Extremophiles
https://doi.org/10.1007/s00792-018-1023-x

ORIGINAL PAPER

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

Amanda M. Williams-Rhaesa[1] · Nanaakua K. Awuku[1] · Gina L. Lipscomb[1] · Farris L. Poole[1] · Gabriel M. Rubinstein[1] · Jonathan M. Conway[2] · Robert M. Kelly[2] · Michael W. W. Adams[1]

Received: 4 December 2017 / Accepted: 2 April 2018

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, cellulolytic 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 (P_{xi}) promoter was utilized to control the expression of the reporter gene (*ldh*) encoding lactate dehydrogenase. The P_{xi}-*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

Communicated by L. Huang.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00792-018-1023-x) contains supplementary material, which is available to authorized users.

✉ Michael W. W. Adams
adamsm@uga.edu

[1] Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA 30602, USA

[2] Department of Chemical and Biomolecular Engineering, North Carolina State University, Raleigh, NC, USA

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

Published online: 24 May 2018

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, IS*Cbe4*, 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 NH_4Cl, 16.5 g KCl, 16.5 g $MgCl_2{\cdot}6H_2O$ and 7 g $CaCl_2{\cdot}2H_2O$. 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 (P_{xi} promoter Fig. 1). The promoter regulatory binding site was

Table 1 Strains of *C. bescii* used and constructed in this study

Strain	Genotype	Parent strain	References
MACB1018	Δ*pyrE*	*C. bescii* DSM 6725	(Lipscomb et al. 2016)
MACB1034	Δ*pyrE*, Δ*ldh*	MACB1018	(Lipscomb et al. 2016)
MACB1066	Δ*pyrE*, Δ*ldh*, P_{xi}-*ldh*	MACB1034	This study

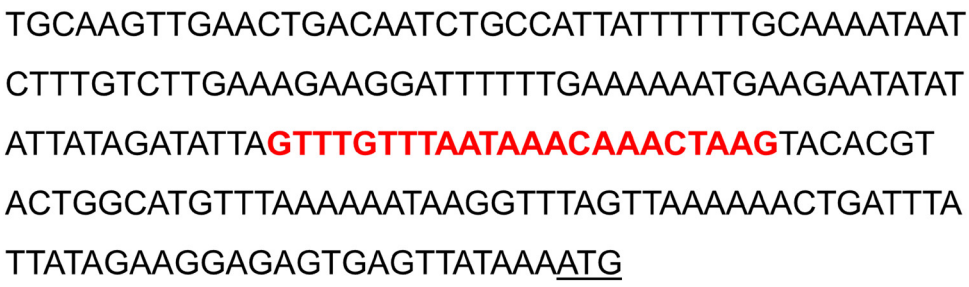

Fig. 1 The 200 bp promoter sequence with the xylose regulator binding site shown in bold red text this was determined through DNA motif recognition software (Virtual Footprint v. 3.0). The transcription start site is shown in underlined text

determined through the 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 the NEBuilder® HiFi DNA assembly (New England Biosciences). Primers used in this study can be found in Table 2. The *ldh* gene, the 200 bp upstream of Athe_0603 constituting the P_{xi} promoter region (note that *C. bescii* 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_{S30} promoter, Cb*htk* 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 Cb*htk* 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

Target	Primer	Sequence
Plasmid construction		
Plasmid backbone	AR015	ATCTAAGAGGTATGATTAAACAAAATAAAAGAGG
	GLCB086	GCATGTGAGCAAAAGGCC
5′ flanking region	AR067	TTTATTGCATATTGCTGTAAGTGCGGCCGCGTTTAAACGGCGCGCCGCATGCTC ACCAAACCTCCTTGTATG
	AR066	ATTTTGTTTAATCATACCTCTTAGATTGCAAGTTTAATAGTAGTTTTAATACTG
Xylose isomerase promoter (P_{xi})	NA08	AAGCCTCCTTTTTGGTAATCTATGCTTGCAAGTTGAACTGACAATCTGCC
	NA04	CAATTTTACCCGGTTTTCTCATTTTATAACTCACTCTCCTTCTATAATAAATCAG
Lactate dehydrogenase (*ldh*)	NA06	ATGAGAAAACCGGGTAAAATTGTAATTATTGG
	NA10	GAGTCAATCTCCCATTGATGTTATAGTTTTAAAGACTCTATCACACTTTTTATTACC
Stem loop terminator	AR069	CATCAATGGGAGATTGACTC
	AR068	ACTTACAGCAATATGCAATAAAGGC
3′ flanking region	AR072	TTGCTGGCCTTTTGCTCACATGCTTTGAGAAAATCTAAGGTATATTGAGAG
	NA09	AGCATAGATTACCAAAAAGGAGGC
Strain validation		
pyrE	GLCB024	AAGGCGGAATGGTCTTTGGTAAAG
	GLCB025	CGTTAATTGGTGCAAACGGTGC
ldh	GLCB003	CTGCAATTAAAGGACCCAAATACG
	GLCB004	GTTTGTGCAACCTTCTATGCC
Athe_0949 genome region	AR031	CTTGCTCTGCTGTTTTGTCTC
	AR032	GAGTATCATTTGCTGCTAATTTATATCC
qPCR		
gapdh	Athe_1406qF	GCTGCAGAAGGCGAATTAAAG
	Athe_1406qR	GGTCTGCAACTCTGTTGGAATA
ldh	AR102	GGCGTAATATGTTGCTCC
	AR103	TGAAATTGCTGCCTGGTCTC
Xylose isomerase	AR106	GGATACAGATCAGTTCCCAACAG
	AR108	CAATGTGACCAATGACCAAGTCTTC

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).

C. bescii transformation and strain construction

Competent cells of strain MACB1034 were inoculated to a density of $6–8 \times 10^5$ cells/mL into 500 mL of LOD-AA medium (Farkas et al. 2013) and grown at 70 °C to an OD_{680} of 0.06–0.07. 500 mL Cultures were cooled for 10–15 min in a room temperature water bath and cells were harvested by centrifugation at 6000*g* for 10 min. Cell pellets were washed twice in 250 mL 10% (wt/vol) sucrose and again centrifuged at 6000*g* for 10 min. Cells were gently re-suspended in 50 mL 10% sucrose and transferred to 50 mL falcon tubes, where they were once again pelleted by centrifugation at 6000*g* for 10 min and again re-suspended in 1 mL of 10% sucrose. They were then transferred to a 1.5 mL microcentrifuge tube and centrifuged for 30 s at 18,000*g*. Most of the supernatant was removed, leaving 150–180 µL into which pellets were gently suspended by pipetting. Plasmid DNA (0.5–1 µg) was added to 50 µL aliquots of competent cells. These were then transferred to 1 mm gap electroporation cuvettes. Cells were electroporated in a Bio-Rad Gene Pulser with a voltage of 1.8–2.2 kV, a resistance of 400–200 Ω and capacitance of 25 µF. Cells were immediately transferred to 20 mL CG516 medium (Lipscomb et al. 2016), pre-heated to 70 °C for recovery and incubated from 1 to 2 h after electroporation. Recovery culture (5 mL) was periodically removed and transferred into CG516 medium containing 50 µg/mL kanamycin for selection. Transformants were screened, as described below, and colony-purified at least once on solid CG516 medium containing 50 µg/mL kanamycin at 70 °C. Counter selection for plasmid loss was performed on CG516 solid medium containing 20 µM uracil and 4 mM 5-FOA. Isolates were further purified at least once on solid CG516 medium containing 20 µM uracil. Insertion of the lactate dehydrogenase (*ldh*) gene was confirmed by sequencing of strain MACB1066.

For preliminary screening of transformants and colonies, culture PCR was used: 1 mL of liquid culture was pelleted and supernatant was removed. The cell pellet was suspended in 100 µL DNase/RNase-free water (Invitrogen) and sonicated briefly to lyse cells. Cell debris was pelleted again by centrifugation at 18,000*g* for 10 min. The supernatant was then used as template for PCR screening reactions using 1 µL in a 10 µL reaction with SpeedSTAR polymerase (Takara). Genomic DNA for final strain verification was purified using the ZymoBead™ Genomic DNA Kit (Zymo Research), with a brief sonication step to improve cell lysis.

Quantitative reverse transcription PCR

RNA was extracted from 50 mL cultures of *C. bescii* strains in mid- to late exponential phase by phenol:chloroform extraction, followed by precipitation with isopropanol. Genomic DNA was removed by digestion with Turbo DNase (Ambion) and subjected to an additional phenol:chloroform extraction. cDNA was synthesized from the purified RNA using the Stratagene/Agilent Affinity Script QPCR cDNA synthesis kit. RNA was quantified using a Thermoscientific NANODROP 2000c spectrophotometer. Real-Time Quantitative PCR (qRT-PCR) 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 6000*g* for 10 min, flash frozen with 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 $MgCl_2$, 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 (P_{xi}) 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 P_{xi} 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 IS*Cbe4* 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 P_{xi} *ldh* gene construct (Fig. 2b).

Xylose-dependence of *ldh* transcription and LDH activity

To test the responsiveness of cells containing the P_{xi} *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 Δ*ldh* 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).

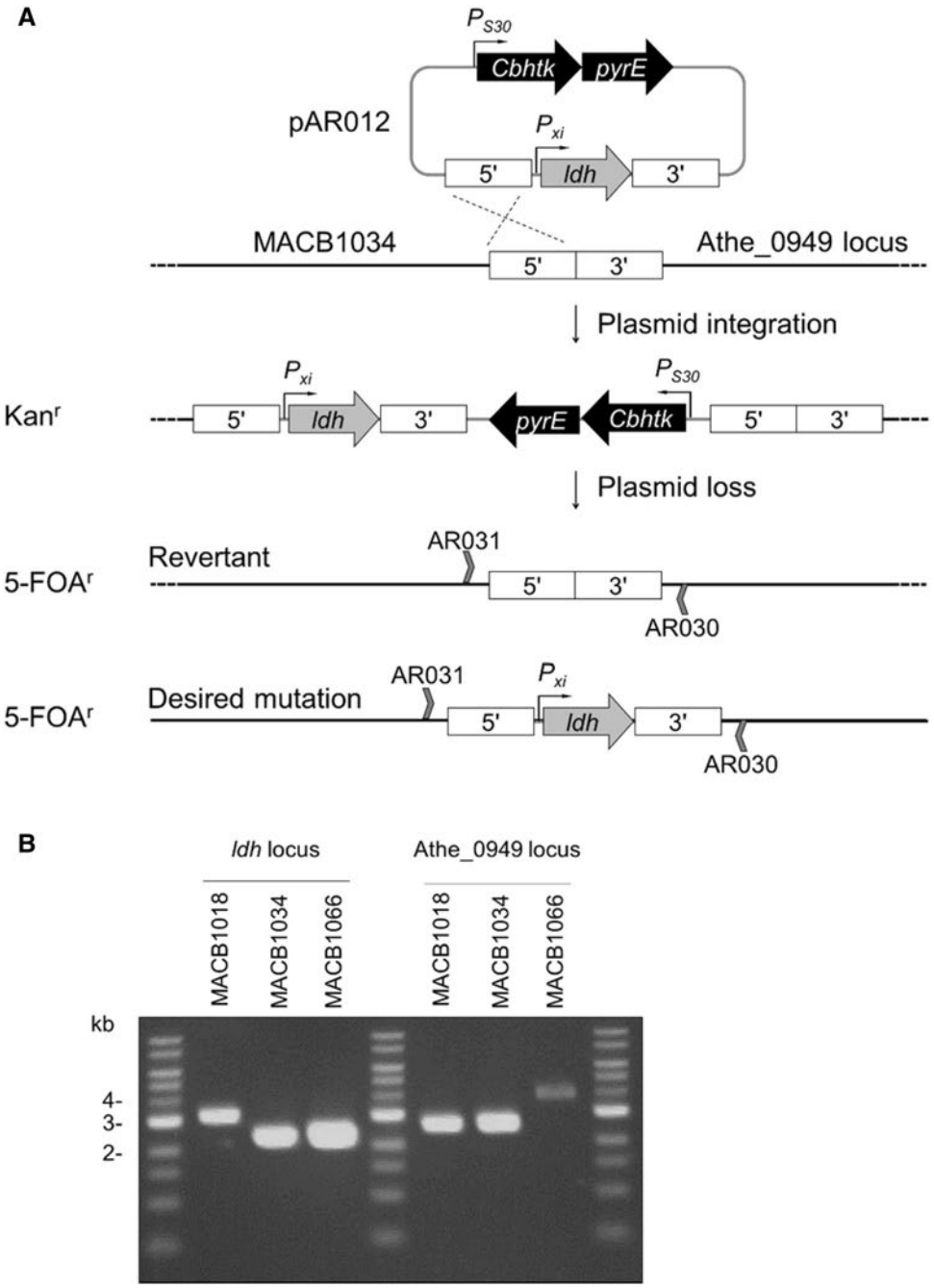

Fig. 2 Confirmation of insertion of P_{xi}-*ldh* construct into strain MACB1066. **a** Scheme of targeted vector, pAR012, integration by homologous recombination (dashed lines) into the Athe_0949 locus of the *C. bescii* strain MACB1034 (*Δldh ΔpyrE*). Plasmid integration is selected for by kanamycin resistance using the *Cbhtk* gene (black). 5-FOA is used for selection of the second crossover event which results in loss of the *pyrE* containing plasmid back bone, resulting in either reversion to the parent type or the desired mutation. A detailed plasmid diagram of pAR012 can be found in Figure S1. Primer binding sites for confirmation of product at the Athe_0949 locus are shown as gray arrows labeled AR031 and AR030. **b** Gel electrophoresis results showing the *ldh* and the P_{xi} *ldh* loci in strains MACB1018, MACB1034 and MACB1066. For the *ldh* locus, MACB1018 gives the 3.2 kb product indicating the presence of the native *ldh* gene while MACB1034 and MACB1066 both give the 2.3 kb product indicating that *ldh* is knocked out. Strain MACB1066 gives a larger, 3.9 kb, product for the Athe_0949 locus indicating insertion of the P_{xi}-*ldh* gene construct at this location in the genome

Interestingly, the LDH-specific activity in the xylose-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 xylose-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)

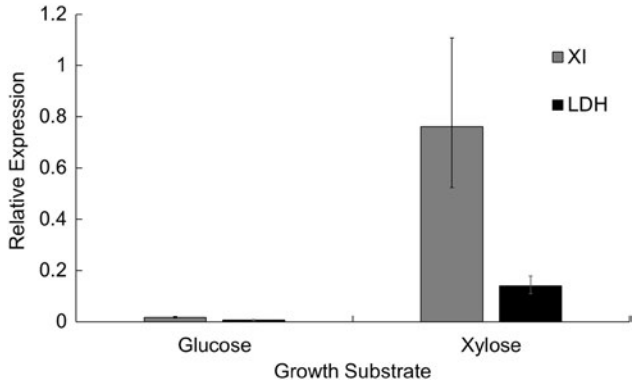

Fig. 3 Relative expression of the genes encoding xylose isomerase (XI) and lactate dehydrogenase (LDH) in strain when grown on 33.3 mM xylose as a carbon source compared to 27.75 mM glucose. Error bars represent propagated error of Ct values for biological duplicates

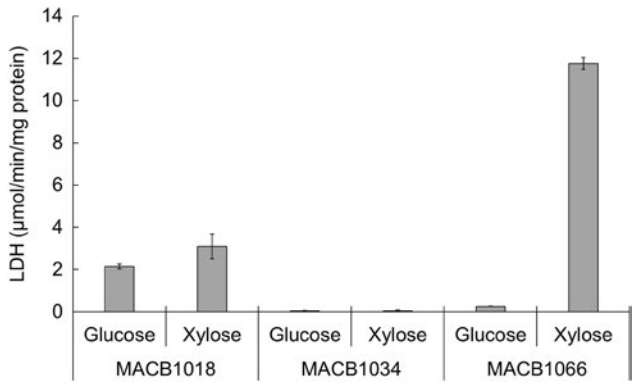

Fig. 4 Specific activity of lactate dehydrogenase (LDH) of strains MACB1018, MACB1034 and MACB1066 grown on both 27.8 mM glucose and 33.3 mM xylose

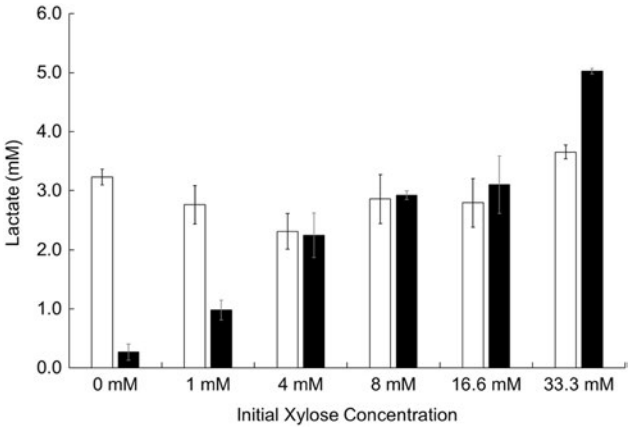

Fig. 5 Lactate production for strains containing the natively controlled *ldh* (MACB1018, white) and the P_{xi} *ldh* (MACB1066, black). Cultures were grown for 24 h and samples were collected for lactate determination. Glucose was used as a supplemental carbon source for cultures with an initial xylose concentration below 33.3 mM as follows: 16.6 mM xylose cultures had an additional 13.9 mM glucose and 0–8 mM xylose cultures had 27.8 mM glucose added

was produced in the absence of added xylose, presumably due to the production of the P_{xi} inducer, which is assumed to be xylose, during the normal metabolism of *C. bescii*. This increased to ~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_{xi} (Fig. 5 and Table 3). In fact, when strain MACB1066 is grown in 16.6 mM xylose in the absence of glucose 5.5 ± 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

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

Initial xylose concentration (mM)	Strain	Glucose consumption (mM)	Xylose consumption (mM)	Total sugar consumption (mM)
0	MACB1018	9.7 ± 0.3	–	9.7 ± 0.3
	MACB1034	11.0 ± 0.4	–	11.0 ± 0.4
	MACB1066	8.3 ± 0.1	–	8.3 ± 0.1
1	MACB1018	10.4 ± 0.2	1.0 ± 0.0	11.4 ± 0.2
	MACB1034	11.0 ± 0.5	1.0 ± 0.0	12.0 ± 0.5
	MACB1066	9.6 ± 0.4	1.0 ± 0.0	10.6 ± 0.4
4	MACB1018	11.8 ± 0.2	4.0 ± 0.0	15.8 ± 0.2
	MACB1034	12.7 ± 0.1	4.0 ± 0.0	16.7 ± 0.1
	MACB1066	10.3 ± 0.5	4.0 ± 0.0	14.3 ± 0.5
8	MACB1018	12.3 ± 0.2	7.1 ± 0.1	19.4 ± 0.3
	MACB1034	13.5 ± 0.5	7.2 ± 0.1	20.7 ± 0.6
	MACB1066	11.3 ± 0.2	7.1 ± 0.1	18.4 ± 0.3
16.6	MACB1018	8.9 ± 0.1	6.6 ± 0.2	15.5 ± 0.3
	MACB1034	9.1 ± 0.2	7.0 ± 0.5	16.1 ± 0.7
	MACB1066	7.7 ± 0.2	6.3 ± 0.3	14.0 ± 0.5
33.3	MACB1018	–	14.8 ± 0.1	14.8 ± 0.1
	MACB1034	–	15.4 ± 0.5	15.4 ± 0.5
	MACB1066	–	14.5 ± 0.3	14.5 ± 0.3

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

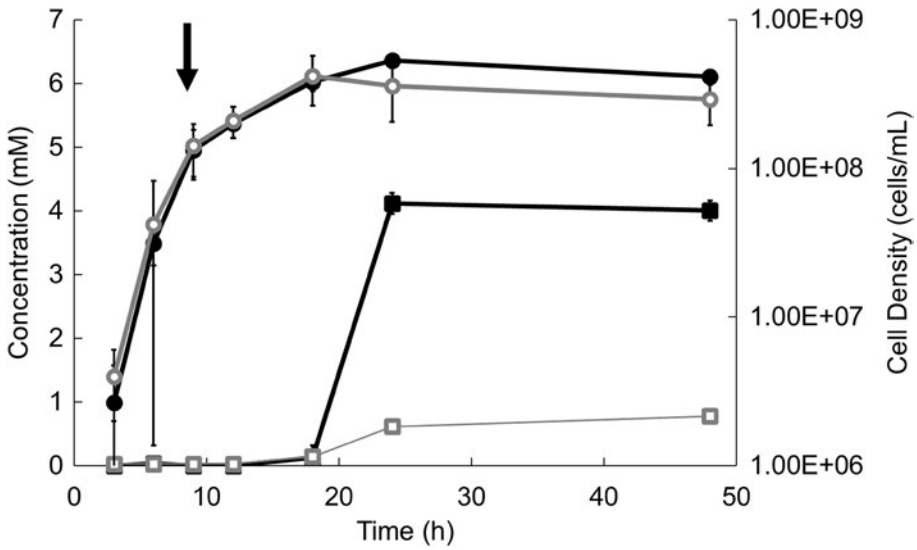

Fig. 6 Growth (circles) and lactate (squares) production for MACB1066 with and without xylose addition (black closed symbols and gray open symbols, respectively). 4 mM xylose was added 9 h after the start of growth which is denoted by the black arrow. Error bars represent standard deviation $n = 3$ biological replicates

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

strain where *ldh* expression is under control of P_{xi}. 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-PS02-06ER64304) 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.

References

Basen M, Sun J, Adams MW (2012) Engineering a hyperthermophilic archaeon for temperature-dependent product formation. MBio 3:e00012–e00053. https://doi.org/10.1128/mBio.00053-12

Blumer-Schuette SE et al (2014) Thermophilic lignocellulose deconstruction. FEMS Microbiol Rev 38:393–448. https://doi.org/10.1111/1574-6976.12044

Cha M, Chung D, Elkins JG, Guss AM, Westpheling J (2013a) Metabolic engineering of *Caldicellulosiruptor bescii* yields increased hydrogen production from lignocellulosic biomass. Biotechnol Biofuels 6:1–8. https://doi.org/10.1186/1754-6834-6-85

Cha M, Wang H, Chung D, Bennetzen JL, Westpheling J (2013b) Isolation and bioinformatic analysis of a novel transposable element, IS*Cbe4*, from the hyperthermophilic bacterium, *Caldicellulosiruptor bescii*. J Ind Microbiol Biotechnol 40:1443–1448. https://doi.org/10.1007/s10295-013-1345-8

Cha M, Chung D, Westpheling J (2016) Deletion of a gene cluster for [Ni–Fe] hydrogenase maturation in the anaerobic hyperthermophilic bacterium *Caldicellulosiruptor bescii* identifies its role in hydrogen metabolism. Appl Microbiol Biotechnol 100:1823–1831. https://doi.org/10.1007/s00253-015-7025-z

Chung D, Farkas J, Huddleston JR, Olivar E, Westpheling J (2012) Methylation by a unique alpha-class N4-cytosine methyltransferase is required for DNA transformation of *Caldicellulosiruptor bescii* DSM6725. PLoS One 7:e43844. https://doi.org/10.1371/journal.pone.0043844

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 6:82. https://doi.org/10.1186/1754-6834-6-82

Chung D, Cha M, Guss AM, Westpheling J (2014a) Direct conversion of plant biomass to ethanol by engineered *Caldicellulosiruptor bescii*. Proc Natl Acad Sci USA 111:8931–8936. https://doi.org/10.1073/pnas.1402210111

Chung D, Pattathil S, Biswal AK, Hahn MG, Mohnen D, Westpheling J (2014b) Deletion of a gene cluster encoding pectin degrading enzymes in *Caldicellulosiruptor bescii* reveals an important role for pectin in plant biomass recalcitrance. Biotechnol Biofuels 7:1–12. https://doi.org/10.1186/s13068-014-0147-1

Chung D, Cha M, Snyder EN, Elkins JG, Guss AM, Westpheling J (2015a) Cellulosic ethanol production via consolidated bioprocessing at 75 °C by engineered *Caldicellulosiruptor bescii*. Biotechnol Biofuels 8:1–13. https://doi.org/10.1186/s13068-015-0346-4

Chung D, Verbeke TJ, Cross KL, Westpheling J, Elkins JG (2015b) Expression of a heat-stable NADPH-dependent alcohol dehydrogenase in *Caldicellulosiruptor bescii* results in furan aldehyde detoxification. Biotechnol Biofuels 8:102. https://doi.org/10.1186/s13068-015-0287-y

Chung D, Young J, Bomble YJ, Vander Wall TA, Groom J, Himmel ME, Westpheling J (2015c) Homologous expression of the *Caldicellulosiruptor bescii* CelA reveals that the extracellular protein is glycosylated. PLoS One 10:e0119508. https://doi.org/10.1371/journal.pone.0119508

Chung D, Young J, Cha M, Brunecky R, Bomble YJ, Himmel ME, Westpheling J (2015d) Expression of the *Acidothermus cellulolyticus* E1 endoglucanase in *Caldicellulosiruptor bescii* enhances its ability to deconstruct crystalline cellulose. Biotechnol Biofuels 8:113. https://doi.org/10.1186/s13068-015-0296-x

Conway JM et al (2016) Multi-domain, surface layer associated glycoside hydrolases contribute to plant polysaccharide degradation by *Caldicellulosiruptor* species. J Biol Chem 291:6732–6747. https://doi.org/10.1074/jbc.M115.707810

Conway JM et al (2017) Functional analysis of the glucan degradation locus (GDL) in *Caldicellulosiruptor bescii* reveals essential roles of component glycoside hydrolases in plant biomass deconstruction. Appl Environ Microbiol. https://doi.org/10.1128/aem.01828-17

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. https://doi.org/10.1007/s10295-012-1202-1

Jones RW, Garland PB (1977) Sites and specificity of the reaction of bipyridylium compounds with anaerobic respiratory enzymes of *Escherichia coli*. Effects of permeability barriers imposed by the cytoplasmic membrane. Biochem J 164:199–211. https://doi.org/10.1042/bj1640199

Lipscomb GL, Conway JM, Blumer-Schuette SE, Kelly RM, Adams MW (2016) A highly thermostable kanamycin resistance marker expands the tool kit for genetic manipulation of *Caldicellulosiruptor bescii*. Appl Environ Microbiol 82:4421–4428. https://doi.org/10.1128/aem.00570-16

Lynd LR, van Zyl WH, McBride JE, Laser M (2005) Consolidated bioprocessing of cellulosic biomass: an update. Curr Opin Biotechnol 16:577–583. https://doi.org/10.1016/j.copbio.2005.08.009

Olson DG, McBride JE, Shaw AJ, Lynd LR (2012) Recent progress in consolidated bioprocessing. Curr Opin Biotechnol 23:396–405. https://doi.org/10.1016/j.copbio.2011.11.026

Schmiedel D, Hillen W (1996) A *Bacillus subtilis* 168 mutant with increased xylose uptake can utilize xylose as sole carbon source. FEMS Microbiol Lett 135:175–178. https://doi.org/10.1111/j.1574-6968.1996.tb07985.x

Scott IM et al (2015) A new class of tungsten-containing oxidoreductase in *Caldicellulosiruptor*, a genus of plant biomass-degrading thermophilic bacteria. Appl Environ Microbiol 81:7339–7347. https://doi.org/10.1128/aem.01634-15

van de Werken HJG et al (2008) Hydrogenomics of the extremely thermophilic bacterium *Caldicellulosiruptor saccharolyticus*. Appl Environ Microbiol 74:6720–6729. https://doi.org/10.1128/aem.00968-08

Van Fossen AL, Verhaart MRA, Kengen SMW, Kelly RM (2009) Carbohydrate utilization patterns for the extremely thermophilic

bacterium *Caldicellulosiruptor saccharolyticus* reveal broad growth substrate preferences. Appl Environ Microbiol 75:7718–7724. https://doi.org/10.1128/aem.01959-09

Williams-Rhaesa AM et al (2017) Genome stability in engineered strains of the extremely thermophilic lignocellulose-degrading bacterium *Caldicellulosiruptor bescii*. Appl Environ Microbiol 83:00417–00444. https://doi.org/10.1128/aem.00444-17

Yang SJ et al (2009) Efficient degradation of lignocellulosic plant biomass, without pretreatment, by the thermophilic anaerobe "*Anaerocellum thermophilum*" DSM 6725. Appl Environ Microbiol 75:4762–4769. https://doi.org/10.1128/Aem.00236-09

Yang SJ et al (2010) Classification of '*Anaerocellum thermophilum*' strain DSM 6725 as *Caldicellulosiruptor bescii* sp. nov. Int J Syst Evol Microbiol 60:2011–2015. https://doi.org/10.1099/Ijs.0.017731-0

Zeldes BM, Keller MW, Loder AJ, Straub CT, Adams MW, Kelly RM (2015) Extremely thermophilic microorganisms as metabolic engineering platforms for production of fuels and industrial chemicals. Front Microbiol 6:1209. https://doi.org/10.3389/fmicb.2015.01209