29 April 19 sbs

Genomic and physiological analyses reveal that extremely thermophilic

Caldicellulosiruptor changbaiensis deploys unique cellulose attachment mechanisms

Asma M.A.M. Khan[‡], Carl Mendoza[‡], Valerie J. Hauk and Sara E. Blumer-Schuetz^{*}

Department of Biological Sciences

Oakland University, Rochester, MI 48309

[‡]A.M.A.M. and C.M. contributed equally to this manuscript

Submitted to: *J Ind Microbiol Biotechnol* (Apr 2019)

Running Title: *Caldicellulosiruptor comparative genomics*

Keywords: *Caldicellulosiruptor*, extreme thermophile, cellulase, tāpirin

Acknowledgements: The authors wish to acknowledge the support of an Oakland University Provost Award, awarded to C. Mendoza.

* Address Correspondence to: Sara E. Blumer-Schuetz
Dept. of Biological Sciences
Oakland University
118 Library Drive
374 Dodge Hall
Phone: (248) 370-3168
Fax: (248) 370-4225
Email: blumerschuetz@oakland.edu

ABSTRACT

The genus *Caldicellulosiruptor* are extremely thermophilic, heterotrophic anaerobes that degrade plant biomass using modular, multifunctional enzymes. Prior pangenome analyses determined that this genus is genetically diverse, with the current pangenome remaining open, meaning that new genes are expected with each additional genome sequence added. Given the high biodiversity observed among the genus *Caldicellulosiruptor*, we have sequenced and added a 14th species, *Caldicellulosiruptor changbaiensis*, to the pangenome. The pangenome now includes 3,791 ortholog clusters, 120 of which are unique to *C. changbaiensis* and may be involved in plant biomass degradation. Comparisons between *C. changbaiensis* and *Caldicellulosiruptor bescii* on the basis of growth kinetics, cellulose solubilization and cell attachment to polysaccharides highlighted physiological differences between the two species which are supported by their respective gene inventories. Most significantly, these comparisons indicated that *C. changbaiensis* possesses unique cellulose attachment mechanisms not observed among the other strongly cellulolytic members of the genus *Caldicellulosiruptor*.

INTRODUCTION

The genus *Caldicellulosiruptor* is comprised of extremely thermophilic, fermentative heterotrophs whose members have been isolated worldwide from terrestrial geothermal springs or other thermal environments [37]. The original isolates from the genus *Caldicellulosiruptor* were identified on the basis of their ability to grow on cellulose at elevated temperatures [56,54], especially temperatures beyond the optimal growth temperature of *Ruminiclostridium thermocellum* [48]. Interest in thermostable enzymes produced by this genus continues, as the initial discovery of their multifunctional, modular enzymes [51,26,57,67] represented an alternate paradigm to cellulosomes [2,52]. Further discoveries on the capabilities of these thermostable enzymes include the unique mode of action used by the central cellulase, CelA, [8], synergistic activity in ionic liquid optimized enzyme mixtures [45,46] and the creation of designer cellulosomes from *Caldicellulosiruptor* catalytic domains [29]. Development of a genetics system for *Caldicellulosiruptor bescii* [14,16] has also expanded the scope of work with this genus, including metabolic engineering [10,12,13,50] and catalytic improvement [18,30,32,31,34,33].

The availability of genome sequences has precipitated deeper insights into the genus *Caldicellulosiruptor*, including comparative studies which have identified biomarkers for plant biomass deconstruction [6,5,23], novel insertion elements [15], genetic tractability [11], diverse mechanisms involved in biomass solubilization [66,37], unique cellulose adhesins (tāpirins) [5,37] and the identification of new combinations of catalytic domains [5,36,23]. Perhaps owing to the unique thermal environments that this genus inhabits, their genomes appear to be dynamic, as the first described *Caldicellulosiruptor* pangenome was predicted to be open [5], and remained open after the addition of five additional genome sequences [36].

Here, we have analyzed the genome sequence of *Caldicellulosiruptor changbaiensis*, isolated from a hot spring in the Changbai Mountains [3], representing the 14th and most recent addition to the *Caldicellulosiruptor* pangenome. Past *Caldicellulosiruptor* pangenomes were comprised of multiple species from most countries of origin, which allowed for prior analysis on

67 the basis of biogeography [5], with the exception of China and Japan [20]. Now with the addition
 68 of the *C. changbaiensis* genome sequence, insights into the biogeography of isolates from
 69 China and how they compare to the global *Caldicellulosiruptor* pangenome is possible.
 70 Furthermore, on the basis of the open *Caldicellulosiruptor* pangenome [20,5], we hypothesize
 71 that the *C. changbaiensis* genome may encode for novel substrate-binding proteins and/ or
 72 plant biomass degrading enzymes. In addition to updating the *Caldicellulosiruptor* pangenome,
 73 we also present differences in the growth physiology of *C. changbaiensis* versus
 74 *Caldicellulosiruptor bescii*, currently the benchmark species against which most
 75 *Caldicellulosiruptor* are compared for their plant biomass degrading capabilities.

MATERIALS AND METHODS

Microbial strains and medium. Freeze-dried stocks of *C. changbaiensis* strain CBS-Z were obtained from the Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures (DSMZ). Glycerol stocks of *C. bescii* DSM-6725 were obtained from the laboratory of Robert M. Kelly, North Carolina State University (Raleigh, NC). Both species were cultured at 75°C on low osmolarity defined (LOD) medium [25] under a nitrogen headspace to maintain anaerobic conditions and supplemented with carbohydrates as a carbon source. Carbohydrates used as a carbon source included cellobiose ($\geq 99\%$, Chem-Impex Int'l, Inc.), pectin (Sigma-Aldrich), xylan (Sigma-Aldrich), glucomannan (NOW Foods), and microcrystalline cellulose (20 μm Sigmacell, Sigma-Aldrich). For genomic DNA isolation, *C. changbaiensis* was cultured anaerobically at 75°C on low osmolarity complex (LOC) medium [25] with cellobiose as a carbon source.

Genomic DNA isolation. Genomic DNA was isolated using the Joint Genome Institute's CTAB-based protocol (<https://jgi.doe.gov/user-programs/pmo-overview/protocols-sample-preparation-information/jgi-bacterial-dna-isolation-ctab-protocol-2012/>), with modifications. In order to isolate enough DNA for sequencing, 500 ml of overnight *C. changbaiensis* culture was harvested by centrifugation at 5000xg, 4°C for 20 minutes and resuspending the cell pellet in 14.8 ml of TE buffer, prior to lysis. Gel electrophoresis in 0.7% agarose was used to assess the quality of genomic DNA and the concentration and purity of the sample for sequencing was quantified using a NanoDrop spectrophotometer, and Qubit fluorometric assay (dsDNA HS assay, Thermo Fisher). Prior to genome sequencing, a 16S rRNA gene fragment was amplified from isolated genomic DNA using oligonucleotide primers (Eton Bioscience) previously designed for identification of *C. changbaiensis* [3], for positive identification of *C. changbaiensis* (**Table 1**). Amplicons were sent for Sanger sequencing (Eton Bioscience), using the same oligonucleotide primers.

C. changbaiensis genome sequencing, assembly and annotation. The genome sequence for *C. changbaiensis* [40] was assembled to 60-fold coverage from long-read Oxford NanoPore (MinION) data generated in house, and short-read Illumina data generated by Molecular Research, LP (MR DNA). Hybrid assembly of the complete *C. changbaiensis* genome used Unicycler v0.4.7 [61], and annotation of the genome used the Prokaryotic Genome Annotation Pipeline v4.7 [55] provided by the National Center for Biotechnology Information (NCBI). The assembled genome and reads used for assembly of the *C. changbaiensis* genome are available through NCBI BioProject accession PRJNA511150.

Phylogenomic analysis. Fourteen genome sequences from the genus *Caldicellulosiruptor* were included in the phylogenomic analyses (see **Table 2** for genome sequence accession numbers). Orthologous protein groups were classified using the GET_HOMOLOGUES v20092018 software package [19], running OrthoMCL v1.4 [39], COGtriangles v2.1 [35], or bidirectional best hits (BDBH) as determined by BLASTP [1,9]. Orthologous protein clusters were determined using the OrthoMCL parameters: 75% pairwise coverage, maximum BLASTP E-value of 1e-5, and MCL inflation of 1.5. GET_HOMOLOGUES was also used to parse the pangenome matrices comparing the *C. changbaiensis* genome inventory against the recent 13 *Caldicellulosiruptor* pangenome [37] or the revised *C. bescii* genome [22]. Core- (**Eq. 1**) and pangenome (**Eq. 2**) parameters were predicted after curve fitting randomly sampled core- or pangenome data to functions previously described by Tettelin et al., [58].

$$coregenes(g) = 1367 + 1668 \exp\left(\frac{-g}{1.75}\right) \quad (1)$$

$$pangenes(g) = 2371 + 63.2(g - 1) + \exp\left(\frac{-2}{2.19}\right) \frac{1 - \exp\left(\frac{-(g-1)}{2.19}\right)}{1 - \exp\left(\frac{-1}{2.19}\right)} \quad (2)$$

Genome-level similarity was quantified as average nucleotide identity (ANIb) from the BLASTN+ alignment of 1,020 nt fragments from the 14 *Caldicellulosiruptor* genomes [49,27]. ANIb were

calculated by Pyani v.0.2.7, (<https://github.com/widdowquinn/pyani>) and percent identities were plotted as a heatmap by the software package.

Growth kinetics on polysaccharides. *C. bescii* or *C. changbaiensis* were revived from -80°C glycerol stocks for growth curve analysis on microcrystalline cellulose, xylan, pectin or glucomannan. Glycerol stocks (1 ml) were subcultured into 50 ml LOD medium for 3 consecutive subcultures using 2% (v/v) inoculum at each passage. Revived cultures were then transferred (2% [v/v] inoculum) to LOD medium containing a 1:1 ratio of maltose (*C. bescii*) or cellobiose (*C. changbaiensis*) to polysaccharide. The 1:1 mixture was then passaged (2% [v/v] inoculum) three times successively in LOD medium with polysaccharide, only. Cultures for growth curves were inoculated at a starting cell density of 1×10^6 cells ml⁻¹ in 200 ml LOD plus the respective polysaccharide. Biological replicates were used for each growth phase experiment. Cell counting used epifluorescence microscopy at 1000x total magnification and a counting reticle as described previously [28]. Cells were fixed in a final volume of 1.1 ml glutaraldehyde (2.5% [v/v] in water) prior to incubation with acridine orange (1 g l⁻¹) and approximately 5 ml sterilized water and thoroughly mixed. Stained cells were then vacuum filtered through a polycarbonate 0.22 µm filter (GE). Samples were counted using a 10x10 reticle a total of ten times. Cell counts were averaged for calculation of cell density (cells ml⁻¹). Doubling times are described as the number of hours per generation during exponential growth, calculated as Δtime divided by the number of generations.

Microcrystalline cellulose solubilization. Solubilization of microcrystalline cellulose followed protocols established by Zurawski *et al.*, [66] with modifications. *C. bescii* or *C. changbaiensis* were cultured in serum bottles with 50 ml of LOD medium supplemented with 0.6g of microcrystalline cellulose (20µm Sigmacell) at a starting cell density of 10^6 cells ml⁻¹. Cultures were then incubated without shaking at 75°C for seven days, after which the remaining microcrystalline cellulose was harvested by centrifugation at 6000 xg, 4°C for 15 min in a swing bucket rotor. The cellulose pellet was washed four times in sterile, deionized water and air dried

at 75°C until the weight of the microcrystalline cellulose did not change. Uninoculated LOD served as an abiotic control. Percent solubilization is reported as the difference in substrate weight divided by the starting weight multiplied by 100. All experimental conditions were measured in triplicate and significance was determined by a t-test (p-value < 0.05).

Cell attachment assays. *C. bescii* and *C. changbaiensis* cell cultures were grown to early stationary phase on either xylan or cellulose (1 g l⁻¹) as the carbon source, and cell densities were calculated before harvesting at 5000 xg for 10 minutes at room temperature. Cells were resuspended and concentrated ten-fold in the binding buffer (50 mM sodium phosphate, pH 7.2) to a 10-fold density of approximately 1-2 x 10⁹ cells ml⁻¹ for cells cultured on xylan or 1 x 10⁸ cells ml⁻¹ for cells cultured on cellulose. For each treatment condition, 1.2 ml of *C. bescii* or *C. changbaiensis* planktonic cells in binding buffer were added to a 1.5 ml microcentrifuge tube, and supplemented with 10 mg of washed substrate (experimental condition: xylan or cellulose), or no substrate for the negative control. All assay tubes were incubated at room temperature for one hour with gentle rotary shaking at 100 rpm. After incubation, planktonic cells were enumerated as described above for the growth curves. Each binding assay was repeated six times. Two-sample t-tests were used to analyze the data using the R studio statistics package v.3.3.3 [47].

RESULTS AND DISCUSSION

Phylogenomic analysis of the *C. changbaiensis* genome. With the addition of the fourteenth *Caldicellulosiruptor* genome [40], we sought to define an updated core- and pangenome. Three different algorithms: OrthoMCL [39], bidirectional best hit and COGtriangles [35] were used to classify orthologous clusters for pangenome analysis (**Table S1**). Of the three, the clusters formed by OrthoMCL resulted in an estimated core- and pangenome with the lowest residual standard errors, and are reported here (**Fig. 1**). Overall, there are 120 unique protein clusters identified in the *C. changbaiensis* genome when compared to the prior *Caldicellulosiruptor* pangenome [37], 75 of which were annotated as hypothetical proteins. Further transcriptomic and proteomic studies may aid in the identification of the function of these unique hypothetical proteins. By adding a 14th genome, the *Caldicellulosiruptor* core genome was reduced to 1,367 orthologous clusters (see **Eq. 1**), however, the pangenome (3,791 genes) continues to expand at an estimated rate of 63.2 genes per additional genome (**Eq. 2, Fig. 1**) highlighting the plasticity of the *Caldicellulosiruptor* pangenome.

In contrast to previously released genome sequences from New Zealand [36], *C. changbaiensis* exhibits a similar pattern of biogeography based on average nucleotide identity (ANIb). As expected, *Caldicellulosiruptor* sp. F32, isolated from compost in China [63], and *C. naganoensis*, isolated from a hot spring in Japan [56] shared higher percent identity levels with *C. changbaiensis*, along with *C. saccharolyticus*, isolated from a hot spring in New Zealand (**Fig. 2, Table S2**). All species that *C. changbaiensis* shared the highest ANI with have been described and confirmed as being strongly cellulolytic, implying that the *C. changbaiensis* genome would also encode for a glucan degradation locus (GDL). Despite the high level of ANIb, based on the open *Caldicellulosiruptor* pangenome, we expected to find new genes involved in carbohydrate metabolism and possibly GDL arrangements.

***C. changbaiensis* exhibits different abilities to grow on polysaccharides versus *C. bescii*.** In order to benchmark the ability of *C. changbaiensis* to grow on plant-related

polysaccharides, we compared its doubling time during exponential growth on representative plant polysaccharides to *C. bescii* (**Table 3**). Doubling times (generation time) were calculated from cell densities measured during exponential growth. Overall, *C. changbaiensis* grows slower on microcrystalline cellulose than *C. bescii*, with a 38% larger doubling time during growth on crystalline cellulose, however, both cultures grew at similar rates on xylan. On both glucomannan, and pectin, *C. changbaiensis* grew faster with 35% lower doubling times (**Table 3**). The differential ability of *C. changbaiensis* and *C. bescii* to grow on pectin and glucomannan is not unexpected, as the differential ability from one species to another to hydrolyze and metabolize plant biomass, comprised of polysaccharides such as xylan, pectin and glucomannan, was previously observed, in one case *C. saccharolyticus* grew slower on plant biomass versus *C. bescii* [62] and *C. kronotskyensis* [66] and another observation where *C. danielii* grew approximately 50% faster than *C. bescii*, *C. morganii* and *C. naganoensis* on plant biomass [36].

When comparing the genomes of *C. changbaiensis* and *C. bescii*, *C. changbaiensis* encodes for 411 genes not shared with *C. bescii*, 120 of which are unique to the genus. We expect that the differences in growth rates on carbohydrates to be related to differences in gene inventory. In fact, the *C. changbaiensis* gene inventory encoding for carbohydrate active enzymes includes 13 genes not found in the *C. bescii* genome, including an annotated β -mannanase (glycoside hydrolase [GH] family 26) and two mannooligosaccharide phosphorylases (GH130). This additional β -mannanase and phosphorylases likely contribute to the enhanced growth of *C. changbaiensis* on glucomannan (**Table 3**).

The lower doubling time on pectin is surprising, however, given that *C. changbaiensis* does not encode for the pectinase cluster that is located in the *C. bescii* genome immediately downstream of the GDL. *C. bescii* gene deletion strains lacking the pectinase cluster were impaired in their growth on both pectin-rich plant biomass and pectin [17], indicating that *C. changbaiensis* has evolved alternate mechanisms to deconstruct or metabolize pectin.

Screening the *C. changbaiensis* genome for pectin-related enzymes did not identify any genes encoding for polysaccharide lyases (PL) that were unique in comparison to *C. bescii*, however genes encoding for representatives from GH family 43, 51 (α -L-arabinofuranosidases) and 95 (α -fucosidase) were present. One scenario is that these enzymes participate in the hydrolysis of carbohydrate sidechains from pectin [44]. Another plausible explanation is that *C. changbaiensis* has evolved to import and efficiently ferment a broader range of carbohydrates released during growth on plant biomass, including uronic acids, and/ or the deoxy sugars fucose and rhamnose. While *C. bescii* may rely on its enzymatic repertoire to deconstruct plant biomass, it may not metabolize all types of carbohydrates that are released, similar to *R. thermocellum* which produces xylanases, but does not metabolize xylose [42,43].

Organization of the *C. changbaiensis* genome degradation locus. *C. changbaiensis* was originally described as strongly cellulolytic [3] and accordingly, its genome encodes for a GDL that shares a similar organization with other strongly cellulolytic members of the genus. Since *C. bescii* was able to grow at a faster rate on microcrystalline cellulose than *C. changbaiensis* (Table 3), we opted to focus on the comparison of GDL between these two species. The GDL from both species is remarkably similar, with only CelD possessing a different arrangement of catalytic and non-catalytic domains (GH10-CBM3-GH5) from *C. changbaiensis*, and truncated versions of CelE (GH9-CBM3-GH5) and CelF (GH74-CBM3) present (Fig. 3). Prior *in vitro* biochemical analyses on the synergy of cellulase mixtures from *C. bescii* had observed that a mixture of three cellulases, CelA, CelC and CelE (ACE cellulases) worked synergistically to hydrolyze cellulose as well as a mixture of all six *C. bescii* cellulases [21]. One could hypothesize, then, that members of the genus *Caldicellulosiruptor* that possess all three of these enzymes would be among the most cellulolytic. Three additional species, *C. kronotskyensis*, *C. danielii*, and *C. naganoensis* also share a similar organization of their GDL [36], including the presence of CelA, CelC and CelE. The contributions of CelD and CelF to

cellulose hydrolysis or solubilization are low [22,21] and likely not to impact the ability of *C. changbaiensis* to efficiently hydrolyze cellulose.

Indeed, *C. changbaiensis* can solubilize microcrystalline cellulose (**Fig. 4**), however the amount of cellulose solubilized was 22.4% lower than the amount solubilized by *C. bescii*, which is similar to the performance of *C. saccharolyticus* when compared to *C. bescii* [24.8% lower, 66]. This result begs the question if the mere presence of the ACE cellulases is sufficient to meet the *C. bescii* benchmark for hydrolysis of cellulose. One explanation could be that the *C. changbaiensis* CelE ortholog may not be as efficient in cellulose hydrolysis since it is lacking two CBM3 modules. However, the nearly equal reduction of cellulose solubilization by both *C. bescii* gene deletion strains incapable of producing CelA-CelC versus CelA-CelE does not support this possibility [22]. Furthermore, CelE truncations that possessed the GH9 catalytic domain and three or two CBM3 domains were equally capable of microcrystalline cellulose hydrolysis [53], making it unlikely that the loss of a CBM3 domain from the *C. changbaiensis* CelA ortholog hampered its activity.

Alternately, sequence divergence of ACE cellulase orthologs may play a larger role in the catalytic capacity of cellulolytic members from the genus *Caldicellulosiruptor*. Of the ACE cellulases, CelA is a key player, supported by its unique hydrolysis mechanism [8], the severe reduction in cellulose hydrolysis by *C. bescii* celA gene deletion mutant [65,22], and biochemical analysis of GDL enzyme synergy [21]. Prior comparison of CelA orthologs from *C. bescii* and *C. danielii* found *CbCelA* to be a superior enzyme [36], indicating that GDL sequences have diverged during speciation, making it likely that the ACE cellulases from *C. changbaiensis* may not demonstrate the same catalytic efficiency as *C. bescii*.

Attachment of *C. bescii* and *C. changbaiensis* to plant polysaccharides. Aside from comparisons of catalytic ability, we also compared the ability of *C. changbaiensis* versus *C. bescii* planktonic cells to bind to insoluble substrates (xylan and cellulose). A decrease in the planktonic cell density (PCD) after exposure to the substrate compared to the PCD of the

negative controls without substrate is indicative of cells binding to the substrate. Surprisingly, we saw no such decrease in PCD for *C. changbaiensis* cultured on xylan after incubation with cellulose or xylan (**Figs. 5A and B**). This inability of *C. changbaiensis* to attach to xylan or cellulose after growth on xylan is surprising, given that xylan is a major polysaccharide constituent of lignocellulose, and would likely serve as a chemical signal. Since no xylan or cellulose attachment proteins are produced in response to growth on xylan, *C. changbaiensis* appears to act as a specialist, responding only to cellulose. Regardless, when *C. changbaiensis* is grown on cellulose, it maintains an ability to attach to cellulose (29% cells attached), which is slightly lower than the relative amount of *C. bescii* cells attached to cellulose (33% attached, **Fig. 5C**). Surprisingly, when *C. bescii* cells cultured on xylan were tested for attachment to either xylan or cellulose there was a significant decrease in (PCD) of indicating that *C. bescii* cells grown on xylan are producing proteins capable of attaching to xylan (33% attachment, **Fig. 5A**) or cellulose (68% attachment, **Fig. 5B**). While we expected to see cells from cultures grown on xylan attaching to xylan, interestingly, *C. bescii* cell attachment was most pronounced when cells were grown on xylan and incubated with cellulose (**Fig. 5B**). The ability of *C. bescii* to attach to cellulose (**Figs. 5B and C**), is in large part due to the presence of tāpirins, since a *C. bescii* tāpirin deletion mutant was severely impaired in cellular attachment to cellulose [37].

The *C. changbaiensis* genome encodes for atypical tāpirin genes. Another notable difference observed between *C. changbaiensis* and *C. bescii* during growth on cellulose is the lack of floc formation by *C. changbaiensis* (**Fig. 6**). Based on this discrepancy between *C. changbaiensis* and *C. bescii*, we examined the genomic context of the type IV pilus locus encoded by the *C. changbaiensis* genome (**Fig. 7**). The T4P locus is found in the genome in all members of the *Caldicellulosiruptor*, and is also located upstream of the GDL in the genomes of strongly cellulolytic species [5,4]. Most notably, while a full T4P locus is present in the *C. changbaiensis* genome, classical tāpirin genes are absent which encode for proteins that bind with high affinity to cellulose [4,37]. Instead, two genes with little, to no homology to the classical

tāpirins are located directly downstream of the T4P locus which we will refer to as atypical tāpirins. The proteins encoded for by these genes are not unique to *C. changbaiensis*, as both *C. acetigenus* and *C. owensensis* also encode for these atypical tāpirins. All three species encode for two atypical tāpirins: a hypothetical protein (Genbank accession: WP_127352232.1) and a von Willebrand Factor A protein (Genbank accession: WP_127352233.1) (yellow arrows, **Fig. 7**). While *C. changbaiensis* shares a similar genomic context at the 3' end of the T4P locus, the atypical *C. changbaiensis* tāpirins are not close orthologs, as they share 74.33% and 68.01% amino sequence similarity with the first and second atypical tāpirins encoded *C. owensensis*. Prior proteomics data collected from cellulose-bound, supernatant and whole cell lysate protein fractions determined that both atypical tāpirins are produced by *C. owensensis* in response to cellulose [5], supporting their potential role in cell attachment to cellulose.

This observed sequence divergence between the atypical tāpirins from strongly and weakly cellulolytic species is similar to the tāpirin encoded for by *C. hydrothermalis* which shares little amino acid sequence homology with classical tāpirins, but shares a similar tertiary structure, and is capable of occupying more sites on crystalline cellulose in comparison to classical tāpirins [37]. Production of tāpirins with an affinity to cellulose likely plays a role in the ability of weakly cellulolytic members of the genus to adhere to cellulose and benefit from the celooligosaccharides released by the action of cellulases [60]. The atypical tāpirins, originally only observed in the genomes of weakly cellulolytic species, may also serve as cellulose adhesins, however, further in-depth biochemical characterization of both atypical tāpirin proteins is required to confirm their function.

CONCLUSIONS

Overall, the *Caldicellulosiruptor* pangenome remains open, and is expected to gain approximately 63 new genes with each additional species sequenced (**Fig. 1A**). The addition of a second species isolated from China indicates that the diversity of *Caldicellulosiruptor* species from this region is higher than those isolated from Iceland, however, the level of observed diversity is not as high as those species isolated from Kamchatka, Russia or New Zealand on the basis of ANIb (**Fig. 2**). *C. changbaiensis* encodes for a GDL (**Fig. 3**) similar in organization as *C. bescii*, however is not as cellulolytic as *C. bescii* on the basis of doubling time (**Table 3**) and cellulose solubilization (**Fig. 4**). However, *C. changbaiensis* does appear to have a broader metabolic appetite for uronic acids or deoxy sugars. *C. changbaiensis* also fails to form a floc during growth on microcrystalline cellulose (**Fig. 6**), a phenotype previously described for *C. bescii* [64], however both species are capable of attaching to cellulose (**Fig. 5**). Interestingly, *C. bescii* retains an ability to attach to cellulose when previously grown on xylan, while *C. changbaiensis* does not (**Fig. 5B**) indicating that the two species respond differently to soluble carbohydrates present in their environment. Tāpirins were previously demonstrated to be key cellulose adhesins for strongly [4] to weakly cellulolytic [37] members of the genus *Caldicellulosiruptor*. Surprisingly, *C. changbaiensis* does not encode for the classical tāpirins, and instead encodes for atypical tāpirins, one of which possesses a von Willebrand type A protein domain (**Fig. 7**). These atypical tāpirins are homologous to those encoded for by weakly cellulolytic *C. owensensis* and *C. acetigenus*, however this may not indicate that the atypical tāpirins are not involved in attachment to cellulose, as the divergent classical tāpirin encoded for by *C. hydrothermalis* binds at a high density to cellulose [37]. The combined lack of classical tāpirins, along with the ability to attach to cellulose indicates that *C. changbaiensis* evolved a unique strategy to attach to cellulose. Further study on the biophysical properties of these atypical tāpirins is warranted to assess their ability to interact with plant polysaccharides, including cellulose.

FIGURE LEGENDS

Figure 1. Core- and pangenome size estimates calculated from random sampling of 14

***Caldicellulosiruptor* genomes. (a)** Fitted curve of the estimated *Caldicellulosiruptor* core

genome from 10 random samples of genomes up to n=14. The current size of the core genome

is 1367 orthologous clusters. **(b)** Fitted curve of the estimated *Caldicellulosiruptor* pangenome

from 10 random samples of genomes up to n=14. The *Caldicellulosiruptor* pangenome remains

open and has increased to 3791 genes. The rate of growth for the pangenome is 63.2 new

genes per genome sequenced. Core- and pangenome estimates were calculated from the

equations reported by Tettelin *et al.*, [58] using GET_HOMOLOGUES software [19].

Figure 2. Heatmap representation of the average nucleotide identity for 14 genome

sequenced species from the genus *Caldicellulosiruptor*. Average nucleotide identity (ANIb)

was calculated on the basis of legacy BLASTn sequence identity over 1020nt sequence

fragments. ANIb values of all 14 genomes are represented by a heat plot ranging from blue

(75% < ANIb < 90%), white (90% < ANIb < 95%) to red (ANIb > 95%). Pyani

(<https://github.com/widdowquinn/pyani>) was used to calculate ANIb values and generate the

clustered heatmap. Hierarchical cluster dendrograms were generated on the basis of similar ANIb

values across each species. ANIb values are reported in Table S1. Calace, *C. acetigenus*;

Cbes, *C. bescii*; Calcha, *C. changbaiensis*; Caldan, *C. danielii*; Calhy, *C. hydrothermalis*; Calkr,

C. kristjanssonii; Calkro, *C. kronotskyensis*; Calla, *C. lactoaceticus*; Calmo, *C. morganii*; Calna,

C. naganoensis; COB47, *C. obsidiansis*; Calow, *C. owensis*; Csac, *C. saccharolyticus*; F32,

C. sp. F32.

Figure 3. Modular multifunctional enzymes encoded for by the glucan degradation locus.

Glucan degradation loci were selected on the basis of the presence of “ACE” cellulases. ACE

cellulases: CelA, CelC and CelE. Circles represent the glycoside hydrolase (GH) domains,

rectangles represent the carbohydrate binding module (CBM) domains. GH5, green circles; GH9, red circles; GH10, violet circles; GH 44, blue circles; GH48, grey circles; GH74, orange circles. CBM3, grey rectangles; CBM22, pink rectangles.

Figure 4. Solubilization of microcrystalline cellulose by *C. bescii* and *C. changbaiensis*.

Uninoculated control, indicates abiotic cellulose solubilization in LOD medium. Error bars represent standard error (n=3). Similar letters over columns denote $p < 0.05$ as determined by a t-test.

Figure 5. Comparison of the ability of *C. bescii* or *C. changbaiensis* planktonic cells to attach to polysaccharides. Titles above bar charts indicate the carbon source for growth/

binding substrate. **(a, b)** When cells are grown on xylan, only planktonic *C. bescii* cells were able to attach to xylan or cellulose. **(c)** Cells grown on cellulose as the carbon source and exposed to cellulose as the binding substrate. Planktonic cell densities (PCD), enumerated by epifluorescence microscopy are plotted on the y-axis. Green columns indicate PCD without binding substrate and purple columns indicate PCD with the binding substrate. * indicates $p < 0.01$ as determined by a t-test. All assays had n=6 biological replicates.

Figure 6. Flocculation of *C. bescii* cells cultured on chemically defined medium and

microcrystalline cellulose. (a) Formation of a floc of *C. bescii* cells around microcrystalline cellulose (diameter, 20 μ m) while planktonic *C. changbaiensis* cells (cloudiness) are visible. **(b)** Same serum bottles as in “A”, however the bottles were vigorously mixed. The *C. bescii* floc remains fairly stable, while both microcrystalline cellulose and cells are mixed in the *C. changbaiensis* culture.

Figure 7. Genomic context for the location of the tāpirins from strongly to weakly cellulolytic *Caldicellulosiruptor* species. Different colors represent the classical versus atypical tāpirins. Blue arrows: Cbes tāpirin 1 (Gen bank accession: YP_002573732) and Cbes tāpirin 2 (Gen bank accession: YP_002573731). Green arrow: Calhy tāpirin 1 (Gen bank accession number: YP_003992006). Yellow arrows: Calcha tāpirin 1 (Gen bank accession: WP_127352232.1) and 2 (Gen bank accession: WP_127352233.1), and Calow tāpirin 1 (Gen bank accession: YP_004002936) and 2 (Gen bank accession r: YP_004002935). Grey rectangles indicate the presence of the GDL downstream of the tāpirins. Atypical tāpirin 1 is annotated as a hypothetical protein and atypical tāpirin 2 is annotated as a von Willebrand factor A protein. Cbes, *C. bescii*; Calhy, *C. hydrothermalis*; Calcha, *C. changbaiensis* and Calow, *C. owensensis*. Peach rectangles represent the type IV pilus locus directly upstream of the tāpirins. Arrows indicate tāpirin 1 and 2. Numbers in the tāpirin arrows indicate the amino acid length.

409 **Conflict of Interest:** A.M.A.M., C.M., V.J.H. and S.E.B.-S. declare that they have no conflict of
410 interest.

411

Table 1. Oligonucleotide primers used for			412
<i>Caldicellulosiruptor</i> 16S rRNA gene fragment amplification			
Primer Name	Primer Sequence (5' to 3')	Source	
8F-207	AGAGTTTGATCCTGGCTCAG	[3]	
Caldi-R-208	GTACGGCTACCTTGTTACG		

Table 2. <i>Caldicellulosiruptor</i> genome sequences included in the updated pangenome analysis		
Species Name	NCBI RefSeq Accession	Reference
<i>C. acetigenus</i>	GCF_000421725.1	[41]
<i>C. bescii</i>	GCF_000022325.1	[22]
<i>C. changbaiensis</i>	GCF_003999255.1	[40]
<i>C. danielii</i>	GCF_000955725.1	[38,36]
<i>C. hydrothermalis</i>	GCF_000166355.1	[7,5]
<i>C. kristjanssonii</i>	GCF_000166695.1	[7,5]
<i>C. kronotskyensis</i>	GCF_000166775.1	[7,5]
<i>C. lactoaceticus</i>	GCF_000193435.2	[7,5]
<i>C. morganii</i>	GCF_000955745.1	[38,36]
<i>C. naganoensis</i>	GCF_000955735.1	[38,36]
<i>C. obsidiansis</i>	GCF_000145215.1	[24]
<i>C. owensensis</i>	GCF_000166335.1	[7,5]
<i>C. saccharolyticus</i>	GCF_000016545.1	[59]
<i>C. str. F32</i>	GCF_000404025.1	[63]

413

414

Table 3. Doubling time of <i>C. changbaiensis</i> or <i>C. bescii</i> grown on plant polysaccharides		
Polysaccharide	g_{Cbes} (hr)	g_{Calcha} (hr)
Microcrystalline cellulose	3.93 ± 0.157	5.43 ± 0.304
Beechwood xylan	2.55 ± 0.211	2.54 ± 0.428
Glucomannan	3.22 ± 0.62	2.08 ± 0.025
Pectin	3.48 ± 0.224	2.26 ± 0.167

References

1. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25:3389-3402
2. Artzi L, Bayer EA, Morais S (2017) Cellulosomes: bacterial nanomachines for dismantling plant polysaccharides. *Nat Rev Microbiol* 15:83-95. doi:10.1038/nrmicro.2016.164
3. Bing W, Wang H, Zheng B, Zhang F, Zhu G, Feng Y, Zhang Z (2015) *Caldicellulosiruptor changbaiensis* sp. nov., a cellulolytic and hydrogen-producing bacterium from a hot spring. *Int J Syst Evol Microbiol* 65:293-297. doi:10.1099/ijms.0.065441-0
4. Blumer-Schuette SE, Alahuhta M, Conway JM, Lee LL, Zurawski JV, Giannone RJ, Hettich RL, Lunin VV, Himmel ME, Kelly RM (2015) Discrete and structurally unique proteins (tāpirins) mediate attachment of extremely thermophilic *Caldicellulosiruptor* species to cellulose. *J Biol Chem* 290:10645-10656. doi:10.1074/jbc.M115.641480
5. Blumer-Schuette SE, Giannone RJ, Zurawski JV, Ozdemir I, Ma Q, Yin Y, Xu Y, Kataeva I, Poole FL, 2nd, Adams MW, Hamilton-Brehm SD, Elkins JG, Larimer FW, Land ML, Hauser LJ, Cottingham RW, Hettich RL, Kelly RM (2012) *Caldicellulosiruptor* core and pangenomes reveal determinants for noncellulosomal thermophilic deconstruction of plant biomass. *J Bacteriol* 194:4015-4028. doi:10.1128/JB.00266-12
6. Blumer-Schuette SE, Lewis DL, Kelly RM (2010) Phylogenetic, microbiological, and glycoside hydrolase diversities within the extremely thermophilic, plant biomass-degrading genus *Caldicellulosiruptor*. *Appl Environ Microbiol* 76:8084-8092. doi:10.1128/AEM.01400-10
7. Blumer-Schuette SE, Ozdemir I, Mistry D, Lucas S, Lapidus A, Cheng JF, Goodwin LA, Pitluck S, Land ML, Hauser LJ, Woyke T, Mikhailova N, Pati A, Kyrpides NC, Ivanova N, Detter JC, Walston-Davenport K, Han S, Adams MW, Kelly RM (2011) Complete genome sequences for the anaerobic, extremely thermophilic plant biomass-degrading bacteria *Caldicellulosiruptor hydrothermalis*, *Caldicellulosiruptor kristjanssonii*,

- 442 *Caldicellulosiruptor kronotskyensis*, *Caldicellulosiruptor owensensis*, and
- 443 *Caldicellulosiruptor lactoaceticus*. J Bacteriol 193:1483-1484. doi:10.1128/JB.01515-10
- 444 8. Brunecky R, Alahuhta M, Xu Q, Donohoe BS, Crowley MF, Kataeva IA, Yang SJ, Resch MG,
- 445 Adams MW, Lunin VV, Himmel ME, Bomble YJ (2013) Revealing nature's cellulase
- 446 diversity: the digestion mechanism of *Caldicellulosiruptor bescii* CelA. Science
- 447 342:1513-1516. doi:10.1126/science.1244273
- 448 9. Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden TL (2009)
- 449 BLAST+: architecture and applications. BMC Bioinformatics 10:421. doi:10.1186/1471-
- 450 2105-10-421
- 451 10. Cha M, Chung D, Elkins JG, Guss AM, Westpheling J (2013) Metabolic engineering of
- 452 *Caldicellulosiruptor bescii* yields increased hydrogen production from lignocellulosic
- 453 biomass. Biotechnol Biofuel 6:1-8. doi:10.1186/1754-6834-6-85
- 454 11. Chung D, Cha M, Farkas J, Westpheling J (2013) Construction of a stable replicating shuttle
- 455 vector for *Caldicellulosiruptor* species: Use for extending genetic methodologies to other
- 456 members of this genus. PLoS One 8:e62881. doi:10.1371/journal.pone.0062881
- 457 12. Chung D, Cha M, Guss AM, Westpheling J (2014) Direct conversion of plant biomass to
- 458 ethanol by engineered *Caldicellulosiruptor bescii*. Proceedings of the National Academy
- 459 of Sciences:201402210. doi:10.1073/pnas.1402210111
- 460 13. Chung D, Cha M, Snyder EN, Elkins JG, Guss AM, Westpheling J (2015) Cellulosic ethanol
- 461 production via consolidated bioprocessing at 75 °C by engineered *Caldicellulosiruptor*
- 462 *bescii*. Biotechnol Biofuel 8:163. doi:10.1186/s13068-015-0346-4
- 463 14. Chung D, Farkas J, Huddleston JR, Olivar E, Westpheling J (2012) Methylation by a unique
- 464 α -class N4-cytosine methyltransferase is required for DNA transformation of
- 465 *Caldicellulosiruptor bescii* DSM6725. PLoS One 7:e43844.
- 466 doi:10.1371/journal.pone.0043844

15. Chung D, Farkas J, Westpheling J (2013) Detection of a novel active transposable element in *Caldicellulosiruptor hydrothermalis* and a new search for elements in this genus. J Ind Microbiol Biotechnol 40:517-521. doi:10.1007/s10295-013-1244-z
16. Chung D, Farkas J, Westpheling J (2013) Overcoming restriction as a barrier to DNA transformation in *Caldicellulosiruptor* species results in efficient marker replacement. Biotechnol Biofuel 6:1-9. doi:10.1186/1754-6834-6-82
17. Chung D, Pattathil S, Biswal AK, Hahn MG, Mohnen D, Westpheling J (2014) Deletion of a gene cluster encoding pectin degrading enzymes in *Caldicellulosiruptor bescii* reveals an important role for pectin in plant biomass recalcitrance. Biotechnol Biofuel 7:147. doi:10.1186/s13068-014-0147-1
18. 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 Biofuel 8:113. doi:10.1186/s13068-015-0296-x
19. Contreras-Moreira B, Vinuesa P (2013) GET_HOMOLOGUES, a versatile software package for scalable and robust microbial pangenome analysis. Appl Environ Microbiol 79:7696-7701. doi:10.1128/AEM.02411-13
20. Conway JM, Crosby JR, Hren AP, Southerland RT, Lee LL, Lunin VV, Alahuhta P, Himmel ME, Bomble YJ, Adams MWW, Kelly RM (2018) Novel multidomain, multifunctional glycoside hydrolases from highly lignocellulolytic *Caldicellulosiruptor* species. AIChE J 64:4218-4228. doi:10.1002/aic.16354
21. Conway JM, Crosby JR, McKinley BS, Seals NL, Adams MWW, Kelly RM (2018) Parsing in vivo and in vitro contributions to microcrystalline cellulose hydrolysis by multidomain glycoside hydrolases in the *Caldicellulosiruptor bescii* secretome. Biotechnol Bioeng 0. doi:10.1002/bit.26773

22. Conway JM, McKinley BS, Seals NL, Hernandez D, Khatibi PA, Poudel S, Giannone RJ, Hettich RL, Williams-Rhaesa AM, Lipscomb GL, Adams MWW, Kelly RM (2017) Functional analysis of the glucan degradation locus in *Caldicellulosiruptor bescii* reveals essential roles of component glycoside hydrolases in plant biomass deconstruction. Appl Environ Microbiol 83:e01828-01817. doi:10.1128/AEM.01828-17
23. Dam P, Kataeva I, Yang S-J, Zhou F, Yin Y, Chou W, 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
24. Elkins JG, Lochner A, Hamilton-Brehm SD, Davenport KW, Podar M, Brown SD, Land ML, Hauser LJ, Klingeman DM, Raman B, Goodwin LA, Tapia R, Meincke LJ, Detter JC, Bruce DC, Han CS, Palumbo AV, Cottingham RW, Keller M, Graham DE (2010) Complete genome sequence of the cellulolytic thermophile *Caldicellulosiruptor obsidiansis* OB47T. J Bacteriol 192:6099-6100. doi:10.1128/JB.00950-10
25. 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 40:1-9. doi:10.1007/s10295-012-1202-1
26. Gibbs MD, Saul DJ, Lüthi E, Bergquist PL (1992) The beta-mannanase from " *Caldocellum saccharolyticum*" is part of a multidomain enzyme. Appl Environ Microbiol 58:3864–3867
27. Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P, Tiedje JM (2007) DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. Int J Syst Evol Microbiol 57:81-91. doi:10.1099/ijs.0.64483-0
28. Hobbie JE, Daley RJ, Jasper S (1977) Use of nuclepore filters for counting bacteria by fluorescence microscopy. Appl Environ Microbiol 33:1225-1228

29. Kahn A, Moraïs S, Galanopoulou AP, Chung D, Sarai NS, Hengge N, Hatzinikolaou DG, Himmel ME, Bomble YJ, Bayer EA (2019) Creation of a functional hyperthermostable designer cellulosome. *Biotechnol Biofuel* 12:44. doi:10.1186/s13068-019-1386-y
30. Kim S-K, 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 Biofuel* 9. doi:10.1186/s13068-016-0588-9
31. Kim S-K, Chung D, Himmel ME, Bomble YJ, Westpheling J (2017) 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. *J Ind Microbiol Biotechnol* 44:1643-1651. doi:10.1007/s10295-017-1982-4
32. Kim S-K, Chung D, Himmel ME, Bomble YJ, Westpheling J (2017) In vivo synergistic activity of a CAZyme cassette from *Acidothermus cellulolyticus* significantly improves the cellulolytic activity of the *C. bescii* exoproteome. *Biotechnol Bioeng* 114:2474-2480. doi:10.1002/bit.26366
33. Kim S-K, Chung D, Himmel ME, Bomble YJ, Westpheling J (2019) Heterologous co-expression of two β -glucanases and a cellobiose phosphorylase resulted in a significant increase in the cellulolytic activity of the *Caldicellulosiruptor bescii* exoproteome. *J Ind Microbiol Biotechnol*. doi:10.1007/s10295-019-02150-0
34. Kim S-K, Himmel ME, Bomble YJ, Westpheling J (2018) Expression of a cellobiose phosphorylase from *Thermotoga maritima* in *Caldicellulosiruptor bescii* improves the phosphorolytic pathway and results in a dramatic increase in cellulolytic activity. *Appl Environ Microbiol* 84:e02348-02317. doi:10.1128/AEM.02348-17
35. Kristensen DM, Kannan L, Coleman MK, Wolf YI, Sorokin A, Koonin EV, Mushegian A (2010) A low-polynomial algorithm for assembling clusters of orthologous groups from

- intergenomic symmetric best matches. *Bioinformatics* 26:1481-1487.
doi:10.1093/bioinformatics/btq229
36. Lee LL, Blumer-Schuetz SE, Izquierdo JA, Zurawski JV, Loder AJ, Conway JM, Elkins JG, Podar M, Clum A, Jones PC, Piatek MJ, Weighill DA, Jacobson DA, Adams MWW, Kelly RM (2018) Genus-wide assessment of lignocellulose utilization in the extremely thermophilic genus *Caldicellulosiruptor* by genomic, pangenomic, and metagenomic analyses. *Appl Environ Microbiol* 84. doi:10.1128/AEM.02694-17
37. Lee LL, Hart WS, Lunin VV, Alahuhta M, Bomble YJ, Himmel ME, Blumer-Schuetz SE, Adams MWW, Kelly RM (2019) Comparative biochemical and structural analysis of novel cellulose binding proteins (tāpirins) from extremely thermophilic *Caldicellulosiruptor* species. *Appl Environ Microbiol* 85:e01983-01918.
doi:10.1128/AEM.01983-18
38. Lee LL, Izquierdo JA, Blumer-Schuetz SE, Zurawski JV, Conway JM, Cottingham RW, Huntemann M, Copeland A, Chen IM, Kyrpides N, Markowitz V, Palaniappan K, Ivanova N, Mikhailova N, Ovchinnikova G, Andersen E, Pati A, Stamatis D, Reddy TB, Shapiro N, Nordberg HP, Cantor MN, Hua SX, Woyke T, Kelly RM (2015) Complete genome sequences of *Caldicellulosiruptor* sp. strain Rt8.B8, *Caldicellulosiruptor* sp. strain Wai35.B1, and "*Thermoanaerobacter cellulolyticus*". *Genome Announc* 3.
doi:10.1128/genomeA.00440-15
39. Li L, Stoeckert CJ, Jr., Roos DS (2003) OrthoMCL: identification of ortholog groups for eukaryotic genomes. *Genome Res* 13:2178-2189. doi:10.1101/gr.1224503
40. Mendoza C, Blumer-Schuetz SE (2019) Complete genome sequence of *Caldicellulosiruptor changbaiensis* CBS-Z, an extremely thermophilic, cellulolytic bacterium isolated from a hot spring in China. *Microbiol Resour Announc* 8. doi:10.1128/MRA.00021-19
41. Mukherjee S, Seshadri R, Varghese NJ, Eloë-Fadrosh EA, Meier-Kolthoff JP, Goker M, Coates RC, Hadjithomas M, Pavlopoulos GA, Paez-Espino D, Yoshikuni Y, Visel A,

- Whitman WB, Garrity GM, Eisen JA, Hugenholtz P, Pati A, Ivanova NN, Woyke T, Klenk HP, Kyrpides NC (2017) 1,003 reference genomes of bacterial and archaeal isolates expand coverage of the tree of life. Nat Biotechnol 35:676-+. doi:10.1038/nbt.3886
42. Ng TK, Ben-Bassat A, Zeikus JG (1981) Ethanol production by thermophilic bacteria: Fermentation of cellulosic substrates by cocultures of *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum*. Appl Environ Microbiol 41:1337-1343
43. Ng TK, Zeikus JG (1981) Comparison of extracellular cellulase activities of *Clostridium thermocellum* LQRI and *Trichoderma reesei* QM9414. Appl Environ Microbiol 42:231-240
44. Numan MT, Bhosle NB (2006) Alpha-L-arabinofuranosidases: the potential applications in biotechnology. J Ind Microbiol Biotechnol 33:247-260. doi:10.1007/s10295-005-0072-1
45. Park JI, Kent MS, Datta S, Holmes BM, Huang Z, Simmons BA, Sale KL, Sapra R (2011) Enzymatic hydrolysis of cellulose by the cellobiohydrolase domain of CelB from the hyperthermophilic bacterium *Caldicellulosiruptor saccharolyticus*. Bioresour Technol 102:5988-5994. doi:10.1016/j.biortech.2011.02.036
46. Park JI, Steen EJ, Burd H, Evans SS, Redding-Johnson AM, Batth T, Benke PI, D'Haeseleer P, Sun N, Sale KL, Keasling JD, Lee TS, Petzold CJ, Mukhopadhyay A, Singer SW, Simmons BA, Gladden JM (2012) A thermophilic ionic liquid-tolerant cellulase cocktail for the production of cellulosic biofuels. PLoS One 7:e37010. doi:10.1371/journal.pone.0037010
47. R Core T (2015) R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria
48. Reynolds PH, Sissons CH, Daniel RM, Morgan HW (1986) Comparison of cellulolytic activities in *Clostridium thermocellum* and three thermophilic, cellulolytic anaerobes. Appl Environ Microbiol 51:12-17

49. Richter M, Rossello-Mora R (2009) Shifting the genomic gold standard for the prokaryotic species definition. *Proc Natl Acad Sci U S A* 106:19126-19131.
doi:10.1073/pnas.0906412106
50. Sander K, Chung D, Hyatt D, Westpheling J, Klingeman DM, Rodriguez M, Engle NL, Tschaplinski TJ, Davison BH, Brown SD Rex in *Caldicellulosiruptor bescii*: Novel regulon members and its effect on the production of ethanol and overflow metabolites. *MicrobiologyOpen* 0:e00639. doi:10.1002/mbo3.639
51. Saul DJ, Williams LC, Grayling RA, Chamley LW, Love DR, Bergquist PL (1990) celB, a gene coding for a bifunctional cellulase from the extreme thermophile "*Caldocellum saccharolyticum*". *Appl Environ Microbiol* 56:3117-3124
52. Smith SP, Bayer EA, Czjzek M (2017) Continually emerging mechanistic complexity of the multi-enzyme cellulosome complex. *Curr Opin Struct Biol* 44:151-160.
doi:10.1016/j.sbi.2017.03.009
53. Su X, Mackie RI, Cann IKO (2012) Biochemical and mutational analyses of a multidomain cellulase/mannanase from *Caldicellulosiruptor bescii*. *Appl Environ Microbiol* 78:2230.
doi:10.1128/AEM.06814-11
54. Svetlichnyi VA, T. P. Svetlichnaya, N. A. Chernykh, and G. A. Zavarzin (1990) *Anaerocellum thermophilum* gen. nov. sp. nov. an extremely thermophilic cellulolytic eubacterium isolated from hot-springs in the Valley of Geysers. *Microbiology* 59:598-604
55. Tatusova T, DiCuccio M, Badretdin A, Chetvernin V, Nawrocki EP, Zaslavsky L, Lomsadze A, Pruitt KD, Borodovsky M, Ostell J (2016) NCBI prokaryotic genome annotation pipeline. *Nucleic Acids Res* 44:6614-6624. doi:10.1093/nar/gkw569
56. Taya M, Hinoki H, Kobayashi T (1985) Tungsten requirement of an extremely thermophilic, cellulolytic anaerobe (strain NA10). *Agric Biol Chem* 49:2513-2515.
doi:<http://dx.doi.org/10.1080/00021369.1985.10867120>

57. Te'o VS, Saul DJ, Bergquist PL (1995) celA, another gene coding for a multidomain cellulase from the extreme thermophile *Caldocellum saccharolyticum*. Appl Microbiol Biotechnol 43:291-296
58. Tettelin H, Massignani V, Cieslewicz MJ, Donati C, Medini D, Ward NL, Angiuoli SV, Crabtree J, Jones AL, Durkin AS, Deboy RT, Davidsen TM, Mora M, Scarselli M, Margarit y Ros I, Peterson JD, Hauser CR, Sundaram JP, Nelson WC, Madupu R, Brinkac LM, Dodson RJ, Rosovitz MJ, Sullivan SA, Daugherty SC, Haft DH, Selengut J, Gwinn ML, Zhou L, Zafar N, Khouri H, Radune D, Dimitrov G, Watkins K, O'Connor KJ, Smith S, Utterback TR, White O, Rubens CE, Grandi G, Madoff LC, Kasper DL, Telford JL, Wessels MR, Rappuoli R, Fraser CM (2005) Genome analysis of multiple pathogenic isolates of *Streptococcus agalactiae*: implications for the microbial "pan-genome". Proc Natl Acad Sci U S A 102:13950-13955. doi:10.1073/pnas.0506758102
59. van de Werken HJG, Verhaart MRA, VanFossen AL, Willquist K, Lewis DL, Nichols JD, Goorissen HP, Mongodin EF, Nelson KE, van Niel EWJ, Stams AJM, Ward DE, de Vos WM, van der Oost J, Kelly RM, Kengen SWM (2008) Hydrogenomics of the extremely thermophilic bacterium *Caldicellulosiruptor saccharolyticus*. Appl Environ Microbiol 74:6720-6729. doi:10.1128/Aem.00968-08
60. Wang Z-W, Hamilton-Brehm SD, Lochner A, Elkins JG, Morrell-Falvey JL (2011) Mathematical modeling of hydrolysate diffusion and utilization in cellulolytic biofilms of the extreme thermophile *Caldicellulosiruptor obsidiansis*. Bioresour Technol 102:3155-3162. doi:10.1016/j.biortech.2010.10.104
61. Wick RR, Judd LM, Gorrie CL, Holt KE (2017) Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput Biol 13:e1005595. doi:10.1371/journal.pcbi.1005595
62. Yang SJ, Kataeva I, Hamilton-Brehm SD, Engle NL, Tschaplinski TJ, Doeppke C, Davis M, Westpheling J, Adams MWW (2009) Efficient degradation of lignocellulosic plant

- biomass, without pretreatment, by the thermophilic anaerobe "*Anaerocellum*
thermophilum" DSM 6725. Applied and Environmental Microbiology 75:4762-4769.
doi:10.1128/Aem.00236-09
63. Ying Y, Meng D, Chen X, Li F (2013) An extremely thermophilic anaerobic bacterium
Caldicellulosiruptor sp. F32 exhibits distinctive properties in growth and xylanases during
xylan hydrolysis. Enzyme Microb Technol 53:194-199.
doi:10.1016/j.enzmictec.2013.04.004
64. Yokoyama H, Yamashita T, Morioka R, Ohmori H (2014) Extracellular secretion of
noncatalytic plant cell wall-binding proteins by the cellulolytic thermophile
Caldicellulosiruptor bescii. J Bacteriol 196:3784-3792. doi:10.1128/JB.01897-14
65. 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 Biofuel 7:142. doi:10.1186/s13068-014-0142-6
66. Zurawski JV, Conway JM, Lee LL, Simpson HJ, Izquierdo JA, Blumer-Schuette S, Nookaew
I, Adams MW, Kelly RM (2015) Comparative analysis of extremely thermophilic
Caldicellulosiruptor species reveals common and unique cellular strategies for plant
biomass utilization. Appl Environ Microbiol 81:7159-7170. doi:10.1128/AEM.01622-15
67. Zverlov V, Mahr S, Riedel K, Bronnenmeier K (1998) Properties and gene structure of a
bifunctional cellulolytic enzyme (CelA) from the extreme thermophile '*Anaerocellum*
thermophilum' with separate glycosyl hydrolase family 9 and 48 catalytic domains.
Microbiology 144:457-465. doi:10.1099/00221287-144-2-457

Figure 1. Core- and pangenome size estimates calculated from random sampling of 14 *Caldicellulosiruptor* genomes. (A) Fitted curve of the estimated *Caldicellulosiruptor* core genome from 10 random samples of genomes up to n=14. The current size of the core genome is 1367 orthologous clusters. **(B)** Fitted curve of the estimated *Caldicellulosiruptor* pangenome from 10 random samples of genomes up to n=14. The *Caldicellulosiruptor* pangenome remains open and has increased to 3791 genes. The rate of growth for the pangenome is 63.2 new genes per genome sequenced. Core- and pangenome estimates were calculated from the equations reported by Tettelin *et al.*, [58] using GET_HOMOLOGUES software [19].

Figure 2. Heatmap representation of the average nucleotide identity for 14 genome sequenced species from the genus *Caldicellulosiruptor*. Average nucleotide identity (ANIb) was calculated on the basis of legacy BLASTn sequence identity over 1020nt sequence fragments. ANIb values of all 14 genomes are represented by a heat plot ranging from blue (75% < ANIb < 90%), white (90% < ANIb < 95%) to red (ANIb > 95%). Pyani (<https://github.com/widdowquinn/pyani>) was used to calculate ANIb values and generate the clustered heatmap. Hierarchical cluster dendrograms were generated on the basis of similar ANIb values across each species. ANIb values are reported in **Table S1**. Calace, *C. acetigenus*; Cbes, *C. bescii*; Calcha, *C. changbaiensis*; Caldan, *C. danielii*; Calhy, *C. hydrothermalis*; Calkr, *C. kristjanssonii*; Calkro, *C. kronotskyensis*; Calla, *C. lactoaceticus*; Calmo, *C. morgani*; Calna, *C. naganoensis*; COB47, *C. obsidiansis*; Calow, *C. owensensis*; Csac, *C. saccharolyticus*; F32, *C. sp. F32*.

Figure 3. Modular multifunctional enzymes encoded for by the glucan degradation locus. Glucan degradation loci were selected on the basis of the presence of “ACE” cellulases. ACE cellulases: CelsA, CelsC and CelsE. Circles represent the glycoside hydrolase (GH) domains, rectangles represent the carbohydrate binding module (CBM) domains. GH5, green circles; GH9, red circles; GH10, violet circles; GH 44, blue circles; GH48, grey circles; GH74, orange circles. CBM3, grey rectangles; CBM22, pink rectangles.

Figure 4. Solubilization of microcrystalline cellulose by *C. bescii* and *C. changbaiensis*.

Uninoculated control, indicates abiotic cellulose solubilization in LOD medium. Error bars represent standard error (n=3). Similar letters over columns denote $p < 0.05$ as determined by a t-test.

Figure 5. Comparison of the ability of *C. bescii* or *C. changbaiensis* planktonic cells to attach to polysaccharides. Titles above bar charts indicate the carbon source for growth/ binding substrate. **(A, B)** When cells are grown on xylan, only planktonic *C. bescii* cells were able to attach to xylan or cellulose. **(C)** Cells grown on cellulose as the carbon source and exposed to cellulose as the binding substrate. Planktonic cell densities (PCD), enumerated by epifluorescence microscopy are plotted on the y-axis. Green columns indicate PCD without binding substrate and purple columns indicate PCD with the binding substrate. * indicates $p < 0.01$. All assays had $n=6$ biological replicates.

Figure 6. Flocculation of *C. bescii* cells cultured on chemically defined medium and microcrystalline cellulose. (A) Formation of a floc of *C. bescii* cells around microcrystalline cellulose (diameter, 20 μ m) while planktonic *C. changbaiensis* cells (cloudiness) are visible. **(B)** Same serum bottles as in “A”, however the bottles were vigorously mixed. The *C. bescii* floc remains fairly stable, while both microcrystalline cellulose and cells are mixed in the *C. changbaiensis* culture.

Figure 7. Genomic context for the location of the tāpirins from strongly to weakly cellulolytic *Caldicellulosiruptor* species. Different colors represent the classical versus atypical tāpirins. Blue arrows: Cbes tāpirin 1 (Gen bank accession: YP_002573732) and Cbes tāpirin 2 (Gen bank accession: YP_002573731). Green arrow: Calhy tāpirin 1 (Gen bank accession number: YP_003992006). Yellow arrows: Calcha tāpirin 1 (Gen bank accession: WP_127352232.1) and 2 (Gen bank accession: WP_127352233.1), and Calow tāpirin 1 (Gen bank accession: YP_004002936) and 2 (Gen bank accession r: YP_004002935). Grey rectangles indicate the presence of the GDL downstream of the tāpirins. Atypical tāpirin 1 is annotated as a hypothetical protein and atypical tāpirin 2 is annotated as a von Willebrand factor A protein. Cbes, *C. bescii*; Calhy, *C. hydrothermalis*; Calcha, *C. changbaiensis* and Calow, *C. owensis*. Peach rectangles represent the type IV pilus locus directly upstream of the tāpirins. Arrows indicate tāpirin 1 and 2. Numbers in the tāpirin arrows indicate the amino acid length.