Assembly of Minicellulosomes on the Surface of *Bacillus subtilis*

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ABSTRACT
To cost-efficiently produce biofuels new methods are needed to convert lignocellulosic biomass into fermentable sugars. One promising approach is to degrade biomass using cellulosomes, which are surface displayed multi-cellulase containing complexes present in cellulolytic Clostridium and Ruminococcus species. In this study we created cellulolytic strains of B. subtilis that display one or more cellulase enzymes. Proteins containing the appropriate cell wall sorting signal are covalently anchored to the peptidoglycan by co-expressing them with the B. anthracis sortase A (SrtA) transpeptidase. This approach was used to covalently attach the Cel8A endoglucanase from C. thermocellum to the cell wall. In addition, a Cel8A-dockerin fusion protein was anchored on the surface of B. subtilis via non-covalent interactions with a cell wall attached cohesin module. We also demonstrate that it is possible to assemble multi-enzyme complexes on the cell surface. A three enzyme containing minicellulosome was displayed on the cell surface that consists of a cell wall attached scaffoldin protein non-covalently bound to three cellulase-dockerin fusion proteins that were produced in E. coli. B. subtilis has a robust genetic system and is currently used in a wide range of industrial processes. Thus, grafting larger, more elaborate minicellulosomes onto the surface of B. subtilis may yield cellulolytic bacteria with increased potency that can be used to degrade biomass.
INTRODUCTION

Dwindling supplies of petroleum have intensified the search for improved methods to produce ethanol from biomass (28). A limiting step in this process is the degradation of lignocellulose into its component sugars (24, 40, 50). Lignocellulose is the main component of biomass and consists of cellulose and hemicellulose carbohydrate fibers that are coated with lignin (21). Although a variety of cellulolysis processes have been demonstrated, commonly used methods first pre-treat lignocellulosic materials with chemicals and/or heat (23, 30, 31, 73). The cellulose is then hydrolyzed into simple sugars by exposing it to a variety of purified cellulases (44, 69, 71). An alternative approach that may improve the efficiency of enzymatic degradation is to employ bacterial cellulosomes: multi-cellulase containing complexes that exhibit extremely potent cellulolytic activity (16). Thus, ongoing research has concentrated on understanding the molecular basis of their cellulolytic activity and sought to engineer cellulosomes for industrial purposes (12, 16, 39).

Anaerobic meso- and thermophilic-bacteria produce cellulosomes that have a common overall architecture in which a central scaffoldin protein coordinates the binding of different cellulolytic enzymes (16). The cellulosome from Clostridium thermocellum is archetypal (2). Its scaffoldin, CipA, has binding sites for nine enzymes (56, 65). Binding is mediated by type I cohesin modules within CipA that interact with sub-nanomolar affinity with type I dockerin modules that are fused to the cellulolytic enzymes (46, 57). CipA also contains a carbohydrate-binding module (CBM) that tethers the cellulosome complex to its substrate, as well as a type II dockerin module located at its C-terminus that anchors the complex to the cell wall by interacting with either the SdbA, Orf2 or OlpB proteins (16). A variety of cellulases with distinct activities are incorporated into the cellulosome: endoglucanases, exoglucanases, xylanases, and pectinases among others. Enzyme co-localization within the cellulosome
enables cultures of *C. thermocellum* displaying these complexes to decompose cellulose at significantly faster rates than purified enzyme solutions (38). The specific enzyme composition within the cellulosome is presumably varied to degrade different types of plant matter as the *C. thermocellum* genome encodes more than sixty dockerin containing enzymes (11, 74). Several other species of anaerobic bacteria also degrade cellulose using cellulosomes that contain the same basic architecture constructed from cohesin-dockerin interactions.

To exploit their potent cellulolytic activity, several research groups have created minicellulosome complexes in which a cohesin containing miniscaffoldin coordinates the binding of cellulase-dockerin fusion proteins (1, 7, 14, 15). Because the cohesin-dockerin interaction is species specific, cohesins from different bacterial species are typically used to construct the miniscaffoldin (20, 49). Ordered and unique multi-protein complexes can then be formed by adding chimeric fusion proteins in which the cellulase enzyme is fused to the appropriate dockerin module. The enzymatic properties of a number of purified designer minicellulosomes have been characterized *in vitro* and the cellulolytic activity of different combinations of endoglucanases, exoglucanases, and β-glucosidases have been tested (8, 14, 15, 45). Even the geometry of the miniscaffoldin protein, altering a linear scaffoldin for one that is circular or rectangular in architecture, has been manipulated to determine the effect of enzyme positioning on cellulolytic activity (45). Combined, this work has produced complexes with more potent and synergistic activity against crystalline cellulose as compared to the isolated enzymes, but the complexes were still less active than naturally occurring cellulolytic cells (14, 15, 43, 45).

Recently, three research groups have created *Saccharomyces cerevisiae* strains that display designer minicellulosomes. These strains are a step towards the construction of a
consolidated bioprocessing microorganism that could produce high levels of ethanol directly from biomass (36, 66, 67). To display the minicellulosome on the surface, each group covalently linked it to the \( \beta \)-1,6-glucan within the cell wall using a glycosyl phosphatidylinositol (GPI) signal motif. Volshenk and colleagues displayed a miniscaffoldin protein containing two cohesin modules by fusing it to the GPI signal motif from the Cwp2 protein (36). The minicellulosome was then successfully assembled by incubating the yeast with distinct cellulase-dockerin fusion proteins. A slightly different approach was used by the Chen and Zhao groups (66, 67). In this work, the Aga1 protein was first covalently anchored to the cell wall via its GPI anchor. Miniscaffoldin proteins containing the Aga2 protein that interacts with Aga1 were then tethered to the cell surface via non-covalent interactions. After incubating the yeast with purified cellulase-dockerin fusion proteins, the Chen group successfully assembled minicellulosomes on the cell surface producing a yeast strain that could produce ethanol from cellulose. The Zhao group also created cellulolytic yeast, but in this case the minicellulosome was constructed by co-expressing the mini-scaffoldin and cellulase-dockerin fusion proteins. However, improved methods to efficiently degrade cellulose are needed as the cellulolytic activity of these engineered yeast strains is significantly lower than the activity of cellulosome displaying bacteria (65). Cellulases have also been displayed in *Escherichia coli* and *B. subtilis* by creating cellulase fusion proteins that are associated with the membrane (17, 29, 32). However, in *B. subtilis* the cellulases were not surface exposed in the intact microbe and required the generation of protoplasts to enable them to degrade cellulose.

Recently it was discovered that cellulolytic *Ruminococcus flavefaciens* uses a transpeptidase enzyme, sortase, to covalently anchor a cellulosome to its cell wall (53). Sortases are widely distributed in Gram-positive bacteria and catalyze a transpeptidation reaction that joins proteins bearing a highly conserved Leu-Pro-X-Thr-Gly (LPXTG, where X
is any amino acid) sorting signal to the cross-bridge peptide of lipid II, a cell wall precursor that is subsequently incorporated into the peptidoglycan. In this study we demonstrate that it is possible to use sortase enzymes to attach a minicellulosome to the surface of *B. subtilis*, a rod shaped Gram-positive bacterium used in a wide-range of industrial processes including the production of antibiotics, vaccines, and pharmaceutically relevant proteins (13, 22, 59, 61, 68). Although the native organism shows minimal cellulolytic activity, *B. subtilis* strains displaying cellulases and a multi-enzyme minicellulosome degrade HCl-treated amorphous cellulose. When assayed using carboxymethyl cellulose, bacteria displaying *C. thermocellum* Cel8A exhibit cellulolytic activity that is as good, or superior to, previously described purified and yeast displayed minicellulosomes that contain several enzymes (8, 66). Thus, *B. subtilis* strains displaying more complicated multi-enzyme minicellulosomes may be even more cellulolytic and useful in degrading biomass.

**MATERIALS AND METHODS**

**Strains and plasmids.** *B. subtilis* JH642 (51) and BAL2238 served as parent strains to produce the strains listed in Table 1. BAL2238 was created by transforming JH642 with the ∆wprA::hyg allele from WB800 (70). The full-length srtA gene from *Bacillus anthracis* str. Ames was cloned downstream from a xylose inducible promoter and integrated into the *B. subtilis* chromosome at the amyE locus using standard methods (4). This work made use of the *E. coli*-*B. subtilis* shuttle plasmid pRDC18 (a gift from F. Arigoni, Institut de Biologie Physico-Chimique, France) to create plasmid pSrtA. The forward primer used to amplify the gene also contains the ribosome binding site from the *B. subtilis* PhrC protein (Table 1S). Standard methods were utilized to create competent JH642 and BAL2238 cells (4), which
were transformed with XhoI linearized pSrtA and plated on LB agar containing 5 µg/ml chloramphenicol. Allelic replacement of \textit{amyE} with P\textsubscript{xybA}\textsuperscript{-srtA} and \textit{chloramphenicol acetyltransferase} (\textit{cat}) was confirmed by PCR amplification of chromosomal DNA and sensitivity to 10 µg/ml spectinomycin. Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible genes encoding proteins that can be anchored to the cell wall by SrtA were inserted into the \textit{thrC} locus using standard methods and \textit{E. coli-}\textit{B. subtilis} shuttle plasmid pBL112 (34). The nucleotide sequences of the primers used to generate plasmids used in this study are provided as supplemental material (Table 1S). Table 1 lists the specific strains that were generated, including the gene names and accession codes, as well as protein amino acid numbers. \textit{E. coli} strain XL2Blue [\textit{recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacZΔM15 Tn10 (Tet') Amy Cam']} was used as the host for all genetic manipulations outside of \textit{B. subtilis}. Expression plasmids producing Cel9G-Doct (pETGf, \textit{C. cellulolyticum} endoglucanase Cel9G fused to the type I dockerin from \textit{R. flavefaciens}) and Cel9E-Doct (pETEc, \textit{C. cellulolyticum} exoglucanase Cel9E fused to its native dockerin) have been described previously (15). A pET28a-based plasmid producing Cel8A-Doct (residues 32-434 of Cel8A from \textit{C. thermocellum} fused at its C-terminus to residues 540-790 of the dockerin from the \textit{C. thermocellum} Xyn10B protein) was created for this study using standard subcloning methods and primers listed in Table 1S. For cloning, \textit{E. coli} and \textit{B. subtilis} cultures were grown in Luria-Bertani (LB) medium supplemented with the appropriate antibiotic (100 µg/ml ampicillin, 1 µg/ml erythromycin, 5 µg/ml chloramphenicol, 50 µg/mL kanamycin or 100 µg/ml hygromycin B).

\textbf{Immunofluorescence microscopy}. Strains TDA02 (Cel8A expressing) and TDA03 (Cel8A and SrtA expressing) were used. A 5 ml culture of each strain was grown overnight in LB
media supplemented with the 1 µg/ml erythromycin. One-half milliliter of the culture was then used to inoculate 50 ml of fresh LB medium (1/100 dilution). The 50 ml cultures were then shaken at 37°C until they reached an OD$_{600}$ of 0.2. Cel8A expression was then induced by adding IPTG to a final concentration of 1 mM. The culture containing strain TDA03 was also induced to express SrtA by adding xylose to the culture when its OD$_{600}$ reached 0.1 (final xylose concentration of 0.5%). When all cell cultures reached an OD$_{600}$ of 2.0, they were centrifuged at 3000 x g for 5 min and then re-suspended in 1 ml of Phosphate Buffered Saline (PBS, 8 g/liter NaCl, 0.2 g/liter KCl, 1.44 g/liter Na$_2$HPO$_4$, 0.24 g/liter KH$_2$PO$_4$, pH 7.4). The cells were then centrifuged and the pellet re-suspended in 800 µl of PBS and 200 µl of Fix buffer (12% formaldehyde, 150 mM NaH$_2$PO$_4$). This solution was incubated at room temperature for 15 min, and then placed on ice for 1 hr. After centrifugation at 3000 x g for 5 min, the pellet was re-suspended in 1 ml of PBS. This washing step was repeated for a total of 3 times. The final pellet obtained from this process was then re-suspended in a volume of GTE buffer (25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM glucose) such that the OD$_{600}$ was ~1.0. Twenty microliters of suspended cells was then aspirated onto a poly-lysine coated microscope slide and dried. The slides were then blocked by adding a 2% solution of Bovine Serum Albumin (BSA) protein dissolved in PBS buffer. After incubating for 15 min, the slides were washed with PBS. Cel8A display was probed using an anti-His$_6$ immunoglobulin G antibody (1.25 µg/µl, Abgent, San Deigo, CA). After incubating for 1 hr, the slides were washed with PBS and incubated for 1 hr with goat anti-mouse immunoglobulin G conjugated with Dylight 488 (0.2 ng/µl, Fisher Scientific). After washing the slides again with PBS, a 10 µl solution containing 70% glycerol and 5 µg/ml 4′,6-diamidino-2-phenylindole (DAPI) was added prior to imaging. Data was collected on an Applied Precision Delta Vision.
Deconvolution microscope (457 nm and 528 nm excitation was used for Dylight 488 and DAPI, respectively).

Whole cell cellulase assays. For each B. subtilis strain a 5 ml LB culture containing 1 µg/ml erythromycin was grown overnight. A total of 0.5 ml from each overnight culture was then added to a 50 ml LB solution containing 1 µg/ml erythromycin. Cells were then shaken at 37°C until they reached an \( \text{OD}_{600} \) of 0.1. If SrtA expression was desired then xylose was added to a final concentration of 0.5%. All cultures were then grown to an \( \text{OD}_{600} \) of 0.2, at which point IPTG was added to a final concentration of 1 mM to induce the expression of the surface displayed protein. Three milliliter samples of each culture were taken periodically, to measure their \( \text{OD}_{600} \) and enzymatic activity. To measure enzymatic activity, the 3 ml sample was centrifuged for 5 min at 3000 x g and the pellet was re-suspended in assay buffer (20 mM Tris-HCl, pH 6.0). The cells were then centrifuged again, and the pellet was re-suspended in 1 ml carboxymethyl cellulose (0.5% CMC, medium viscosity (Sigma), 20 mM Tris-HCl, pH 6.0). Each cell suspension was then incubated at 37°C for 1 hr, and centrifuged at 20,000 x g for 1 min. Activity was determined by adding 3 ml of dinitrosalicylic acid (DNSA) to the supernatant (the DNSA solution contained: 1% DNSA, 1% NaOH, 0.2% phenol, and 0.05% Na\(_2\)SO\(_3\)). Samples were then boiled for 10 min and the absorbance was recorded at 575 nm. The amount of sugar released was quantified using a glucose standard curve. All whole cell enzymatic assays were performed in triplicate. To control for different growth rates, the enzymatic activity values obtained for each 3 ml culture was normalized by dividing this data by the \( \text{OD}_{600} \) value determined for each culture prior to centrifugation.

When measuring cellulase activity of minicellulosomes assembled on the surface of B. subtilis, cells displaying Cel8A-Doct, Cel9E-Doctc, and/or Cel9G-Doct were incubated in 1 ml
of 0.5% HCl-treated amorphous cellulose. The suspensions were then incubated at 37°C for 1 hr. After incubation, the suspensions were centrifuged at 5,000 x g for 5 min and the amount of reducing sugar released was determined as described above. HCl-treated amorphous cellulose was prepared as described by Hsu and Penner, except that Whatman No.1 filter paper was substituted for Avicel PH101 (25).

**Immunoblot analysis of cell fractions.** Samples used to monitor protein expression were created in an identical manner as samples used to monitor whole cell cellulase activity (described above). In this work, the 50 ml cultures were grown for 3 hrs after the addition of IPTG and then centrifuged for 5 min at 3,000 x g. The cell pellet was then re-suspended in 1 mL STM buffer (25% sucrose, 50 mM Tris-HCl pH 8.0, 5 mM MgCl₂) and re-centrifuged. The cell pellets were then re-suspended in a volume of STM, such that each had an OD₆₀₀ value of 1 (typically 1 ml of STM was used). The STM solution also contained lysozyme enzyme at a final concentration of 500 µg/ml. The re-suspension was incubated at 37°C for 30 min, and then centrifuged for 10 min at 20,000 x g. The supernatant contains solubilized cell wall proteins and was subjected to immunoblot analysis. The pellet contains protoplasts, whose proteins were released by re-suspending the pellet in 0.1N NaOH such that the solution had an OD₆₀₀ of ~1. The protoplast solution was then centrifuged for 10 min at 20,000 x g. The membrane and cytoplasmic proteins were collected in the supernatant after centrifugation. To precipitate proteins that had been secreted into the growth medium, trichloroacetic acid (TCA) was added to the LB supernatant obtained by centrifuging the 50 ml cell culture (final concentration of 10% w/v TCA). The solution was then centrifuged and the pellet was re-dissolved in water for immunoblot analysis. The solutions containing the cell wall, protoplast
and secreted protein fractions were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane using standard procedures. The membrane was then blocked by soaking it for 1.5 hrs in Tris Buffered Saline Plus Tween (TBST, 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% Tween 20) supplemented with 5% BSA. The membrane was then incubated with anti-His$_6$ immunoglobulin G (0.25 µg/µl) for 1 hr, washed with TBST for 30 min and incubated with a horseradish peroxidase (HRP) conjugated rabbit anti-mouse immunoglobulin G secondary antibody (1:50,000 dilution for 1 hr., Sigma, cat. No. A9044). The blot was then washed and incubated with Pierce ECL Western Blotting substrate (0.125 ml/cm$^2$) for 1 min and visualized by exposing to an autoradiography film (Fisher Scientific). A similar immunoblot analysis was performed using strain TDA08 that expressed the Cel8A-GST protein (Table 1, Fig. 2C) to track the fate of the processed and unprocessed protein using an anti-His$_6$ immunoglobulin G primary antibody. The details used to perform this procedure have been described previously (5).

**Protein purification and complex assembly on the cell surface.** The Cel8A, Cel8A-Doct, Cel9E-Docc, and Cel9G-Docf proteins were expressed in E. coli and purified to homogeneity. Methods used to produce Cel9E-Docc and Cel9G-Docf have been described previously (15). The histidine tagged Cel8A and Cel8A-Doct were produced from a 1 L culture of LB and purified as specified by Novagen and using a Co-NTA resin (HisPur cobalt resin, Fisher Scientific). The mobility of the purified Cel8A-Doct, Cel9E-Docc and Cel9G-Docf proteins on an SDS-PAGE gel is compatible with their predicted molecular weights (81,061 Da, 95,012 Da, and 78,604 Da, respectively). Purified enzymes were dissolved in binding buffer (25 mM Tris-HCl, pH 7.0, 200 mM NaCl, 5 mM CaCl$_2$). For *in vitro* cellulase assays, purified Cel8A
was dissolved in assay buffer (20 mM Tris-HCl, pH 6.0). Minicellulosomes were constructed on the surface of 

*B. subtilis* by incubating purified enzymes with cells containing either Coh or Scaf attached to their cell wall. Procedures used to display these proteins are identical to those described above. A 50 ml culture of cells displaying either Coh or Scaf were grown for varying amounts of time in the presence of 1 mM IPTG. A 3 ml sample of each culture was then centrifuged and re-suspended in binding buffer. The suspension was then centrifuged for a second time and re-suspended in 25 µl of binding buffer containing 100 µM purified cellulase enzyme (Cel8A-Doct, Cel9E-Docc, and/or Cel9G-Docf). After incubating on ice for 1 hr, the suspensions were centrifuged to remove unbound protein. The cells were then re-suspended in binding buffer and centrifuged at 3,000 x *g* for 5 min. This washing step was repeated 3 times.

**RESULTS**

Construction of a sortase mediated protein display system in *B. subtilis*. Previously, it has been shown that the Gram-positive anaerobe *R. flavefaciens* anchors a cellulosome to its cell wall using a sortase enzyme (53). We wondered whether a similar mechanism could be used to attach an artificial minicellulosome to the surface of *B. subtilis*. Towards this goal we developed a system in which to anchor proteins to the peptidoglycan. The display system works by co-expressing the *B. anthracis* sortase A enzyme (SrtA) with a protein that it covalently anchors to the cell wall (Fig. 1A) (18). This enzyme was chosen because its second substrate, lipid II, is conserved between *B. subtilis* and *B. anthracis*, suggesting that SrtA could function properly in both organisms (42). Homologous recombination was used to introduce the *srtA* gene into the *amyE* locus under the control of a xylose inducible P*xyLA*
promoter. The protein to be anchored by SrtA was introduced through similar methods into the thrC locus and is expressed from an IPTG inducible P_spachy promoter. Appended to the beginning of the protein substrate is the N-terminal secretory peptide derived from the B. subtilis PhrC protein and a hexahistidine (His₆) tag. The protein also contains at its C-terminus a portion of the Staphylococcus aureus Fibronectin Binding Protein B, which consists of a 123 amino acids spacer segment and a cell wall sorting signal (CWS) (Fig. 1B). This S. aureus CWS sequence is identical to those found in many B. anthracis surface proteins that are anchored to the cell wall by SrtA (18).

Initially, the Cel8A endoglucanase from C. thermocellum (Fig. 1B) was displayed on the surface of B. subtilis. Cel8A was used because its in vitro activity has been well characterized and because it has previously been displayed on the surface of yeast (60, 66) Homologous recombination was used to construct strain TDA03, which expresses srtA and Cel8A under the inducible control of xylose and IPTG, respectively. Following protein induction, the cells were grown to an OD₆₀₀ of 2.0 and protein display was visualized using immunofluorescence microscopy (Fig. 2A). Antibody staining of the N-terminal hexahistidine tag within the Cel8A protein reveals that it is located on the bacterial surface (right panel, Fig. 2A). Cells in control experiments in which SrtA expression was not induced showed significantly smaller amounts of displayed enzyme (center panel, Fig. 2A). Moreover, minimal display is observed for strain TDA02, which lacks the srtA gene (Fig. 2A, left panel). This indicates that sortase is required to display Cel8A and that the expressed Cel8A protein does not associate with the cell surface non-specifically in the absence of sortase.

To substantiate that Cel8A is linked to the cell wall we fractionated TDA03 cells and performed immunoblot experiments to determine its location. As shown in Fig. 2B, a significant amount of Cel8A is localized to the cell wall when both SrtA and Cel8A are
expressed. In particular, when whole cells are washed and treated with lysozyme, significant amounts of protein are released from the cell wall (lane 7). However, no detectable Cel8A is found in the cytoplasm or membrane fractions (lane 8). Furthermore, only a small amount of Cel8A is observed when whole cells are lysed, presumably because most of the protein is attached to the cell wall and thus cannot be separated by SDS-PAGE (lane 6). Interestingly, the display system is not 100% efficient, as a significant amount of Cel8A is secreted into the supernatant (lane 9). Additional control experiments were performed in which SrtA expression was not induced. Unexpectedly, this work yielded generally similar results and showed that Cel8A is targeted to the cell wall in the absence of SrtA, albeit at reduced levels (lane 3). This suggests that some of the Cel8A protein produced in the absence of SrtA non-specifically binds to the cell wall. However, this protein is not functional (see below) and it is not exposed to the growth medium based on our inability to detect it by immunofluorescence microscopy (Fig. 2A).

To more thoroughly investigate the sortase anchoring reaction, we performed additional experiments that measured the abundance of a Cel8A fusion protein in which glutathione S-transferase (GST) is positioned downstream from the CWS (Fig. 2C) (5). Based on its molecular weight, the addition of GST allows the mature form of the protein to be distinguished from precursors of the protein in which either the signal peptide or CWS have been cleaved. These precursors correspond to forms of the protein that have not been processed by SrtA and include the intact protein (P1) and protein in which only the N-terminal signal peptide has been cleaved by the signal peptidase (P2). As shown in the top panel of Fig. 2D, when SrtA is not expressed, unprocessed Cel8A accumulates in the membrane and cytoplasmic fractions of B. subtilis and little mature Cel8A is located in the cell wall (middle panel). In contrast, when SrtA is expressed, precursor forms of Cel8A are diminished and
mature Cel8A is found in the cell wall. Importantly, mature Cel8A appears to be covalently anchored to the cell wall, since the cell wall fraction was washed extensively with SDS prior to treatment with mutanolysin to cleave the glycan. It should be noted that data presented in Figs. 2B and 2D are not contradictory, since the cell wall preparations analyzed in Fig. 2D were subjected to SDS treatment to stringently remove non-covalently bound protein, whereas the cell wall fractions analyzed in Fig. 2B were not.

Functional endoglucanase is displayed on the surface of *B. subtilis*. Strain TDA03 displaying Cel8A was tested for its ability to degrade carboxymethyl cellulose (CMC). Samples from cultures expressing SrtA and Cel8A were collected periodically, washed, and their endo-glucanolytic activity determined using a standard dinitrosalicylic acid assay (66). When both SrtA and Cel8A are induced, a cell pellet derived from a 3 ml culture of cells produces 70 mg/L of reducing sugar when the pellet is re-suspended in a 1 ml solution of 0.5% CMC and incubated for 1 hour (Fig. 3A, closed diamonds). The cellulolytic activity is due to cell wall attached Cel8A as cultures in which SrtA is not induced with xylose do not efficiently degrade CMC (Fig. 3A, closed diamonds). This indicates that the cellulolytic activity of strain TDA03 is dependent on the presence of SrtA. Moreover, it suggests that residual Cel8A protein retained in the cell wall when SrtA is not induced (Fig. 2B, lane 3) is not competent to degrade CMC, presumably because it is not sufficiently exposed or improperly folded. Finally, the cellulolytic activity of TDA03 is not caused by an endogenous *B. subtilis* cellulase, as strain TDA01 lacking *Cel8A* does not degrade CMC (data not shown).

Inspection of Fig. 3A reveals that maximal cellulolytic activity is achieved ~3 hours after induction of Cel8A expression. The activity then decreases substantially, with only 20% remaining after 6 hours. A growth analysis reveals that cells exponentially growing have
maximal enzymatic activity and that the activity decreases as they transition into the
stationary phase (Fig. 3C). As B. subtilis expresses several extracellular proteases, we
wondered whether Cel8A was being proteolyzed (67). To investigate this issue, whole cell
activity experiments were repeated, but tetracycline was added to the cultures three hours
post-induction to stop the production of proteases. This treatment preserved cell wall
associated Cel8A activity (data not shown). The B. subtilis cell wall associated WprA protease
could be degrading Cel8A because it is expressed throughout growth and it is well known that
it degrades heterologously expressed proteins (34, 40, 61, 69). We therefore introduced the
cell wall attachment system into a wprA- background (B. subtilis str. BAL2238) to create strain
TDA05. These cells show increased enzyme activity and stability relative to TDA03 cells (Fig.
3B, closed boxes). In particular, the maximal activity obtained is 30-fold greater than that of
TDA03 and only modestly decreases after 70 hours, even throughout stationary growth (Fig.
3C). We were unable to determine if cells displaying Cel8A can grow on HCl-treated
amorphous cellulose because the current system requires the addition of xylose to induce
protein expression and xylose can be used by B. subtilis as a carbon source. Combined,
these data indicate that Cel8A is attached to the cell wall by SrtA and that the deletion of the
WprA protease dramatically increases cell wall attached enzyme activity.

Assembly of a functional surface displayed cohesin-cellulase complex. We next
determined the feasibility of assembling a cohesin-cellulase complex on the surface of B.
subtilis. A protein containing a cohesin module (Coh) was anchored to the cell wall, and its
ability to tether a fusion protein containing the Cel8A enzyme and a type I dockerin (Cel8A-
Doct) was investigated. The Coh protein corresponds to the second cohesin module from the
C. thermocellum scaffoldin protein CipA and contains the appropriate N- and C-terminal
sequence elements for SrtA mediated anchoring to the cell wall (Fig. 1B). Cel8A-Doct contains the aforementioned Cel8A enzyme with the type I dockerin from the *C. thermocellum* Xyn10B xylanase fused to its C-terminus. The Xyn10B derived polypeptide also contains a family-22 carbohydrate-binding module (CBM) (Fig. 1C). This fragment of the Xyn10B polypeptide was chosen because it has previously been shown to bind with high affinity in vitro to the CipA cohesin module (52). The CBM of Xyn10B may not be optimal for cellulose binding as it is a family-22 CBM whose members typically bind to xylan. It should also be noted that native Cel8A encodes a dockerin module that can be bound by the cohesin of CipA (52). The *wprA* strain TDA06 expressing SrtA and Coh was grown for varying lengths of time and the cells were then harvested by centrifugation. The cells were then re-suspended in a binding buffer containing 100 μM purified Cel8A-Doct protein. After washing, the ability of the cells to degrade CMC (after one hour of incubation) was determined. As shown in Fig. 4A, supplying purified Cel8A-Doct to cells that are displaying Coh on the surface yields functional cellulolytic complexes. The cellulolytic activity is correlated with the stationary phase of growth, as it is maximal when Cel8A-Doct is supplied to TDA06 cells that have been expressing Coh and SrtA for more than ten hours. A maximum of 1,200 mg/L reducing sugar is released after five hours of Coh and SrtA expression, and remains stable for at least an additional ten hours. Importantly, cultures in which SrtA expression is not induced with xylose show minimal activity after incubation with purified Cel8A-Doct (TDA06 (-SrtA/+Coh)). A similar result is also obtained for the isogenic control strain BAL2238 that does not contain the *srtA* and *coh* genes. Immunoblot analysis was used to further substantiate that the Coh:Cel8A-Doct complex had assembled on the cell surface. TDA06 cultures were harvested and exposed to Cel8A-Doct as previously described. After washing, the cells were treated with lysozyme to release the cell wall attached Coh:Cel8A-Doct complex. Since both Cel8A-
Doct and Coh contain an N-terminal His$_6$ tag their presence in the complex was detected using an anti-His$_6$ antibody. As shown in Fig 4C, when SrtA and Coh are expressed, the cell walls contain both Cel8A-Doct and Coh (lane 2). However, when SrtA is not expressed by the cells, only Coh is detected in the cell wall (lane 1). This species migrates at a higher molecular weight than the SrtA-dependent band, and presumably corresponds to non-specifically bound protein in which the C-terminal CWS has not been cleaved. The lack of enzymatic activity in cells not expressing SrtA suggests that this non-specifically bound form of Coh is incapable of productively interacting with Cel8A-Doct. Thus, functional Cel8A-Doct can only be tethered to the cell surface via non-covalent interactions with Coh that is covalently attached to the cell wall by SrtA.

To avoid having to add purified enzymes to Coh displaying cells, we investigated whether the Coh:Cel8A-Doct complex could be assembled on the cell surface by co-expressing its components. Strain TDA07 was generated in which the Coh and Cel8A-Doct-(sec) proteins are co-expressed as a single transcript under the control of IPTG. Cel8A-Doct-(sec) and Cel8A-Doct are identical, except Cel8A-Doct-(sec) contains an N-terminal signal sequence that enables it to be secreted from the cell. Cultures of TDA07 expressing SrtA, Coh and Cel8A-Doct-(sec) possess ~2-fold more cellulolytic activity than cells in which the Coh:Cel8A-Doct complex was produced by adding purified Cel8A-Doct (compare Figs. 4A and B, closed boxes). As much as 2,300 mg/L of reducing sugar is released using strain TDA07 within five hours of protein induction and the activity remains stable for at least fifteen hours. Assembly of the Coh:Cel8A-Doct-(sec) complex depends on sortase, as cells are unable to degrade CMC when only the Coh and Cel8A-Doct-(sec) proteins are expressed (Fig. 4B, open boxes). An immunoblot of the cell wall fraction of TDA07 further substantiates that the complex is assembled in a sortase-dependent manner (Fig. 4C, lanes 3 and 4). Interestingly, compared
to complexes created by the addition of purified CelA-Doct, co-expressing the components increases the amount of cell wall associated CelA-Doct-(sec), which may explain why these cells exhibit greater cellulolytic activity.

Display of a functional minicellulosome. We next investigated whether it was possible to display a functional minicellulosome on the surface of B. subtilis that contained three different cellulase enzymes. The minicellulosome possesses a scaffoldin (Scaf) that contains three cohesin modules that have distinct binding specificities: (1) the cohesin from the C. thermocellum CipA protein (Coht), (2) the cohesin from C. cellulolyticum CipC1 (Cohc), and (3) the cohesin from R. flavefaciens ScaB (Cohf) (Fig. 1B). It also contains the family-3 CBM from C. thermocellum CipA which binds cellulose, as well as a C-terminal CWS that enables it to be anchored to the cell wall by SrtA. Scaf was used because it had previously been shown to successfully assemble a minicellulosome both in vitro and on the surface of yeast (15, 66).

Cells were induced to co-express Scaf and SrtA, and then grown for varying amounts of time. At various times during growth a sample of cells was collected, centrifuged, and the cell pellets were re-suspended in solutions that contained different purified cellulase proteins. The C-termini of each cellulase protein is fused to a distinct dockerin module. Three purified cellulase-dockerin fusions were added which should each bind to a distinct cohesin module within Scaf (Fig. 1C). These include: (1) the aforementioned Cel8A-Doct protein that binds to the Coht module, (2) Cel9E-Docc, that contains the C. cellulolyticum exoglucanase Cel9E enzyme and its native dockerin that binds to the Cohc module, and (3) Cel9G-Docf, that contains the Cel9G endoglucanase from C. cellulolyticum fused to a dockerin module from the R. flavefaciens ScaA protein which binds to the Cohf module. In separate experiments,
cells displaying Scaf were incubated with each of the cellulase-dockerin proteins and subjected to immunoblot analysis that confirmed enzyme binding to Scaf (Fig. 5A, lanes 5-7). In addition, an immunoblot of cells incubated with all three fusion proteins is compatible with the enzymes interacting with Scaf on the cell surface to form a minicellulosome (lane 8). As expected, association of each fusion protein with the cell wall is dependent upon the presence of SrtA anchored Scaf (lanes 1-4).

Cells displaying a minicellulosome, as well as single enzymes, were tested for their ability to degrade HCl-treated amorphous cellulose (Fig. 5B). The methods used to determine cell associated enzymatic activity were identical to those used to study the surface associated Cel8A-cohesin complex (Fig. 4). Separate incubation of Scaf displaying cells with each cellulase-dockerin fusion protein yields similar overall activity (~100-200 mg/L sugar produced). However, when all three enzymes are incubated with Scaf displaying cells, a ~4 fold increase in activity is observed (~800 mg/L sugar produced). Interestingly, the enzymes appear to be working synergistically, as the activity of cells containing a minicellulosome with all three enzymes is greater than the sum of the enzymatic activities of cells harboring only a single enzyme (~1.3 fold more active). Importantly, the activity differences are due to the amount of displayed enzyme on each cell as the cell density of each sample tested is identical. Taken together, these data indicate that a minicellulosome containing three enzymes can assemble on the surface of *B. subtilis* and that these cells are more cellulolytic than cells that display only a single enzyme. Future experiments will characterize in greater detail whether the enzymes in this complex function synergistically.
Cellulosic biomass is the most abundant source of carbon in the biosphere and it could function as an inexpensive feedstock to produce biofuels if improved methods were developed to degrade it into metabolically accessible sugars (6, 9, 19, 40, 55). Recently, studies of the Gram-positive anaerobe *R. flavefaciens* revealed that it uses a sortase transpeptidase enzyme to attach a cellulolytic cellulosome complex to its cell wall (53). This finding has suggested that *B. subtilis*, an industrially useful microbe that has an established genetic system, could be engineered to degrade biomass by using a sortase enzyme to display minicellulosomes on its surface. Towards this goal, we initially constructed *B. subtilis* cells that display the Cel8A cellulase from *C. thermocellum*. The Cel8A enzyme was covalently anchored to the peptidoglycan by co-expressing it with the *B. anthracis* Sortase A transpeptidase (SrtA). SrtA mediates the display of Cel8A on the surface of *B. subtilis* as evidenced by immunofluorescence microscopy, immunoblot analyses and the ability of the cells to degrade CMC. Both the stability and enzymatic activity of surface displayed Cel8A was improved when the cell wall WprA protease was genetically deleted. The improvement was substantial, with nearly 50% more protein anchored to each cell as compared to cells containing a full-complement of proteases (47). We estimate that ~300,000 Cel8A proteins may be displayed per cell. This estimate was made by measuring the enzymatic activity of cultures in which the colony forming units had been experimentally determined. It also assumed that the cell wall attached proteins had similar enzymatic activity as the purified enzyme whose specific activity was determined experimentally (data not shown). Strains in which additional proteases are deleted will likely exhibit better protein display properties and will be explored in the future.
Although SrtA anchored large amounts of protein to the cell wall, the process appears inefficient as ~70% of the expressed Cel8A protein was secreted into the medium. Similar inefficiency was observed by Schumann and colleagues, who used the sortase enzyme from *Listeria monocytogenes* to anchor the α-amylase enzyme to the cell wall of *B. subtilis* (47). In marked contrast, *B. anthracis* SrtA is highly efficient in its native organism, anchoring nearly all of its protein substrates to the cell wall and very little protein is secreted (18). The inefficiency of the *B. anthracis* enzyme in *B. subtilis* could be caused by the over expression of the protein substrate relative to the sortase enzyme. This was supported by an immunoblot analysis which revealed that even in the presence of SrtA, unprocessed Cel8A precursors were present. It is also possible that the protein substrates were missing features not yet identified that are required for enzyme activity. The genome of *B. subtilis* contains two putative sortase encoding genes whose functions have not been characterized (47). As functional Cel8A protein was not displayed when SrtA was absent, these endogenous enzymes were presumably unable to anchor Cel8A to the cell wall. The reason for this is not known, but it could be because the endogenous sortases were not expressed during the growth conditions used in our experiments and/or the enzymes were unable to recognize the cell wall sorting signal present in the Cel8A substrate.

Cellulose derived from biomass is significantly more complex and heterogeneous than HCl-treated amorphous cellulose, suggesting that in order to efficiently degrade it using *B. subtilis*, multiple enzymes will need to be displayed on its surface (e.g. endoglucanases, exoglucanases, β-glucosidases, xylanases, and pectinases). Towards this objective we investigated whether it was possible to assemble a cohesin:cellulase complex on the surface of *B. subtilis*. The cohesin:cellulase complex (Coh:Cell8A-Doct) was formed by covalently attaching a cohesin module to the cell wall, which in turn coordinated the non-covalent
binding of a Cel8A-dockerin fusion protein (Cel8A-Doct). The complex could be assembled by either co-expressing the components or by adding purified exogenous Cel8A-Doct to cells displaying Coh. Interestingly, co-expression yielded cells that have ~2-fold more enzymatic activity. The reason for this is unknown, but could occur if the Cel8A-Doct proteins produced in *E. coli* were less active, or if complex assembly was initiated as the proteins were secreted.

Importantly, we also demonstrated that a similar strategy could be used to assemble a three enzyme minicellulosome in which *B. subtilis* cells containing a sortase attached scaffoldin coordinated the binding of three distinct enzyme-dockerin fusion proteins that had been produced in *E. coli*. Bacterial cells displaying minicellulosomes exhibited increased activity against HCl-treated amorphous cellulose, suggesting that more elaborate complexes can be engineered to degrade different types of more complex biomass.

Several studies have shown that *B. subtilis* can be used as a host to secrete heterologous cellulases, and naturally occurring strains have been identified that secrete cellulases (24, 26, 53, 62). Cellulase enzymes have also been targeted to the membrane, enabling protoplasts of *B. subtilis* to degrade CMC (29). However, to the best of our knowledge, this was the first example of a cellulolytic *B. subtilis* strain in which cellulases and cellulase containing complexes were attached to the peptidoglycan. Interestingly, *B. subtilis* cells that displayed anchored Cel8A protein degraded CMC as well as, or better than, two previously described minicellulosomes that contained similar endoglucanases. Direct comparisons are problematic as minicellulosomes produced in different labs can have distinct enzyme components, different substrates can be used, and the experimental conditions to measure activity can differ. However, two previous studies used CMC to measure the activity of a minicellulosome and thus serve as a useful benchmark. Doi and colleagues measured the CMC activity of a purified minicellulosome that contained two copies of the EngB protein bound to a scaffoldin
containing two cohesin modules (8). As compared to this system, engineered *B. subtilis*
displaying only the Cel8A-cohesin complex was ~4-fold more effective at degrading CMC
after an incubation time of 30 minutes. *B. subtilis* displaying a single enzyme was also slightly
more active (~30%) and more effective at degrading CMC than a previously reported
engineered yeast strain (66). Although the cell densities used in this study were not reported,
it is tempting to speculate that the elevated levels of cellulase activity in *B. subtilis* were due
to a greater number of complexes being anchored to its cell wall; in yeast it is estimated that
only 10,000-100,000 molecules can be displayed via the Aga1-Aga2 interaction used to
anchor the minicellulosome (10).

In conclusion, we have created cellulolytic *B. subtilis* that contained a minicellulosome
covalently attached to the cell wall by a heterologous sortase enzyme. Future work may yield
industrially useful strains that display minicellulosomes with multiple enzymes that
synergistically degrade different types of biomass. The cellulolytic activity of *B. subtilis* was
also quite stable, which is in marked contrast to non-covalently attached cellulosomes in *C.
thermocellum* that detach from the cell as it enters stationary phase (3, 53). The thermophilic
Cel8A enzyme used in this study is optimally active at 75°C and is significantly less active at
the temperatures used in this study (60). In the future, the display of orthologous mesophilic
enzymes on the surface of *B. subtilis* may therefore yield significant improvements in
cellulolytic activity. Finally, the robust genetic system of *B. subtilis* may make it feasible to use
it as a consolidated bioprocessor in which both cellulolytic and biofuel producing metabolic
pathways are genetically introduced into a single microorganism.
ACKNOWLEDGMENTS

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REFERENCES


57. Schaeffer, F., M. Matuschek, G. Guglielmi, I. Miras, P. M. Alzari, and P. Beguin. 2002. Duplicated dockerin subdomains of Clostridium thermocellum endoglucanase CelID bind to a cohesin domain of the scaffolding protein CipA.
with distinct thermodynamic parameters and a negative cooperativity.

Biochemistry 41:2106-14.


Figure 1. Schematic showing proteins used in this study. (A) A generalized substrate of the SrtA sortase that contain an N-terminal secretory peptide (SP) and C-terminal cell wall sorting signal (CWS) with the LPXTG sorting motif. (B) Specific surface proteins that were anchored to the cell wall by SrtA. Each protein contains the SP derived from B. subtilis PhrC, followed by a hexahistidine (His$_6$) or human influenza hemagglutinin (HA) tag. At their C-termini, each protein contains the CWS from the S. aureus Fibronectin Binding Protein B (Fib). Cel8A is the endoglucanase from C. thermocellum and contains the family 8 glycoside hydrolyase (GH) module. Coh is the type I cohesin from the C. thermocellum CipA protein. Scaf contains several domains and has been described previously (15). It contains: the
type I cohesin from \textit{C. cellulolyticum} CipC (Cohc), a Carbohydrate Binding Module (CBM), a type I cohesin from \textit{C. thermocellum} CipA (Cohf), and a type I cohesin from \textit{R. flavefaciens} ScaB (Cohf). (C) Schematic of the dockerin containing cellulase enzymes that were displayed on the surface of \textit{B. subtilis}. Proteins purified from \textit{E. coli} include: Cel9E-Docc, the \textit{C. cellulolyticum} exoglucanase Cel9E fused to its native dockerin and contains a family 9 GH, an Immunoglobulin like module (IG) and CBM (15); Cel9G-Doct, \textit{C. cellulolyticum} endoglucanase Cel9G, that contains a family 9 GH and CBM, fused to a type I dockerin from \textit{R. flavefaciens} ScaA (15); and Cel8A-Doct, the Cel8A endoglucanase from \textit{C. thermocellum} fused to the CBM and dockerin modules derived from the \textit{C. thermocellum} xylanase Xyn10B protein. CelA-Doct-(sec) was used to assemble the cohesin-cellulase complex through co-expression.
Figure 2. Cel8A is successfully displayed on the surface of *B. subtilis*. (A) Immunofluorescence micrographs of *B. subtilis* strain TDA03 displaying His$_6$-tagged Cel8A. Left panel: Cells of strain TDA02 expressing Cel8A. Middle panel: Cells of strain TDA03 expressing only Cel8A. Right panel: Cells of strain TDA03 expressing both SrtA and Cel8A. Cells were probed for the presence of Cel8A on
the surface with mouse anti-His\textsubscript{6} serum and fluorescently stained anti-mouse IgG conjugated to Dylight-488. 4',6-diamidino-2-phenylindole (DAPI) was used to stain the DNA. In images containing larger numbers of cells, a similar display pattern was observed. (B) Immunoblot analysis of the cellular localization of Cel8A in strain TDA03. Lane: 1: purified Cel8A. 2,6: lysed whole cells. 3,7: lysozyme solubilized cell wall. 4,8: membrane/cytosol. 5,9: precipitated secreted protein. Samples were probed with a mouse anti-His\textsubscript{6} antibody. Lanes 2-5 represent samples in which SrtA was not expressed. Lanes 6-9 represent samples in which SrtA was expressed. (C) Diagram showing the Cel8A-GST protein used to track processing by SrtA. The expected forms of the protein include: P1, the unprocessed full length precursor; P2, the precursor protein after cleavage by the signal peptidase; and M, the mature protein after cleavage of the CWS by SrtA. (D). Immunoblots of cell fractions of strain TDA08 expressing Cel8A-GST and/or SrtA. Top Panel: SDS-released cytoplasmic fractions in cells in which SrtA expression has not been induced (SrtA U, left column) or has been induced (SrtA I, right column). Middle Panel: Blot of cell wall extracts that had been digested with mutanolysin. Bottom Panel: detection of SrtA expression in the SDS-treated cytoplasmic and membrane fractions using an anti-FLAG antibody.
Figure 3. Eliminating the WprA protease increases the cellulolytic activity of B. subtilis cells displaying the Cel8A cellulase. (A) Cellulase activity of TDA03 cells during growth. Growth cultures of cells displaying Cel8A (+SrtA/+Cel8A) or not displaying CelA (-SrtA/+Cel8A) were periodically collected, washed and their ability to degrade carboxymethyl cellulose determined by measuring the amount of reducing sugars that were released. (B) Identical to panel A, except that strain TDA05 was used in which
the WprA cell wall associated protease has been genetically deleted. (C)

Corresponding growth curves of TDA03 and TDA05 as a function of time. Activity profiles were performed in triplicate. The reported error is the standard deviation of these measurements.
Figure 4. Assembly of a cohesin:cellulase complex on the surface of *B. subtilis* by either adding purified cellulase or by co-expressing each component. (A) Display of the cohesin:cellulase complex after adding purified cellulase enzyme. Cultures of TDA06 induced to display Coh were grown for varying amounts of...
time. Purified Cel8A-Doct was then added, and the ability of washed cells to
degrade CMC determined. (B) Display of the cohesin:cellulase complex by co-
expressing its components. Strain TDA07 was induced to express SrtA, Coh and
Cel8A-Doct-(sec) and periodically collected during the expression, and the ability
of the cells to degrade CMC determined. Experiments in panels A and B were
performed in triplicate and the error reported is the standard deviation. (C)
Immunoblot of cell wall fractions of strain TDA06 (lanes 1-2) exposed to purified
Cel8A-Doct and strain TDA07 (lanes 3-4) expressing Coh and Cel8A-Doct-(sec).
Lane 1: TDA06 -SrtA/+Coh/+Cel8A-Doct. Lane 2: TDA06 +SrtA/+Coh/+Cel8A-
Doct. Lane 3: TDA07 -SrtA/+Coh:Cel8A-Doct-(sec). Lane 4:TDA07
Samples were probed using a mouse anti-His6 antibody.
Figure 5. Assembly of a surface displayed minicellulosome that contains three enzymes. (A) Immunoblot analysis of the cell wall of cells of strain TDA09 expressing Scaf (lanes 1-4) only, or both SrtA and Scaf (lanes 5-8). Cells were incubated individually with Cel8A-Doct (lanes 1 and 5), Cel9E-Docc (lanes 2 and 6), Cel9G-Docf (lanes 3 and 7) or all three cellulases (lanes 4 and 8). The cell walls were then solubilized and the proteins probed with an anti-His$_6$ antibody. (B) Whole cell activity of cells displaying individual enzymes or a minicellulosome. Cultures of strain TDA09 expressing Scaf and/or SrtA were periodically collected and incubated with purified Cel8A-Doct, Cel9E-Docc, and/or Cel9G-Docf protein. After washing, activity against HCl-treated amorphous cellulose was determined. The curve labeled “Sum” is the sum of the enzymatic activities of cells incubated with only a single type of enzyme. Whole cell cellulase assays were performed in triplicate and the standard deviation of these measurements used to represent the error.
Table 1. *Bacillus subtilis* strains used in this study

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<th>Phenotype a</th>
<th>Reference/construction</th>
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<td>thrC::(P*staphy-sp-ha-scaf-fib cat)</td>
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a. Proteins expressed by the strains.
b. Full length sortase A transpeptidase from *B. anthracis* str. Ames (GenBank accession AE016879.1).
c. CelA surface protein consists of a secretory peptide derived from *B. subtilis* PhrC (sp, residues 1-35, GenBank accession ZP_03590039), a hexahistidine tag (His6), *C. thermocellum* ATCC 27405 (obtained from the ATCC endoglucanase A (family 8 endoglucanase, celA, residues 32-434, GenBank accession K03088), and the C terminal portion of *S. aureus* NCTC 8325 (fib, residues 756-917, GenBank accession CP000253).
d. Coh surface protein is identical to CelA, except that a cohesin domain from *C. thermocellum* ATCC 27405 (coh, residues 182-328, GenBank accession ABN54273) has replaced the CelA polypeptide.
e. CelA-Doct protein is identical to CelA, except that fib has been replaced with a CMB and dockerin module from *C. thermocellum* ATCC 27405 Xyn10B (residues 540-790, GenBank accession ABN52146).
f. CelA-GST surface protein is identical to CelA, except that GST from plasmid pGEX-4t (GE Life Sciences) has been appended to the C-terminus.
g. Scal surface protein is identical to CelA, except that the CelA polypeptide has been replaced by a three cohesin containing polypeptide (type I cohesins from *C. thermocellum* CipA, *C. cellulolyticum* CipC, and *R. flavefaciens* ScaB) and a family 3 CMB (15).
h. *erm*, erythromycin; *cat*, chloramphenicol acetyltransferase; *amyE*, these genes have been integrated into the *amyE* locus in the chromosome; *thrC*, these genes have been integrated into the *thrC* locus in the chromosome.