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RESEARCH ARTICLE

Cohesin-dockerin microarray: Diverse specificities between two complementary families of interacting protein modules

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The cellulosome is an intricate multienzyme complex, designed for efficient degradation of plant cell wall polysaccharides, notably cellulose. The supramolecular cellulosome architecture in different bacteria is the consequence of the types and specificities of the interacting cohesin and dockerin modules, borne by the different cellulosomal subunits. In this study, we describe a microarray system for determining cohesin-dockerin specificity, which allows global comparison among the interactions between various members of these two complementary families of interacting protein modules. Matching recombinant fusion proteins were prepared that contained one of the interacting modules: cohesins were joined to an appropriate cellulose-binding module (CBM) and the dockerins were fused to a thermostable xylanase that served to enhance expression and proper folding. The CBM-fused cohesins were immobilized on cellulose-coated glass slides, to which xylanase-fused dockerin samples were applied. Knowledge of the specificity characteristics of native and mutated members of the cohesin and dockerin families provides insight into the architecture of the parent cellulosome and allows selection of suitable cohesin-dockein pairs for biotechnological and nanotechnological application. Using this approach, extensive cross-species interaction among type-II cohesins and dockerins is shown for the first time. Selective intraspecies binding of an archaeal dockerin to two complementary cohesins is also demonstrated.

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Abbreviations: CBM, cellulose-binding module; **CBM-Coh**, CBM-fused cohesin; **SPR**, surface plasmon resonance; **Xyn**, xylanase; **XynDoc**, xylanase-fused dockerin

1 Introduction

Cellulosomes are multicomponent, multienzyme machines, produced by anaerobic bacteria for the efficient digestion of cellulose and other plant cell wall polysaccharides [1–3]. The various enzymes and other cellulosome components are

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integrated into a central multimodular scaffolding subunit (*scaffoldin*) [4, 5], by virtue of a specialized type of *cohesin* module, present in multiple copies on the scaffoldin, which binds tightly to a complementary *dockerin* module borne by each of the cellulosomal components. The scaffoldin also harbors a cellulose-binding module (CBM), which targets the cellulosome to its substrate [6, 7], and a divergent dockerin of its own, which binds to selected cohesins of alternative scaffoldin(s) that anchor the cellulosome complex to the bacterial cell surface [8–12]. Cellulosome architecture is determined by specificities of the different cohesin-dockerin interactions, and their number and arrangement in the different scaffoldins and other cellulosomal subunits.

The high-affinity cohesin-dockerin interaction has been shown to be calcium dependent, owing to the calcium-binding motif of the dockerin module, whose ~70-residue sequence bears strong resemblance to the EF-hand loop-helix motif of eukaryotic calcium-binding proteins, *e.g.*, calmodulin and troponin C [13–15]. The ~140-residue cohesin module folds into a structurally stable 9-stranded β-sandwich [16–19], and the molecular bases for two different types cohesin-dockerin interactions have been elucidated [20, 21]. Broad interspecies sequence divergence is evident in both the dockerin and especially the cohesin family [1, 22].

The phylogenetic distribution of the various scaffoldin-borne cohesin sequences is shown in Fig. 1. Historically, the separation of the cohesins into types I and II resulted from the divergent cohesins found in the cellulosomal scaffoldin of *Clostridium thermocellum* (Fig. 1) [9, 10]. The nine type-I cohesins of this bacterium are components of the major cellulosome-integrating protein, CipA, and interact exclusively with the complementary dockerins, borne by the enzymes and other cellulosomal subunits. An additional type-I cohesin appears in a single copy on a curious type of cell-surface anchoring protein, OlpA, which also binds to the same set of enzyme-borne dockerins [23, 24]. In contrast, the type-II cohesins are found in assorted anchoring proteins (Orf2p, OlpB, and SdbA) that interact with the lone C-terminal CipA dockerin, thereby incorporating the scaffoldin and its complement of enzymes onto the cell surface [10, 11, 25–27].

The location on the phylogenetic tree reflects the relationship of the amino acid sequences among the different cohesins. As additional cohesins were discovered, their intimacy with the type-I or -II kindred determined their classification. Thus, the cohesins of *Acetivibrio cellulolyticus* scaffoldin ScaA clearly belong to type-I [28], whereas the cohesins of ScaB belong to type-II [29]. The cohesins of the *A. cellulolyticus* anchoring scaffoldin ScaC are also closely related to their type-I homologs.

The simplistic view of the scaffoldins, however, became somewhat more complicated with the discovery of the cohesins from *Bacteroides cellulosolvens*. In contrast to the set of the cohesins in *C. thermocellum* and *A. cellulolyticus,* the arrangement in *B. cellulosolvens* is the reverse: the enzyme-integrating ScaA scaffoldin carries type-II cohesins, whereas the anchoring scaffoldin ScaB bears type-I cohesins [30, 31]. Additional intricacy awaited the discovery of the *A. cellulolyticus* anchoring scaffoldin ScaD, which harbors both types of cohesin in the same protein [32].

Subsequent identification and analysis of the scaffoldins in *Ruminococcus flavefaciens* led to their classification in yet another type [33, 34]. Cohesins from *R. flavefaciens* ScaA, ScaB, and ScaE emanate from a single branch of the phylogenetic tree, which differs from their type-I and -II relatives, and are collectively grouped into type-III. In contrast, the single ScaC cohesin from *R. flavefaciens* resembles those of the *A. cellulolyticus* ScaC and the closely related type-I cohesins [35]. Finally, the cohesins of the *Clostridium acetobutylicum* scaffoldin [36] and those of the archaeon, *Archaeoglobus fulgidus* [37], are unlike all other cohesins yet described and map on separate branches of the phylogenetic tree.

The dockerin type is commonly classified according to the designated type of cohesin(s) with which they interact. The phylogenetic tree of the various dockerin sequences usually reflects the complementary cohesin tree [1].

The sequences of hundreds of different cohesins and dockerins from about a dozen different cellulosome-producing bacterial species are now known. Moreover, ongoing genome-sequencing projects of such bacteria guarantee ever-increasing numbers of new types of cohesins and dockerins, whose discovery merits additional research into their individual specificities. The information gained by these studies will enable broad comparative evaluation of cellulosome architecture and/or cohesin-dockerin function in newly described bacteria. In addition, the high-affinity cohesin-dockerin interaction is proving to be a useful component for self-assembly of "designer cellulosomes" and for production of other types of biotechnological and nanotechnological assemblies [38–41].

In the present article, we developed and applied a protein microarray system that is useful for evaluating the specificity(ies) of test dockerin modules for large numbers of cohesins. For this purpose, we have employed a matching fusion-protein system [42], in which the cohesin is fused with a CBM and the dockerin is paired with a xylanase (Xyn) carrier. Both fusion partners – the CBM and Xyn – are thermostable, high-expression components. Both types of fusion protein emulate the native setting of the test module: cohesins are natural neighbors of CBMs in the native scaffoldin, and dockerins are natural appendages of cellulosomal enzymes. Fabrication of the microarray is based on the tenacious affinity of the CBM for its 3-D cellulosic substrate, for which cellulose-coated glass slides were employed [43]. CBM-mediated binding to the slide dictates proper presentation of the cohesins, thereby enabling the desired interaction with the target dockerins and decreasing background due to nonspecific binding. The displayed cohesin contrasts with that of the ELISA-based system, in which cohesin orientation is undefined and the surface-adsorbed proteins may be partially unfolded.

2 Materials and methods

2.1 Materials

All fine biochemicals and solvents were obtained from Sigma–Aldrich (St. Louis, MO). Cy5 Mono-Reactive Dye was purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Horseradish peroxidase-conjugated goat antirabbit IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA). Regenerated cellulose-coated slides were obtained from Zephyr ProteomiX (Kiryat-Shemona, Israel).

2.2 Methods

2.2.1 Strains and primers

A. cellulolyticus ATCC 33288 *A. fulgidus DSM 4304*, *B. cellulosolvens* ATCC 35603, *C. acetobutylicum* ATCC 824, *Clostridium cellulolyticum* ATCC 35319, *C. thermocellum* YS, and *R. flavefaciens* strain 17 were either those described in previous work [22, 28, 30, 33, 44] or obtained from the relevant culture collections. Genomic DNA was prepared from cells of the designated bacterial strains as described previously [15, 22, 45]. Lists of primers used to prepare the various cohesin- and dockerin-containing DNA constructs are available in Supporting Information online at the PROTEOMICS website (www.proteomics-journal.com).

2.2.2 Construction and bacterial expression of CBM-fused cohesin (CBM-Coh)

A PCR product containing the CBM from the *C. thermocellum* scaffoldin CipA [7] was inserted into the pET28a vector at the *NcoI* and *BamHI* sites, allowing insertion of any desired cohesin at sites between the *BamHI* and *XhoI* of this vector creating a CBM-Coh fusion protein [46]. Protein expression and purification was carried out by transforming BL21 (λDE3) pLysS cells with the designated plasmids and growing them on Luria-Bertani medium at 37°C to an A_{600} ~ 1. Isopropyl thio-β-D-galactoside (IPTG) was added to a final concentration of 0.1 mM. Following induction, cells were incubated for 3 h at 37°C, centrifuged, sonicated, and the supernatant fluids were incubated with amorphous cellulose for 1–2 h at 4°C to allow binding of the CBM-Coh. The amorphous cellulose was washed three times with TBS, pH 7.4, containing 1 M NaCl and three times with TBS. The protein was eluted with 1% v/v triethylamine, and neutralized with MES buffer [42].

2.2.3 Construction and bacterial expression of Xyn-Doc

A PCR construct of *Geobacillus stearothermophilus* Xyn T-6 [47] with a His-tag at the 5′-terminus and a *KpnI* site at the 3′-terminus was ligated at the *KpnI* site with the PCR product of a *C. thermocellum* CelS (Cel48S) dockerin [48] and inserted into the pET9d vector at *NcoI* and *BamHI* sites as described by Barak *et al.* [42]. This plasmid allows facile replacement of the CelS dockerin (termed DocS) with any other desired dockerin. The resultant expressed product constitutes a His-tagged Xyn T-6 fusion-protein bearing a dockerin at the C-terminus.

Plasmids were transformed into BL21 (λDE3) pLysS cells and grown at 37°C to an A_{600} ~1 in Luria-Bertani medium, supplemented with 2 mM $CaCl_2$. IPTG was added to a final concentration of 0.1 mM, and growth was continued at 16°C for 16 h for protein expression. Cells were centrifuged for 15 min at 6000 rpm, the pellet was resuspended in 20 mL of binding buffer, consisting of TBS supplemented with 5 mM imidazole and protease-inhibitor cocktail (1 mM PMSF, 0.4 mM benzamidine, and 0.06 mM benzamide). Cells were disrupted by sonication, and the sonicate was centrifuged for 30 min at 15000 rpm at 4°C. When dockerins from thermophilic bacteria were used, a heat treatment step (30 min, 60°C) was included before centrifugation. The supernatant fluids were loaded onto a Ni-NTA column (GE Healthcare Bio-Sciences AB), and eluted from the column using a linear gradient of imidazole using an AKTA-prime System (GE Healthcare Bio-Sciences AB).

2.2.4 Antibody production and preparation

Polyclonal rabbit anti-Xyn T-6 from *G. stearothermophilus* production was carried out in the antibody unit of the Weizmann Institute of Science (as described previously [7]). The Ig fraction was purified on a protein A column (GE Healthcare Bio-Sciences AB). N-Hydroxysuccinimide-ester-activated Cy-5 dye (300 μg) was resuspended in 0.1 M sodium carbonate buffer, pH 9, and mixed with the antibody (1 mg in 0.5 mL), according to the manufacturer's instructions. Free dye was removed by dialysis against PBS. The fluorescence-labeled antibody was stored in 50% glycerol at –20°C.

2.2.5 Affinity-based ELISA

Analysis of the dockerin of *C. thermocellum* family-11 XynB (*Ct*-Doc11B) using immobilized cohesins was performed essentially as described by Barak *et al.* [42]. The ELISA plates were coated with 15 nM of the desired CBM-Coh and the interaction with xylanase-fused dockerin (XynDoc) was performed at concentrations ranging from 6 pM to 6 nM.

2.2.6 Printing CBM-based microarray

A Micro Grid II arrayer (BioRobotics, Woburn, MA) was utilized to print proteins onto the cellulose-coated glass slides at the Microarray unit of the Weizmann Institute. Protein samples were diluted in PBS, pH 7.4, to a final concentration of 8 μM and applied to the plates in five successive two-fold dilutions. The proteins were printed in spot diameters of 0.2 mm and at 0.375 mm intervals, center-to-center. The

printed microarrays were kept at room temperature in the absence of desiccant prior to application.

To assist in locating the positions of the protein samples on the microarray, CBM-conjugated Xyn T6 was spotted as a marker in triplicate beneath the lowest concentration of each sample. The conjugate was prepared by mixing an aliquot of 0.475 mL of CBM (5 mg/mL PBS) with 0.475 mL of Xyn T-6 (1.7 mg/mL PBS) followed by addition of 50 μL bis(sulfosuccinimidyl)suberate (Pierce Chem., Rockford, IL) (9 mg/mL in DMSO). The mixture was allowed to interact for 1 h at room temperature, and the sample was dialyzed overnight against PBS in 4°C. The conjugate was analyzed by SDS-PAGE.

2.2.7 Microarray probing

Printed microarrays were blocked by incubating the slides in blocking buffer (1% BSA in TBS with 10 mM $CaCl_2$ and 0.05% Tween 20) at room temperature for 30 min. The slides were then incubated at room temperature with the desired Xyn-Doc at a concentration of 2 nM (0.1 μg/mL) in blocking buffer for 45 min. In some cases, 10 mM EDTA was substituted for the $CaCl_2$ in all solutions to determine calcium dependence of the interaction. After washing with blocking buffer, fluorescence-labeled anti-Xyn T-6 antibody in blocking buffer was added for an incubation period of 30 min. The probed slides were washed four times with washing buffer (TBS with 10 mM $CaCl_2$ and 0.05% Tween 20), air-dried and scanned for fluorescence signals using a Typhoon 9400 Variable Mode Imager GE Healthcare Bio-Sciences AB. Quantification of the spots was carried out using Image Quant 5.2 software (GE Healthcare Bio-Sciences AB).

2.2.8 Phylogenetic analysis

Multiple sequence alignment and neighborhood joining (N-J) phylogenetic trees were generated using the ClustalW program (http://www2.ebi.ac.uk/clustalw/) [49] with bootstrapping of 1000 data sets. The radial consensus tree in Fig. 1 was rendered using TreeView 1.6.0 [50], resulting in an unrooted tree radiating from a central point. A complementary phylogram (see Supporting Information) was

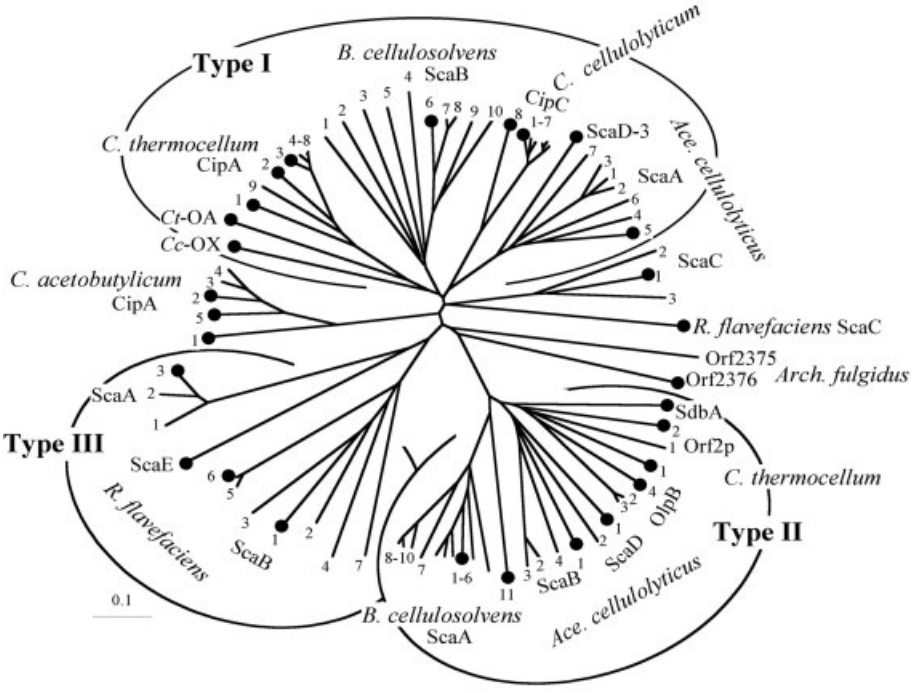


Figure 1. Phylogenetic relationship of the various cohesins used in this work. Closed circles indicate the cohesins used to produce the CBM-Coh fusion proteins for fabrication of the microarray. Their relationship to the other (unmarked) cohesins on the same protein is shown. Numbers refer to the position of the given cohesin on the designated scaffoldin, relative to the N terminus. Cohesins belonging to the three recognized types are grouped as indicated. Scale bar indicates the percentage (0.1) of amino acid substitutions. GenBank, EMBL or Swiss-Prot accession numbers for scaffoldin sequences, used to generate the radial consensus cohesin tree, are as follows: *A. cellulolyticus* ScaA (AF155197), ScaB (AY221112) ScaC (AY221113), and ScaD (AY221114); *A. fulgidus* Orfs 2375 and 2376 (AE001112), *B. cellulosolvens* ScaA and ScaB (AF224509); *C. acetobutylicum* CipA (AE007607); *C. cellulolyticum* CipC (U40345) and OrfX (*Cc*-OX, AF081458); *C. thermocellum* primary scaffoldin, CipA (Q06851), and anchoring scaffoldins, OlpA (*Ct*-OA, Q06848), SdbA (U49980), OlpB (Q06852), and Orf2p (Q06853); *R. flavefaciens* ScaA (AJ278969), ScaB (AJ278969), ScaC (AJ585075), and ScaE (AJ810899). A complementary N-J phylogram with bootstrapping values is available online at the PROTEOMICS website (www.proteomics-journal.com) in the Supporting Information for this article.

displayed with bootstrapping values using N-J plot (M. Gouy, Univ. Lyon; mgouy@bioserv.univ-lyon1.fr). In all of the above analyses, amino acid (rather than nucleotide) sequences were used.

3 Results and discussion

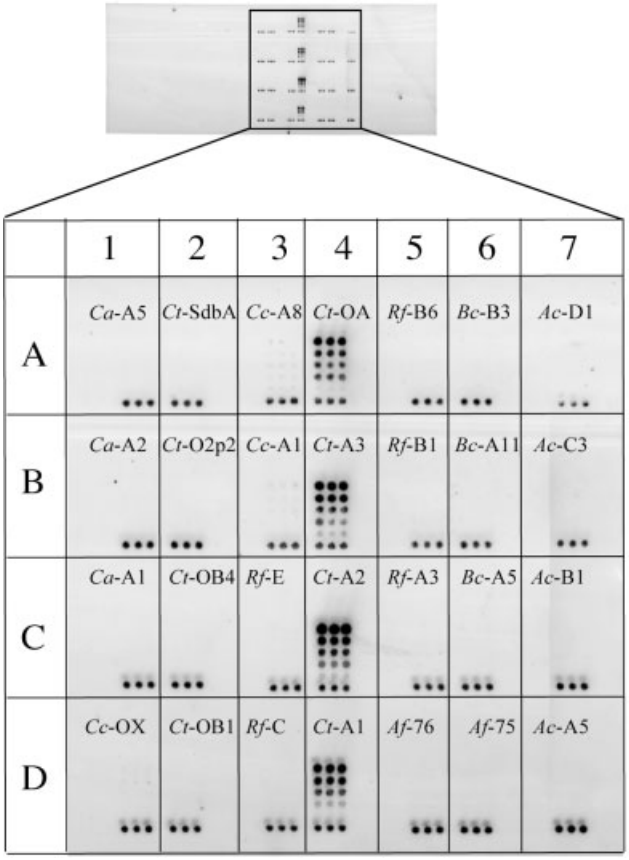
3.1 Design of the protein microarray

An area of the cellulose-coated glass slide was printed in triplicate with a variety of CBM-Coh samples (see Fig. 2 and key for abbreviations and coordinates of samples). These include type-I, -II, -III, and nontyped cohesins derived from scaffoldins of various cellulosome-producing bacteria. Altogether, 28 different cohesins from 20 different proteins from six different bacteria and one archaeon were used to fabricate the microarray designed in this study. Each sample was applied in triplicate at five successive dilutions, ranging from 0.5 to 8 μM (19–300 μg/mL). CBM-conjugated Xyn was also spotted in triplicate, as a marker placed beneath the lowest concentration of each protein sample to assist in locating their positions on the microarray.

As described previously, the dockerins were all secured as Xyn-Doc fusion proteins with a Xyn T6 carrier, derived from the thermophilic bacterium, *G. stearothermophilus*. The Xyn carrier helps stabilize the fused dockerin module and enables the detection of the proteins using the Cy-5-labeled anti-Xyn T6 antibodies. It should be noted that recombinant cohesin- and dockerin-bearing fusion proteins were used in these experiments, which consequently represented non-glycosylated forms of the native modules.

3.2 Specificity of the *C. thermocellum* type-I cohesin-dockerin interaction

In order to validate the use of this microarray system for determining cohesin-dockerin interactions, we selected the well-characterized *C. thermocellum* type-I interaction as a reference. The intraspecies fidelity of the nine type-I cohe-



Key

Term	Coordi-nates	Organism	Scaffoldin	Cohesin (N°)
Ac-A5	D-7	*Acetivibrio cellulolyticus*	ScaA	5
Ac-B1	C-7	*Ace. cellulolyticus*	ScaB	1
Ac-C3	B-7	*Ace. cellulolyticus*	ScaC	3
Ac-D1	A-7	*Ace. cellulolyticus*	ScaD	1
Af-75	D-6	*Archaeoglobus fulgidus*	Orf2375p	
Af-76	D-5	*Arc. fulgidus*	Orf2376p	
Bc-A5	C-6	*Bacteroides cellulosolvens*	ScaA	5
Bc-A11	B-6	*B. cellulosolvens*	ScaA	11
Bc-B3	A-6	*B. cellulosolvens*	ScaB	3
Ca-A1	C-1	*Clostridium acetobutylicum*	ScaA	1
Ca-A2	B-1	*C. acetobutylicum*	ScaA	2
Ca-A5	A-1	*C. acetobutylicum*	ScaA	5
Cc-A1	B-3	*Clostridium cellulolyticum*	CipC	1
Cc-A8	A-3	*C. cellulolyticum*	CipC	8
Cc-OX	D-1	*C. cellulolyticum*	OrfX	
Ct-O2p2	B-2	*Clostridium thermocellum*	Orf2p	2
Ct-A1	D-4	*C. thermocellum*	CipA	1
Ct-A2	C-4	*C. thermocellum*	CipA	2
Ct-A3	B-4	*C. thermocellum*	CipA	3
Ct-OA	A-4	*C. thermocellum*	OlpA	
Ct-OB1	D-2	*C. thermocellum*	OlpB	1
Ct-OB4	C-2	*C. thermocellum*	OlpB	4
Ct-SdbA	A-2	*C. thermocellum*	SdbA	
Rf-A3	C-5	*Ruminococcus flavefaciens*	ScaA	3
Rf-B1	B-5	*R. flavefaciens*	ScaB	1
Rf-B6	A-5	*R. flavefaciens*	ScaB	6
Rf-C	D-3	*R. flavefaciens*	ScaC	
Rf-E	C-3	*R. flavefaciens*	ScaE	

Figure 2. Interaction of the *C. thermocellum* Cel48S dockerin (*Ct*-Doc48S) with the cohesin microarray. The area of the cellulose layer of the glass slide is shown above, and the magnified portion immediately below shows the area wherein the samples were applied. CBM-Coh fusion proteins, as listed in the Key, were applied in triplicate in successive two-fold dilution, starting from a maximum concentration of 8 μM (~5 nL). The XynDoc fusion protein (10 mL *per* slide) was then applied at a final concentration of 2 nM, and the amount of dockerin bound to the cohesin samples was visualized by immunofluorescence. The triplet of spots at the base of each sample area denotes a marker, composed of a Xyn-CBM conjugate, which indicates the location of the samples on the cellulose slide.

sins from the CipA scaffoldin of this bacterium has been demonstrated by various methods (including, nondenaturing gel-retardation assay, ELISA, surface plasmon resonance (SPR), and isothermal titration calorimetry) to interact indiscriminately with its various enzyme-borne dockerins [15, 39, 51]. However, the same cohesins and dockerins failed to recognize the orthologous modular counterparts from a similar cellulosome-producing bacterium, *C. cellulolyticum* [22], thus indicating cross-species stringency of the cohesin-dockerin system.

Using the experimental setup described above, we were able, in a single experiment, to challenge a test dockerin (*Ct*-Doc48S) from the prominent *C. thermocellum* family-48 cellobiohydrolase Cel48S against a greater number of cross-scaffoldin and cross-species cohesins of different types. Indeed, as predicted, *Ct*-Doc48S recognized all of the type-I cohesins examined in this work from *C. thermocellum*, including representative cohesins of the CipA scaffoldin as well as the single OlpA cohesin (Fig. 2). No interaction was observed with any of the type-II cohesins from the same bacterium or any of the cohesins from other species (including the different type-I cohesins), thus indicating the conservation of specificity among a very wide range of different species of cohesins. The presence of EDTA served to block the interaction (not shown), in line with the known calcium-dependence of the dockerin structure.

Most of the other type-I cohesins used in the microarray displayed exquisite specificity for their own (test) dockerins only (not shown). As expected, the type-I cohesins also failed to exhibit cross-type recognition of any of the other dockerins derived from other scaffoldins of the same species or from other species.

Although early work favored the exquisite specificity of the type-I cohesins, within a given scaffoldin for its own enzyme-borne dockerins, some exceptions to this rule have recently been noted. Specifically, it was determined by SPR [52] and ELISA [42] that two *C. thermocellum* Xyn-borne dockerins also recognize the type-I CipC cohesins from *Clostridium josui* and *C. cellulolyticum* (the sequences of the cohesins and dockerins between the latter species are very similar), although the observed binding was clearly weaker than the intraspecies interaction. It was therefore of interest to see whether such interspecies interaction would be detected using the cohesin-dockerin microarray. For this purpose, one of the cross-specific dockerins (*Ct*-Doc11B, the dockerin of the *C. thermocellum* family-11 XynB) was employed as the test dockerin (Fig. 3). The microarray results were entirely consistent with the published SPR and ELISA data, whereby *Ct*-Doc11B was recognized by all of its own type-I cohesins examined as well as the type-I cohesins from *C. cellulolyticum*. The cross-species interaction was weaker than the intraspecies recognition, as can be seen in Fig. 3 (compare coordinates A-3 and B-3 with the samples in column 4). Moreover, the interaction of *Ct*-Doc11B was stronger with one of the *C. cellulolyticum* cohesins (*Cc*-A1) compared to its homolog (*Cc*-A8). This result may reflect the divergence of

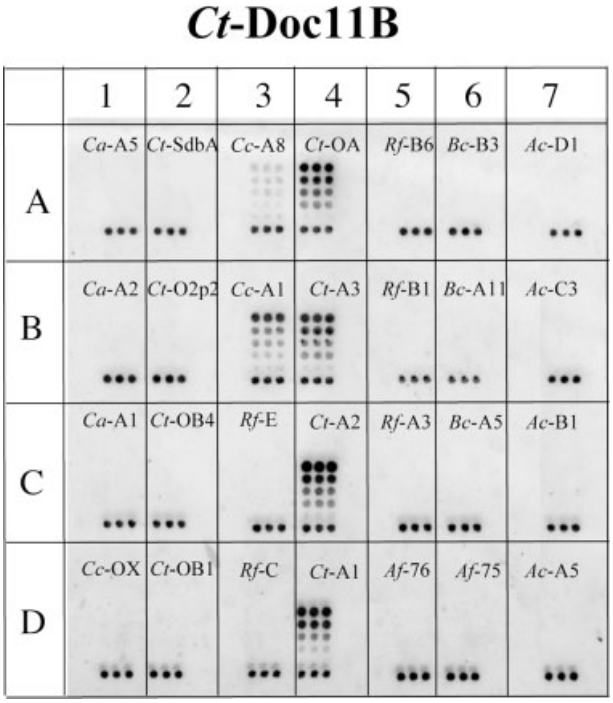


Figure 3. Interaction of the *C. thermocellum* Xyn11B dockerin (*Ct*-Doc11B) with the cohesin microarray. The experiment was carried out as described in the legend to Fig. 2, except the XynDoc fusion protein contained the dockerin from Xyn11B. Note the additional cross-species interaction of this *C. thermocellum* dockerin with cohesins from *C. cellulolyticum* (A3 and B3).

the latter *C. cellulolyticum* cohesin from the other seven cohesins carried by the CipC scaffoldin. Notably, the cross-species type-I interaction was limited to these two species. *Ct*-Doc11B displayed no binding with any of the other type-I cohesins, nor with any of the more distantly related (type-II, -III, *etc.*) cohesins.

The spots on the microarray can also be quantified by densitometry using the Image Quant 5.2 software (GE Healthcare Bio-Sciences AB). For demonstration purposes, the positive interactions shown in Fig. 3 for *Ct*-Doc11B were analyzed quantitatively. The results (Fig. 4) were in general accord with those obtained by ELISA, although some differences were evident. In particular, the interaction of *Ct*-Doc11B with *Cc*-A8 was much more pronounced in the ELISA experiment; only very low (but detectable) levels of binding were observed in the microarray. It should be noted that the experiments are innately different: in the microarray, various quantities of cohesin are applied to the slide and the dockerin concentration in solution is constant. In contrast, in the ELISA experiment, a constant concentration of cohesin is applied to the wells of microtiter plates and various concentrations of test dockerin are then added. Moreover, the cohesin in the microarray is properly displayed to the medium, owing to the interaction of the CBM with the cellulose, whereas the orientation of the cohesin in the ELISA plate is

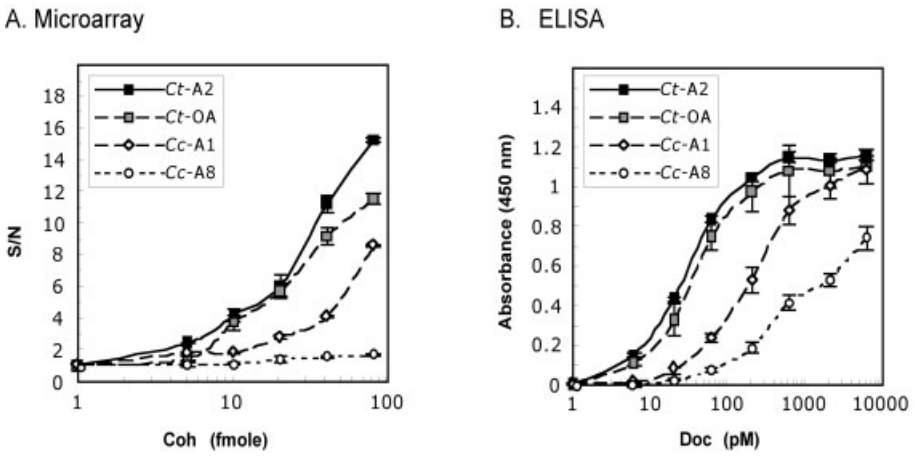


Figure 4. Quantification of the dose-response curves for the cohesin-dockerin interaction of *Ct*-Doc11B (see Fig. 3): ELISA *versus* microarray. (A) Quantitative evaluation of fluorescence signal intensity (S/N) using Image Quant 5.2 software *versus* concentration of the printed CBM-Coh samples. (B) ELISA plates were coated with the designated CBM-borne cohesins as in panel A at a concentration of 15 nM. Dockerin-Xyn-T6 fusion protein (*Ct*-Doc11B), derived from *C. thermocellum* was added at incremented concentrations, and the resultant cohesin-dockerin interaction was detected using anti-Xyn primary antibody and HRP-labeled secondary antibody preparations. Error bars indicate SD from the mean of duplicate (ELISA) or triplicate (microarray) samples from two separate experiments.

arbitrary. Quantification of the microarray data can thus be used as an initial estimate of the interaction between a given reactive cohesin and its dockerin counterpart, subject to subsequent analysis and comparison by ELISA and/or SPR.

3.3 Interactions of type-II dockerins with type-II cohesins

The type-II cohesins of *C. thermocellum* were differentiated from the type-I cohesins on the basis of sequence homology as well as specificity [8]. Phylogenetic trees, generated from collections of type-I and -II cohesins, indeed reveal considerable divergence in their distribution (Fig. 1). However, as observed for the type-I cohesin-dockerin interaction, the specificity of the type-II interaction may differ within and between species. It is thus important to determine the intra- and interspecies specificities of the different type-II cohesins and dockerins.

Xyn-Doc fusion proteins were constructed using the three known type-II dockerins. In the case of *C. thermocellum* and *A. cellulolyticus*, the adjacent X module was also included (*Ct*-XDoc and *Ac*-XDoc, from the CipA and ScaA scaffoldins, respectively), as its presence enhances considerably the relevant cohesin-dockerin interaction [21, 53]. Since the enzyme-borne type-II dockerin (*Bc*-48A) lacks such a module [31], it was thus fused alone as XynDoc construct. Eight representative type-II cohesins, derived from six different scaffoldins from the three bacterial species, were used in this analysis.

The results, presented in Fig. 5, demonstrated that the interscaffoldin interspecies specificity appears to be much less strict for the type-II cohesin-dockerin interaction than that observed for type-I. In the case of the *C. thermocellum* type-II interaction, *Ct*-XDoc indeed appeared to recognize all four of the tested type-II cohesins, derived from the three relevant scaffoldins (SdbA, Orf2p, and OlpB). In this particular case, cross-species interaction is not observed. The situation is very different, however, with the *A. cellulolyticus* type-II dockerin. As can be seen in Fig. 5, *Ac*-XDoc bound to its own type-II cohesins from ScaB and ScaD (*Ac*-B1 and weakly to *Ac*-D1), but it also bound the SdbA cohesin and cohesin 1 (but not cohesin 4) from OlpB. In fact, the observed interspecies interaction of *Ac*-XDoc with the *C. thermocellum* SdbA cohesin appears to be stronger than its interaction with its own cohesins. The SdbA cohesin also bound to the remaining known type-II dockerin, harbored by the *B. cellulosolvens* family-48 cellulase (*Bc*-48A), indicating that it might serve as a general type-II cohesin. In addition to the SdbA cohesin, *Bc*-48A bound to its own type-II cohesins from the ScaA scaffoldin (*Bc*-A5 and *Bc*-A11) but not to any of the other type-II cohesins examined in this study. None of the type-II cohesins, including the SdbA cohesin, bound to any of the type-I or -III dockerins (or any of the other dockerins tried in this study). Likewise, none of the type-II dockerins interacted with cohesins of any other type (including types I and III); the cross-reactivity was limited to interactions with other interspecies type-II cohesins. The apparent plasticity in the specificity of some of the *C. thermocellum* type-II cohesins, particularly of SdbA, had not been addressed previously. The microarray results for the type-II cohesin-dockerin interaction have been substantiated in general by ELISA assays (data not shown), although future research is needed to verify these findings and to address the observed subtle interspecies cross-reactivities in more detail.

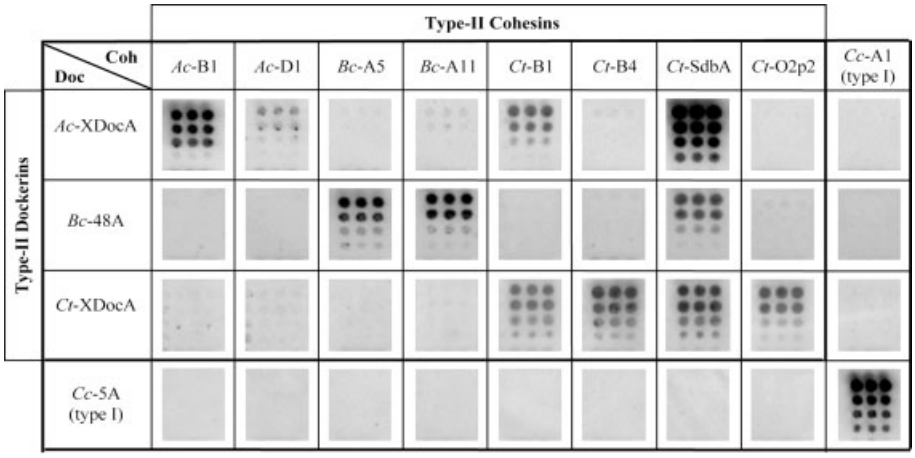


Figure 5. Cross-species type-II cohesin-dockerin interaction. Experiments were carried out as described in the legend to Fig. 2, with the designated XynDoc fusion proteins. Three type-II dockerins: *Ac*-XDocA, *Bc*-48A, *Ct*-XDocA and one control type-I dockerin (*Cc*-5A) were chosen for interaction with the printed cohesins. Shown are all the interactions with the type-II cohesins from *A. cellulolyticus* ScaB and ScaD (*Ac*-B1, *Ac*-D1), from *B. cellulosolvens* ScaA (*Bc*-A5 and *Bc*-A11), and from *C. thermocellum* OlpB (*Ct*-B1 and *Ct*-B4), Orf2p (*Ct*-O2p2), and SdbA (*Ct*-SdbA), as well as one control type-I cohesin from *C. cellulolyticum* (*Cc*-A1). Note the cross-species interaction of the *A. cellulolyticus* and *B. cellulosolvens* dockerins with the cohesins. The *C. thermocellum* SdbA cohesin interacted with all three test dockerins.

3.4 Cohesin-dockerin interactions in *R. flavefaciens*

A variety of enzyme-borne dockerins and scaffoldin-borne cohesins has been discovered in the cellulosome-producing rumen bacterium, *R. flavefaciens*. The cohesins were thus far found on 4 different scaffoldins: ScaA, ScaB, ScaC, and ScaE, and the genes that encoded for the latter scaffoldins appeared in a gene cluster on the chromosome [54, 55]. The cohesins from ScaA, ScaB, and ScaE map together on a distinctive branch of the phylogenetic tree (Fig. 1) and were thus designated type-III. The *R. flavefaciens* ScaC cohesin appears on its own branch, in a position immediately adjacent to the three *A. cellulolyticus* ScaC cohesins and the type-I cohesins from other bacteria. The *R. flavefaciens* dockerins used to generate XynDoc fusion proteins for this study included the Cel44A enzyme-borne dockerin [56], and the dockerins of ScaA and ScaC [33, 35]. As in the type-II *C. thermocellum* CipA and *A. cellulolyticus* ScaA, the ScaB scaffoldin of *R. flavefaciens* harbors a C-terminal X module-dockerin dyad [54], although the sequences of its X module and dockerin components are both markedly different from those of the type-II XDoc dyads.

The specificity characteristics of the *R. flavefaciens* type-III cohesin-dockerin interactions are all very well defined and consistent with those reported in the literature (Fig. 6). ScaA cohesin 3 bound selectively to the dockerin of the enzyme Cel44A as well as to the ScaC dockerin; ScaB cohesins 1 and 6 interacted very strongly with the dockerin of ScaA; the ScaE cohesin was selective for the ScaB XDoc. The ScaC cohesin, however, failed to interact with any of the *R. flavefaciens* dockerins thus far described or any other dockerin in our collection. We are still searching for cellulosomal components that bear a dockerin, which will interact selectively with the *R. flavefaciens* ScaC cohesin.

3.5 Cohesin-dockerin interactions in *A. fulgidus*

Early bioinformatics-based work [37] served to detect cohesin and dockerin sequences in two consecutive ORFs of the sequenced genome of the archaeon *A. fulgidus*. One of the cohesin-containing ORFs also harbored a dockerin module, but the intraspecies specificity(ies) of the modules was never examined. In the present work, the individual *A. fulgidus* cohesin and dockerin modules were cloned in the appropriate plasmid cassettes to produce the respective CBM-Cohs and XynDoc constructs. The latter were expressed; the cohesins were immobilized on the cellulose-coated glass slides and thus included in the microarray study.

The microarray data revealed that the ORF 2375 dockerin of *A. fulgidus* interacted with both its own cohesin and the recombinant cohesin of the tandem ORF 2376, with a notable preference for the latter (Fig. 7). The dockerin failed to interact with any of the type-I, -II, or -III cohesin tested in this study. Likewise, neither of the *A. fulgidus* cohesins displayed any interaction with any of the dockerins examined in this study. It therefore appears that the intraspecies cohesin-dockerin recognition in *A. fulgidus* is very specific, although the biological rationale for its interaction with both the cohesin of its parent protein and a second protein remains a mystery.

4 Concluding remarks

In this article we have employed a microarray system designed to assess the specificity characteristics of large numbers of cohesins and dockerins, thereby providing a global view of the specificity patterns among members of two

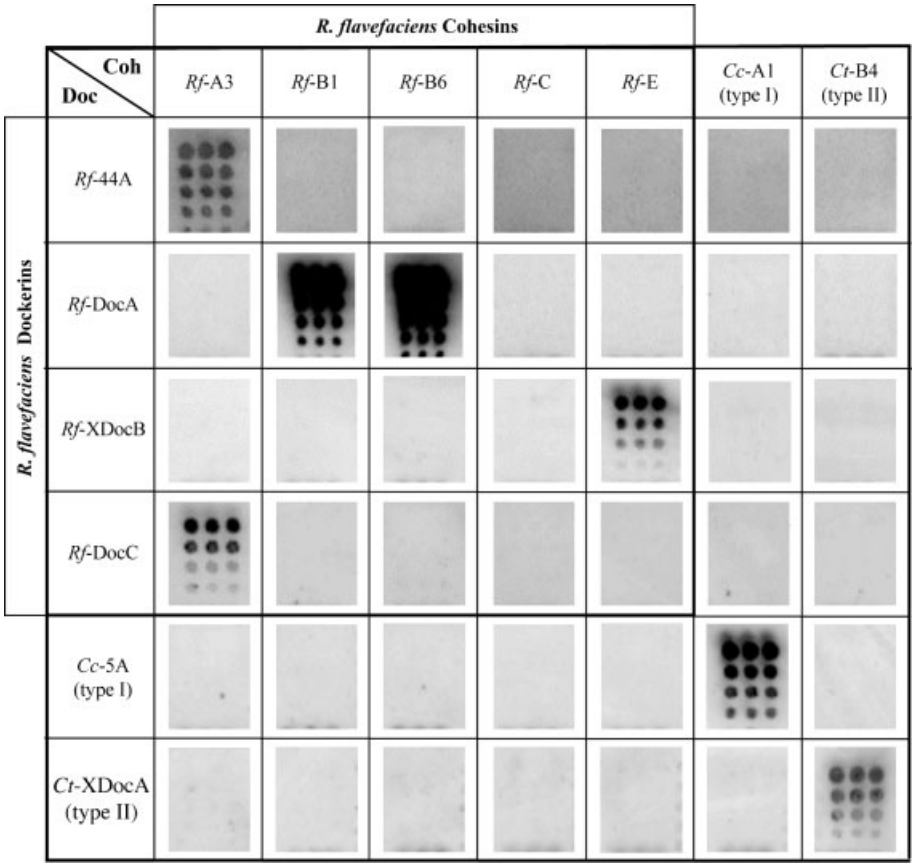


Figure 6. Specificity characteristics of cohesin-dockerin interactions in *R. flavefaciens* strain 17. Experiments were performed as described in the legend to Fig. 2, with the designated XynDoc fusion proteins. Four *R. flavefaciens* dockerins: the type-III *Rf*-44A, *Rf*-DocA, and *Rf*-XDocB dockerins, and the pseudo type-I *Rf*-DocC dockerin, were chosen for interaction with the printed cohesins. Shown are the interactions with five *R. flavefaciens* cohesins, derived from ScaA (*Rf*-A3), ScaB (*Rf*-B1 and *Rf*-B6), ScaC (*Rf*-C), and ScaE (*Rf*-E). Representative cohesins from type-I (*Cc*-A1) and type-II (*Ct*-B4) and type-I and type-II dockerins (*Cc*-5A and *Ct*-XDocA, respectively) are shown as controls.

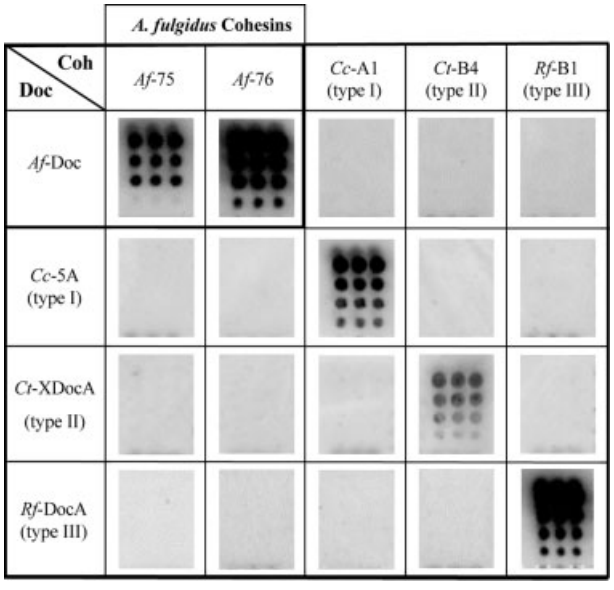


Figure 7. Selective intraspecies interaction of the lone *A. fulgidus* dockerin with the two cohesin modules. The experiment was conducted essentially as described in the legend to Fig. 2, using the XynDoc construct of the *A. fulgidus* dockerin. No interspecies interaction of the *A. fulgidus* dockerin or cohesins with any other type-I, -II, or -III cohesin or dockerin, respectively, was detected.

complementary families of interacting protein modules. The approach is a natural marriage of two recently designed approaches: an ELISA-based matching fusion-protein system for analysis of the cohesin-dockerin interaction [42] and the development of a CBM-based protein microarray [43]. In theory, the described microarray system provides a qualitative edge over the ELISA technique, since the interaction of the CBM with the cellulose surface serves to properly display the cohesin for subsequent interaction with the dockerin. The newly described methodology allows facile examination of the calcium-dependent interaction between a set of dockerins with vast numbers of cohesins. A method employing alternative immobilization and detection strategies was recently reported for identification of interacting proteins with members of the distantly related EF-hand-containing calcyclin (S100) family with a library of 80 recombinant proteins [57]. The cohesin-dockerin microarray system should provide an answer to the growing numbers of newly discovered cellulosomal and noncellulosomal cohesins and dockerins. Hundreds of different cohesins and dockerins are now known, and information regarding their specificity profiles will be required for future engineering of self-assembling designer cellulosome constructs [38–41, 58, 59]. The system will also be beneficial for analysis of cohesin and dockerin mutation libraries [46, 60–65]. All of the advantages

of the previously described approaches are inherent in the current cohesin-dockerin microarray system for an initial screening of the cohesin-dockerin interactions. In this context, slides containing hundreds or thousands of CBM-Cohs can be replicated concurrently, and the printed slides can be stored for extended time periods at room temperature pending subsequent interaction with the dockerins. Minute amounts of samples are required for fabricating the slides, and extensive isolation steps can be avoided. Several dozen slides can be examined *per* day by a single investigator, and the slides can be analyzed days or weeks after interaction, using conventional fluorescent scanners as opposed to specialized types of microarray scanners. Moreover, novel details concerning the specificity of the different types of cohesin-dockerin interactions, particularly those of type-II and the archaeal system, have been revealed in this work, thus establishing the sensitivity, reliability, and utility of the approach.

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