

Functional heterologous expression of an engineered full length CipA from *Clostridium thermocellum* in *Thermoanaerobacterium saccharolyticum*

Currie *et al.*



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# Functional heterologous expression of an engineered full length CipA from *Clostridium thermocellum* in *Thermoanaerobacterium saccharolyticum*

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## Abstract

**Background:** Cellulose is highly recalcitrant and thus requires a specialized suite of enzymes to solubilize it into fermentable sugars. In *C. thermocellum*, these extracellular enzymes are present as a highly active multi-component system known as the cellulosome. This study explores the expression of a critical *C. thermocellum* cellulosomal component in *T. saccharolyticum* as a step toward creating a thermophilic bacterium capable of consolidated bioprocessing by employing heterologously expressed cellulosomes.

**Results:** We developed an inducible promoter system based on the native *T. saccharolyticum* *xynA* promoter, which was shown to be induced by xylan and xylose. The promoter was used to express the cellulosomal component *cipA\**, an engineered form of the wild-type *cipA* from *C. thermocellum*. Expression and localization to the supernatant were both verified for CipA\*. When a  $\Delta$ *cipA* mutant *C. thermocellum* strain was cultured with a CipA\*-expressing *T. saccharolyticum* strain, hydrolysis and fermentation of 10 grams per liter SigmaCell 101, a highly crystalline cellulose, were observed. This trans-species complementation of a *cipA* deletion demonstrated the ability for CipA\* to assemble a functional cellulosome.

**Conclusion:** This study is the first example of an engineered thermophile heterologously expressing a structural component of a cellulosome. To achieve this goal we developed and tested an inducible promoter for controlled expression in *T. saccharolyticum* as well as a synthetic *cipA*. In addition, we demonstrate a high degree of hydrolysis (up to 93%) on microcrystalline cellulose.

**Keywords:** *Thermoanaerobacterium saccharolyticum*, *Clostridium thermocellum*, Cellulosome, Thermophile, Anaerobe, Ethanol, Consolidated bioprocessing

## Background

A long sought goal in the cellulosic ethanol field is one-step solubilization and fermentation without added enzymes [1,2]. Such consolidated bioprocessing, or CBP, is considered to be the ultimate low cost approach for cellulose hydrolysis and fermentation [2]. A successful CBP organism must be capable of solubilizing both cellulose and hemicellulose, and also fermenting the resulting sugars to a useful product (e.g., ethanol) at high

yield and titer. Unfortunately, no single organism has yet been found or developed that combines these two essential characteristics [3].

Two saccharolytic bacteria of interest for development of CBP-enabling microbes are *Clostridium thermocellum* and *Thermoanaerobacterium saccharolyticum*, both Gram-positive, thermophilic anaerobes. *C. thermocellum* exhibits among the highest growth rates on cellulose among described microbes [1], but lacks the ability to ferment hemicellulose. *C. thermocellum*'s ability to solubilize crystalline cellulose, as well as other insoluble components of plant biomass, results from its elaborate, multi-protein cellulase complex or cellulosome [4-7]. *T. saccharolyticum*

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readily solubilizes hemicellulose and ferments all common sugars in biomass, but does not solubilize cellulose. This bacterium is highly amenable to genetic manipulation, indeed exhibiting natural competence, and has been engineered to make ethanol at high yields and titers [8-11].

The component of the *C. thermocellum* cellulosome with the highest molecular weight is the scaffoldin protein, CipA, which has been implicated in mediating the enzymatic synergy seen in the cellulosome [7,12,13]. The structure of the *C. thermocellum* CipA includes one cellulose binding domain or CBD, one type II dockerin which is used to associate with cell wall anchoring proteins, and 9 highly conserved type I cohesins interspaced by flexible linker regions [7]. The type 1 cohesin domains bind with high affinity to type 1 dockerin domains present in over 70 catalytically-active enzymes [14].

Previous studies have largely focused on expressing mini cellulosomes, to date with 4 or fewer cohesin regions, or chimeric “designer” cellulosomes in which cohesin-dockerin pairs from different organisms are used to form complexes with a specified sequence of catalytic proteins [15-31]. Recently a paper reporting *in vitro* assembly of cellulosomes. To date, there have been no reported attempts to engineer thermophiles to heterologously express a cellulosome, although one attempt has been made to express cellulases [32].

In *C. thermocellum* the presence or absence of CipA has little effect on activity on phosphoric acid swollen cellulose (PASC), carboxymethyl cellulose (CMC), or  $\beta$ -Glucan, but when absent, results in over an order of magnitude decrease in activity on microcrystalline cellulose [27,33]. With this in mind, microcrystalline cellulose was chosen as a test substrate for cellulosome assembly and complementation.

Heterologous expression of a functional cellulosome system in *T. saccharolyticum* is of interest both for fundamentally-oriented studies of microbial cellulose utilization and as a strategy for developing a CBP-enabling microorganism. The logical point of departure for this endeavor is expression of CipA. Here we endeavor to develop an inducible gene expression system in *T. saccharolyticum*, synthesize a gene (*cipA\**) coding for the same amino acid sequence as CipA but with more diversity in the DNA sequences for ease of use, express CipA\* in *T. saccharolyticum*, and demonstrate functionality by complementing  $\Delta$ *cipA* mutants of *C. thermocellum*.

## Results

### Construction and testing of xylose/xyylan inducible promoter system

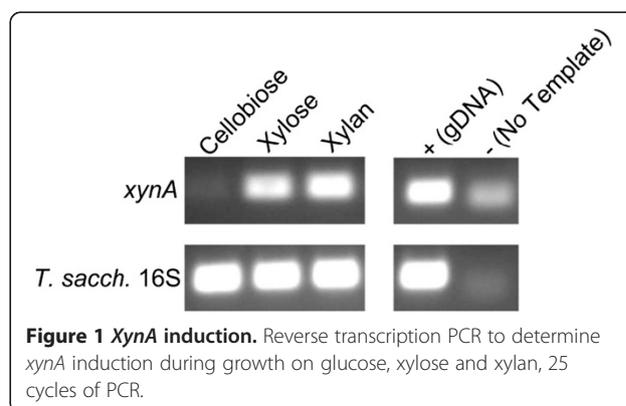
We hypothesized that the promoter and leader sequences of the family 10 endoxylanase *xynA* (Tsac\_1459) might be useful in expressing and secreting heterologous proteins in *T. saccharolyticum*. To test this hypothesis, we performed

RT-PCR to determine gene transcription during growth on cellobiose, xylose, and xylan (Figure 1). The results show that *xynA* is indeed expressed during growth on xylose or xylan and not glucose. In addition it was also observed to be catabolite repressed when glucose and xylose were both present (data not shown). Next, pMC200, which was designed to remove the *xynA* coding region, was transformed into *T. saccharolyticum* M1442 to confirm that the regions of homology and resistance cassette were sufficient to drive integration and the removal of the *xynA* open reading frame. *XynA* mutants were tested for growth on xylan and xylose with no substantial defect in growth, in agreement with previous results [34].

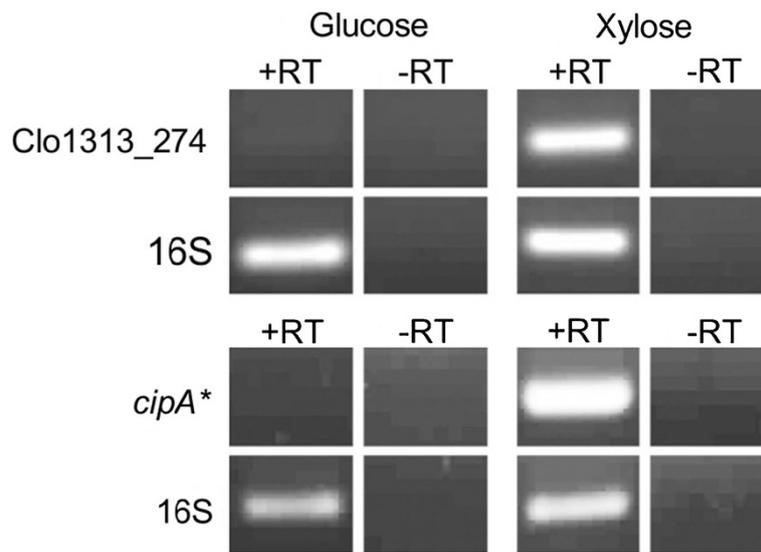
A second test of the system was performed by replacing *xynA* with the His tagged coding region for the secreted *C. thermocellum* family 48 cellulase *cel48S* (Clo1313\_2747), via pMC212. Expression was confirmed via RT-PCR (Figure 2) and was shown to still be regulated by xylose. Unfortunately, the strain did not produce detectable quantities of Cel48S, and as a result no further work was done with the strain. From these data we concluded the *xynA* promoter would function well as an inducible promoter for heterologous gene expression. Furthermore, it allowed cloning of potentially toxic gene products in *E. coli*. For example, it was found that when *cipA\**, discussed in detail below, was placed downstream of the native *T. saccharolyticum* *pta/ack* promoter, this resulted in substantial toxic effects to *E. coli*. However, no toxicity issues were seen with the *xynA* promoter upstream of *cipA\**. This indicates that unlike the *pta/ack* promoter the regions of the promoter found on pMC200 were insufficient to drive expression in *E. coli* in the absence of inducer (xylose).

### Design and synthesis of CipA\*

The native *cipA* sequence has large repeated regions likely to be problematic for sequencing as well as genetic stability once introduced into *T. saccharolyticum*. To optimize and homogenize *cipA* for expression and sequencing in *T. saccharolyticum*, we first identified repeated regions,



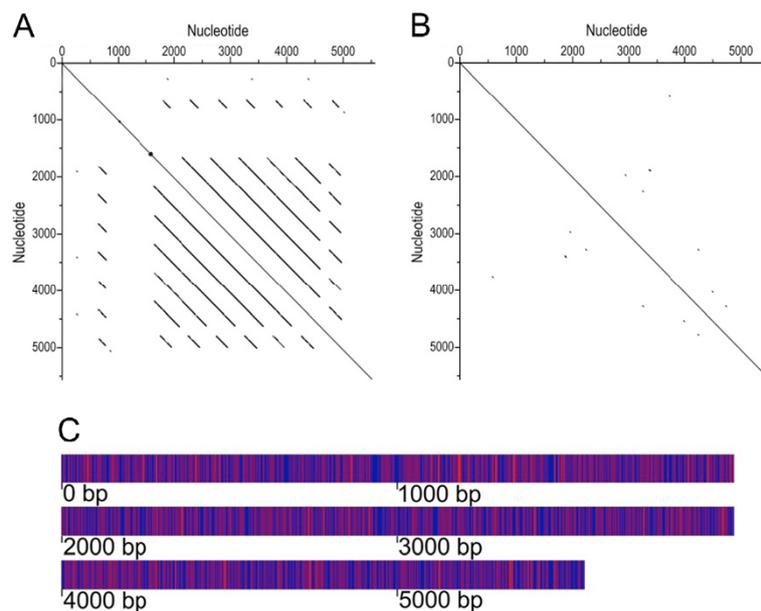
**Figure 1** *XynA* induction. Reverse transcription PCR to determine *xynA* induction during growth on glucose, xylose and xylan, 25 cycles of PCR.



**Figure 2** *XynA*'s upstream region as an inducible promoter for use in *T. saccharolyticum*. Reverse Transcriptase PCR analysis of Clo1313\_274 and *cipA\** under the control of the *xynA* promoter with positive (16S) and negative (no reverse transcriptase added) controls.

the largest 4 of these being identical repeats of almost 500 base pairs (Figure 3). To introduce silent mutations to remove this homology we utilized alternate codons with a minimum usage of 15% for a given amino acid in predicted *T. saccharolyticum* open reading frames. By manually and iteratively randomizing which alternate codon was used in a given location we were able to break up the repeated

regions without generating extended new homologous sequences. It should also be noted that a standard codon optimization would, rather than rectifying the problem, only serve to enhance the redundancy. The result is *cipA\** which has 1325 nucleotide mismatches to the wild type and no unbroken repeats longer than 19 nucleotides while maintaining a wild type amino acid sequence (Figure 3).



**Figure 3** A comparison between the nucleotide sequence of *C. thermocellum*'s *cipA* and the synthetic *cipA\**. **A)** A dot matrix analysis of the wild-type *cipA* with window size of 15 and 0 acceptable mismatches. Positions at which the two sequences are identical at a given position are indicated with a dot. Consecutive identities appear as diagonal lines. **B)** A dot matrix analysis of the engineered *cipA\** with window size of 15 and 0 acceptable mismatches. **C)** Map of single nucleotide polymorphisms between *cipA* and *cipA\** with red representing SNPs.

### Expression and localization of CipA\*

CipA, like other cellulosomal components, is secreted via the sec pathway which utilizes an N-terminal signal peptide which is cleaved to liberate the mature protein. In order to assure the His tag's presence in the mature protein, *cipA\** was tagged at the C-terminus with a 10X His tag via a linker (GGGTGHHHHHHHHHH) for detection via western blot.

A number of methods to concentrate *T. saccharolyticum* supernatant were tested including His purification with Ni beads and FPLC. However, the best results were obtained using molecular weight cut off spin columns. Initial attempts included bacterial or mammalian protease inhibitors, but after it became clear that proteolysis was not an issue, their inclusion was discontinued. Xylose was used as an inducer since it was as effective as xylan (Figure 1) but was more practical to use with spin columns. Unlike in *E. coli* no negative cellular effects were seen as the result of the presence, or induction, of *cipA\**. The concentrated protein was washed with 20 mM sodium citrate buffer (pH 5.7) to remove residual sugars to prevent heavy warping of the protein bands during migration on a SDS-PAGE gel.

Protein isolated from the supernatant and pellet was subject to western blot analysis, probing for the 10X-His tags. CipA\* was observed in both the cytoplasm and in the supernatant under inducing conditions (Figure 4) and was absent when grown on glucose (data not shown). To further confirm active secretion and to rule out the possibility of cell lysis as a means of generating CipA\* in the supernatant, a second strain was constructed lacking the predicted native sec tag [35] (Additional file 1: Figure S2) and the localization of *cipA\** in the two strains was compared. The CipA\* derivative lacking a sec tag was only detected in the pellet fraction whereas the CipA\* with its sec tag intact was found in both the pellet and the supernatant suggesting lysis was not responsible for presence of CipA\* in the supernatant (Figure 4).

Our initial attempts using the standard 100°C denaturation in preparation for running an SDS-PAGE resulted

in protein cleavage as others have also observed for *cipA* [36,37]. However, when the denaturation temperature was dropped, or time was decreased, we obtained a single intact band.

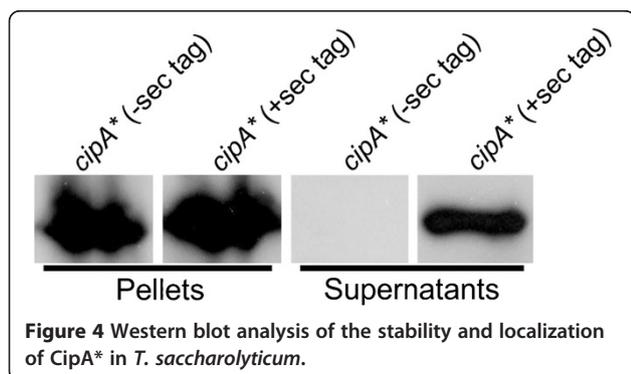
### Interspecies complementation

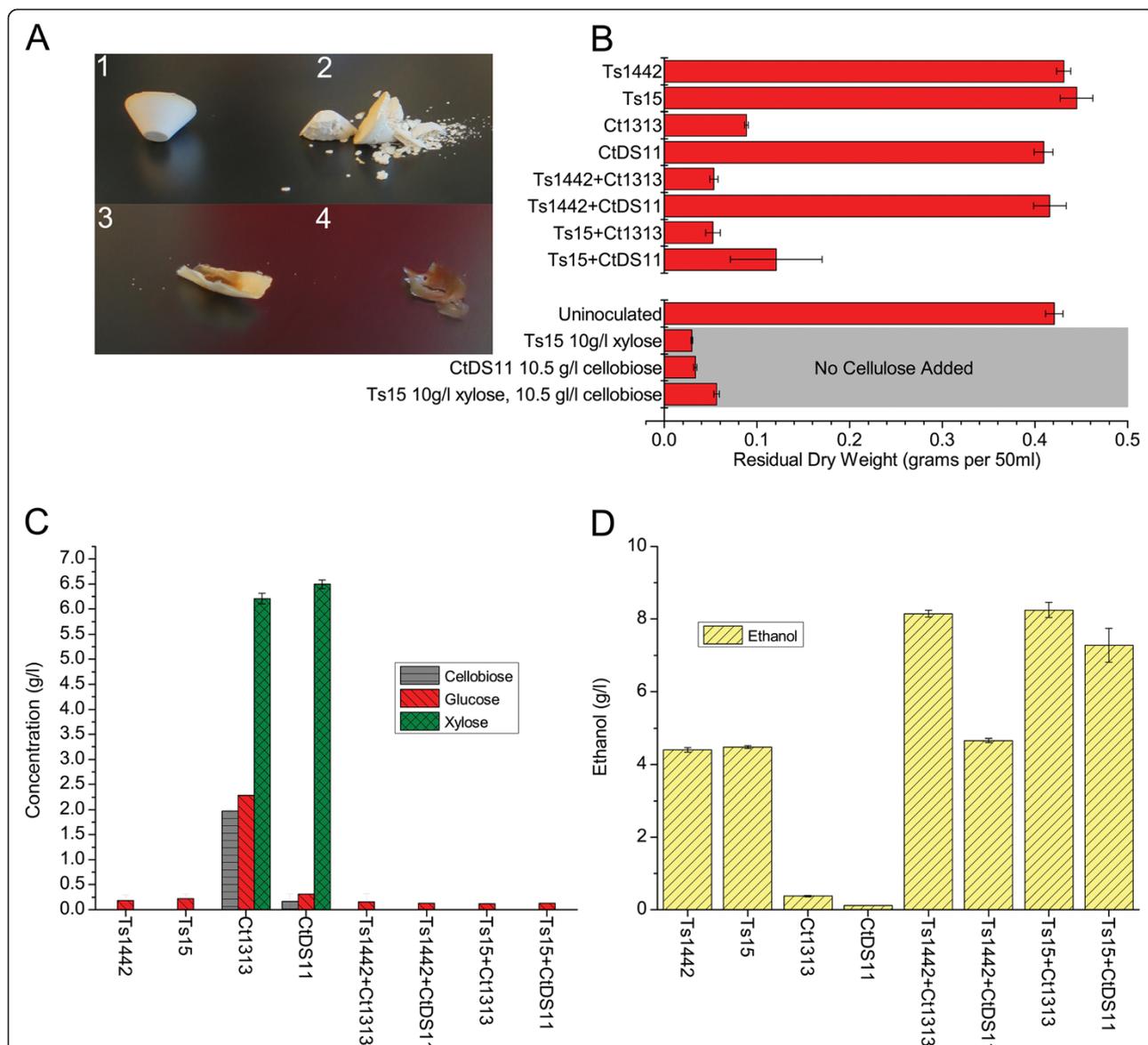
Co-cultures of *C. thermocellum* and *T. saccharolyticum* were used to test the functionality of heterologously expressed CipA\*. We utilized a *cipA* deletion strain of *C. thermocellum* that shows markedly impaired growth on microcrystalline cellulose but still produces cellulosomal components other than CipA when cultivated on cellobiose. By co-culturing *C. thermocellum*  $\Delta$ *cipA* with the strain of *T. saccharolyticum* that expresses CipA\*, we hypothesized that CipA\*, if functional, would complement the deletion in *C. thermocellum* and act as a scaffold thus restoring the ability to hydrolyze cellulose. To evaluate the co-culture's ability to hydrolyze cellulose we determined residual cellulose/dry weight and product formation (Figure 5).

The wild type *C. thermocellum* performed well with respect to cellulose hydrolysis as measured by dry weight. The observed low product formation and unfermented sugars seen with wild type *C. thermocellum* are most likely the result of the low starting pH and the lack of pH control resulting in a discontinuation of metabolic, but not enzymatic hydrolysis activity later in growth. No strain of *T. saccharolyticum* alone appeared to have any effect on cellulose, but grew entirely on the supplied xylose, nor did the wild type strain of *T. saccharolyticum* rescue the cellulose hydrolysis defect in *C. thermocellum* strain DS11. Only *T. saccharolyticum* expressing *cipA\** (strain DHC15) was able to restore cellulose hydrolysis functionality to the *C. thermocellum*  $\Delta$ *cipA* (strain DS11).

Finally, we wished to confirm that populated cellulosomes were being formed. Cellulosomes were purified via affinity digestion from co-cultures between DHC15 and DS11 and compared via native PAGE to those from wild type *C. thermocellum* and concentrated supernatants from DHC15 and DS11 grown individually (Additional file 2: Figure S3). As expected neither DHC15 nor DS11 were able to form cellulosomes when grown independently. When DHC15 and DS11 were grown together cellulosomes were produced with an identical native PAGE migration as those from wild type *C. thermocellum*.

These data demonstrate that the *T. saccharolyticum*-produced CipA\* is capable of gathering and displaying functional cellulosomal enzymes. In addition, the appearance of the pellet further supported the removal of cellulose from the wild type *C. thermocellum* and the co-cultures of *cipA\** and  $\Delta$ *cipA* as these dry pellets were nearly translucent, suggesting only cell debris, rather than the white cellulose observed in the other samples.





**Figure 5 Results from mono and co-cultures of *T. saccharolyticum* and *C. thermocellum*.** Strain names are given in an abbreviated form for ease of reading. **A)** Representative residual solids with increasing degrees of cellulose solubilization. #1 uninoculated, #2 *T. saccharolyticum* M1442, #3 co-culture between *T. saccharolyticum* DHC15 and *C. thermocellum* DS11, #4 co-culture between wild-type *C. thermocellum* and *T. saccharolyticum* M1442. **B)** The dry weights of residual solids from each of the mono or co-cultures. The three final bars labeled 'No Cellulose Added' were not supplied with cellulose and are present to show the maximum contribution made by cell mass alone. Panels **C** and **D** show HPLC data for remaining sugars and ethanol formation, respectively for the parent strain of *T. saccharolyticum* (Ts1442), the CipA\* expressing strain of *T. saccharolyticum* (Ts15), the wild type *C. thermocellum* (Ct1313), and the *C. thermocellum* *cipA* deletion mutant (CtDS11).

## Discussion

Here we report the expression of an engineered CipA\* under the control of a novel inducible promoter in *T. saccharolyticum* which allowed for the assembly of active cellulosomes when co-cultured with a *cipA* deletion strain of *C. thermocellum*.

The wild-type *cipA* gene has multiple sections with essentially identical DNA sequences, corresponding to the 9 type-I dockerin regions [7]. These repeated sequences

can be problematic by complicating sequence verification via routine sequencing technology and could also lead to unwanted partial gene deletion via homologous recombination. Many strains of *E. coli* used to heterologously express cellulosomal proteins for biochemical studies are *recA*- [24,27,38]. However, studies such as this one which seek to integrate cellulase genes into the chromosome via native host machinery must use *recA*+ strains thereby exacerbating the challenge of homologous recombination.

By using *cipA\**, designed to avoid repeated sequences, routine sequencing proceeded without difficulty and homologous recombination was not observed.

Toxicity has been observed by other groups working with heterologous expression of *cipA*, and were solved, at least in part, by using inducible promoters in *E. coli* and *Lactococcus lactis* [23,27]. For this and other reasons we wished to express *cipA\** under the control of an inducible promoter. As there have been no inducible promoter systems described for *T. saccharolyticum* we designed and tested one based on *xynA*'s promoter. We chose this promoter due to the fact that XynA has been shown to be non-essential [34] and thus could be replaced with a gene of interest. We found that the *xynA* promoter avoided toxicity effects in both *E. coli* and *T. saccharolyticum*, although apparent toxicity was encountered using the *pta/ack* promoter.

In past *in vitro* heterologous cellulosome expression reports significant hydrolysis of microcrystalline cellulose was either not achieved or not tested, with one exception reaching 45% hydrolysis [15,17,18,23,25,28-31]. In the data reported here if we remove the contribution of cell mass to the dry weights and compare that to the uninoculated bottles we see that total cellulose solubilization is between 98 and 100 percent for the co-culture of the *CipA\** expressing *T. saccharolyticum* and the wild-type *C. thermocellum*, and between 71 and 93 percent for the *CipA\** expressing *T. saccharolyticum* and *C. thermocellum ΔcipA*. While the trans-complementation co-culture can achieve close to wild type co-culture solubilization in some cases, it is rather variable. The lower hydrolysis from the *CipA\** expressing *T. saccharolyticum* and *C. thermocellum ΔcipA* co-culture, as compared to that of wild-type *C. thermocellum*, may be the result of one or more effects. First, while the cohesin dockerin interaction is quite robust, it is possible that when produced by two different strains the assembly of mature cellulosomes occurs less efficiently than if the components are being secreted simultaneously from the same cell [39,40]. Second, the assembled cellulosomes are incapable of adhering to the surface of the more metabolically active *T. saccharolyticum*, and thus are either present free in the media or bound to the surface of *C. thermocellum*'s via the native anchor proteins [41-43]. This could result in local product inhibition, and may contribute to the lower than expected hydrolysis [3,44-46].

Krauss *et al.* found that *CipA* purified from *E. coli* was populated with cellulosomal enzymes present in *C. thermocellum* supernatants and had near wild-type activity on microcrystalline cellulose [27]. These results led the authors to conclude that the cohesin dockerin interactions are the primary means of cellulosome assembly, an interpretation which our work also supports. Whereas Krauss *et al.* reported cellulosome assembly

*in vitro*, we demonstrate here assembly from components produced by growing cultures with heterologous production using a host that has a temperature optimum compatible with that of the *C. thermocellum* cellulosome.

In both Krauss *et al.* and this study, cellulosomes derived from components produced in separate organisms but otherwise unmodified are found to exhibit similar, although somewhat lower, activity on crystalline cellulose as compared to controls with components produced by a single organism. By contrast, studies involving "designer cellulosomes", in which specific catalytic components bind in a specific order to chimeric scaffoldins, either report several-fold lower activity on crystalline cellulose compared to controls or do not report activity on crystalline cellulose at all. This difference could be because of the importance of a diverse population of cellulosomes with randomly-combined catalytic components, the smaller size of chimeric scaffoldins used in designer cellulosome work, or a combination. The model system reported here, featuring full-sized engineered scaffoldins, is a promising platform for understanding these effects.

An unexpected result of the expression and purification of *CipA\** in *T. saccharolyticum* was the observed instability upon high temperature treatment with SDS-PAGE loading buffer present. While a similar effect has been reported by Morag and Lamed in *C. thermocellum*, the conditions used to achieve this result are markedly different [36,37]. In the previous reports, purified protein was subjected to low pH (3.5) or low ionic strength (dialyzed against double distilled water overnight) which resulted in the cleavage of an Asp-Pro peptide bond present in the cohesin domain of *CipA*. In contrast, no harsh conditions were applied to *CipA\** from *T. saccharolyticum* with supernatant pHs staying above 5.8 and with no dialysis treatment applied. This may indicate a considerable difference between the extracellular environment developed by cultures of *C. thermocellum* compared to that of *T. saccharolyticum*, and could be important in future attempts at heterologous cellulosome expression.

## Conclusion

Combined with the native ability of *T. saccharolyticum* to utilize hemicellulose and the availability of engineered strains that produce ethanol at high yield and titer [8,11], a strain of *T. saccharolyticum* with the ability to solubilize cellulose would be a strong candidate organism for CBP. Our results, including expression and secretion of a functional, engineered, full-length *CipA*, represent a step toward developing such an organism. In addition, we demonstrate a model system in which understanding cellulolytic organisms and their enzyme systems can be tested by systematically reconstructing them.

## Methods

### Microorganisms and growth media

The parent strain for all *T. saccharolyticum* strains is M1442 [47], engineered with deletions of the genes for phosphotransacetylase, acetate kinase, and lactate dehydrogenase and expressing genes for urea utilization from *C. thermocellum* which serve to buffer acid production [11]. *C. thermocellum* DSM1313 was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany). *C. thermocellum* strain DS11, a *cipA* deletion mutant, was generated in our laboratory and is derived from *C. thermocellum* DSM1313  $\Delta$ *hpt* and was supplied by D. G. Olson [48]. All *T. saccharolyticum* strains were grown in modified DSMZ M122 medium [9] with 10 g/l xylose or xylan where noted, 0.5 g/l urea at pH 6.3 and 55°C unless otherwise stated. *C. thermocellum* strains were grown in M122 with 10 g/l cellobiose, pH 7.0 at 55°C. All cultures were grown in a Coy anaerobic chamber under a nitrogen, carbon dioxide, and hydrogen gas mix unless otherwise noted.

### Plasmid and strain construction

All plasmids were constructed in *Saccharomyces cerevisiae* FY2 [49] via yeast mediated homologous recombination [50], isolated from yeast with a Zymoprep Yeast Plasmid Miniprep II (Zymo Research, Irvine, CA) and transformed into chemically competent Invitrogen *E. coli* TOP10 (Invitrogen Corp, Carlsbad, CA) to generate sufficient quantities of plasmid for transformation into *T. saccharolyticum*. Plasmids were then isolated from *E. coli* with the QIAGEN Plasmid Mini kit (QIAGEN Inc, Hilden, Germany). *T. saccharolyticum* was transformed with plasmid DNA as previously described in a Coy anaerobic chamber [9]. Plasmids and strains are listed in Table 1. Genotypic confirmation for modified strains was obtained via PCR and sequencing. *CipA\** was synthesized by GeneArt (Additional file 3: Figure S1) [51].

### Reverse transcription PCR

RNA was isolated from cultures of *T. saccharolyticum* incubated overnight at 55°C in modified M122 [9] with

**Table 1 Strains, plasmids, and primers used in this study**

Strain	Description and characteristics	Reference
<i>T. sacch.</i> M1442	High titer ethanol producing strain	Lee et al., 2011 [47]
<i>T. sacch.</i> DHC6	<i>T. sacch</i> $\Delta$ <i>xynA</i> ::Clo1313_2747	This study
<i>T. sacch.</i> DHC15	<i>T. sacch</i> $\Delta$ <i>xynA</i> :: <i>cipA*</i> (wild type sec tag)	This study
<i>T. sacch.</i> DHC16	<i>T. sacch</i> $\Delta$ <i>xynA</i> :: <i>cipA*</i> (with a deletion in the sec tag)	This study
<i>C. therm.</i> DSM 1313	<i>C. therm</i> wild type obtained from DSMZ culture collection	DSMZ
<i>C. therm.</i> DS11	<i>C. therm</i> 1313 $\Delta$ <i>cipA</i>	Olson et al. 2013 [48]
<i>E. coli</i> TOP 10	Chemically competent	Invitrogen
<i>S. cerevisiae</i> FY2	Uracil Auxotroph used for homologous recombination	Winston et. al, 1995 [49]
Plasmid	Description and characteristics	Reference
pMC200	Deletes <i>xynA</i> and replaces it with a kanamycin resistance gene and a removable marker	This study
pMC212	Replaces <i>xynA</i> with Clo1313_2747, a kanamycin resistance gene, and a removable marker	This study
pMC213	Replaces <i>xynA</i> with <i>cipA*</i> , a kanamycin resistance gene, and a removable marker	This study
pMC223	Replaces <i>xynA</i> with $\Delta$ sec tag <i>cipA*</i> , a kanamycin resistance gene, and a removable marker	This study
Primer	Sequence	Reference
<i>xynA</i> _RT_F	TACTTCAGGATGGGTTGGAACAGG	This study
<i>xynA</i> _RT_R	TCCAATTAGCTGTTCTCCCTGTCCG	This study
<i>gamA</i> _RT_F	ATATTCACCAGCAACGCTGGCTTC	This study
<i>gamA</i> _RT_R	AATAAGCCTTTGCCAGTTGTCGCG	This study
Clo1313_2747_RT_F	AGTGGCGTTATACAAACGCTCCTG	This study
Clo1313_2747_RT_R	ATACAACGGAAGCAACGGCAGAAC	This study
<i>cipA*</i> _RT_F	ACGACTATCTTTGCCGCTATGATCCC	This study
<i>cipA*</i> _RT_R	ACTTTAGATCCTACGGCAGCAGTGAC	This study
pMC200_up_F	gtcttcgactgagccttctgtttatttgatgctggatctttctgaccttaattggcg	This study
pMC200_up_R	cagctgaagcttccccgggatcctctagagaattcgagctctcttacttccctcagtaaatattattattg	This study
pMC200_down_F	ctagataggggtccccgagcgcctacgaggaattgtatcgaaaaaacaataatcttaagtaaaaggcagagagg	This study
pMC200_down_R	ccgtcagtagctgaacaggaggacagctgtagagaagtcaaatcgcaaaaaaacgccc	This study

10 g/l glucose, xylose, or xylan. RNA was purified with the QIAGEN RNeasy Mini Kit and stored at  $-80^{\circ}\text{C}$ . cDNA was generated with the QIAGEN QuantiTect Reverse Transcription Kit. The resulting cDNA was examined for the presence of the transcripts of interest using the primers listed in Table 1.

#### Insertion of *cipA*\* into the chromosome

Insertion of *cipA*\* into the chromosome under the control of the *xynA* promoter was achieved via double homologous recombination, selected for by the presence of a kanamycin resistance marker on a nonreplicative plasmid pMC213. The sites of recombination were directly upstream of the start codon and downstream of the stop codon in the *xynA* open reading frame. The size of these regions of homology was 1000 base pairs each, and left the ribosome binding site from *xynA* intact.

#### Western blot analysis of his-tagged proteins

*T. saccharolyticum* strains were grown to 2/3 maximum OD600. Cells were pelleted, supernatants were filter sterilized and concentrated with Vivaspin 20, PES 10,000 molecular weight cut off centrifugal concentrators (Sartorius Stedim Biotech) as per the manufacturer's instructions. Samples were washed with two volumes of sodium citrate buffer (20 mM, pH 5.7) at  $15^{\circ}\text{C}$ . Pellets were treated with 20 mg/ml lysozyme in SET buffer (40 mM EDTA, 50 mM Tris-HCl, pH 8.0, 0.75 M sucrose) for 10 minutes to remove the cell wall, pelleted and resuspended in lysis buffer (10 mM Tris pH 7, 0.2% SDS, 1 mM DTT) and incubated at  $55^{\circ}\text{C}$  for 15 minutes. Proteins were denatured at  $55^{\circ}\text{C}$  in loading buffer (5X loading buffer: 6.25 ml 1 M Tris pH 6.8, 2 ml glycerol, 7.3 g SDS, bromophenol blue 0.1%, final pH 6.8). Total protein from either supernatants or lysed cell pellets were analyzed via Western blot with mouse Penta-His (Cat. No. 34660, QIAGEN Inc.) primary, and goat anti-mouse peroxidase conjugate (Cat. No. 31439, Thermo Sci.) secondary antibody. Detection was performed with Western Lightning ECL substrate (PerkinElmer, Waltham, MA) and detected on Kodak X-ray film.

#### Co-cultures

50 ml co-cultures were grown in 115 ml nitrogen flushed anaerobic serum bottles agitated in an incubator at  $55^{\circ}\text{C}$ . The medium for the co-cultures was modified DSMZ M122 [9] with 10 g/l xylose and 10 g/l Sigmacell 101 (a microcrystalline cellulose similar to avicel) and 0.5 g/l cellobiose to assist in the initial growth of *C. thermocellum*. The initial pH was pH 6.3, previously demonstrated to be suitable for co-cultures between these two organisms [52]. Co-cultures were allowed to grow for 5 days. Cellulosomes were purified via affinity digestion of PASC [27].

#### Product formation and dry weight

Dry weight was determined by pelleting the remaining cellulose via centrifugation for 10 minutes at 8000 g, washing twice with deionized water, and drying at  $45^{\circ}\text{C}$  under vacuum for 2 days. Dried pellets were then weighed to determine residual solids. The contribution of cell mass was taken into account with controls containing 10 g/l cellobiose in place of Sigmacell. Residual sugars and ethanol in the supernatants were quantified by HPLC using an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA). Nucleotide sequence accession number. The sequences reported in this paper have been deposited in the GenBank database (accession no. KC675188 [pMC200], KC675189 [pMC212], KC675190 [pMC213], and KC675191 [pMC223]).

#### Additional files

**Additional file 1:** Sequence of *cipA*\* minus the predicted signal peptide with a C-terminal 10X his tag and linker region.

**Additional file 2:** Coomassie stained native PAGE of trans-species formed cellulosomes.

**Additional file 3:** Sequence of *cipA*\* with a C-terminal 10X his tag and linker region.

#### Abbreviations

CBP: Consolidated bioprocessing; CBD: Cellulose binding domain; PASC: Phosphoric acid swollen cellulose; CMC: Carboxymethyl cellulose.

#### Competing interests

This research was supported in part by Mascoma Corporation, Lebanon NH, with which authors DHC, CDH, DAH and LRL are affiliated. Mascoma Corporation has a commercial interest in the organisms used in this study. The authors DHC and AMG are listed on the international and national pending patents for the sequence of *cipA*\* [52].

#### Authors' contributions

DHC performed the work presented herein and drafted the manuscript. DGO assisted in drafting the manuscript. CDH, AMG, DAH and LRL supervised the work and assisted in drafting the manuscript. All authors read and approved the final manuscript.

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#### References

- Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS: **Microbial cellulose utilization: fundamentals and biotechnology.** *Microbiol Mol Biol Rev* 2002, **66**:506–577. table of contents.
- Lynd LR, van Zyl WH, McBride JE, Laser M: **Consolidated bioprocessing of cellulosic biomass: an update.** *Curr Opin Biotechnol* 2005, **16**:577–583.
- Olson DG, McBride JE, Joe Shaw A, Lynd LR: **Recent progress in consolidated bioprocessing.** *Curr Opin Biotechnol* 2012, **23**:396–405.
- Lamed R, Setter E, Bayer EA: **Characterization of a cellulose-binding, cellulase-containing complex in *Clostridium thermocellum*.** *J Bacteriol* 1983, **156**:828–836.
- Bayer EA, Setter E, Lamed R: **Organization and distribution of the cellulosome in *Clostridium thermocellum*.** *J Bacteriol* 1985, **163**:552–559.
- Beguín P, Lemaire M: **The cellulosome: an exocellular, multiprotein complex specialized in cellulose degradation.** *Crit Rev Biochem Mol Biol* 1996, **31**:201–236.
- Bayer EA, Belaich JP, Shoham Y, Lamed R: **The cellulosomes: multienzyme machines for degradation of plant cell wall polysaccharides.** *Annu Rev Microbiol* 2004, **58**:521–554.
- Shaw AJ, Podkaminer KK, Desai SG, Bardsley JS, Rogers SR, Thorne PG, Hogsett DA, Lynd LR: **Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield.** *Proc Natl Acad Sci U S A* 2008, **105**:13769–13774.
- Shaw AJ, Hogsett DA, Lynd LR: **Natural competence in *Thermoanaerobacter* and *Thermoanaerobacterium* species.** *Appl Environ Microbiol* 2010, **76**:4713–4719.
- Shaw AJ, Covalla SF, Hogsett DA, Herring CD: **Marker removal system for *Thermoanaerobacterium saccharolyticum* and development of a markerless ethanologen.** *Appl Environ Microbiol* 2011, **77**:2534–2536.
- Shaw JA, Covalla SF, Miller BB, Firliet BT, Hogsett DA, Herring CD: **Urease expression in a *Thermoanaerobacterium saccharolyticum* ethanologen allows high titer ethanol production.** *Metab Eng* 2012, **14**:528–532.
- Bayer EA, Shimon LJ, Shoham Y, Lamed R: **Cellulosomes-structure and ultrastructure.** *J Struct Biol* 1998, **124**:221–234.
- Gilbert HJ: **Cellulosomes: microbial nanomachines that display plasticity in quaternary structure.** *Mol Microbiol* 2007, **63**:1568–1576.
- Zverlov VV, Kellermann J, Schwarz WH: **Functional subgenomics of *Clostridium thermocellum* cellulosomal genes: identification of the major catalytic components in the extracellular complex and detection of three new enzymes.** *Proteomics* 2005, **5**:3646–3653.
- Hyeon JE, Yu KO, Suh DJ, Suh YW, Lee SE, Lee J, Han SO: **Production of minicellulosomes from *Clostridium cellulovorans* for the fermentation of cellulosic ethanol using engineered recombinant *Saccharomyces cerevisiae*.** *FEMS Microbiol Lett* 2010, **310**:39–47.
- Tsai SL, Goyal G, Chen W: **Surface display of a functional minicellulosome by intracellular complementation using a synthetic yeast consortium and its application to cellulose hydrolysis and ethanol production.** *Appl Environ Microbiol* 2010, **76**:7514–7520.
- Hyeon JE, Jeon WJ, Whang SY, Han SO: **Production of minicellulosomes for the enhanced hydrolysis of cellulosic substrates by recombinant *Corynebacterium glutamicum*.** *Enzyme Microb Technol* 2011, **48**:371–377.
- Goyal G, Tsai SL, Madan B, DaSilva NA, Chen W: **Simultaneous cell growth and ethanol production from cellulose by an engineered yeast consortium displaying a functional mini-cellulosome.** *Microb Cell Fact* 2011, **10**:89.
- Anderson TD, Robson SA, Jiang XW, Malmirchegini GR, Fierobe HP, Lazazzera BA, Clubb RT: **Assembly of minicellulosomes on the surface of *Bacillus subtilis*.** *Appl Environ Microbiol* 2011, **77**:4849–4858.
- Sun J, Wen F, Si T, Xu JH, Zhao H: **Direct conversion of xylan to ethanol by recombinant *Saccharomyces cerevisiae* strains displaying an engineered minihemicellulosome.** *Appl Environ Microbiol* 2012, **78**:3837–3845.
- Caspi J, Irwin D, Lamed R, Li Y, Fierobe HP, Wilson DB, Bayer EA: **Conversion of *Thermobifida fusca* free exoglucanases into cellulosomal components: comparative impact on cellulose-degrading activity.** *J Biotechnol* 2008, **135**:351–357.
- Caspi J, Barak Y, Haimovitz R, Irwin D, Lamed R, Wilson DB, Bayer EA: **Effect of linker length and dockerin position on conversion of a *Thermobifida fusca* endoglucanase to the cellulosomal mode.** *Appl Environ Microbiol* 2009, **75**:7335–7342.
- Wieczorek AS, Martin VJ: **Engineering the cell surface display of cohesins for assembly of cellulosome-inspired enzyme complexes on *Lactococcus lactis*.** *Microb Cell Fact* 2010, **9**:69.
- Fierobe HP, Bayer EA, Tardif C, Czjzek M, Mechaly A, Belaich A, Lamed R, Shoham Y, Belaich JP: **Degradation of cellulose substrates by cellulosome chimeras. Substrate targeting versus proximity of enzyme components.** *J Biol Chem* 2002, **277**:49621–49630.
- Mingardon F, Perret S, Belaich A, Tardif C, Belaich JP, Fierobe HP: **Heterologous production, assembly, and secretion of a minicellulosome by *Clostridium acetobutylicum* ATCC 824.** *Appl Environ Microbiol* 2005, **71**:1215–1222.
- Morais S, Barak Y, Caspi J, Hadar Y, Lamed R, Shoham Y, Wilson DB, Bayer EA: **Cellulase-xylanase synergy in designer cellulosomes for enhanced degradation of a complex cellulosic substrate.** *MBio* 2010, **1**:e00285–00210.
- Krauss J, Zverlov VV, Schwarz WH: **In vitro reconstitution of the complete *Clostridium thermocellum* cellulosome and synergistic activity on crystalline cellulose.** *Appl Environ Microbiol* 2012, **78**:4301–4307.
- Wen F, Sun J, Zhao H: **Yeast surface display of trifunctional minicellulosomes for simultaneous saccharification and fermentation of cellulose to ethanol.** *Appl Environ Microbiol* 2010, **76**:1251–1260.
- Cho HY, Yukawa H, Inui M, Doi RH, Wong SL: **Production of minicellulosomes from *Clostridium cellulovorans* in *Bacillus subtilis* WB800.** *Appl Environ Microbiol* 2004, **70**:5704–5707.
- Lilly M, Fierobe HP, van Zyl WH, Volschenk H: **Heterologous expression of a *Clostridium* minicellulosome in *Saccharomyces cerevisiae*.** *FEMS Yeast Res* 2009, **9**:1236–1249.
- Fan LH, Zhang ZJ, Yu XY, Xue YX, Tan TW: **Self-surface assembly of cellulosomes with two miniscaffoldins on *Saccharomyces cerevisiae* for cellulosic ethanol production.** *Proc Natl Acad Sci U S A* 2012, **109**:13260–13265.
- Mai V, Wiegel J: **Advances in development of a genetic system for *Thermoanaerobacterium* spp.: expression of genes encoding hydrolytic enzymes, development of a second shuttle vector, and integration of genes into the chromosome.** *Appl Environ Microbiol* 2000, **66**:4817–4821.
- Zverlov VV, Klupp M, Krauss J, Schwarz WH: **Mutations in the scaffoldin gene, *cipA*, of *Clostridium thermocellum* with impaired cellulosome formation and cellulose hydrolysis: insertions of a new transposable element, IS1447, and implications for cellulase synergism on crystalline cellulose.** *J Bacteriol* 2008, **190**:4321–4327.
- Podkaminer KK, Guss AM, Trajano HL, Hogsett DA, Lynd LR: **Characterization of xylan utilization and discovery of a new endoxylanase in *Thermoanaerobacterium saccharolyticum* through targeted gene deletions.** *Appl Environ Microbiol* 2012, **78**:8441–8447.
- Petersen TN, Brunak S, von Heijne G, Nielsen H: **SignalP 4.0: discriminating signal peptides from transmembrane regions.** *Nat Methods* 2011, **8**:785–786.
- Morag E, Bayer EA, Lamed R: **Unorthodox intrasubunit interactions in the cellulosome of *Clostridium-Thermocellum* - identification of structural transitions induced in the S1-subunit.** *Appl Biochem Biotechnol* 1992, **33**:205–217.
- Lamed R, Kenig R, Morag E, Yaron S, Shoham Y, Bayer EA: **Nonproteolytic cleavage of aspartyl proline bonds in the cellulosomal scaffoldin subunit from *Clostridium thermocellum*.** *Appl Biochem Biotechnol* 2001, **90**:67–73.
- Yaron S, Shimon LJ, Frolow F, Lamed R, Morag E, Shoham Y, Bayer EA: **Expression, purification and crystallization of a cohesin domain from the cellulosome of *Clostridium thermocellum*.** *J Biotechnol* 1996, **51**:243–249.
- Mechaly A, Fierobe HP, Belaich A, Belaich JP, Lamed R, Shoham Y, Bayer EA: **Cohesin-dockerin interaction in cellulosome assembly: a single hydroxyl group of a dockerin domain distinguishes between nonrecognition and high affinity recognition.** *J Biol Chem* 2001, **276**:9883–9888.

40. Demishtein A, Karpol A, Barak Y, Lamed R, Bayer EA: **Characterization of a dockerin-based affinity tag: application for purification of a broad variety of target proteins.** *J Mol Recognit* 2010, **23**:525–535.
41. Leibovitz E, Beguin P: **A new type of cohesin domain that specifically binds the dockerin domain of the *Clostridium thermocellum* cellulosome-integrating protein CipA.** *J Bacteriol* 1996, **178**:3077–3084.
42. Leibovitz E, Ohayon H, Gounon P, Beguin P: **Characterization and subcellular localization of the *Clostridium thermocellum* scaffoldin dockerin binding protein SdbA.** *J Bacteriol* 1997, **179**:2519–2523.
43. Zhao G, Li H, Wamalwa B, Sakka M, Kimura T, Sakka K: **Different binding specificities of S-layer homology modules from *Clostridium thermocellum* AncA, Slp1, and Slp2.** *Biosci Biotechnol Biochem* 2006, **70**:1636–1641.
44. Lynd LR, Zhang Y: **Quantitative determination of cellulase concentration as distinct from cell concentration in studies of microbial cellulose utilization: analytical framework and methodological approach.** *Biotechnol Bioeng* 2002, **77**:467–475.
45. Lu Y, Zhang YH, Lynd LR: **Enzyme-microbe synergy during cellulose hydrolysis by *Clostridium thermocellum*.** *Proc Natl Acad Sci U S A* 2006, **103**:16165–16169.
46. Gefen G, Anbar M, Morag E, Lamed R, Bayer EA: **Enhanced cellulose degradation by targeted integration of a cohesin-fused beta-glucosidase into the *Clostridium thermocellum* cellulosome.** *Proc Natl Acad Sci U S A* 2012, **109**:10298–10303.
47. Lee JM, Venditti RA, Jameel H, Kenealy WR: **Detoxification of woody hydrolyzates with activated carbon for bioconversion to ethanol by the thermophilic anaerobic bacterium *Thermoanaerobacterium saccharolyticum*.** *Biomass Bioenerg* 2011, **35**:626–636.
48. Olson DG, Giannone RJ, Hettich RL, Lynd LR: **Role of the CipA Scaffoldin protein in cellulose solubilization, as determined by targeted gene deletion and complementation in *Clostridium thermocellum*.** *J Bacteriol* 2013, **195**:733–739.
49. Winston F, Dollard C, Ricupero-Hovasse SL: **Construction of a set of convenient *Saccharomyces cerevisiae* strains that are isogenic to S288C.** *Yeast* 1995, **11**:53–55.
50. Shanks RM, Kadouri DE, MacEachran DP, O'Toole GA: **New yeast recombineering tools for bacteria.** *Plasmid* 2009, **62**:88–97.
51. Currie DH, McBride J, Guss A: **Modified *cipA* gene from *Clostridium thermocellum* for enhanced genetic stability.** Filed: 4/21/2010. Patent Application Number PCT/US2010/031907.
52. Argyros DA, Tripathi SA, Barrett TF, Rogers SR, Feinberg LF, Olson DG, Foden JM, Miller BB, Lynd LR, Hogsett DA, Caiazza NC: **High ethanol titers from cellulose by using metabolically engineered thermophilic, anaerobic microbes.** *Appl Environ Microbiol* 2011, **77**:8288–8294.

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