RESEARCH LETTER

Exchange of type II dockerin-containing subunits of the Clostridium thermocellum cellulosome as revealed by SNAP-tags

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Abstract

Clostridium thermocellum is a thermophilic anaerobic bacterium which efficiently hydrolyzes and metabolizes cellulose to ethanol through the action of its cellulosome, a multiprotein enzymatic complex. A fluorescent protein probe, consisting of a type II dockerin module fused to a SNAP-tag, was developed in order to gain insight into the quaternary configuration of the cellulosome and to investigate the effect of deleting cipA, the protein scaffold on which the cellulosome is built. Fluorescence microscopy suggested that the probe had localized to polycellulosomal protuberances on the cell surface. Surprisingly, fluorescence intensity did not substantially change in the cipA deletion mutants. Sequential labeling experiments suggested that this was a result of bound type II dockerins from CipA being replaced by unbound type II dockerins from the fluorophore-SNAP-XDocII probe. This mechanism of dockerin exchange could represent an efficient means for modifying cellulosome composition.

Introduction

Clostridium thermocellum is a thermophilic, gram-positive bacterium which is of interest for biofuel production due to its high rate of cellulose utilization (Lynd et al., 2002). This ability is due in part to its cellulosome, a multiprotein enzymatic complex tethered to the cell surface. The cellulosome consists of many repeated enzymatic subunits organized around a noncatalytic polypeptide, the primary scaffoldin, CipA. CipA has nine type I cohesin modules, one type II dockerin module, and a cellulose binding module that mediates attachment of the cellulosome to its substrate. The type I cohesins of CipA bind to type I dockerin modules on enzymatic subunits that possess diverse hydrolytic activities. The type II dockerin of CipA binds to a type II cohesin on secondary anchoring scaffoldins tethered to the cell surface by an S-layer protein which interacts noncovalently with the peptidoglycan layer of the bacterial cell wall. Anchoring scaffoldins SdbA, Orf2p and OlpB bind 1, 2, and 7 CipAs, respectively, allowing incorporation of up to 63 enzymatic subunits into a single complex that acts synergistically at the cell surface (Bayer et al., 2008).

The expression of both catalytic and structural components of the cellulosome change during growth on different substrates, indicating that C. thermocellum regulates its cellulosome composition in response to the available substrate and that the ability to exchange these subunits is important for efficient metabolism (Gold & Martin, 2007; Raman et al., 2009). A bicistronic system of carbohydrate-sensing antisigma and sigma factors has been shown to be able to regulate cellulase gene expression and respond to changes in substrate (Nataf et al., 2010). Polypeptide sequences of the cellulosome components contain typical surface signal peptides, suggesting that the components are secreted individually, and the cellulosome is assembled on the cell surface (Beguin & Aubert, 1994). The cellulosome subunits are invariably found in the complexed form, suggesting a strong interaction between cohesins and dockerins (Bayer et al., 1985). The interaction between cohesins and dockers is one of the strongest reported in nature with dissociation constants < 10^-9 M (Mechaly & Fierobe, 2001). During active growth, the cellulosome tightly adheres to the cell surface and also to the solid substrate forming a complex between cells, cellulosome, and cellulose. However, C. thermocellum is known to release cellulosomes throughout growth and en-masse in late-stationary phase (Demain et al., 2005; Raman et al., 2009). This turnover and release of cellulo-
somes during fermentation may be necessary to allow for the creation of new cellulosomes with modified composition. It has also been suggested that the controlled release of cellulosomes during growth may function as a mechanism to release C. thermocellum from its substrate, leaving deployed cellulosomes to continue hydrolyzing cellulose (Bayer & Lamed, 1986).

Although extensive work has been performed analyzing the composition of purified cellulosomes, the composition of the cellulosome in its native microbial context is not well understood. There is an increasing interest in building artificial cellulosomes, which is currently limited by a lack of understanding of structural elements in native cellulosomes (Krauss et al., 2012).

In order to increase understanding of the cellulosome in its native microbial context, we undertook work to develop a fluorescent probe for labeling type II cohesins based on the commercially available SNAP-tag labeling system (Kepper et al., 2003). The SNAP-tag system was developed by Kepper et al. as a method of covalently labeling fusion proteins in vivo. SNAP-tag is a mutant of the O\(^{-}\)-alkylguanine-DNA alkyl transferase human DNA repair protein which has increased activity against its substrate O\(^{-}\)-benzyguanine. The mutated protein binds covalently with benzylguanine-derived fluorophores. To create the probe, we fused a type II dockerin with the commercially available SNAP-tag. We then used this probe to visualize localization of type II cohesins modules in the cellulosome for both wild type and mutants of the cipA scaffolding protein (Supporting Information, Fig. S1).

**Methods**

**Strains and media**

Clostridium thermocellum DSM 1313 (WT) was grown in modified DSM 122 broth (Olson et al., 2010) with the addition of 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS) sodium salt and 3 g L\(^{-1}\) trisodium citrate (Na\(_3\)-C\(_6\)H\(_5\)O\(_7\)*2H\(_2\)O). All manipulations of C. thermocellum were carried out inside an anaerobic chamber (Coy Laboratory Products Inc.) with an atmosphere of 85% nitrogen, 10% carbon dioxide, 5% hydrogen, and < 5 parts per million oxygen. Clostridium thermocellum was grown at 55 °C with vigorous shaking. When the optical density (600 nm) reached a value of 0.6, the incubation temperature was reduced to 37 °C with vigorous shaking. The optical density (600 nm) reached a value of 0.6, the incubation temperature was reduced to 37 °C with vigorous shaking.

<table>
<thead>
<tr>
<th>Table 1. Strains</th>
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<tr>
<td>Strain</td>
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<tr>
<td>DSM1313</td>
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<tr>
<td>M1354</td>
</tr>
<tr>
<td>DS16</td>
</tr>
<tr>
<td>LL347</td>
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<tr>
<td>LL348</td>
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**Table 2. Plasmids**

<table>
<thead>
<tr>
<th>Plasmid name</th>
<th>Genbank accession number</th>
<th>Description</th>
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<tbody>
<tr>
<td>pDGO-03</td>
<td>JX489218</td>
<td>CipA deletion vector used for making strain DS11</td>
</tr>
<tr>
<td>pDGO-34</td>
<td>JX489219</td>
<td>CipA deletion vector used for making strain DS16</td>
</tr>
<tr>
<td>pAMG270</td>
<td>JX477172</td>
<td>Allelic replacement vector used for making strain LL347</td>
</tr>
<tr>
<td>pAMG269</td>
<td>JX477171</td>
<td>Allelic replacement vector used for making strain LL348</td>
</tr>
<tr>
<td>pDGO-54</td>
<td>JX500710</td>
<td>Escherichia coli SNAP-XDocII expression vector. The type II dockerin from Clostridium thermocellum (including the X-module required for solubility) were inserted downstream of and in-frame with the SNAP-tag protein from the pSNAP-tag (T7)-2 Vector from New England Biolabs</td>
</tr>
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**Expression and purification of the fusion protein**

A SNAP-XDocII fusion protein was created by cloning the XDocII region from the cipA gene into the pSNAP-tag\(^{®}\) (T7)-2 Vector in-frame with the SNAP-tag coding sequence using the In-Fusion cloning kit (Clontech) to create plasmid pDGO-54. This plasmid was transformed into T7 Express lysY/Iq competent Escherichia coli (New England BioLabs). A 0.1% inoculum was used, and cell cultures were incubated aerobically at 37 °C with vigorous shaking. When the optical density (600 nm) reached a value of 0.6, the incubation temperature was reduced to...
30 °C, and expression of the fusion protein was induced with 0.1 mM IPTG for 15 min. Cells were collected by centrifugation for 30 min at 6000 g, supernatant was discarded, and pellets were frozen overnight. Cell pellets were resuspended 10× in Lysis Buffer containing 25 mM Tris–HCl, pH 7.4, 250 mM NaCl, 8 M Urea. A final volume of 3 mL was sonicated for 5 min total process time (30 s on, 30 s off) using Misonix S-4000 (Misonix Inc.) with the amplitude set to 55%. Cell debris was removed by centrifugation for 30 min at 6000 g, and the supernatant containing the fusion protein was collected for further analysis.

Supernatant was dialyzed to Dockerin Reaction Buffer (25 mM Tris–HCl, pH 7.4, 50 mM NaCl, 1 mM CaCl2, 1 mM DTT, 0.1% Tween 20). The sample was centrifuged for 30 min at 6000 g to remove precipitates formed during dialysis, and pellet was discarded. Supernatant containing the SNAP-XDocII fusion protein in Dockerin Reaction Buffer was used in all subsequent labeling experiments.

Expression of the SNAP-XDocII fusion protein was optimized to include a short induction period of 15 min at 30 °C. Protocols for recovery of the SNAP-XDocII fusion protein were adapted from Adams et al. (2004). Under these conditions, the soluble SNAP-XDocII fusion protein was recovered at a final concentration of 2.5 mM. The SNAP-XDocII fusion protein exhibited covalent binding to the SNAP fluorophore, as determined by SDS-PAGE analysis. Optimized parameters for labeling the fusion protein with SNAP fluorophore resulted in complete labeling of the fusion protein, with unbound fluorophore remaining in solution at < 50% of the concentration of the fusion protein.

**Fluorescent labeling of the SNAP-XDocII fusion protein**

Fusion proteins for flow cytometry and microscopy were labeled with SNAP-Surface® Alexa Fluor® 647 and SNAP-Cell® 505 fluorescent dyes (New England BioLabs) by incubation of 2.5 mM fluorescent dye with fusion protein at 37 °C for 1 h. The resulting fluorescent proteins are referred to as 505-SNAP-XDocII and 647-SNAP-XDocII (Fig. S1). Before incubation with *C. thermocellum*, the labeling reaction was centrifuged to remove nonfluorescent precipitates that formed during 37 °C incubation. For fluorescent SDS-PAGE analysis, fusion protein was labeled with SNAP-Vista® Green according to the manufacturer’s instructions (New England BioLabs).

**Labeling *C. thermocellum* with fluorescent fusion protein**

Volumes of *C. thermocellum* culture, grown to an OD600 nm of 0.5 were harvested by centrifugation for 2 min at 15 000 g. Cell pellets were resuspended with an equal volume of 0.4 mM fusion protein in Dockerin Reaction Buffer and incubated for < 1 min at room temperature (25 °C) under aerobic conditions. Cells were washed three times with Dockerin Reaction Buffer. In negative control experiments, cells were labeled with mixtures containing purified SNAP-tag® protein (missing the XDocII fusion partner) or fluorescent dye (with no fusion protein) at 0.4 mM concentration under the same conditions. Varying the ratio of *C. thermocellum* cells to fluorescent fusion protein showed complete saturation at 0.83 pmol of fluorescent fusion protein per μL cells at an approximate 600 nm optical density of 0.5.

**Microscopy**

Microscopy was performed using a Nikon Optiphot-2 microscope. Fluorescence microscopy used a Prior Lumen 2000 for illumination set at 100%. A Nikon G-2A filter (EX 510-560, DM 575, EF 590) was used for visualizing SNAP-Cell® 505 fluorescence. A Chroma 49006 filter (EX 620, DM 660, EF 700) was used for visualizing SNAP-Surface® Alexa Fluor® 647. Images were captured using NIS-ELEMENTS Basic Research version 3.07 software Auto-Capture settings. Exposure time was kept constant for all images in a series.

**Flow cytometry**

Nonsorting flow cytometry experiments were performed using a Becton Dickinson 5-Color FacScan. Flow cytometry sorting was performed using a Becton Dickinson FacsAria. Data were collected using Becton Dickinson CELLQUEST software. Flow cytometry data were further analyzed using FLOWING Software 2 (www.flowingsoftware.com). Graphs were prepared using ORIGIN LABS ORIGIN PRO 8.6 software.

**SDS-PAGE**

Samples were mixed with an equal volume of Novex 2× SDS Sample Buffer and incubated at 99 °C for 5 min. Twenty-five microlitre of sample was loaded into each well. Gels were 4–20% Mini-PROTEAN® TGX precast gels (Bio-Rad). SDS-PAGE gels were stained with Simply-Blue SafeStain (Invitrogen) according to the manufacturer’s instructions. SDS-PAGE gels with samples labeled with SNAP-Vista® Green were visualized using 302 nm UV transillumination on a Bio-Rad XR+ system. Images were captured and analyzed with QUANTITY ONE version 4.6.9 software (Bio-Rad).

**Results and discussion**

In order to test the specificity of labeling type II cohesins with our 505-SNAP-XDocII protein, we attempted to
label both *C. thermocellum* and *E. coli* cells. *Clostridium thermocellum* cells were labeled by SNAP-XDocII, but not the *E. coli* cells, indicating that our protein binds specifically to *C. thermocellum* (Fig. 1). Although fluorescent signals were observed in the labeling reactions containing *E. coli* cells, they did not correspond with the position of cells, as determined by phase contrast microscopy. Instead, they may represent aggregations of the SNAP-XDocII protein, because the XDocII module is known to form homodimers in solution (Adams *et al.*, 2010).

The ability of SNAP-XDocII to bind to *C. thermocellum* suggests that type II cohesins are available for binding in the wild type strain. However, it was unclear whether this availability was due to a subpopulation of unoccupied anchor proteins or whether CipA was being displaced from occupied anchors. Therefore, we examined whether the SNAP-XDocII probe bound differentially to the *C. thermocellum* wild type strain and ΔcipA. Comparison of cells with and without cipA did not show any clear differences in fluorescent labeling (Fig. 1). In both cases, some cells were labeled quite strongly, and some cells were not labeled at all.

To focus on the effects of the removing the XDocII module, instead of the whole CipA protein, we extended

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**Fig. 1.** Fluorescence microscopy of *Escherichia coli* and *Clostridium thermocellum* cells labeled with 505-SNAP-XDocII fusion protein. Images of cells after incubation with 505-SNAP-XDocII fusion protein. Column 1, phase contrast; column 2, fluorescence; column 3, merged image of the phase contrast and fluorescence with the fluorescence layer false-colored red. Bars indicate 10 μm.
our investigation to a strain where just the XDocII module of CipA had been deleted. Unfortunately, cipA contains extensive regions of DNA repeats (Gerngross et al., 1993), making genetic manipulation problematic. Therefore, the wild type allele of cipA was synthesized with extensive synonymous mutations, such that the regions of DNA identity were removed while maintaining the amino acid sequence. Two forms of this allele were created: cipA* and cipA*ΔxdocII (cipA* with the DocII module deleted). These alleles were used to replace the wild type cipA allele on the chromosome, resulting in C. thermocellum strains LL347 (cipA*) and LL348 (cipA*ΔXDocII). These strains provide a more controlled platform for testing the role of the dockerin because they differ only by the presence or absence of the XDocII module. Similar to the comparison between wild type and ΔcipA, microscopy of strains cipA* and cipA*ΔXDocII did not reveal any clear differences in fluorescent labeling (Fig. 1).

It is difficult to get quantitative data from microscopy experiments; therefore, the labeling intensity of the wild type and ΔcipA strains was measured by flow cytometry. Both strains displayed similarity in distribution of fluorescence intensity. The relative mean fluorescence intensity (RMFI) of wild type cells was 1014 ± 10 (99% confidence interval), and the RMFI of ΔcipA cells was 1011 ± 44 (99% confidence interval).

Interestingly, microscopy revealed that the label was not evenly distributed along the length of the cell, but localized to specific regions including cell extremities and some cell–cell interfaces (Fig. 2). Cellulosome protuberances have been observed to protract and form fibrous corridors between cells and between cell and substrate under certain conditions (Bayer & Lamed, 1986). The size and shape of the labeled regions is similar to that of polycellulosomes (Lemaire et al., 1995), although it is notable that most cells contain dozens of polycellulosomes but fewer labeled regions.

Next, the specificity of the labeling was quantified by flow cytometry. We attempted to label C. thermocellum cells with SNAP-XDocII protein and SNAP protein missing the XDocII module. Labeling cells with the SNAP protein missing the XDocII module did not result in labeling of C. thermocellum cells, indicating that binding was mediated by the XDocII module, as expected (Fig. 3). In the absence of the fluorophore, the SNAP protein or the XDocII module, a mean fluorescence intensity of c. 10 was observed. In the presence of all three components, a mean fluorescence intensity of c. 1000 was observed, further indicating the binding specificity of the SNAP-XDocII protein and ruling out possible background fluorescence from components of the E. coli lysate, nonspecific binding of the SNAP protein or SNAP-fluorophore. Taken together with the subcellular localization of the labeling, these data indicate that the SNAP-XDocII fusion protein fluorescently labeled C. thermocellum via the cohesin–dockerin interaction.

Three mechanisms could explain why the presence of native CipA protein did not affect fluorescent labeling intensity. First, a significant excess of type II cohesins in proportion to CipA could mask the differences in cohesin availability between wild type and ΔcipA. Indeed, transcript and proteomic analyses have suggested that C. thermocellum has an excess of type II cohesin modules at the cell surface in relation to the number of CipA scaffolds (Dror et al., 2003; Raman et al., 2009). A second possibility is that levels of cohesin-containing proteins were different in wild type and ΔcipA. A third possibility is that SNAP-XDocII fusion proteins could displace native CipA proteins in the wild type by competitive dockerin-replacement, masking the differences in cohesin availability between wild type and ΔcipA. We refer to this third possibility as the ‘dockerin-replacement’ hypothesis.
To investigate the possibility of dockerin-replacement, wild-type *C. thermocellum* cells were subjected to sequential incubations in the presence of SNAP-XDocII fusion protein bound to different fluorophores. The fluorescent intensity of the labeled cells was analyzed by flow cytometry. The RMFI of the population was normalized to 1.00 based on the single-labeling reaction, using either the SNAP-Cell 505 or 674 fluorophores. After labeling the cells with SNAP-Cell 505, a second labeling reaction was performed with the SNAP-Cell 647 fluorophore. The RMFI of the SNAP-Cell 647 label was 1.63, and the RMFI of the SNAP-Cell 505 label had decreased to 0.67. A third labeling reaction (with the same SNAP-Cell 505 label used in the first labeling reaction) resulted in an increase in the RMFI of the SNAP-Cell 505 label to 1.46 and a decrease of the RMFI of the SNAP-Cell 647 label to 0.73. Each additional label substantially decreased the intensity of the previous label (Fig. 4), indicating that the SNAP-XDocII proteins were capable of displacing each other, and supporting a role for the dockerin-replacement hypothesis. It is interesting that subsequent labeling reactions increased the fluorescence intensity of supposedly saturated samples (RMFI values > 1 in Fig. 4). One possible explanation is that cellulosomal protuberances may prolapse during the washing procedure exposing additional unbound cohesins that were not accessible to the SNAP-XDocII probe during the initial reaction.

In all samples, 30–70% of the flow cytometry ‘events’ did not display any fluorescence, which is in agreement with the proportion of cells that did not display fluorescence in

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**Fig. 3.** Relative mean fluorescence intensity of wild type cell populations labeled with 505-SNAP-XDocII protein as determined by flow cytometry. Wild type *Clostridium thermocellum* cells were incubated with various preparations containing different components of the SNAP-XDocII protein labeling scheme. Bars indicate the mean fluorescence, at 505 nm, of wild type cell populations analyzed by flow cytometry, measured in relative units of fluorescence (RFUs). Error bars indicate 99.9% confidence intervals. ‘*Escherichia coli* lysate’ indicates lysate from *E. coli* BL21(DE3) cells and was used to control for nonspecific fluorescence from the lysate. ‘505-SNAP’ indicates purified SNAP protein bound to the SNAP-Cell 505 fluorophore and was used to control for nonspecific binding of the SNAP protein to *Clostridium thermocellum*. ‘*Escherichia coli* lysate with 505-SNAP-XDocII’ indicates a lysate of *E. coli* BL21(DE3) cells where expression of the SNAP-XDocII protein had been induced. Subsequently, the lysate was labeled with SNAP-Cell 505 fluorophore (to generate 505-SNAP-XDocII) before being added to *C. thermocellum* cells.

To investigate the possibility of dockerin-replacement, wild-type *C. thermocellum* cells were subjected to sequential incubations in the presence of SNAP-XDocII fusion protein bound to different fluorophores. The fluorescent intensity of the labeled cells was analyzed by flow cytometry. The RMFI of the population was normalized to 1.00 based on the single-labeling reaction, using either the SNAP-Cell 505 or 674 fluorophores. After labeling the cells with SNAP-Cell 505, a second labeling reaction was performed with the SNAP-Cell 647 fluorophore. The RMFI of the SNAP-Cell 647 label was 1.63, and the RMFI of the SNAP-Cell 505 label had decreased to 0.67. A third labeling reaction (with the same SNAP-Cell 505 label used in the first labeling reaction) resulted in an increase in the RMFI of the SNAP-Cell 505 label to 1.46 and a decrease of the RMFI of the SNAP-Cell 647 label to 0.73. Each additional label substantially decreased the intensity of the previous label (Fig. 4), indicating that the SNAP-XDocII proteins were capable of displacing each other, and supporting a role for the dockerin-replacement hypothesis. It is interesting that subsequent labeling reactions increased the fluorescence intensity of supposedly saturated samples (RMFI values > 1 in Fig. 4). One possible explanation is that cellulosomal protuberances may prolapse during the washing procedure exposing additional unbound cohesins that were not accessible to the SNAP-XDocII probe during the initial reaction.

In all samples, 30–70% of the flow cytometry ‘events’ did not display any fluorescence, which is in agreement with the proportion of cells that did not display fluorescence in
the microscopy experiment. In multiple labeling experiments, however, this value changed by < 2% points between labeling reactions, suggesting that the unlabeled populations are stable. The results do not rule out the possibility of the other hypotheses. Resolving which mechanism is predominant remains an unresolved question. However, dockerin replacement may explain the surprising result that cells with and without the cipA gene showed similar levels of fluorescence after labeling with the SNAP-XDocII fusion protein, because the necessity of displacing CipA protein in the wild type and cipA* strains did not reduce fluorescence intensity.

We have shown that the SNAP-tag system can be used to fluorescently label C. thermocellum via the cohesin–dockerin interaction. Previous studies have visualized cellulosomes by transmission electron microscopy (Bayer et al., 1985); however, the ability to specifically label the cellulosome in aqueous solution could lead to the ability to observe cellulosome operation in-vivo.

Although much is known about the interaction between free dockerins and free cohesins, the interaction between free dockerins and bound cohesin–dockerin pairs has been less well studied. Dockerin exchange suggests a mechanism for compositional change of the cellulosome. Clostridium thermocellum is known to release cellulosomes in the late-stationary phase of growth; as well as optimize the composition of cellulosomes attached to its surface in response to substrate changes (Bayer & Lamed, 1986; Raman et al., 2009). It has been suggested that detachment of intact cellulosomes in these processes is achieved by proteolytic cleavage of the cohesin-II containing anchor proteins (Raman et al., 2009). The results of this study suggest an alternative or complementary mechanism, wherein the mere production of CipA molecules can effect turnover by dockerin exchange. Similar experiments could be used to probe interactions between type I cohesins and dockerins.

In this study, we have demonstrated displacement of bound dockerin-containing proteins with free dockerin-containing proteins. This result sheds light on a possible mechanism for the natural turnover and reordering of cellulose subunits within the poycellulose. Furthermore, the methods of this article have established the SNAP-tag system as a valuable tool for labeling components and sub-components of the cellulosome.

Acknowledgements

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References


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** (a) Description of individual components of the SNAP-XDocII labeling system. (b) Components of SNAP-XDocII labeling system in microbial context.