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Enhanced microbial cellulose utilization of recalcitrant cellulose by an *ex vivo* cellulosome-microbe complex

Running title: Cellulosome-microbe synergy

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ABSTRACT

A cellulosome-microbe complex was assembled *ex vivo* on the surface of *Bacillus subtilis* displaying a mini-scaffoldin that can bind with three dockerin-containing cellulase components -- an endoglucanase Cel5, a processive endoglucanase Cel9, and a cellobiohydrolase Cel48. The hydrolysis performance of the synthetic cellulosome bound to living cells, the synthetic cellulosome, a non-complexed cellulase mixture with the same catalytic components, and a commercial fungal enzyme mixture was investigated on low-accessibility recalcitrant Avicel and high-accessibility regenerated amorphous cellulose. The cell-bound cellulosome exhibited 4.5- and 2.3-fold higher hydrolysis ability than cell-free cellulosome on Avicel and RAC, respectively. The cellulosome-microbe synergy was not completely explained by the removal of hydrolysis products from the bulk fermentation broth by free living cells, and appeared due to substrate channeling of long chain hydrolysis products assimilated by the adjacent cells located in the boundary layer. Our results implied that long-chain hydrolytic products in the boundary layer may inhibit cellulosome activity greater than the short-chain products in bulk phase. The findings that cell-bound cellulosome expedited microbial cellulose utilization rate by 2.3-4.5 fold would help develop better consolidated bioprocessing microorganisms (e.g., *B. subtilis*) that can hydrolyze recalcitrant cellulose rapidly at low secretory cellulase levels.

Keywords: *Bacillus subtilis*, cellulase, consolidated bioprocessing, cellulosome-microbe synergy, cellulose-cellulosome-microbe complex, synthetic cellulosome

INTRODUCTION

Biofuels and commodity chemicals produced from cellulosic biomass are of interest as sustainable substitutes for functionally-similar molecules based on petroleum. The primary obstacle to biological production of such products is cost-effective technology to overcome the recalcitrance of cellulosic biomass (19, 22, 37).

Consolidated bioprocessing (CBP) - in which saccharolytic enzyme production, plant cell wall solubilization, and fermentation occur in a single step – is widely seen as a promising low cost processing route (18, 22, 24, 37). CBP microorganisms can be developed according to three strategies: (i) engineering naturally-occurring cellulolytic microorganisms for improved product formation-related properties, such as *Clostridium thermocellum* (6), *Clostridium cellulovorans* (29), and *Clostridium phytofermentans* ISDg (30), (ii) engineering natural high-yield product-forming microorganisms by expressing recombinant cellulases, such as *Saccharomyces cerevisiae* (16, 31, 34), and (iii) engineering one host with both recombinant product-forming and cellulose-utilizing abilities, such as *Escherichia coli* (15) and *Bacillus subtilis* (2, 26, 37).

Nature has evolved two distinctive cellulase systems for degrading cellulosic material: non-complexed cellulase mixtures and complexed cellulases, called cellulosomes. Aerobic fungi (e.g., *Trichoderma reesei*) usually secrete high levels (e.g. ≥1-10 g protein/L) of several different functionally-distinct cellulase components. By contrast, some anaerobic bacteria, such as *C. thermocellum* and *C. cellulovorans*, produce low levels of cellulosomes (i.e., ~0.1 g/L), in which many glycoside hydrolases are linked

67 together by non-hydrolytic scaffoldins through the high-affinity interaction between
68 cohesins in scaffoldins and enzyme-borne dockerins (3, 7-10). *C. thermocellum* exhibits
69 among the highest growth rates on cellulose among described microbes (24), although it
70 produces less cellulase per cell mass than aerobic microorganisms. This observation
71 raises an interesting question – how anaerobic cellulolytic microorganisms can hydrolyze
72 cellulose rapidly and effectively without the production of ample secretory cellulase,
73 where the biosynthesis of cellulase means a large bioenergetic burden for anaerobic
74 cellulolytic bacteria. Recently, *in vitro* evidence pertaining to designer cellulosomes
75 suggests that designer cellulosomes exhibit faster hydrolysis rate than their non-
76 complexed counterparts due to an enzyme proximity synergy (25, 32, 41). Zverlov et al.
77 (43) reported that a *C. thermocellum* mutant featuring a completely defective scaffoldin
78 protein exhibited a 15-fold reduction in specific cellulase activity on crystalline cellulose.
79 Furthermore, Lynd et al. (21) found out that *C. thermocellum* along with cell-bound
80 cellulosome exhibited *ca.* 2.8-4.7-fold enhanced cellulose hydrolysis rates on Avicel
81 compared to purified cellulosome in the presence of another soluble sugar-utilizing
82 microorganism (21). Several recent studies have expressed mini-cellulosomes on the
83 surface of microorganisms, such as *B. subtilis* (1, 5) and *S. cerevisiae* (31, 34) but did not
84 quantitatively evaluate the enzyme-microbe synergy.
85
86 In this study, mini-CipA was displayed on the cell surface of *B. subtilis* through a cell
87 wall-binding module of a *B. subtilis* cell wall hydrolase, LytE. A tri-functional mini-
88 cellulosome was assembled *ex vivo* on the cell surface of *B. subtilis*. The hydrolysis
89 performance of a three-enzyme mixture, cell-free mini-cellulosome, cell-bound mini-

cellulosome as well as a commercial fungal cellulase mixture was compared on low-accessibility Avicel and high-accessibility regenerated amorphous cellulose (RAC).

MATERIALS AND METHODS

Chemicals. All chemicals were reagent grade or higher, purchased from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA), unless otherwise noted. Microcrystalline cellulose – Avicel PH105 (20 µm) – was purchased from FMC (Philadelphia, PA). RAC was prepared from Avicel as previously described (19, 38). The oligonucleotides were synthesized by Integrated DNA Technologies (Coraville, IA). The PCR enzyme was high-fidelity Phusion DNA polymerase from New England Biolabs (Ipswich, MA). A commercial *Trichoderma* cellulase mixture (50013) was gifted from Novozymes North America (Franklinton, NC). The purified fungal enzymes of cellobiohydrolase I (CBH I, Cel7A) and endoglucanase II (EG II, Cel5) from *Trichoderma* spp. were purchased from Megazyme (Wicklow, Ireland).

Strains and media. The strains and plasmids used in this study are listed in Table 1. *E. coli* JM109 was used as a host cell for DNA manipulation. *E. coli* BL21 Star (DE3) (Invitrogen, Carlsbad, CA) and *B. subtilis* WB600 (35) were used as the hosts for recombinant protein expression. *B. subtilis* was transformed through a new simple and fast transformation technology as described elsewhere (38). The Luria-Bertani (LB) medium was used for *E. coli* cell culture and recombinant protein expression. 2 X Mal medium was used for *B. subtilis* recombinant protein expression (38). The final concentrations of antibiotics for *E. coli* were 100 mg/L ampicillin and 25 mg/L chloramphenicol. The chloramphenicol concentration for *B. subtilis* was 5 mg/L.

Construction of plasmids. The primers used in this study are listed in Table 2. For constructing pNWP43N-LysM, the DNA sequence encoding the *B. subtilis* cell wall hydrolase (LysM, GenBank Accession number: U38819, 25-230 amino acids) was amplified from the genomic DNA of *B. subtilis* 168 by a primer pair of LysM_For and LysM_Rev_Flag; the DNA sequence encoding a vector pNWP43N was amplified from pNWP43N-BsCel5 (38) by a primer pair of pNWP43N_For and pNWP43N_Rev. The two PCR products were both digested with NheI/XhoI and then ligated, yielding pNWP43N-LysM. For constructing pNWP43N-LysM-mini-cipA (pNWP43N-LMC), the DNA sequence encoding LysM was amplified by using a primer pair of LysM_For and LysM_Rev based on the *B. subtilis* genomic DNA by PCR, followed by double digestion by XhoI/EcoRV. The DNA sequence encoding truncated mini-CipA (GenBank Accession number: L08665, 26-723 amino acids) was amplified from the genomic DNA of *C. thermocellum* by a primer pair of MC_For and MC_Rev_Flag, followed by double digestion by EcoRV/NheI. The two resultant fragments were ligated into the XhoI/NheI-digested vector pNWP43N to produce pNWP43N-LMC. The DNA sequence encoding truncated mini-CipA was amplified from the genomic DNA of *C. thermocellum* ATCC27405 by a primer pair of mini-CipA_For and mini-CipA_Rev. The PCR product was digested with NdeI/XhoI and then ligated into the NdeI/XhoI-digested vector pET20b (Novagen, Madison, WI), yielding pET20b-mini-CipA.

pET20b-Bscel5' was obtained by using the overlap extension PCR. The DNA sequence encoding mature BsCel5 (GenBank Accession number: CAA82317) was amplified from genomic DNA of *B. subtilis* 168 by a primer pair of BsCel5_For / BsCel5'_Rev. The DNA fragment encoding a dockerin module (DocK, 821-895 amino

136 acids) of *C. thermocellum* CelK (NCBI Reference Sequence: YP_001036843) was
137 amplified from the genomic DNA of *C. thermocellum* by a primer pair of DocK_For
138 /DocK_Rev. The two resultant fragments were assembled by using a primer pair of
139 BsCel5_For/DocS_Rev through overlap extension PCR. These resultant fragments were
140 cloned into NdeI/XhoI- digested pET20b, generating pET20b-Bscel5'. pET20b-Ctcel9
141 was obtained by using PCR amplification and overlap extension PCR. The DNA
142 encoding the mature *C. thermocellum* Cel9 (GenBank Accession number: CAA43035)
143 was amplified from the genomic DNA of *C. thermocellum* by a primer pair of
144 CtCelF_For /CtCelF_Rev. The PCR product was digested with NdeI/XhoI and ligated
145 into the corresponding sites of the vector pET20b, yielding pET20b-Ctcel9. The DNA
146 sequence encoding a mature *C. phytofermentans* Cel48 (GenBank Accession number:
147 ABX43721) were amplified from pP43N-Cpcel48 (39) by a primer pair of CpCel48_For
148 /CpCel48_Rev. Plasmid pET20b-Cpcel48 was constructed in the same way as that of
149 pET20b-Bscel5'. The dockerin of Cpcel48 was DocS of the *C. thermocellum* Cel48S
150 (GenBank Accession number: L06942, 673-741 amino acids). All plasmid sequences
151 were verified by DNA sequencing. The resulting plasmids are listed in Table 1.
152 **Production of dockerin-containing cellulases in *E. coli.*** The strain *E. coli* BL21 Star
153 (DE3) containing the protein expression plasmid was cultivated in the LB medium
154 supplemented with 1.2% glycerol at 37 °C. Protein expression and purification was
155 conducted routinely as published elsewhere (19, 37, 38).
156 **Removal of the *B. subtilis* surface proteins by LiCl.** *B. subtilis* cells harboring
157 pNWP43N-LMC were pre-cultured in the LB medium at 37^{o}C till the A_{600} reached about
158 1.2, which remained at a logarithmic growth phase. Two hundred microliter of the cell

culture was inoculated into 50 mL of 2X Mal medium and then grown at 30°C till A_{600} reached three. Two mL culture of the *B. subtilis* cells were washed two times in Buffer A (50 mM HEPES buffer, pH 7.5, containing 50 mM NaCl and 10 mM $CaCl_2$). The cell pellets were resuspended in 80 µL of Buffer B (50 mM HEPES buffer, pH 7.5, containing 5 M LiCl, 50 mM NaCl, and 10 mM $CaCl_2$). After incubation for 20 min on ice followed by centrifugation at 8,000 g at 4°C for 10 min, a fraction of the supernatant after 10% trichloroacetic acid precipitation was loaded in SDS-PAGE. The other fraction of the supernatant was diluted in Buffer A by five folds, and then mixed with 50 µg RAC. After centrifugation, the adsorbed LMC by RAC was examined by SDS-PAGE, as described elsewhere (37, 38). For validation of the formation of *ex vivo* mini-cellulosome, the resuspended cells with bound LMC were mixed with 0.05 mg purified dockerin-tagged cellulases (BsCel5' or cellulase mixture with equimolar of BsCel5', CtCel9 and CpCel48) at 4°C for 1 h. The cells were washed in Buffer A two times. The cell-bound mini-cellulosome was eluted by LiCl, adsorbed by RAC, and examined by SDS-PAGE, as described above.

Confocal immunofluorescence microscopy. *B. subtilis* cells (200 µL of cell culture at A_{600} = 3.0) having surface-displayed LMC or cell-bound mini-cellulosome were washed in ice-cold phosphate-buffered saline (PBS, 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, and 0.24 g/L KH_2PO_4) two times, and then mixed with 4% para-formaldehyde at 4°C for 30 min. After wash in 1 mL of PBS two times, the cells were resuspended in 250 µL of PBS containing 1 mg/mL of bovine serum albumin (BSA) and 0.5 µg of monoclonal anti-Flag M2 (Sigma F1804) or monoclonal anti-His (Sigma H1029) antibody with occasional mixing for 2 h. The cells were washed in 1 mL of PBS for two times, followed by

resuspension in 250 µL of PBS containing 1 mg/mL BSA and 0.5 µg anti-mouse IgG conjugated with FITC (Sigma F9137). After incubation for 2 h, cells were washed with 1 mL of PBS two times, and then resuspended in PBS to obtain the cell solution with A_{600} = 1. The cells were examined by the ZEISS LSM 510 confocal LASER microscope (Thornwood, NY).

RAC and Avicel hydrolysis. All cellulose hydrolysis experiments were conducted in 50-mL serum bottles with a rotary shaking rate of 250 rpm at 37 ^{o}C. Equimolar of BsCel5', CtCel9 and CpCel48 were pre-mixed together, where molecular weights of BsCel5', CtCel9 and CepCel48 were 44 918, 80 108 and 107 184, respectively. Equimolar of mini-CipA was mixed with the three-cellulase mixture for the formation of tri-functional mini-cellulosome. Similarly, the number of LMC on the surface of *B. subtilis* was determined as described elsewhere (4). The cell culture containing equimolar of LMC was mixed with the three-cellulase mixture for the formation of cell-bound cellulosome. For RAC hydrolysis, the LMC-displayed *B. subtilis* cells collected from 33.3 mL of the cell culture with A_{600} = 3.0 were mixed with 0.1 mg (total) three-cellulase mixture in 10 mL of the ice-cooled Buffer A containing 0.4% RAC followed by hydrolysis at 37^{o}C. The LysM-displayed *B. subtilis* cells collected from 33.3 mL of the cell culture with A_{600} = 3.0 were mixed with tri-functional mini-cellulosome containing 0.1 mg (total) three-cellulase mixture in 10 mL of the ice-cooled Buffer A containing 0.4% RAC followed by hydrolysis at 37^{o}C. For Avicel hydrolysis, the LMC-displayed *B. subtilis* cells collected from 133.3 mL of the *B. subtilis* cell culture with A_{600} = 3.0 were mixed with 0.4 mg of the three-cellulase mixture in 10 mL of the ice-cooled Buffer A containing 0.4% Avicel followed by hydrolysis at 37^{o}C. The LysM-displayed *B. subtilis*

cells collected from 133.3 mL of the cell culture with A_{600} = 3.0 were mixed with tri-functional mini-cellulosome containing 0.4 mg (total) three-cellulases in 10 mL of the ice-cooled Buffer A containing 0.4% Avicel followed by hydrolysis at 37°C. The cellulose hydrolysis by the same amount of the LMC-displayed *B. subtilis* cells or LysM-displayed *B. subtilis* cells without heterologously-added cellulase was performed as a negative control. The same cellulase (mass) concentrations of mini-cellulosome and the three-cellulase mixture were also used to hydrolyze RAC and Avicel in 10 mL Buffer A in the presence of 60 units of β-glucosidase (Bgl, Novozymes 188 from Sigma) per gram of cellulose, respectively. A commercial Novozymes cellulase and a two-enzyme *Trichoderma* fungal cocktail containing EG II and CBH I at the same mass concentration as the bacterial three-cellulase mixture were used to hydrolyze RAC and Avicel at 37°C in 50 mM citrate buffer (pH 5.0) containing 50 mM NaCl and 10 mM $CaCl_2$ in the presence of 60 units of β-glucosidase per gram of cellulose, respectively. In the two-enzyme cocktail, the mass amount of EG II was the same as the sum of BsCel5' and CtCel9 and the mass amount of CBH I was the same as CpCel48. The cellulose hydrolysis by the same amount of β-glucosidase was performed as a negative control. One mL of the reaction sample was withdrawn at indicated time intervals. The concentration of soluble sugars in the supernatant was measured by using the phenol–sulfuric acid method with glucose as the standard; while the residual cellulose was determined by quantitative saccharification with glucose as the standard (42). All hydrolysis experiments were performed in triplicate.

Other assays. Protein mass concentration was measured by the Bio-Rad Bradford protein dye reagent method as a reference of bovine serum albumin. Their protein

masses based on the Bradford method were calibrated by their UV absorbance at 280 nm in 6 M guanidine hydrochloride (38). The purity of protein samples was examined by SDS-PAGE followed by Coomassie Blue staining. The activity of individual cellulase was measured as described elsewhere (19).

RESULTS

Functional display of mini-CipA on the *B. subtilis* cell surface

Mini-CipA, a fragment of *C. thermocellum* CipA containing three cohesins and one CBM3b (10, 13), was expressed in *B. subtilis* using the *B. subtilis* - *E. coli* shuttle vector pNWP43N-LMC. This vector had an expression cassette containing a NprB signal peptide-encoding sequence, a *B. subtilis* cell wall-binding module (LysM) from a *Bacillus subtilis* cell wall hydrolase LytE (4, 36), a mini-CipA, and a C-terminal Flag-tag, called LMC, under the control of a strong constitutive P43 promoter. Because cell wall hydrolase LytE is located at cell separation sites and poles of *B. subtilis* through its cell wall-binding module (LysM) (4, 36), LMC can be displayed on the cell wall of *B. subtilis*. Controls included plasmid pNWP43N-LysM that expressed a surface displayed LysM with a C-terminal Flag tag and plasmid pNWP43N that did not produce any related surface displayed protein.

After cell cultivation, *B. subtilis* cells harboring pNWP43N-LMC and pNWP43N-LysM produced cell surface-bound LMC and LysM, respectively. Through LiCl elution, the cell wall protein solutions containing cell surface-displayed LMC and LysM were examined in SDS-PAGE (Fig. 2A, Lane 1 and Lane 2), respectively. By the addition of

RAC that binds with high specificity to CBM3b-containing LMC, the LMC (Fig. 2A, Lane 4) was easily separated from other cell wall proteins. The apparent molecular masses for LMC (~ 105,000) and LysM (~30,000) determined by SDS-PAGE were a little higher than their calculated values (96,711 and 23,215) based on their deduced amino acid sequences, perhaps due to the serine-rich linker sequence in LysM (20). The LMC concentration was estimated to be 1.2 mg/L of the cell culture (A_{600} = 3.0) based on the band intensity in the SDS-PAGE, as described elsewhere (4). Approximately 20,000 molecules of LMC were estimated to be displayed on the surface of each *B. subtilis* cell.

Expression and purification of cellulases and mini-CipA in *E. coli*

Cellulases used for the assembly of tri-functional mini-cellulosome were (i) a non-cellulosomal *B. subtilis* family 5 endoglucanase (BsCel5), (ii) a cellulosomal *C. thermocellum* family 9 processive endoglucanase (CtCel9), and (iii) a non-cellulosomal *C. phytofermentans ISDg* family 48 cellobiohydrolase (CpCel48) (Fig. 1). BsCel5 contains a catalytic module, a dockerin module from *C. thermocellum*, and a C-terminal His tag; CtCel9 contains a catalytic module, CBM3c, a dockerin module, and a C-terminal His tag; and CpCel48 contains a catalytic module, CBM3b, a dockerin module, and a C-terminal His tag. Mini-CipA, a truncated mini-scaffoldin from CipA of *C. thermocellum*, contains three cohesins and one CBM3b. Mini-CipA and three cellulase components expressed in *E. coli* BL21 were purified to a homogeneous protein (Fig. 2B).

The cellulases used in this study were the same as those in our previous work except CpCel9 (19). Since the activities of CBM-free BsCel5 and CBM-containing CpCel48

were higher than those of CBM-containing BsCel5 and CBM-free CpCel48, respectively (data not shown), CBM-free BsCel5 and CBM-containing CpCel48 were used. In addition, it was found that CBM-containing CpCel48 was expressed at a much higher level than its CBM-free counterpart in *E. coli* and *B. subtilis* (data not shown). In this study, family 9 cellulase (CtCel9F) from *C. thermocellum* was used instead of CpCel9 due to: (i) CtCel9F was expressed at higher levels than CpCel9, (ii) these two enzymes exhibited comparable activities at the temperatures tested, and (iii) CtCel9F contained its own dockerin. Two other dockerin modules from two dockerin-containing cellulases of *C. thermocellum* were added to the C-terminal of BsCel5 and CpCel48, respectively, because these three cellulases used in this study will be co-expressed in developing consolidated bioprocessing *B. subtilis* strains (19).

Ex vivo assembly of mini-cellulosomes on the _B. subtilis_ cell surface

The LMC-displayed *B. subtilis* cells were mixed with excess Cel5' or a three-enzyme cellulase mixture containing equimolar Cel5', Cel48, and Cel9. After LiCl elution followed by RAC specific adsorption, LMC + Cel5 exhibited only two bands responsible for LMC and Cel5 at an approximately molar ratio of 1:3, examined by SDS-PAGE (Fig. 3, Lane 2), indicating that one LMC can bind with about three Cel5 molecules. When the cells were mixed with the three-cellulase mixture, LMC bound with the three cellulase components nearly equally (Fig. 3, Lane 3), indicating that each dockerin-containing cellulase component was non-selectively bound with three cohesins of LMC. A negative control LysM-displayed *B. subtilis* cells did not bind any dockerin-containing cellulase (data not shown).

The *ex vivo* assembly of mini-cellulosome on *B. subtilis* cell surface was also examined by confocal immunofluorescence microscopy. When the primary anti-Flag antibody aiming at the C-terminal Flag tag in LMC- or LysM-displayed cells was used, the green fluorescence signals were observed on the surface of the cells displaying LMC and LysM but not on a negative control (*B. subtilis* WB600/pNWP43N) (Fig. 4A). These results indicated that LMC and LysM were displayed on the *B. subtilis* cell surface. LMC- and LysM-displayed *B. subtilis* cells were mixed with excess CtCel9, followed by the primary anti-His antibody that can bind with the His-tag of CtCel9. LMC-displayed *B. subtilis* cells with CtCel9 exhibited a strong green fluorescence signal (Fig. 4B), suggesting the *ex vivo* formation of a LMC-CtCel9 complex. By contrast, LysM-displayed *B. subtilis* cells, a negative control, did not present a detectable fluorescence signal (Fig. 4B). It was noted that the fluorescence signal for LMC-CtCel9 in Fig. 4B was much stronger than that in Fig. 4A because three anti-His antibodies can bind with three CtCel9 linked by one LMC while one anti-Flag antibody can bind with one LMC.

Comparative hydrolysis experiments

Cellulose hydrolysis activity in the presence of the same mass concentrations of cellulase were compared for: living cell-bound mini-cellulosome, cell-free mini-cellulosome, and a (bacterial) three-cellulase mixture with a BsCel5': CtCel9: CpCel48 molar ratio of 1:1:1 on two model cellulosic materials, RAC and Avicel. Since mini-cellulosome can tightly bind on cellulose, the cellulose-enzyme-microbe (CEM) complex was formed. Cell-bound mini-cellulosome hydrolyzed RAC more rapidly than cell-free mini-cellulosome

and the three-enzyme mixture (Fig. 5A). At 72 h, a digestibility of 28.4% was achieved by the mini-cellulosome, about 1.57-fold higher than that of the three-cellulase mixture. This phenomenon was attributed to the enzyme proximity effect (25, 32, 41). More notable, the cell-bound mini-cellulosome hydrolyzed RAC 2.25-fold higher in digestibility than the mini-cellulosome (Fig.5A). The similar hydrolysis trend in an increasing order of the cellulase mixture, cellulosome, and cell-bound mini-cellulosome was observed on Avicel (Fig. 5B). The cell-bound mini-cellulosome exhibited 4.54-fold higher in Avicel digestibility than did the mini-cellulosome (Fig. 5B). The comparison of the CEM synergy (Fig. 6) indicated that the cellulosome-microbe complex expedited cellulose hydrolysis rate more significantly on recalcitrant Avicel than on RAC.

To understand the causes that the CEM complex hydrolyzed cellulose more rapidly than the mini-cellulosome, two control experiments were conducted: (1) non-active mini-cellulosome-displayed *B. subtilis* cells by the addition of 1 g/L NaN_3 inhibiting cells' sugar uptake ability; and (2) the mini-cellulosome plus active LysM-displayed *B. subtilis* cells, which were able to assimilate all soluble sugars in the bulk phase. The non-active cells associated with cell-bound cellulosome did not hydrolyze cellulose as rapidly as active cellulosome-bound cells (Fig. 5) possibly due to accumulated sugars in the supernatant, which inhibited mini-cellulosome activity. The mini-cellulosome plus active LysM-displayed *B. subtilis* cells where no significant soluble sugars were accumulated in the supernatant (data not shown) exhibited less hydrolysis ability than the active cellulosome-bound cells (Fig. 5).

The hydrolysis performance of bacterial cellulase systems was compared to those of a commercial fungal cellulase mixture and a two enzyme cocktail made of purified *Trichoderma* CBH I and EG II at the same protein mass concentration. The two-fungal-enzyme cocktail hydrolyzed cellulosic materials more efficiently than the three-bacterial-cellulase cocktail and the tri-functional mini-cellulosome at 72 h, although each bacterial cellulase components exhibited much higher specific activities at short reaction timeframes (e.g., 10 min to 1 h) (data not shown). The commercial fungal mixture worked better than the two fungal-cellulase mixture, possibly due to its optimized enzyme ratio. Although the non-complexed three-bacterial-cellulase mixture or bacterial mini-cellulosome exhibited less ability in hydrolyzing solid cellulosic materials than the commercial fungal cellulase, the cell-bound cellulosome showed equal hydrolytic ability on RAC and approximately 30% higher hydrolytic ability on Avicel (Fig. 6).

DISCUSSION

We assembled the *ex vivo* tri-functional mini-cellulosome on the surface of the Gram-positive *B. subtilis* strain through the high-affinity interaction between the dockerin modules of cellulase components and the three cohesin modules of mini-CipA. This enabled the comparison of cellulose hydrolysis rate by the cellulose-enzyme-microbe (CEM) complex relative to the non-complexed cellulase mixture or cellulosome (Fig. 5). The CEM synergy was not primarily due to removal of hydrolysis products from the bulk fermentation broth, as suggested by control experiments (Fig. 5). For enzymatic hydrolysis occurring on the surface of solid cellulosic substrate, the concentration of hydrolysis products in the boundary layer was thought to be much higher than that in

366 bulk phase according to the boundary layer theory (11). Such high concentration
367 hydrolysis products, especially for long chain cellodextrins, in the boundary layer was
368 expected to inhibit cellulase activity more strongly than glucose and cellobiose in the
369 bulk phase because beta-glucosidase that does not have a CBM usually works in the bulk
370 phase. Because the distance between the cell and mini-cellulosome through a LMC (i.e.,
371 20-50 nm) is much shorter than the thickness of boundary layer on solid substrate
372 cellulose for cellulolytic microorganisms (e.g., 10-100 μm) (33), the adjacent cells
373 located in the boundary layer can assimilate long chain hydrolysis products before their
374 diffusion to the bulk phase so to effectively eliminate product inhibition to cellulases and
375 cellulosomes (41). This explanation was partially supported by the observance of some
376 polycellulosomal protuberance between cellulose and *C. thermocellum* cell under
377 transmission electron microscope (27) and the fast assimilation of long chain
378 cellodextrins by adjacent cellulolytic cells rather than further hydrolysis to cellobiose and
379 glucose by cellulases in the bulk phase (41, 42).
380
381 The CEM synergy was more significant on recalcitrant Avicel than on highly-reactive
382 amorphous cellulose (Fig. 6). This difference may be explained by stronger boundary-
383 layer product inhibition on crystalline cellulose than amorphous cellulose. Because
384 cellobiohydrolase is more sensitive to product inhibition than endoglucanase (i.e., $K_{I,CBH}$
385 << $K_{I,EG}$) and endoglucanase exhibits more hydrolysis ability on amorphous cellulose
386 than on Avicel (19), the aggregated cellulosome exhibited less product inhibition on
387 amorphous cellulose than on recalcitrant Avicel (17). Displaying cellulosome on the

surface of a microorganism would be effective to enhance the cellulolytic host's ability to effectively hydrolyze recalcitrant cellulosic fragment of pretreated heterologous biomass.

Both *B. subtilis* and *S. cerevisiae* are important industrial microorganisms. As a potential CBP host, *B. subtilis* could be better than *S. cerevisiae* due to (i) a natural ability to take up long-chain cellodextrins, (ii) a natural ability to co-utilize C5 and C6 sugars, (iii) an inherent ability to secrete a large amount of proteins, and (iv) a small size cell (0.7 × 2 μm) *vs.* a large size yeast (2.5 ~ 10 × 4.5 ~ 21 μm) (i.e., a better mass transfer for a smaller cell). The first two features have been introduced into recombinant yeasts (12, 28). In spite of intensive efforts, real recombinant cellulose-utilizing yeasts that can produce ample cellulase and hydrolyze cellulose to support cell growth and cellulase synthesis without the help of other soluble organic nutrient are not yet available (18). By contrast, a recombinant cellulose-utilizing *B. subtilis* has been created to produce lactate from cellulose without the addition of exogenous cellulase and any water-soluble organic nutrients (37). Since anaerobic cellulolytic microorganisms must produce more secretory cellulase than do aerobic counterparts based on weight ratio of cellulase to cellular protein for supporting their growth on cellulose (23), cellulase synthesis always represents a significant bioenergetic burden for anaerobic microorganisms (42). The bacterium *C. thermocellum*, for example, produces ~10-20% (wt/wt) cellulase relative to cellular proteins for fast cellulose hydrolysis with nearly all of cellulosome displayed on its cell surface (40). It appears that cellulolytic, anaerobic bacteria evolved cell-bound cellulosomes so to increase specific cellulase activity and to decrease their bioenergetic burden (42). However, this cellulase evolution mechanism is speculated not to occur to

411 fungi and yeasts because a) ATP supply is much more plentiful and b) relatively large
412 cellulolytic fungi and yeasts may not have enough cell surface to display 10%-20% wt.
413 cellulase relative to cellular protein due to low ratios of surface/volume, where
414 surface/volume is inversely proportion to a radius of a cell. Therefore, in nature
415 cellulolytic fungi is hypothesized to evolve to secrete a large amount of cellulases.
416
417 For high-yield biofuels production from cellulosic material, it is vital to increase
418 carbohydrate allocation of desired biofuels by decreasing carbohydrate allocation to
419 synthesis of cellulase and cell mass under anaerobic conditions (14, 22). This study
420 showed that displaying cellulosome on the surface of a microbe can enhance microbial
421 cellulose hydrolysis rate by several-fold without increasing cellulase synthesis burden.
422 Since fungal cellulases exhibited higher hydrolysis ability in a long time range (19), the
423 co-expression of dockerin-containing fungal cellulases by recombinant cellulolytic *B.*
424 *subtilis* strains may be another worthy direction. Another potential direction would be *in*
425 *vitro* assembly of dockerin-containing fungal cellulases produced by *Trichoderma* spp.
426 and a recombinant yeast or bacterium that can produce a cell-surface displayed scaffoldin.
427

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434

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558 **FIGURE LEGENDS**

559 **Fig. 1**. Schematic representation of the recombinant proteins used in this study.

560

561 **Fig. 2**. SDS-PAGE of cell wall proteins eluted from the cell-surface of *B. subtilis* strains
562 (A) and the purified recombinant cellulases and mini-CipA produced by *E. coli* (B). A:
563 Lane 1, the LiCl-eluted supernatant from the *B. subtilis* (pNWP43N-LMC) cells; Lane 2,
564 the LiCl-eluted supernatant from the *B. subtilis* (pNWP43N-LysM) cells; Lane 3, the
565 LiCl-eluted supernatant from the *B. subtilis* (pNWP43N) cells; Lane 4, adsorbed LMC
566 eluted supernatant from the *B. subtilis* (pNWP43N-LMC) cells by using RAC. B: Lane 1,
567 mini-CipA; Lane 2, CpCel48; Lane 3, CtCel9; and Lane 4, BsCel5'.

568

569 **Fig. 3**. SDS-PAGE of RAC affinity pull-down for cell-wall proteins eluted from *B.*
570 *subtilis* strains. Lane 1, cell-bound LMC from the *B. subtilis* (pNWP43N-LMC) cells;
571 Lane 2, cell-bound unifunctional mini-cellulosome from the *B. subtilis* (pNWP43N-LMC)
572 cells premixed with BsCel5'; and Lane 3, cell-bound tri-functional mini-cellulosome
573 from the *B. subtilis* (pNWP43N-LMC) cells premixed with BsCel5', CtCel9 and
574 CpCel48.

575

576 **Fig. 4**. Confocal fluorescence microscopy images of LMC, a negative control (blank
577 plasmid), and LysM on the surface of *B. subtilis* cells (A) as well as the cell-bound mini-
578 cellulosome on the surface of *B. subtilis* cells relative to a negative control (B). The cells
579 displaying Flag-tag LMC and LysM were probed with the anti-Flag antibody followed by
580 the rabbit anti-mouse IgG conjugated with FITC (A). The mini-cellulosome containing

581 LMC + CtCel9 was probed with the anti-His6 antibody followed by the rabbit anti-mouse
582 IgG conjugated with FITC, where CtCel9 contains a His6 tag (B).
583

584 **Fig. 5.** Hydrolysis of RAC (A) and Avicel (B) by the enzyme mixtures supplemented
585 with excess β-glucosidase: the bacterial cellulase mixture (▲), the mini-cellulosome (■),
586 the Novozymes fungal cellulase mixture (⬥), the two-enzyme *Trichoderma* fungal
587 mixture (EG II and CBH I) (△), as well as the cell-bound mini-cellulosome (●), the mini-
588 cellulosome in the presence of LysM-displayed *B. subtilis* cells (□), and the cell-bound
589 mini-cellulosome in the presence of 1 g/L NaN_3 (○).
590

591 **Fig. 6**. Comparison of cellulose digestibility by the bacterial cellulase mixture, the cell-
592 free mini-cellulosome, the cell-bound mini-cellulosome, the commercial fungal cellulase
593 mixture, and the two-fungal-enzyme cocktail on RAC (A) and Avicel (B) at hour 72.
594 The error bars represent the standard deviation of triplicate samples.

Table 1. Strains and plasmids in this study.

Stains or plasmids	Characteristics	References
E. coli		
JM109	*rec*A1, *sup*E44 *end*A1 *hsd*R17 (r^-k,m^+k) *gyr*A96 *rel*A1 *thi* (*lac-proAB*) F'[*tra*D36 *pro*AB$^+$ *lacI*^{q}*lacZ* ΔM15]	
BL21 Star (DE3)	F$^-$*omp*T *hsd*SB (rB$^-$mB$^-$) *gal dcm rne131* (DE3)	Invitrogen, Carlsbad, CA
B. subtilis		
WB600	*nprE aprA epr bpf mpr nprB*	(35)
Plasmids		
pNWP43N	Cm^R, pNW33N derivate	(38)
pNWP43N-LysM	Cm^R, with LysM expression cassette cloned	This work
pNWP43N-LysM-mini-cipA	Cm^R, with LysM-mini-cipA expression cassette cloned	This work
pET20b	Amp^R, overexpression vector containing T7-dependent promoter	Novagen, Madison, WI
pET20b-mini-cipA	Amp^R, with mini-cipA expression cassette cloned	This work
pET20b-Bscel5'	Amp^R, with Bscel5' expression cassette cloned	This work
pET20b-Ctcel9	Amp^R, with Ctcel9 expression cassette cloned	This work
pET20b-Cpcel48	Amp^R, with Cpcel48 expression cassette cloned	This work

1 **Table 2**. The primers used to amplify gene fragments.

Gene	Template	Primer name	Sequence	RE*
LysM	Genomic DNA of *B.subtilis* 168 ATCC 23857	LysM_For	GAGCAG**CTCGAG**GCACAAA GCATTAAGGTGAAAAAAGG	XhoI
		LysM_Rev_Flag	GCTGCT**GCTAGC**TTATTATT TGTCATCGTCATCTTTATAA TCGACTAACGCTTTTGCATC AGAAACCAGCTTG	NheI
LysM-mini-CipA	Genomic DNA of *B. subtilis* 168 (ATCC 23857)	LysM_For	GAGCAG**CTCGAG**GCACAAA GCATTAAGGTGAAAAAAGG	XhoI
		LysM_Rev	GCTGCT**GATATC**GACTAAC GCTTTTGCATCAGAAACC	EcoRV
	Genomic DNA of *C. thermocellum* (ATCC 27405)	MC_For	GTAAGTA**GATATC**GTATCG GCGGCCACAATGACAGTCG	EcoRV
		MC_Rev_Flag	GCAGTA**GCTAGC**TTATTATT TGTCATCGTCATCTTTATAA TCATTCGAATCATCTGTCGG TGTTGTTACAGG	NheI
pNWP43N	pNWP43N-BsCel5	pNWP43N_For	GCCGAC**GCTAGC**TTAAGCT TTTTTTTGGCGGACATCAGT AAC	NheI
		PNWP43N_Rev	GACTAT**CTCGAG**ACCTGCA GCTGAGGCATGTGTTACAAA AAC	XhoI
Mini-CipA	Genome of *Clostridium thermocellum*	Mini-CipA_For	GTAGTA**CATATG**GTATCGG CGGCCACAATGACAG	NdeI
		Mini-CipA_Rev	GCAGTA**CTCGAG**ATTCGAA TCATCTGTCGGTGTTG	XhoI
BsCel5’	Genome of *Bacillus subtilis*	BsCel5_For	CCTCAG**CATATG**GCAGGGA CAAAAACGCC	NdeI
		BsCel5’_Rev	CTCCGGTTCTTCTGGGTCTA CTCCTCCAGAAATACCATTT TCCTGTGTGGGTTTATC	
	Genome of *Clostridium thermocellum*	DocK_For	GGAGGAGTAGACCCAGAAG AACCGGAGGTTATTTATG	
		DocK_Rev	GCCGCC**CTCGAG**TTTATGTG GCAATACATCTATC	XhoI
CtCel9	Genome of *Clostridium thermocellum*	CtCelF_For	GCTTCA**CATATG**GCGGATTT CAACTATGGTGAGGCAC	NdeI
		CtCelF_Rev	GGACCAT **CTCGAG** CTGTTC AGCCGGGAATTTTTCAATAA	XhoI

			G	
CpCel48	pP43N-Cpcel48	Cpcel48_For	CCTCTG**CATATG**GGTGAAA CTGAGCAAGC	NdeI
		Cpcel48_Rev	GTAGAGGACCCACCTCCTCC AGATCCTGGTTCGATACCCC AATTAAGTTTTCC	
	Genome of *Clostridium thermocellum*	DocS_For	GGATCTGGAGGAGGTGGGT CCTCTACTAAATTATACGGC GACGTC	
		DocS_Rev	GCATTA**CTCGAG**GTTCTTGT ACGGCAATGTATC	XhoI

*RE, restriction enzyme site. Restriction enzyme sites included in primer sequences for cloning purposes are indicated in bold, and the Flag tag sequences are indicated by underlined text.

Plasmids	Gene products Notation M.W.	Modular structure
pNWP43N-lysM-mini-cipA	LysM-Mini-CipA (LMC) (96,711 Da)	
pNWP43N-lysM	LysM (23,215 Da)	
pET20b-mini-cipA	Mini-CipA(MC) (75,790 Da)	
pET20b-Bscel5'	BsCel5' (44,918 Da)	
pET20b-Ctcel9	CtCel9 (80,108 Da)	
pET20b-Cpcel48	CpCel48 (107,184 Da)