Biosystems).

### Lactate dehydrogenase activity

Cells were grown to late exponential phase in 500 mL of both CG516 and CX516 media at 75 °C. Cell pellets were collected by centrifugation at 6000g for 10 min, resuspended in liquid nitrogen, and stored at −80 °C prior to preparation of cell lysates. Cell lysates were prepared anaerobically in a COY chamber by suspending *C. bescii* cells in 0.5 mL of 25 mM phosphate buffer pH 7.0 with 2 mM dithionite, 1% trehalose and 1% 6-aminohexanoic acid. Cells were disrupted by sonication for 3 × 30 s at a maximum of 40 W with 2 min between each round of sonication. Lactate dehydrogenase (LDH) activity was determined by the pyruvate-dependent oxidation of NADH. The assay was performed under anaerobic conditions in glass cuvettes at 75 °C, containing 50 mM MOPS buffer, pH 7.0, and 0.5 mM NADH. The reaction was started by the addition of 5 mM pyruvate and an extinction coefficient of 6.22 mM⁻¹ cm⁻¹ was used for NADH at 340 nm. In cases where no LDH activity was observed, the integrity of the protein extract was confirmed...
by assaying for pyruvate oxidoreductase (POR) activity by the pyruvate-dependent reduction of methyl viologen (MV). The assay solution contained 0.2 mM CoASH, 0.4 mM thiamine pyrophosphate, 2.0 mM MgCl₂, 2.0 mM MV. Anaerobic conditions were maintained by the addition of trace amounts (<2 mM) of sodium dithionite. The reaction was started by the addition of 10 mM pyruvate; an extinction coefficient of 13.0 mM⁻¹ cm⁻¹ was used for reduced MV at 600 nm (Jones and Garland 1977). All activity was normalized to protein concentration in each extract as determined by the Bradford Method using bovine serum albumin as a standard.

**Lactate, glucose and xylose quantification**

Lactate, glucose and xylose were measured at the end of growth by high-performance liquid chromatography (HPLC) on a 2690 separations module (Waters, Milford, MA), using a Bio-Rad fermentation column, a photodiode array detector (model 996; Waters) and a refractive index detector (model 410; Waters). The mobile phase was 5 mM sulfuric acid at a flow rate of 0.5 mL per min. Samples were taken periodically throughout growth or at the end point of growth for cultures at the 25 and 5 mL scale, respectively. Samples were prepared by centrifugation at 18,000 g for 5 min to remove cells and particulate, followed by acidification with a final concentration of 0.1 M sulfuric acid.

**Results and discussion**

**Strain generation**

Based on work in the related organism, *Caldicellulosiruptor saccharolyticus*, where the gene encoding an annotated xylose isomerase (XI), was shown to be differentially expressed under growth of a variety of sugars (Van Fossen et al. 2009). We determined that the homologous gene in *C. bescii* (Athe_0603) has increased expression when xylose is used as the carbon source (data not shown). These results suggested that the xylose isomerase promoter (Pxi) could be used as an inducible promoter in *C. bescii*. The 200 bp promoter sequence used is shown in Fig. 1 with a xylose regulator binding site characterized in *Bacillus subtilis* mapped 75 bp upstream from the transcription start site (Schmiedel and Hillen 1996).

To test use of Pxi as an inducible promoter, we selected the gene (Athe_1918) encoding lactate dehydrogenase from *C. bescii* as the reporter. This gene had been previously used in *Pyrococcus furiosus* as a reporter gene for testing a cold inducible promoter (Basen et al. 2012). Additionally, lactate dehydrogenase deletion strains (Δldh) of *C. bescii* have been developed in multiple genetic lineages, allowing for the use of ldh as a reporter in an ldh null background (Cha et al. 2013a; Lipscomb et al. 2016). The Δldh strain MACB1034 was used as the parent strain for this study as ldh is a known target of the ISCbe4 and, thus, using the lineage with few IS elements was advantageous (Cha et al. 2013b; Lipscomb et al. 2016; Williams-Rhaesa et al. 2017). Plasmid pAR012 was constructed (Fig. 2a) containing the colE1 origin for cloning in *E. coli*, upstream and downstream flanking regions of ~1 kb for targeting plasmid integration at the intergenic region between Athe_0949 and Athe_0950, the Cbhtk gene for selection of kanamycin resistance in both *E. coli* and *C. bescii*, and the pyrE gene for counter selection of plasmid loss from *C. bescii* (Fig. 2a). Between the flanking regions is the *C. bescii* ldh gene (Athe_1918) under the control of a 199 bp region from directly upstream of the xylose isomerase gene, Athe_0603. The plasmid, pAR012, was used to transform *C. bescii* strain MACB1034. After final strain development, MACB1066 was confirmed to encode the Pxi ldh gene construct (Fig. 2b).

**Xylose-dependence of ldh transcription and LDH activity**

To test the responsiveness of cells containing the Pxi ldh gene construct to xylose, we grew strains MACB1018, MACB1034 and MACB1066 (see Table 1) in media containing either glucose or xylose as the carbon source. RNA was collected from exponentially growing cells and qRT-PCR was performed (Table S1). Native expression of the ldh by strain MACB1018 did not change due to the presence of xylose and no amplification product could be detected for the ldh gene from strain MACB1034, which is the Δldh parent strain (Table S1). In Fig. 3, we show the relative expression for the xi gene and ldh. Expression of both genes is increased during growth on xylose, 60- and 30-fold for xi and ldh, respectively. Expression of the ldh gene was lower than that of the xi gene in its native context, suggesting that the 199 bp selected for the XI promoter region is sufficient for regulating the expression of a downstream reporter gene, ldh in strain MACB1066 though other more distal elements may be necessary for maximal expression from this promoter.

Having analyzed the transcriptomic levels of the ldh reporter gene in MACB1066, the next goal was to determine if there was a measurable difference in LDH activity in this strain when it was grown on xylose, 33.3 mM, or glucose, 27.75 mM, as the carbon source. As shown in Fig. 4, LDH-specific activity in the cell-free extract increases 32-fold in MACB1066 when it is grown on xylose compared to glucose. There was no detectable LDH activity in the Δldh strain MACB1034, as expected, and the LDH-specific activity for the original parent strain MACB1018 showed no significant response to changes in carbon source (Fig. 4).
Interestingly, the LDH-specific activity in the xylene-inducible MACB1066 strain was 3 times higher than that in the original parent strain MACB1018 (Fig. 4).

**Xylose-dependent lactate production**

The extent to which the production of lactate by xylene-inducible strain MACB1066 was dependent upon the initial xylose concentration in the growth medium was determined using xylose concentrations up to 33.3 mM. The original parent strain MACB1018, where its ldh is under the control of the native promoter, when grown on glucose produces approximately 3 mM lactate; this amount is independent of whether xylose is also present in the growth medium (Fig. 5). As expected, the Δldh parent strain, MACB1034, did not produce lactate when grown on glucose regardless of whether xylose was also present (data not shown). However, the MACB1066 strain did exhibit xylose-dependent lactate generation (Fig. 5). Indeed, the responsiveness to the initial xylose concentration was very specific, such that the amount of lactate produced increased almost proportionally with xylose. A very low concentration of lactate (~0.25 mM)
was produced in the absence of added xylose, presumably due to the production of the \( P_{xli} \) inducer, which is assumed to be xylose, during the normal metabolism of \( C. bescii \). This increased to \( \approx 5 \) mM lactate when 33 mM xylose was present, which is almost twice the lactate produced by the original parent strain MACB1018 in which \( ldh \) expression is under native control. In general, these data correlated with the approximately threefold higher LDH-specific activity when MACB1066 was grown in the presence of 33.3 mM xylose compared to the parent strain (Fig. 5).

Under all conditions, the strains consumed similar total amounts of sugar, 12.5–16.5 mM total, with 50–56% of the total substrate remaining in excess at the end of growth (Table 3). For cultures with initial xylose concentrations of 16.6 and 8, 6.3–7.0 and 7.0–7.2 mM, xylose was consumed, respectively, leaving 9–10 and 1 mM xylose in excess (Table 3). This would explain the similar results for these two conditions with both resulting in 2.8–3.0 mM lactate for strain MACB1066 (Table 3). For both 4 and 1 mM initial xylose concentrations, no xylose was detected at the end of growth for all three strains (Table 3). In cultures with both glucose and xylose present, slightly less glucose was consumed overall. However, there is no evidence of preferential consumption of either sugar, as neither was fully depleted prior to utilization of the other except when xylose concentrations were very low (i.e., 4 and 1 mM initial xylose) (Table 3). This result is in agreement with the study in \( C. saccharolyticus \) which showed simultaneous utilization of six monosaccharides (Van Fossen et al. 2009). The decrease in lactate production for both 16.6 and 8 mM initial xylose, where xylose was still in excess, compared to 33.3 mM xylose, where no glucose was present, suggests that the presence of glucose may result in slight down regulation of \( P_{xli} \) (Fig. 5 and Table 3). In fact, when strain MACB1066 is grown in 16.6 mM xylose in the absence of glucose \( 5.5 \pm 0.4 \) mM lactate is generated similar to the 33.3 mM xylose growth condition. It is possible that this effect is due to inefficient sugar transport, which is not well characterized for any member of this genus. However, there is evidence that for \( C. saccharolyticus \) glucose and xylose are transported by the same uptake systems (van de Werken et al. 2008; Van Fossen et al. 2009).

To confirm the utility of this promoter for more traditional induction of gene expression, strain MACB1066 was grown in glucose medium with and without xylose addition during exponential growth, at 9 h of incubation (Fig. 6). There was no significant difference in growth under the two conditions (Fig. 6). We found no difference in the induced and uninduced cultures up to 9 h
Extremophiles

1 3

post-induction. However, at the 24 h of culture time, the induced cultures had generated 4.1 mM lactate while the culture without xylose addition generated only 0.6 mM lactate. These data indicate that $P_{xi}$ will be of use for induction of gene expression either with xylose present from the beginning of growth or added later. Efforts will be needed to optimize induction and expression of individual gene constructs in the future utilizing this promoter.

**Conclusions**

Here, we present a tunable xylose-inducible promoter for use in *C. bescii*. We used the lactate dehydrogenase gene, *ldh*, native to this organism as the reporter gene and demonstrated the xylose-dependent production of *ldh* mRNA, expression of LDH protein (as measured by specific activity), and lactate product formation in the recombinant

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**Table 3** Glucose and xylose consumption after 24 h by strains MACB1018, MACB1034, and MACB1066 on increasing initial xylose concentrations

<table>
<thead>
<tr>
<th>Initial xylose concentration (mM)</th>
<th>Strain</th>
<th>Glucose consumption (mM)</th>
<th>Xylose consumption (mM)</th>
<th>Total sugar consumption (mM)</th>
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<td>15.5±0.3</td>
</tr>
<tr>
<td></td>
<td>MACB1034</td>
<td>9.1±0.2</td>
<td>7.0±0.5</td>
<td>16.1±0.7</td>
</tr>
<tr>
<td></td>
<td>MACB1066</td>
<td>7.7±0.2</td>
<td>6.3±0.3</td>
<td>14.0±0.5</td>
</tr>
<tr>
<td>33.3</td>
<td>MACB1018</td>
<td>–</td>
<td>14.8±0.1</td>
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<tr>
<td></td>
<td>MACB1034</td>
<td>–</td>
<td>15.4±0.5</td>
<td>15.4±0.5</td>
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<tr>
<td></td>
<td>MACB1066</td>
<td>–</td>
<td>14.5±0.3</td>
<td>14.5±0.3</td>
</tr>
</tbody>
</table>

All values are given in millimolar with standard deviation from biological triplicates. Dashes (–) indicate no glucose or xylose added under these conditions.

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**Fig. 6** Growth (circles) and lactate (squares) production for MACB1066 with and without xylose addition (black closed symbols and gray open symbols, respectively). 4 mM xylose was added 9 h after the start of growth which is denoted by the black arrow. Error bars represent standard deviation $n=3$ biological replicates.
strain where \(ldh\) expression is under control of \(P_{ax}\). This demonstrated use of this promoter opens the door to inducible and regulated control of genes within this organism and greatly expands the molecular genetics tool kit available. Future use of this promoter will allow conditional deletions to confirm gene essentiality as well as controlled inducible expression of genes for heterologous pathways in \(C. bescii\). Likely candidates that will need to be tested include the bifurcating hydrogenase, shown to be the major hydrogen production enzyme in \(C. bescii\), and other genes involved in glycolysis (Cha et al. 2016). Future studies to characterize the molecular mechanisms responsible for induction of this promoter will also increase our understanding of \(C. bescii\) and its metabolism.

**Acknowledgements** This research was supported by a Grant (DE-FG02-06ER64304) from the Bioenergy Science Center (BESC), Oak Ridge National Laboratory, a US Department of Energy (DOE) Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science.

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