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Matching fusion protein systems for affinity analysis of two interacting families of proteins: the cohesin–dockerin interaction

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Cellulosomes are multi-enzyme complexes that orchestrate the efficient degradation of cellulose and related plant cell wall polysaccharides. The complex is maintained by the high-affinity protein–protein interaction between two complementary modules: the cohesin and the dockerin. In order to characterize the interaction between different cohesins and dockerins, we have developed matching fusion-protein systems, which harbor either the cohesin or the dockerin component. For this purpose, corresponding plasmid cassettes were designed, which encoded for the following carrier proteins: (i) a thermostable xylanase with an appended His-tag; and (ii) a highly stable cellulose-binding module (CBM). The resultant xylanase–dockerin and CBM–cohesin fusion products exhibited high expression levels of soluble protein. The expressed, affinity-purified proteins were extremely stable, and the functionality of the cohesin or dockerin component was retained. The fusion protein system was used to establish a sensitive and reliable, semi-quantitative enzyme-linked affinity assay for determining multiple samples of cohesin–dockerin interactions in microtiter plates. A variety of cohesin–dockerin systems, which had been examined previously using other methodologies, were revisited applying the affinity-based enzyme assay, the results of which served to verify the validity of the approach. Copyright © 2005 John Wiley & Sons, Ltd.

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INTRODUCTION

The cellulosome concept was initially described over two decades ago as a highly active cellulase complex, secreted by the Gram-positive, anaerobic, thermophilic, cellulolytic bacterium, *Clostridium thermocellum*, which mediates the efficient hydrolysis of cellulose and other plant cell wall polysaccharides (Bayer *et al.*, 1983; Lamed *et al.*, 1983a, b). The enzymatic subunits of the cellulosome are incorporated into a scaffolding protein (scaffoldin), which also promotes binding of the complex to the cellulose substrate (Poole *et al.*, 1992; Shoseyov *et al.*, 1992; Fujino *et al.*, 1993; Gerngross *et al.*, 1993). The *C. thermocellum* scaffoldin contains nine highly similar cohesin modules that recognize and bind tightly to complementary dockerin modules harbored by each of the catalytic subunits (Bayer *et al.*, 1994). Subsequent studies indicated the existence of cellulosomes in other cellulolytic bacteria, and distinctive cellulosomes were firmly established for *C. cellulovorans* (Doi and Tamura, 2001), *C. cellulolyticum* (Belaich *et al.*, 1997), *C. josui* (Kakiuchi *et al.*, 1998), *Acetivibrio cellulolyticus* (Ding *et al.*, 1999), *Bacteroides cellulosolvens* (Ding *et al.*, 2000), *Ruminococcus albus* (Ohara *et al.*, 2000) and *R. flavefaciens* (Kirby *et al.*, 1997).

The cohesin–dockerin pair represents a high-affinity, protein–protein interaction, in which calcium ions are critical to the interaction. The calcium dependence of the interaction has been attributed to the calcium-binding motif of the dockerin domain, whose sequence resembles the EF-hand motif of eukaryotic calcium-binding proteins, e.g. calmodulin and troponin C (Chauvaux *et al.*, 1990; Yaron *et al.*, 1995; Choi and Ljungdahl, 1996; Lytle *et al.*, 2000).

The cohesin–dockerin interaction tends to be non-specific within a given species but specific between species (Pagès *et al.*, 1997). Thus, a scaffoldin-borne cohesin of one species would be expected to recognize and bind to most or all of

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Abbreviations used: CBM, cellulose-binding module 3a derived from the *Ct* scaffoldin; *Cc, Clostridium cellulolyticum*; Coh, cohesin; *Ct, Clostridium thermocellum*; Doc, dockerin; cELIA, competitive enzyme-linked interaction assay; HRP, horseradish peroxidase; IPTG, isopropyl-1-thio-β-D-galactoside; MES, 2-(*N*-morpholino) ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate; SPR, surface-plasmon resonance; TBS, Tris-buffered saline; Xyn, xylanase.

the enzyme-borne dockerins from the same species, but not with the dockerins from a different species. However, this rule is based on the limited number of cohesin–dockerin pairs tested. The rule indeed appears to apply to several bacterial species, such as *C. thermocellum* and *C. cellulolyticum*, but recent evidence from other systems has indicated that in some systems the status of the cohesin–dockerin interaction may be much more intricate than originally considered (Salamitou *et al.*, 1994; Leibovitz and Béguin, 1996; Rincon *et al.*, 2003, 2004; Bayer *et al.*, 2004; Xu *et al.*, 2004). As hundreds of cohesins and dockerins have already been sequenced, many more cohesin–dockerin pairs should be tested in order to verify the generality of this rule. In addition, mutagenesis studies of cohesins and dockerins have been reported in the past few years. Rapid, efficient and reliable screening methods would thus be useful for assessing this high-affinity protein–protein interaction.

One of the major obstacles in comparing different types of cohesin–dockerin interaction is the production of purified recombinant proteins that include the individual modules. Efficient production of the native cohesin and dockerin constructs has been hindered by low expression levels, low solubility and degradation of the expressed proteins. This is particularly apparent with respect to attempts to produce free dockerins in the absence of the parent protein (Fierobe *et al.*, 1999; Lytle *et al.*, 2001; Adams *et al.*, 2005). In order to circumvent these difficulties, we have developed expression cassettes that encode two different carrier proteins: (i) a thermostable xylanase with an appended His-tag for expression and purification of the dockerin modules (Lapidot *et al.*, 1996); and (ii) a highly stable cellulose-binding module (CBM) for expression and purification of the cohesin modules (Morag *et al.*, 1995). Both of these complementary fusion proteins—the xylanase–dockerin (XynDoc) and the CBM–cohesin (CBM-Coh)—showed very high expression levels of soluble protein in *E. coli*. In addition, the matching fusion proteins facilitated standardization of the expression and purification of these proteins, thus enabling their rapid and reliable production. The expressed proteins were extremely stable, and the functionality of the cohesin or dockerin component was retained. The matching fusion protein systems were used to develop a semi-quantitative assay system for screening large numbers of cohesin- and dockerin-containing samples. The xylanase T6 and CBM fusion partners can be more generally employed to assess the interaction of other protein pairs unrelated to cohesins and dockerins.

MATERIALS AND METHODS

Materials

Restriction endonucleases were purchased from New England Biolabs Inc. (Beverly, MA, USA). Luria-Bertani Broth Lennox (LB) was obtained from BD Diagnostic Systems (Sparks, MD, USA). Isopropyl-1-thio-β-D-galactoside (IPTG) and protein molecular weight markers were products of Fermentas UAB (Vilnius, Lithuania). Antibiotics, phenylmethylsulfonyl fluoride (PMSF), benzamidine and benzamide were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Imidazole was obtained from Merck KGaA (Darmstadt, Germany). TMB + Substrate-Chromogen was purchased from Dako Corp. (Carpinteria, CA, USA). Ni-NTA agarose was a product of Qiagen GmbH (Hilden, Germany). Polyclonal antibodies against CBM and xylanase-T6 were prepared as described earlier (Morag *et al.*, 1995; Lapidot *et al.*, 1996). Secondary antibody-enzyme conjugate (HRP-labeled goat anti-rabbit IgG) was a product of Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA).

Bacterial strains and vectors

Escherichia coli strain XL1-Blue was obtained from Stratagene Co. (La Jolla, CA, USA). *E. coli* strain BL21 pLysS(λDE3) was used as a host for protein expression. Vectors pET9d and pET28a were obtained from Novagen Inc. (Madison, WI, USA).

Design of plasmid cassettes for expression of fusion proteins

XynDoc cassette. A construct of *G. stearothermophilus* xylanase T-6 with a His-tag and a *Bsp*HI site at the 5′-terminus and a *Kpn*I site at the 3′-terminus was produced using PCR (Lapidot *et al.*, 1996; Handelsman *et al.*, 2004a). This construct was ligated at the *Kpn*I site with the PCR product of a *C. thermocellum* CelS (Cel48A) dockerin (Wang *et al.*, 1993), containing a 5′-terminal *Kpn*I site and a 3′-terminal *Bam*HI site, and inserted into the pET9d vector at *Nco*I and *Bam*HI sites. This plasmid allows facile replacement of the CelS dockerin (termed DocS) with any other desired dockerin by digesting with *Kpn*I and *Bam*HI, and the resulting expressed product constitutes a His-tagged xylanase T-6 fusion protein bearing a dockerin at the C-terminus (termed in this case XynDocS).

CBM-Coh cassette. A PCR product containing the CBM3a from the *C. thermocellum* scaffoldin CipA (Morag *et al.*, 1995) was inserted into the pET28a vector at the *Nco*I and *Bam*HI sites, allowing insertion of any desired cohesin at sites between the *Bam*HI and *Xho*I of this vector, creating a CBM-Coh fusion protein (Handelsman *et al.*, 2004a).

Expression and purification of fusion proteins

XynDoc constructs. BL21(λDE3) pLysS cells were transformed with the desired pETXynDoc plasmid and plated on LB–kanamycin plates. Four milliliters of LB were added to the plate to resuspend cells; the resuspended cells were added to 500 ml LB, supplemented with 50μg/ml kanamycin and 2 mM $CaCl_2$. The culture was grown at 37°C to an $A_{600}\approx1$. 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 binding buffer, consisting of Tris-buffered saline (TBS, 137 mM NaCl, 2.7 mM KCl, 25 mM Tris–HCl, pH 7.4) 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 was then loaded onto an Ni-NTA column, equilibrated with binding buffer, and purified using an AKTA-prime System (Amersham Pharmacia Biotech). The column was washed with 3 column volumes of binding buffer followed by a similar amount of binding buffer supplemented with 20 mM imidazole. The recombinant protein was eluted from the column using a linear gradient (25 mM to 1 M) of imidazole in TBS supplemented with protease-inhibitor cocktail, and fractions were analyzed by SDS–PAGE. The fractions containing pure recombinant protein were pooled, and $CaCl_2$ (10 mM final concentration) was added. The proteins were stored in 50% (v/v) glycerol at −20°C.

CBM-Coh constructs. BL21 (λDE3) pLysS cells were transformed with the desired pETCBMCoh plasmid and treated as above for cells transformed with the pETXynDoc plasmid, except that the kanamycin-supplemented medium had no added $CaCl_2$, and, following induction, cells were incubated for 3 h at 37°C. When cohesins from thermophilic bacteria were used, a heat-treatment step (30 min, 60°C) was included before centrifugation. Fifteen milliliters of amorphous cellulose were then added to the supernatant and incubated for 1–2 h on a rotator at 4°C. The amorphous cellulose was pelleted by centrifugation (5 min, 4000 rpm, 4°C). The pellet was washed three times with 45 ml TBS, containing 1 M NaCl and three times with 45 ml TBS. The protein was eluted from the pellet with 2 vol (12 ml each) of 1% (v/v) triethylamine, and the eluted fractions were neutralized with 0.6–1.2 ml of 1 M MES at pH 5.5. The exposure of the cohesin construct to triethylamine does not impair its subsequent binding to dockerin. Purity was assessed by SDS–PAGE, and proteins were stored in 50% (v/v) glycerol at −20°C.

Protein concentration

The concentration of the purified proteins was estimated by absorbance (280 nm) based on the known amino acid composition of the desired protein using the Protparam tool (www.expasy.org/tools/protparam.html) on the EXPASY Server (Gasteiger *et al.*, 2005). The extinction coefficients at 280 nm for the different type-I CBM-Coh constructs were typically between 36 000 and 40 000 M^{-1} cm^{-1} and higher values (up to ~60 000 $M^{-1}cm^{-1}$ were calculated for the type-II CBM-Coh constructs, owing to the presence of Trp residues in the type II cohesins, lacking in their type I analogs. Specifically, for the 342-residue type I *C. thermocellum* and 344-residue *C. cellulolyticum* CBM-Coh constructs (Coh-*Ct* and Coh-*Cc*, respectively), $\varepsilon_{280\,nm} = 40\,800$ and 36 960 M^{-1} cm^{-1}. Those of the XynDoc constructs were generally around 80 000 M^{-1} cm^{-1}. For the 462-residue *C. thermocellum* XynDocS construct $\varepsilon_{280\,nm} = 83\,210\,M^{-1}$ cm^{-1}, and for the 454 residue *C. cellulolyticum* XynDocA construct $\varepsilon_{280\,nm} = 84\,490\,M^{-1}$ cm^{-1}.

Affinity-based ELISA protocols

Analysis of divergent dockerins using immobilized cohesins. MaxiSorp ELISA plates (Nunc A/S, Roskilde, Denmark) were coated overnight at 4°C with predetermined concentrations (designated below) of the desired CBM-Coh (100 µl/well) in 0.1 M sodium carbonate (pH 9). The following steps were performed at room temperature with all reagents at a volume of 100 µl/well. The coating solution was discarded and blocking buffer (TBS, 10 mM $CaCl_2$, 0.05% Tween 20, 2% BSA) was added (1 h incubation). The blocking buffer was discarded, and incremental concentrations of the desired XynDoc constructs, diluted in blocking buffer, were added. After a 1 h incubation period, the plates were washed three times with wash buffer (blocking buffer without BSA), and the primary antibody preparation (rabbit anti-xylanase T-6 antibody, diluted 1:10 000 in blocking buffer) was added. Following another 1 h incubation period, the plates were washed three times with wash buffer and the secondary antibody preparation (HRP-labeled anti-rabbit antibody diluted 1:10 000 in blocking buffer) was added. After another 1 h incubation, the plates were again washed (four times) with wash buffer and 100 µl/well TMB + Substrate-Chromogen were added. Color formation was terminated upon addition of 1 M H_2SO_4 (50 µl/well), and the absorbance was measured at 450 nm using a tunable microplate reader.

Absorbance was plotted as a function of XynDoc concentration, usually resulting in a sigmoidal (dose–response) curve. For presentation of results in bar-graph form, two alternative and complementary methods were used: (i) the pEC_{50} was determined for the binding curve of the test XynDoc constructs (Motulsky and Christopoulos, 2003) and compared with that of the XynDoc standard; and (ii) the 'reference concentration' of a XynDoc standard that generates a maximum response was employed for comparison of the level of response produced by other test XynDoc constructs at that concentration. In the latter case, the data can be normalized as a percentage (relative binding) of maximum response by the reference XynDoc. The two methods produced very similar results.

Analysis of divergent cohesins using immobilized dockerins. The assay for divergent cohesins was essentially the converse of that described above for the divergent dockerins: instead of immobilizing a cohesin construct, a suitable dockerin construct was substituted. The following modifications were then introduced: coating was performed with 20 nM of the desired XynDoc construct, the desired CBM-Coh(s) was diluted to concentrations of 10 pM to 10 nM, and rabbit anti-CBM antibody (diluted 1:10 000) was used as the primary antibody preparation. Subsequent steps were performed as described in the previous section.

RESULTS AND DISCUSSION

In order to validate the affinity-based ELISA approach with previously published reports, we developed corresponding CBM-containing cohesin constructs and examined their relative interaction with the appropriate

XynDoc construct as part of a general fusion protein strategy for characterizing problematic types of protein–protein interactions.

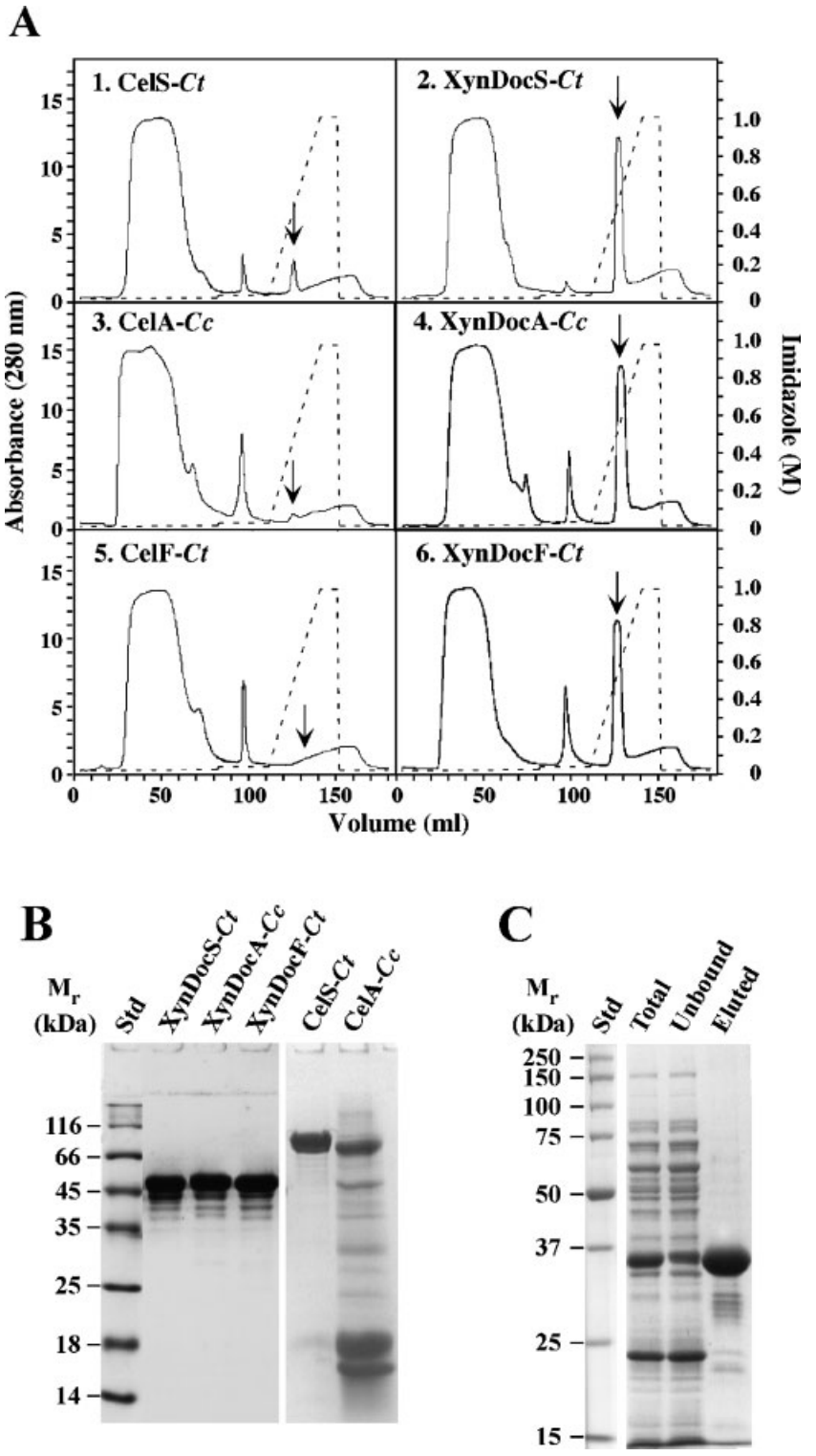
Preparation and purification of fusion proteins

XynDoc fusion proteins. In previous affinity experiments with dockerins, the expressed protein used for such studies has usually included either truncated or complete parent enzyme constructs that harbor the desired dockerin domain. Alternative attempts to produce the dockerin domain alone (without the catalytic domain) have proved problematic, since the dockerin is usually unstable in solution. Moreover, only very limited quantities of the recombinant dockerin or dockerin-containing protein are usually produced. For these reasons, we decided to establish a 'fail-safe' model system, which involved the design of a 'universal' carrier protein for the dockerin. For this purpose, we chose xylanase T6 from *Geobacillus stearothermophilus*, which is known for its exceptionally high propensity towards expression in *E. coli* host cell systems (Lapidot *et al.*, 1996; Mechaly *et al.*, 2000a). *G. stearothermophilus* does not produce a cellulosome and the native xylanase-T6 does not bear a dockerin. Nevertheless, we were able to purify xylanase-dockerin fusion proteins (XynDoc) at reasonably high levels of *purified* protein (>150 mg/l of cell culture) at a final storage concentration typically between 3 and 6 mg/ml after dilution in 50% glycerol. Another desirable feature of this enzyme is its high thermostability, which facilitates its isolation and resistance to denaturation. Xylanase T6 was His-tagged to allow purification of the dockerin-containing fusion protein by using Ni-NTA column chromatography [Fig. 1(A)].

In order to establish the validity of this approach, we compared the preparation and purification of various XynDoc constructs with those of three different recombinant wild-type dockerin-containing cellulase preparations: the recombinant wild-type *C. thermocellum* (CelS-*Ct*), the CelF enzyme from the same bacterium (CelF-*Ct*), and the CelA enzyme from a related cellulosome-producing bacterium, *C. cellulolyticum* (CelA-*Cc*). The corresponding XynDoc fusion proteins using the dockerins of the three latter enzymes were also prepared and purified. All constructs (both the wild-type enzymes and XynDoc fusion proteins) were designed to contain an N-terminal His-tag, and the preparation and purification of all proteins followed an identical protocol. As is evident from Fig. 1, the different wild-type recombinant cellulases show great variation in their purification. While we achieved moderate amounts of highly purified CelS-*Ct* (3.5 mg/l of cell culture), the purification of CelA-*Cc* resulted in much lower amounts of impure protein while in the case CelF-*Ct* virtually no His-tagged protein could be purified [Fig. 1(B)]. The advantages of the Xyn-Doc fusion protein approach can be clearly seen in their purification. Comparatively large amounts of purified protein were consistently produced, irrespective of the source of dockerin. As dockerin sequences are highly conserved and represent a comparatively small portion of the XynDoc chimaera, they can usually be exchanged with little effect on the amount and purity of the resulting chimaera. The Xyn-Doc system has thus allowed us to generate a standard protocol for the preparation and purification of large amounts of soluble, stable dockerins.

CBM-Coh fusion proteins. The cohesins are much more stable and easier to purify than the dockerins. Nevertheless, wide variations in purity and/or yield have been observed for His-tagged cohesins derived from different sources (unpublished observations). Consequently, we searched for an alternative approach that would both provide a standard, rapid purification procedure for the cohesins and be appropriate for use in the affinity-based ELISA approach described in this work. For this purpose, desired cohesins were fused to the scaffoldin-based CBM3a of *C. thermocellum* (Morag *et al.*, 1995). The resultant hybrid proteins were amenable for affinity-purification on cellulose [Fig. 1(C)]. SDS–PAGE of the eluted CBM-Coh fusion proteins showed a purified product consistent with the calculated molecular weight (36 538 for Coh-*Ct*). This purification procedure could be accomplished by batch adsorption to and desorption from cellulose matrices, allowing simultaneous isolation of several different proteins. This approach resulted in highly purified proteins in relatively large yields (>35 mg/l of cell culture), frequently approaching those of the Xyn-Doc fusion proteins, albeit at much lower final storage concentrations (typically 0.2–0.4 mg/ml). In addition, the CBM is a particularly logical carrier protein for cohesins, since it occurs adjacent to cohesins in its native state as a component of the *C. thermocellum* scaffoldin. Moreover, as a modular component of a thermophilic bacterial protein, the CBM is highly stable to different conditions (e.g. heat, salt, solvents, chemicals, pH, etc.) and has been used previously to stabilize neighboring fusion protein partners (Berdichevsky *et al.*, 1999).

Figure 1. Purification of dockerin- and cohesin-bearing fusion proteins. (A) Comparative purification of XynDoc fusion proteins with that of the recombinant wild-type cellulases. Three different His-tagged wild-type cellulase constructs—CelS and CelF from *C. thermocellum* (CelS-*Ct*, CelF-*Ct*) and CelA from *C. cellulolyticum* (CelA-*Cc*)—and the XynDoc chimeras of these three cellulases were similarly expressed in an *E. coli* host cell system and subjected to purification on a Ni-NTA column, using an AKTA-prime System. The solid line shows absorbance at 280 nm. The dashed line shows imidazole concentration. The relevant peak, representing the purified protein, is indicated by an arrow. (B) SDS–PAGE separation of purified XynDoc fusion proteins. The purified fractions shown in (A) were pooled, and similar amounts of the purified proteins were subjected to SDS–PAGE on 10% gels. The eluted XynDoc constructs were remarkably similar in their purity, and their mobility on the gels were consistent with their calculated molecular size (~53 000). The recombinant wild-type CelS-*Ct* appeared as a major purified protein band, at a position consistent with its calculated molecular weight (81 884) but the alleged CelA-*Cc* product was impure, and only a minor band was consistent with its calculated molecular weight (51 793). Std, molecular weight standards. (C) Batchwise purification of an expressed CBM-Coh fusion protein. CBM-borne cohesin_2, derived from *C. thermocellum* CipA scaffoldin (Coh-*Ct*, representative of CBM-Coh fusion proteins) was expressed in the *E. coli* host-cell system. The cells were sonicated, the soluble proteins were applied batchwise to cellulose resin, and the adsorbed proteins were eluted with 1% triethylamine. Total protein (the cell sonicate prior to application to the cellulose resin) and the unbound (proteins that failed to bind to cellulose) and eluted fractions were subjected to SDS–PAGE on 10% gels.



Affinity-based ELISAs: immobilized CBM-Coh vs immobilized XynDoc

In order to analyze the specificity of divergent dockerin domains, CBM-containing target cohesins were adsorbed to microtiter plates, and the desired dockerin-borne fusion proteins (Xyn-Doc) were applied. The amount of interacting dockerin was then determined immunochemically using anti-xylanase primary antibody and HRP-labeled secondary antibody. Alternatively, xylanase activity can be measured directly using an appropriate substrate (e.g. *p*-nitrophenyl derivatives of xylobiose or cellobiose), as reported previously (Handelsman *et al.*, 2004a, b). The ELISA-based assay system used in the present study provides increased sensitivity and the capacity to screen larger numbers of cohesin–dockerin pairs.

Dockerin domains from two representative cellulosomal enzymes of *C. thermocellum* and *C. cellulolyticum* (CelS and CelA, respectively) were subjected to the affinity-based ELISA procedure using the corresponding immobilized cohesins [(Fig . 2(A) and (B)]. The dockerins from these two enzymes are known to interact with their respective cohesins in a species-specific manner. Indeed, under the conditions of the assay, the *C. thermocellum* cohesin interacted exclusively with the *C. thermocellum* dockerin [Fig. 2(A)] and vice versa [Fig. 2(B)]. The half-maximal response for the *C. thermocellum* interaction was about 6 pM as opposed to about 20 pM for the *C. cellulolyticum* interaction, reflecting the differences in affinities for these two species of cohesin–dockerin interactions.

In some cases, it appeared worthwhile to immobilize the dockerin components for examination of cohesin samples, such as for comparing the interaction of cohesin mutants for a given dockerin module. We thus tested the efficacy of adsorbing XynDoc constructs to microtiter plates and assayed their interaction with model CBM-Coh. The amount of interacting cohesin was then determined using an anti-CBM antibody and HRP-labeled secondary antibody. The same representative cohesin and dockerin constructs derived from *C. thermocellum* and *C. cellulolyticum* were again employed as model components for analysis of the dockerin-immobilized protocol [Fig. 2(C) and (D)]. As expected, under the conditions of the assay and in accordance with the results obtained for the immobilized cohesin component, the interaction between the immobilized dockerin and solution-state cohesin was species-specific. However, the effective response of this system was 20- to 30-fold lower than that of the immobilized cohesins. In this context, the structural continuity or accessibility to solvent of the relatively small dockerin component may be impaired, following immobilization of the XynDoc construct to the microtiter plate, owing to its dynamic conformation and hydrophobic character. Thus, immobilization of the relatively stable cohesin construct is preferred, particularly in cases of relatively low-affinity cohesin–dockerin interactions.

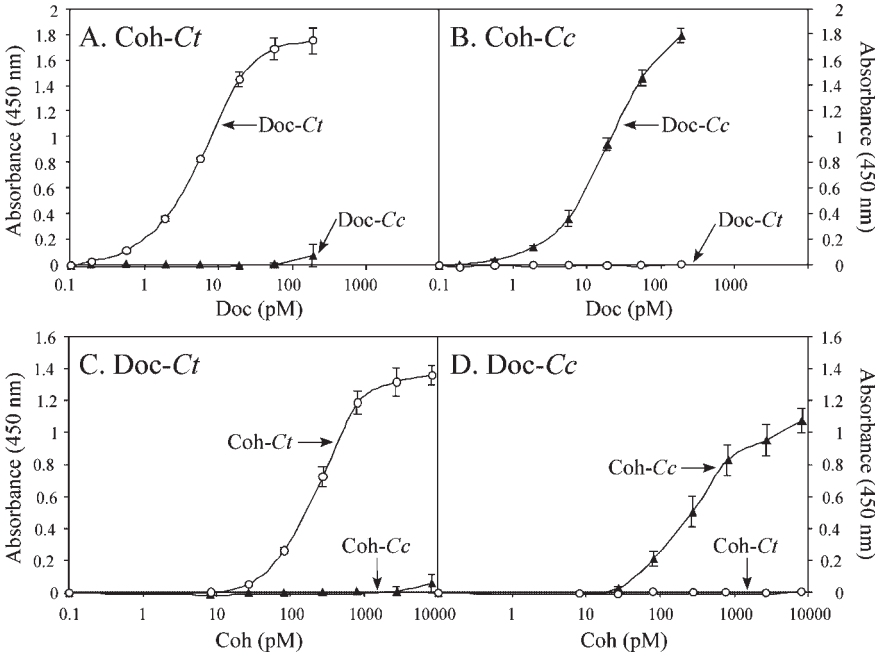


Figure 2. Affinity-based ELISA of divergent species of dockerins and cohesins: consequence of the immobilized component. (A and B) Assay of divergent dockerins on immobilized cohesins. (A) Immobilized Coh-*Ct*: ELISA plates were coated with the CBM-borne cohesin_2 from *C. thermocellum* at a concentration of 3 nM. Dockerin-containing xylanase-T6 fusion proteins, derived from either *C. thermocellum* CelS (Doc-*Ct*: ○) or *C. cellulolyticum* CelA (Doc-*Cc*: ▲) were added at incremented concentrations, and the resultant cohesin–dockerin interaction was detected using anti-xylanase primary antibody and HRP-labeled secondary antibody preparations. (B) Immobilized Coh-*Cc*: the ELISA assay was performed as in (A) except that the plates were coated with the CBM-borne cohesin_1 from *C. cellulolyticum* instead of Coh-*Ct*. (C and D) Assay of divergent cohesins on immobilized dockerins. (C) Immobilized Doc-*Ct*: ELISA plates were coated with the dockerin-containing xylanase-T6 fusion protein, derived from *C. thermocellum* CelS at a concentration of 20 nM. The appropriate CBM-borne cohesins from either *C. thermocellum* (Coh-*Ct*: ○) or *C. cellulolyticum* (Coh-*Cc*: ▲) were added at incremental concentrations, and the resultant cohesin-dockerin interaction was detected using anti-CBM primary antibody and HRP-labeled secondary antibody preparations. (D) Immobilized Doc-*Cc*: the ELISA assay was performed as in (C) except that the plates were coated with the dockerin-containing xylanase-T6 fusion protein, derived from *C. cellulolyticum* CelA instead of Doc-*Ct*.

Optimization of the immobilized component

The amount of CBM-borne cohesin to be used for coating the microtiter plates proved critical to the performance of the assay, and the reliability of the assay benefited from its optimization. In order to examine this phenomenon, solutions containing various concentrations of *C. thermocellum* or *C. cellulolyticum* cohesin constructs were used for adsorption to the microtiter plates and their interaction with the two species of XynDoc constructs (Fig. 3). In the case of the immobilized *C. thermocellum* cohesin, the specificity for its own dockerin was unconditional for all concentration ranges examined (1–300 nM in the coating solution). Even at the lowest concentrations tested, near-maximum signals were observed for the *C. thermocellum* cohesin–dockerin interaction. In contrast, the *C. cellulolyticum* cohesin was specific for its own dockerin at relatively low coating concentrations (<3 nM), above which cross-species reactivity was observed (Fig. 3). As for *C. thermocellum*, near-maximum signals were observed for the *C. cellulolyticum* interaction at low coating concentrations with little or no recognition of the rival dockerin. The optimum coating concentration for both species of cohesin was thus determined to be 3 nM (100 µl/well). Similar studies for the dockerin-immobilized protocol (not shown) determined that the optimal dockerin concentration required for coating was 20 nM.

Compatibility of affinity-based ELISA with previous approaches

In order to verify that the affinity-based ELISA system developed in this communication is in line with previous approaches, we revisited several previously examined cohesin–dockerin systems. These include: (i) the interaction of dockerins derived from different *C. thermocellum* enzymes; (ii) the interaction of mutated dockerins with two rival species of wild-type cohesins; (iii) the interaction of different *C. cellulolyticum* cohesins with its own dockerin; and (iv) the type-II cohesin–dockerin interaction in *C. thermocellum*. The results of the latter experiments will be described in the following sections.

Interaction of different *C. thermocellum* enzyme-borne dockerins. Previous work on dockerin specificity, mainly carried out using dockerins from *C. thermocellum* cellulase CelS and *C. cellulolyticum* CelA, has indicated a limited number of specificity residues suspected to govern their species-specific interaction with the cohesins (Pagès *et al.*, 1997; Mechaly *et al.*, 2000b, 2001a, b). Recent biochemical evidence (Jindou *et al.*, 2004b) has shown that, although species specificity was generally conserved among the dockerins derived from two different species (i.e. *C. thermocellum* and *C. josui*), there was at least one exception to the rule: one of the *C. thermocellum* dockerins, derived from xylanase XynA, recognized cohesins from both species. Since *C. josui* and *C. cellulolyticum* are very closely related species, and their dockerin and cohesin sequences are remarkably alike, it was of interest to investigate whether our methodology could identify a similar phenomenon among the homologous enzyme-borne dockerins with the *C. cellulolyticum* cohesin.

For this purpose, appropriate XynDoc constructs using dockerins from selected *C. thermocellum* cellulases and xylanases were produced (derived from cellulases Cel48S, Cel5O and Cel9F and xylanases Xyn10Z, Xyn11A and Xyn11B). As expected, all tested dockerins interacted similarly with the *C. thermocellum* cohesin and most of these representative dockerins failed to interact with the

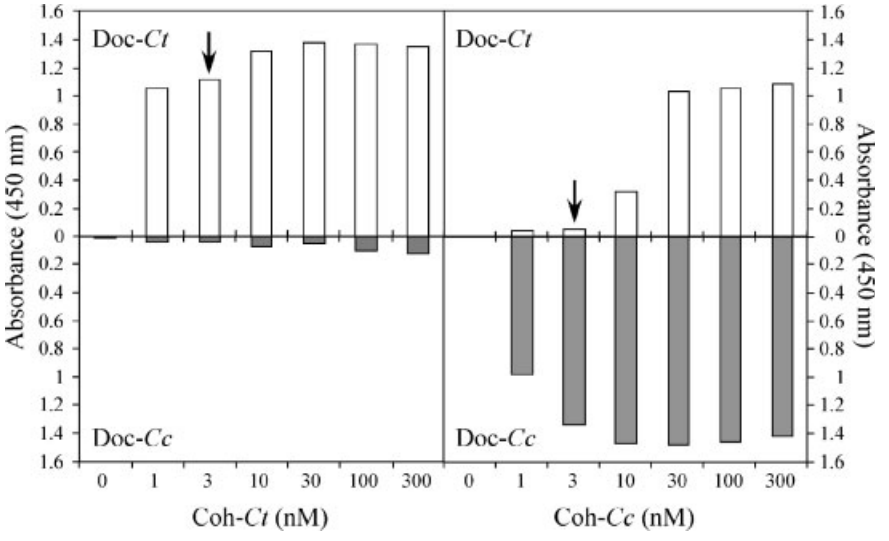


Figure 3. Optimization of cohesin concentration used for coating the microtiter plates. ELISA plates were coated with incremented concentrations of CBM-containing cohesins derived from either *C. thermocellum* or *C. cellulolyticum* (Coh-*Ct* and Coh-*Cc,* respectively). Dockerin-borne xylanase-T6 fusion proteins derived from *C. thermocellum* (Doc-*Ct*: white bars) or *C. cellulolyticum* (Doc-*Cc*: gray bars) were examined at a reference concentrations of 0.5 nM, and the resultant cohesin–dockerin interaction was detected using anti-xylanase primary antibody and HRP-labeled secondary antibody. Absorbance was measured at 450 nm. The optimum conditions for coating concentration (arrows) were chosen to promote intra-species fidelity.

C. cellulolyticum cohesin (Fig. 4). However, in line with the results of Jindou *et al.* (2004b), xylanase 11A (as well as its paralog Xyn11B, shown here for the first time) interacted significantly with the *C. cellulolyticum* cohesin.

Mutant dockerins of a *C. thermocellum* cellulosomal enzyme. In previous work (Mechaly *et al.*, 2000b, 2001a, b; Schaeffer *et al.*, 2002), the involvement of several key dockerin residues, located within the duplicated segments of the dockerin sequence, was demonstrated. In the present study, we re-examined a set of CelS mutants to assess their relative affinity for the *C. thermocellum* cohesin vs that of *C. cellulolyticum*. In the earlier study, the mutants consisted of the wild-type recombinant CelS enzyme, modified at desired positions in the dockerin module (see Table II of Mechaly *et al.*, 2001a), whereas in the present work, the corresponding XynDoc fusion protein was mutated accordingly in the dockerin module at the equivalent positions (Table 1). The results (Fig. 5) showed a similar graded effect in that the mutated dockerins exhibited intermediate levels of recognition of both species of cohesin, as reported earlier using affinity blotting and SPR (Fierobe *et al.*, 1999; Mechaly *et al.*, 2000b; Schaeffer *et al.*, 2002). As in the previous work, mutant m8, in which eight residues were swapped, showed a preference for the rival cohesin.

Table 1. Mutations of potential specificity residues in the dockerin module of *C. thermocellum* CelS[a]

	Duplicated segment 1				Duplicated segment 2			
	Positions				Positions			
Sample	10	11	18	22	10′	11′	18′	22′
Doc-*Ct*	S	T	R	R	S	T	R	K
m1	A							
m2		L						
m3	A	L						
m6	A	L	K		A	L	K	
m8	A	L	K	G	A	L	K	G
Doc-*Cc*	A	L	K	A	A	F	K	G

[a] The designated residues at the given positions of the *C. thermocellum* CelS dockerin (Doc-*Ct*) were swapped to match or emulate those of its rival wild-type dockerin of *C. cellulolyticum* CelA (Doc-*Cc*). Blank spaces indicate no mutation at the designated residue.

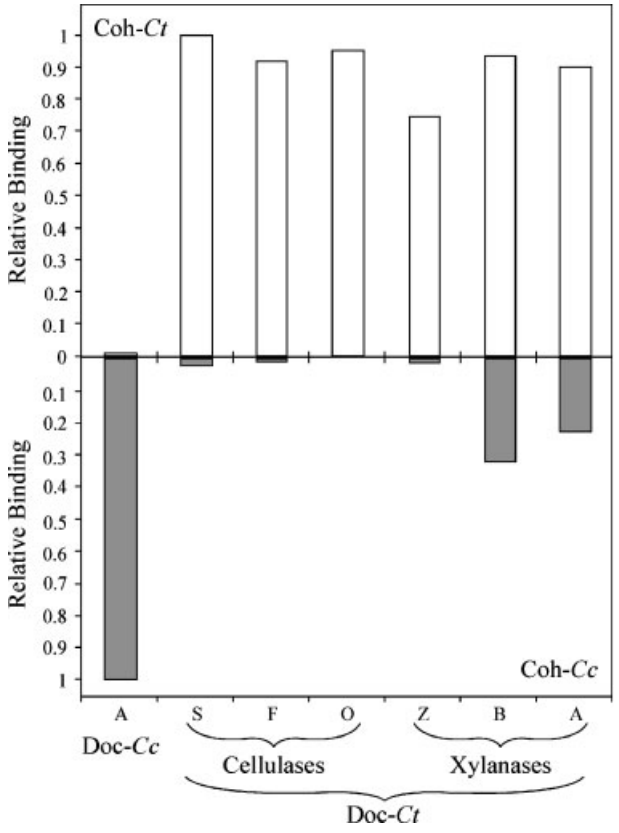


Figure 4. Specificity of *C. thermocellum* dockerins. Affinity-based ELISA standards were carried out using immobilized CBM-Coh constructs from *C. thermocellum* (white bars) or *C. cellulolyticum* (gray bars). The *C. thermocellum* and *C. cellulolyticum* dockerin fusion proteins (Doc-*Ct* and Doc-*Cc*) used in Figs 2 and 3 were again used here as standards. Additional XynDoc fusion proteins were also produced, comprising the dockerins from *C. thermocellum* Cel5O (O), Cel9F (F), Xyn10Z (Z), XynB (B) and XynA (A). XynDoc constructs were examined at a reference concentration of 0.2 nM.

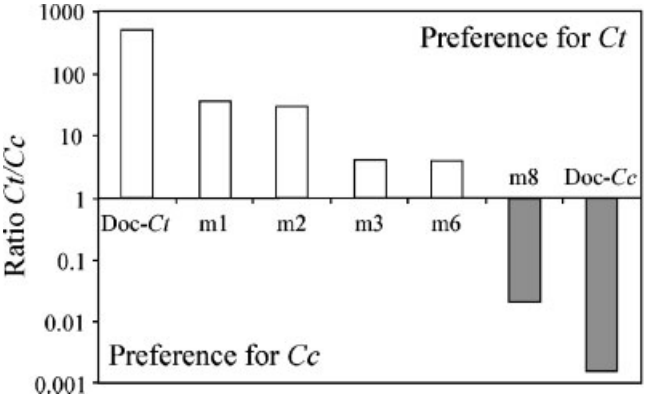


Figure 5. Specificity of mutant *C. thermocellum* dockerins. Affinity-based ELISA assays were carried out using XynDoc fusion proteins bearing the wild-type dockerins from *C. thermocellum* and *C. cellulolyticum* (Doc-*Ct* and Doc-*Cc*) as defined in the legends to Figs 2 and 3. The test dockerins comprised various mutations of the native dockerin derived from the *C. thermocellum* CelS (Doc-*Ct*), in which suspected recognition residues were swapped with homologous residues of the rival species (Doc-*Cc*). See Table 1 for description of mutants. XynDoc constructs were examined at a reference concentration of 0.2 nM.

Interaction of different *C. cellulolyticum* cohesins. Previous studies on the cohesins of the *C. cellulolyticum* CipC scaffoldin (Pagès *et al.*, 1999) have shown that its most divergent cohesins (Coh1 and Coh8) interact similarly with the *C. cellulolyticum* dockerin. The latter studies also described another more divergent cohesin, termed CohX, derived from the product of another gene in the cellulosome gene cluster, i.e. *orfX*. In this case, CohX displayed a much weaker (almost 20-fold) interaction with the *C. cellulolyticum* dockerin as measured by surface plasmon resonance

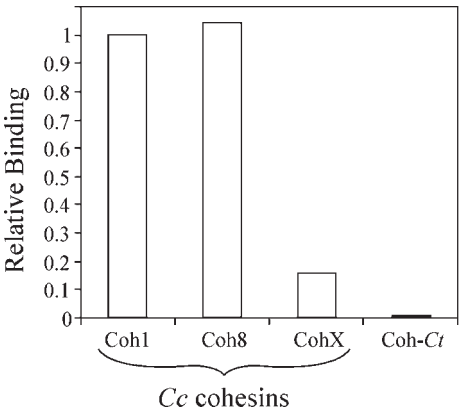


Figure 6. Specificity of *C. cellulolyticum* cohesins. The interaction of three different *C. cellulolyticum* CBM-Coh fusion proteins: cohesins 1 and 8 from scaffoldin CipC and the sole cohesin from OrfXp were examined using the XynDoc construct derived from *C. cellulolyticum* Cel5A. The results were standardized against the Coh1 construct. A *C. thermocellum* cohesin construct was used as a negative control. ELISA plates were coated with the cohesins at a concentration of 30 nM, and XynDoc constructs were examined at a reference concentration of 0.2 nM.

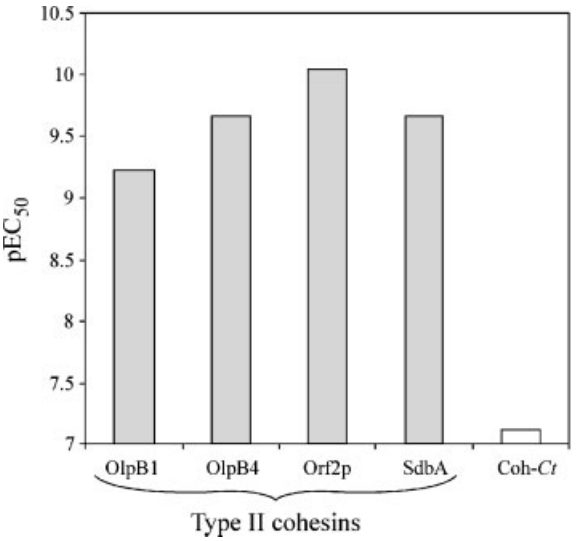


Figure 7. Type II cohesin–dockerin interaction. *C. thermocellum* type II CBM-Coh fusion proteins, containing cohesins 1 and 4 from OlpB, cohesin 2 from Orf2p and the lone cohesin of SdbA, were tested for their interaction with the XynDoc construct, comprising the type II dockerin from the *C. thermocellum* CipA scaffoldin (gray bars). A type I *C. thermocellum* cohesin construct (Coh-*Ct*) was used as a negative control (white bar). ELISA plates were coated with the CBM-appended cohesins at a concentration of 30 nM, and the scaffoldin-derived XynDoc construct was examined at different concentrations as described in the legend to Fig. 2. The pEC_{50} was determined as described by Motulsky and Christopoulos (2003).

(SPR). It was thus of interest to examine the relative interaction between the corresponding CBM-containing cohesin constructs with the appropriate XynDoc construct using the affinity-based ELISA analysis. Indeed, the interaction between the matching fusion proteins mirrored the SPR results reported earlier (Fig. 6). The relative binding of Coh1 and Coh8 for Doc-*Cc* were very similar, but that of CohX was reduced by about one order of magnitude, in accord with the SPR results.

Type II cohesin–dockerin interactions. Cohesins and dockerins can be divided by sequence similarity into different types (i.e. type I, type II, type III, etc.) on the basis of sequence alignment and their mapping on the respective phylogenetic tree (Bayer *et al.*, 2004). Most of the previous analyses of the cohesin–dockerin interactions have focused on those of type I. Only a few reports have appeared in which the type II interaction has been studied (Salamitou *et al.*, 1994; Lemaire *et al.*, 1995; Leibovitz and Béguin, 1996; Schaeffer *et al.*, 2002; Jindou *et al.*, 2004a). Type II cohesins have been described for three anchoring proteins of *C. thermocellum*, SdbA, Orf2p and OlpB, which bear 1, 2 and 4 type II cohesins, respectively. Cohesin components of SdbA and OlpB were shown originally, on the basis of radiolabeling experiments (Leibovitz and Béguin, 1996), to bind to the type II dockerin of the primary scaffoldin CipA. More recently, the type II interaction between the SdbA cohesin and scaffoldin-derived dockerin was quantified using SPR (Jindou *et al.*, 2004a).

The affinity-based ELISA approach was used to extend the latter studies and in doing so to further examine the validity of the approach. In this regard, various *C. thermocellum* type II cohesins were produced as CBM-borne fusion proteins to be examined against the XynDoc construct derived from the *C. thermocellum* CipA dockerin. The test cohesins included OlpB cohesins 1 and 4, Orf2p cohesin 2 and the SdbA cohesin. As a negative control we used the type I cohesin 2 from the *C. thermocellum* CipA scaffoldin. As expected, all four type II cohesins tested interacted similarly with the CipA dockerin, whereas the type I cohesin did not (Fig. 7).

FUTURE PERSPECTIVES

Protein–protein interactions are responsible for a wide variety of biological processes. The cohesin–dockerin couple represents the interaction between two complementary families of protein modules that exhibit divergent specificities and affinities, ranging from one of the highest known affinity constants ($K_a > 10^{11}$ M^{-1}) between two proteins to relatively low-affinity interactions.

The increasing numbers of divergent cohesins and dockerins that have emerged from newly sequenced bacterial and archaeal genomes warrant new tools for the analysis of their specificities and function. The structural relationship of the dockerin module is of special interest, as a primordial form of the calcium-binding EF-hand motif of eukaryotic calcium-dependent protein–protein interactions. Indeed, the dockerins may be a variant of a more general structural context for calcium-binding proteins (Rigden and Galperin, 2004). In fact, several of the newly sequenced bacterial genomes (e.g. *Rhodopirellula baltica, Clostridium perfringens, Bacillus cereus*, etc.) possess genes that encode for proteins that bear a dockerin-like module, although genes encoding for cohesin-like sequences are intrinsically more difficult to identify. Several of the parent bacteria are clearly

non-cellulosomal, since relevant glycoside hydrolase enzymes are lacking.

The matching fusion protein systems described in this communication were used to establish an ELISA-like affinity assay for the cohesin–dockerin interaction. The same XynDoc and CBM-Coh components can also be used for competitive enzyme-linked assays, protein microarrays, surface plasmon resonance, isothermal titration calorimetry, etc. Moreover, the system can be extended, since the hyperstable xylanase T6 and CBM carrier proteins can be employed as complementary fusion partners to assess the interaction of other unrelated protein pairs.

Our current library of CBM-Coh and XynDoc constructs includes 28 cohesins from 16 different scaffoldins and 35 dockerins. The latter cohesins and dockerins were derived from six different species of bacteria and archaea. Our library is designed as a database for future proteomics characterization of the binding specificity of newly discovered cohesins and dockerins.

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