Post-translational processing of modular xylanases from *Streptomyces* is dependent on the carbohydrate-binding module.

Margarita Díaz[1*], José M. Fernández-Ábalos[1], Juan Soliveri[2], José L. Copa-Patiño[2], and Ramón Santamaría[1]

[1]Instituto de Microbiología Bioquímica/Departamento de Microbiología y Genética. CSIC/Universidad de Salamanca, Salamanca, Spain.

[2]Departamento de Microbiología y Parasitología, Facultad de Farmacia, Universidad de Alcalá, Alcalá de Henares, Spain.

[1*]Corresponding author:

M. Díaz. Instituto de Microbiología Bioquímica /Departamento de Microbiología y Genética. CSIC/Universidad de Salamanca, Edificio Departamental. Campus Miguel de Unamuno. 37007-Salamanca, Spain. Email: mardi@usal.es; Telephone: +34-923-294732; Fax: +34-923-224876;

Abstract

Xylanases are very often modular enzymes composed of one or more catalytic domains and a carbohydrate-binding module connected by a flexible linker region. Usually, when these proteins are processed they lose their carbohydrate-binding capacity.

Here, the role of the linker regions and cellulose- or xylan-binding domains in the processing of Xys1L from *Streptomyces halstedii* JM8 and Xyl30L from *Streptomyces avermitilis* UAH30 was studied. Xys1 variants with different linker lengths were tested, these being unable to avoid protein processing. Moreover, several fusion proteins between the Xys1 and Xyl30 domains were obtained and their proteolytic stability was studied.

We demonstrate that carbohydrate-binding module-processing takes place even in the complete absence of the linker sequence. We also show that the specific carbohydrate module determines this cleavage in the proteins studied.

Key words

Xylanase, protein processing, carbohydrate module, *Streptomyces*, linker.

Introduction

Xylanases (EC 3.2.1.8.) are the most important enzymes responsible for the hydrolysis of the main hemicellulosic component of plants, namely xylan. They may be used in different industrial applications, such as in the food industry (juice clarification, fruit maceration, coffee extraction…), the paper industry (prebleaching, the refining of pulp fibre, the de-inking of recycled fibres…), the processing of residues (ethanol production) and in animal nutrition (forage predigestion) [2-4, 9].

Enzymes with xylanase activity mainly belong to families 10 and 11 of ß-glycanases but they have also been identified in families 5, 8, 26 and 43 [9] out of the 118 families

described to date (http://www.cazy.org/Glycoside-Hydrolases.html). Many of these xylanases are modular enzymes composed of one or more catalytic domains (CD) and a carbohydrate-binding module (CBM) connected by a flexible linker region (LK) rich in proline, glycine, and hydroxy amino acids. CBMs may be located either at the N-terminal, the C-terminal, or both. To date, 59 families of CBMs have been described (http://www.cazy.org/Glycoside-Hydrolases.html). The carbohydrate-binding domain is important for degrading complex substrates [7]. Nevertheless, many of these xylanases are processed to their CD, and they lose this degradation capacity.

An example of these modular xylanase is Xys1 from *Streptomyces halstedii* JM8 (EMBL AAC4554.1), which belongs to family 10, harbouring a cellulose-binding domain (CBD) [19]. This protein is produced in *Streptomyces halstedii* JM8 as a large 45 kDa protein (Xys1L) that is secreted to the supernatant and processed to a small 33.7 kDa variant (Xys1S) and its CBD by proteolytic cleavage (Fig. 1). The same processing occurs when the gene is cloned in other *Streptomyces* species, such as *S. lividans* 66 or *S. parvulus* and both enzymes are fully active, although Xys1S loses its cellulose-binding capacity [20]. The 3-D structure of the catalytic domain has been resolved [8].

Another example of a modular xylanase is Xyl30 from *Streptomyces avermitilis* UAH30 (CECT3339) (NCBI AAD32560.1), composed of a catalytic domain plus a xylan-binding domain (XBD) [12] (Fig. 1). In this case, the Xyl30 protein is poorly processed in comparison with Xys1L when expressed in *S. lividans* 66, and the secreted large version of 42.8 kDa is much more stable in the supernatants of 6-day-old cultures (Figure 1b).

Previous work carried out in our laboratory showed that at least five *Streptomyces* serine proteases were able to cleave Xys1L *in vitro*, such as SpB and SpC from *S. lividans* 66, and SAM-P20, SAM-P26, and SAM-P45 from *S. albogriseolus*. This processing was almost completely inhibited when the serine protease inhibitor *slpI* gene from *S. lividans* was co-

expressed with the xylanase *xysA* gene in *S. lividans*. In contrast, none of these proteases was able to process Xyl30 *in vitro* [10].

Preliminary studies performed with the xylanase Xys1 in poultry feed have been carried in collaboration with NOREL SA (a Spanish company dedicated animal nutrition), showing the ability of this enzyme to improve digestibility in animals and to increase their weight to levels even better than the commercial enzymes used for this purpose (data not shown). One improvement in the Xys1L enzyme would be to prevent CBD domain processing in order to increase the binding of the enzyme to forage. Thus, here we made several attempts to eliminate the cleavage of Xys1L. First, we constructed different *xysA* variants (the gene that encodes Xys1) with progressive deletions in the linker region sequence, and second we constructed chimerical proteins between the CDs and the CBD or XBD of Xys1 and Xyl30 respectively.

The results showed that the processing of Xys1L is not linker region length-specific and therefore that protease activity depends more on the structure of the CD domain and, mainly in the sequences present in the carbohydrate-binding domain (CBD or XBD).

Materials and Methods

Bacterial strains and DNA manipulation

Escherichia coli strain DH5α [11] was grown in Luria –Bertani (LB) liquid broth or on LB agar. R2YE and the MSA sporulation medium were used for *S. lividans* JI66. Liquid cultures of *S. lividans* JI66 were performed in 10 ml of YES medium [20] supplemented with 1% xylose in 100-ml three-baffled flasks. 10^6 spores ml^{-1} were used as inoculum. Cultures were carried out at 30º C and 200 rpm for four days. When necessary, the medium was supplemented with antibiotics (100 µg ml^{-1} for ampicillin for *E. coli*, 50 µg ml^{-1} for kanamycin for *E. coli* or *S. lividans* JI66). DNA manipulations of *E. coli* and *Streptomyces* were done as indicated by Sambrook et al. [22] and Hopwood et al. [13].

Plasmid constructions

All oligonucleotide sequences used in this work are shown in Table I. The *xysA* gene variants were generated by PCR using a common reverse oligonucleotide including an XbaI site (LK3') and several forward oligonucleotides adding an XhoI site (LKM0, LKM1, LKM2, LKM3, LKM4, and LKM5). The PCR fragments were cloned in the XhoI/XbaI sites of the *E. coli* pSK^+ plasmid, obtaining pSHA2vo, v1, v2, v3, v4 and v5. Then, all amplifications were cloned into the *E. coli/Streptomyces* shuttle vector pN702GEM3 [10] in a triple ligation to construct the *xysA* derivatives: HindIII/BglII fragment from pN702GEM3 + BglII/XhoI fragment from pXHis1 [1] + XhoI/HindIII fragment from the corresponding pSHA2 (v0-v5). In these constructions, all modifications were under the control of *xysA* promoter and flanked by *mmrt* and *fdt* transcriptional terminators, affording the different *xysA* plasmid versions (pVR055 to pVR060) (Fig. 2a).

Chimerical genes between *xysA* and *xyl30* were generated using the SalI restriction enzyme site present at the end of the CD coding region of both genes. The chimerical genes were obtained in several steps (not detailed) and cloned into the pN702GEM3 vector, affording plasmid pNX1/X30-SalI (273 amino acids Xys1 CD + 76 amino acids Xyl30 CD + 88 amino acids Xyl30 XBD) and plasmid pNX30/X1-SalI (272 Xyl30 CD amino acids + 74 Xys1 CD amino acids + 108 amino acids Xys1 CBD).

A new set of recombinant genes between *xyl30* and *xysA* was obtained as follows. The CD module of Xyl30 was amplified with primers MRG24 and MRG25 (Table I), including the NdeI and XhoI sites respectively. The PCR fragment thus obtained was cloned by replacing the *xysA* CD module in plasmid pVR055, yielding pNX30/X1.A (containing the whole CBD *xysA* module with the linker region), and in plasmid pVR059 obtaining pNX30/X1.B (containing the v4 CBD *xysA* module variant without the linker region). Additionally, the XBD domain of Xyl30 was amplified with and without its linker region, using the primers MRG21 and MRG22

or MRG20 and MRG22, respectively (Table II). The corresponding PCR fragments obtained were cloned into plasmid pVR055, replacing the CDB plus the linker region of *xysA* gene by Xyl30 XBD (with and without the linker), obtaining plasmids pNX1/X30.A and pNX1/X30.B.

The entire DNA *xysA* variant constructs and *xysA-xyl30* fusions were sequenced in both strands using a Perkin Elmer ABI Prism 377 DNA sequencer. The plasmids obtained (Table II) were transformed in *S. lividans* JI66 and protein production was analyzed. Manipulation was accomplished with the Gene Construction Kit (GCK, Textco)).

Protein analysis

Protein proteolytic events were assessed by SDS–PAGE (15% acrylamide in a MiniProtean II system, BioRad). Low-molecular weight standards from Bio-Rad were used as size markers. Coomassie blue R was used for protein staining. Protein was quantified by the method of Peterson [18] with bovine serum albumin as the standard.

Immunodetection of Xys1 was performed with anti-Xys1 antibodies on proteins transferred to Immobilon-P (Millipore), with anti-rabbit alkaline-phosphatase-conjugated antibodies (Promega) as secondary antibodies.

The amino-terminal amino acid sequence was determined in proteins separated by SDS-PAGE, blotted onto Immobilon-P (Millipore) membranes, and cut off. The amino terminus was sequenced with an Applied Biosystems 470A Protein Sequenator.

Enzyme activity assays.

The dinitrosalicylic acid (DNS) method, using xylose as standard [5, 6], was used to measure xylanase activity in culture supernatants. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of reducing sugars (expressed as xylose equivalents) in one minute. All data shown are averages of at least three different experiments.

Xylan and cellulose binding assays

The ability of the proteins to bind cellulose or xylan was studied by incubating the supernatant containing the proteins for 2 hours at room temperature with 4 % avicel (Merck) or with 4 % insoluble oatspelt xylan (Sigma) respectively. The mix was centrifuged for 5 minutes at 13000 rpm and the insoluble fraction was washed twice with water. Following this, 1x SDS-loading buffer was added to the insoluble fraction and this solution was boiled for 5 minutes to elute the retained protein. The proteins present in each fraction were analyzed by SDS-PAGE.

Results

Xys1 processing is not dependent on specific linker size

The processing of Xys1L to produce Xys1S was mainly observed in aged cultures (older than 48 h) and Xys1L xylanase cleavage occurred after the D362 residue in *S. lividans* [21]. This residue forms part of a linker of 15 amino acids that separates the catalytic domain and the CBD. To test the importance of linker length in protein processing, several variants were obtained by progressive deletion of this region (see Materials and Methods). As a result of the cloning process, an extra E residue was present in all the constructs (in bold in Figure 2a) but this did not affect the proteolytic process, as tested in the v0 variant (Fig. 2b). The v1 variant lacked the first 8 amino acids of the linker region. Variant v2 had a deletion of the first 12 aa and contained only the last 3 amino acids, and v3 was a version lacking the whole linker region (15 aa). Additionally, we obtained a v4 variant without the 15 aa of the linker plus the first two amino acids of the CBD domain, and a v5 variant without the linker plus 10 amino acids of the CDB, involving the loss of C365, which forms a disulphide bond with C458 [21].

The different plasmids (pVR055(v0) to pVR060(v5)) were introduced into *S. lividans* and liquid cultures were performed and analysed after 72 h of culture. As shown in Figure 2b, all the Xys1 protein variants generated were processed. Proteins with deletions in the linker region (v1, v2, and v3) underwent a proteolytic event similar to that observed in the wild-type

protein (v0). When the first two amino acids (v4) or the first 10 amino acids (v5) of CBD were also eliminated, all the Xys1L protein was immediately processed to Xys1S, which was accumulated in the supernatant. The use of anti–Xys1 antibodies revealed that the Xys1L form was not detected in the v4 and v5 variants in 24-h-old cultures (Fig. 2c). Nevertheless, the CBD was detected by the antibodies in v4 but not in v5 (Fig. 2c), indicating the stability of the CBD in v4 and the instability of this domain in the v5 variant, presumably due to the lack of the disulfide bond between C365 and C458.

Xys1 and Xyl30 protein fusions are processed in different ways depending of the modules used.

Significantly different processing rates of the xylanase Xys1 (X1) and Xyl30 (X30) proteins expressed in *S. lividans* were observed under our culture conditions. While X1 was almost totally processed after 6 days of culture, X30 had not undergone much processing at this culture time (Fig. 1b). Blast analysis indicated that the catalytic domains of both proteins shared 58 % identity and 69 % similarity, whereas the carbohydrate binding domains were quite different (23 % identity and 32 % similarity). In fact, X1 has a CBD (family CBM-2) while X30 has an XBD (family CBM-13). Additionally, the linker region that separates both domains was shorter in X30 than in X1 (Fig. 1a).

With the final aim of obtaining an X1 protein more resistant to proteolytic cleavage, we studied the importance of the linker region and CBM of both proteins in processing. Different fusions between the genes encoding X30 and X1 were generated by interchanging the DNA sequences that encode these regions.

First, we used a SalI restriction site present in the same frame in both genes and situated at the carboxy terminus of the CD coding sequence (Fig. 3a). The F1 and F2 fusions were obtained in plasmids pNX1/NX30-SalI and pNX30/NX1-SalI respectively, as described in

Materials and Methods. *S. lividans* cells harbouring these plasmids were grown in order to analyze the stability of the proteins produced after 72 hours of culture. The cultures carrying F1 accumulated a protein of only 17 kDa but no protein bands of the expected size (45 kDa) were observed (Fig. 3b upper panel). Moreover, neither the L nor the S forms of the chimerical F1 xylanase were detected, even when anti-Xys1 was used in Western-blot assays (Fig. 3b middle panel). Also, no xylanase activity was detected in these cultures (Fig. 3b lower panel). The N-terminal sequence (GDPXXE) of the P17 protein corresponded to the XBD of X30 and was specifically retained by oatspelt xylan but not by avicel when carbohydrate-binding experiments were performed (Fig. 3c). This result showed that this P17 was a functional xylan-binding module. Production of the L-form by F1 was detected in 1-day culture supernatants with only anti-Xys1, but not with Coomasie Blue (Fig. 3c). This demonstrated that the F1 protein was produced but that it was quickly processed to its CD-X1/X30 and XBD domains. In this case, the fusion CD was extremely unstable and was degraded after processing. However, the XBD, not recognised by anti-Xys1 antibodies, was very stable and accumulated in the supernatant.

The F2 protein, which has the catalytic domain mainly from X30 and the carboxy terminus of the CD, the linker region, and the CBD of X1, was accumulated in the supernatant and underwent a processing similar to X1 and to a much greater extent than that observed for X30 after 3 days of culture (Fig. 3b). Both the L and S forms were detected with anti-Xys1, as was the processed CBD. Nevertheless, the enzyme activity of this F2 fusion was about 65 % of the original activity (X1 and X30), showing that the chimerical X30/X1 catalytic domain was less active (Fig. 3b).

More precise fusion proteins were generated by interchanging the carbohydrate modules immediately after the last CD amino acid of X1 and X30, affording the F3 and F4 protein variants, as described in Materials and Methods. Two versions were generated for each protein:

one with the linker region and another without it: versions A and B respectively (Fig. 4a). *S. lividans* transformed with the different plasmids was grown in liquid medium and the supernatants were analyzed in SDS-PAGE and detected with Coomasie Blue or anti-Xys1.

Analysis of 72-h-old supernatants revealed that both F3 fusion proteins were mainly accumulated in their S form (Fig. 4b lower panel), while no accumulation of the L form was observed at this time. Anti-Xys1 antibodies, used on supernatants of 1-day-old cultures, allowed us to detect the L form and a large number of degradation bands between the L and S sizes (Fig. 4b upper panel). This result suggested an imprecise cut in the fusion proteins and their XBD degradation that were independent of the presence or absence of the X30 linker region, finally accumulating the S form (3-day-old cultures) (Fig. 4b lower panel). A different type of behaviour was observed for both F4 fusions. Thus, when the 3-day-old culture supernatants were analyzed a processing similar to that undergone by the original X1 was obtained for the fusion F4-A, the L and S forms being observed (Fig. 4c lower panel). However, the F4-B fusion, which did not have any standard linker region between either domain, was processed completely and only the S form was accumulated at this culture time. The use of anti–Xys1 antibodies on supernatants from 1-day-old cultures allowed us to detect the L form in both fusions, and no protein degradation bands, as in the case of the F3 fusions, were observed, pointing to a precise site of proteolysis. Again, this cleavage was not dependent on the amino acids present in the linker region and these F4 fusions permitted us to obtain processed forms of the X30 protein (Fig. 4c lower panel).

Discussion

The broad potential applications of xylanases in industrial processes encompass all three sectors of the industrial markets (food, feed, and technical). The discovery of new enzymes (such as extremophilic xylanases) and the basic research carried out to improve the

characteristics of already described xylanases is currently an active field of research. Thus, the United States Patents and Trademark Office (http://www.uspto.gov/) lists 468 patents referring to xylanases since 2001 [9]

As stated above, the elimination of the proteolytic cleavage that occurs in the linker region separating the catalytic domain from the carbohydrate-binding module would be useful for certain applications of xylanase, such as in animal feed, where the digestion of complex substrates is necessary. The collection of chimerical enzymes combining the catalytic and sugar-binding domains from different organisms is another way to eliminate this processing and improve the properties of the enzymes. Some examples that support this observation are that the fusion of family 2b of the carbohydrate-binding module from *S. thermoviolaceus* STX-II to the carboxyl-terminus of XynB from *Thermotoga maritima*, (XynB-CBM2b) increases the catalytic activity of the original enzyme against soluble xylan [14] and that the addition of a family 6 CBM to *Bacillus halodurans* xylanase enhances activity against insoluble xylan [17]. CBM modules are usually joined to the catalytic domain by a flexible linker region that permits the proper packaging of both domains and that could play a role in protein stability, as occurs with XynAS27 from *Streptomyces* sp. S27 [15]. The linker region has been used to construct bifunctional fusions such as ß-glucanase and xylanase [16].

Here we studied the role of the linker regions and substrate-binding modules of Xys1L (X1-L) and Xyl30L (X30-L) in their processing. The initial goals of this study were to improve basic knowledge about this event and, if possible, to obtain unprocessed versions of Xys1L protein, which could improve its effectiveness in animal feed.

The deletion of different numbers of amino acids in the linker region (LK) of X1-L was expected to originate unprocessed forms of this xylanase. However, not only did the different deletions of this LK region fail to prevent the processing of the protein (vo-v3) but, also, deletions that eliminated the entire linker region and the first two or ten amino acids of the

CBD (v4, and v5 respectively) originated proteins that were processed immediately. The accumulation of the X1-S form was observed in these v4 and v5 deletions, while it was very difficult to detect the large X1L protein in young cultures (24 h old). Therefore, CD and/or CDB, and not the LK, must determine the proteolytic event that occurs in this xylanase. This result was corroborated by obtaining fusion proteins in which the CD of X30, a xylanase that is scarcely processed, was linked to the CBD of X1 with or without X1-LK (Fusions F4A and F4B respectively). In both constructions, the proper processing of both domains occurred in a similar way to the original X1. Thus, the linker region is not necessary for the proteolytic processing of the L form of X1.

We also observed that the first cysteine (C365) of the X1-CBD was essential for the correct conformation of the binding module, because the disulfide bond that this residue establishes with C458 was eliminated. Consequently, the binding domain of v5 is degraded in the supernatant while this degradation does not occur when C365 is present (v4).

The presence of an in-frame SalI restriction site in the genes that encode X1 and X30 (upstream from the sequences that encode V274 and V273 respectively) facilitated the collection of CD hybrids of the X1 and X30 peptides. Both proteins shared 66 % identity and 72 % similarity in this region (from V274 to L347 in X1 and from V273 to L342 in X30). However, although the similarity between the CDs of both proteins in the interchanged region was very high (SalI peptides) and the xylanase processing of the chimerical proteins was expected to be similar, the instability of the hybrid CD of one of the fusions (F1), which carried the N-terminus part of the CD of Xys1 and the SalI carboxyl part of X30, suggests the importance of the carboxyl part of the X1-CD in the stability of this domain. This result was corroborated in the CD of other fusions carried out (F2) harbouring the N-terminus part of the CD of X30 and the SalI carboxyl part of X1 that was stable and was processed in a similar way to the original X1. The importance of the carboxy SalI fragment of Xys1CD stabilization was

again corroborated in the fusion of the entire X1-CD with the XBD of X30, with or without the X30 linker region (F3A and F3B), where an accumulation of the complete CD was observed (S form). Interestingly, no specific processing from F3L to yield F3S was observed, and a non-specific proteolytic degradation not observed from F4L to yield F4S was detected. This indicates that degradation was not dependent on the presence or absence of the linker region (F3A and F3B respectively).

In conclusion, although it is generally believed that CBMs processing mainly depends on the preceding linker sequence, here we demonstrate that this assumption should not be taken for granted because, at least in the proteins studied, it occurs even in the complete absence of this linker sequence. We also show that the CBD of Xys1 is able to determine the cleavage in the Xyl30 protein, which initially is barely processed, opening new possibilities for the generation of modified proteins (Figure 5).

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Figure legends

Figure 1: a) Clustal W alignment of Xys1 and Xyl30 mature L-form proteins. The catalytic domains (CD) and carbohydrate-binding modules (CBD and XBD respectively) are marked in a box; the linker region (LK) is between both domains. SalI: denotes the SalI position in the corresponding coding genes. b) Schematic representation of Xys1 and Xyl30 proteolytic processing of their CDs and CBM modules (upper part) and SDS-PAGE protein gels stained with Coomasie blue showing the proteins in 10 µl of culture supernatant after 3 and 6 days of culture (lanes 3d and 6d respectively) (lower part). (L: L-form; S: S-form).

Figure 2: a) Schematic representation (upper part) and peptide sequences of the different variants of Xys1 obtained in this work: pVR055(v0) to pVR060 (v5) (lower part). All the variants contain an extra E347 amino acid in the junction region of the construct (in bold). The last amino acid of the linker region corresponds to D362. C365, which is important for the stability of CBD, is also marked in bold. b) SDS-PAGE protein gel stained with Coomasie blue showing the processing of the protein variants in 10 µl of culture supernatant after 3 days culture. c) SDS-PAGE protein gel stained with Coomasie blue (left) and Western blot with anti-Xys1 (right) showing the processing of the protein variants (v0, v3, v4, and v5) in 10 µl of culture supernatant after 1 day of culture.

Figure 3: a) Schematic representation of the different domains of the F1 and F2 protein fusions from Xys1 (X1) and Xyl30 (X30). They were constructed using the SalI restriction enzyme site

present in-frame in both coding genes. b) SDS-PAGE protein gel stained with Coomasie blue (upper part), Western blot with anti-Xys1 (middle part), and xylanase activity (lower part) showing the processing of the protein fusions (F1 and F2) in 10 µl of culture supernatant after 3 days of culture together with their enzyme activities. c) Retention assay in xylan (left) or avicel (right) of the protein accumulated in a 3-day supernatant of the F1 fusion-producing strain (S: 10 µl of supernatant, NR: non-retained fraction; R; retained fraction). d) SDS-PAGE protein gel stained with Coomasie blue (left) and Western blot with anti-Xys1 (right), showing the production of the L form in X1 and F1 in 10 µl of culture supernatant after 1 day of culture. (L: L-form; S: S-form).

Figure 4: a) Schematic representation of the different F3 and F4 fusions between Xys1 (X1) and Xyl30 (X30) CDs and CBMs. A-versions have the corresponding linker region and B-versions do not. b) Western blot with anti-Xys1 after 1 day of culture (upper part), and SDS-PAGE protein gel stained with Coomasie blue of a 3-day culture (lower part) showing the processing of the protein fusions (F3A and F3B) in 10 µl of culture supernatant as compared to X1. b) Western blot with anti-Xys1 after 1 day culture (upper part), and SDS-PAGE protein gel stained with Coomasie blue of a 3-day culture (lower part) showing the processing of the protein fusions (F4A and F4B) in 10 µl of culture supernatant compared to X1 and X30. (L: L-form; S: S-form)

Figure 5: Protein scheme of the different forms (L and S) and CBMs produced by the constructs studied (X1, X30, F1, F2, F3, and F4). The accumulated proteins in the supernatants are filled with grey. (✹: protein degradation).

Tables

Table I – Primers used in this work

Primers	Sequence (5´-3´)	Function
LK3'	ATTATTTCTAGAGTCAGGAAGCGGTGCAGGCACCC	Amplification of LK + CBD of *xysA* (reverse)
LKM0	ATTATTCTCGAGGGCGGTTCCGGCGGAGGCGGTG	Amplification of LK + CBD of *xysA* (forward)
LKM1	ATTATTCTCGAGGACGGCGGGGAGGGCGGCGAC	Deletion 1 of LK of *xysA* (forward): v1
LKM2	ATTATTCTCGAGGGCGGCGACGGCGCCTGCACG	Deletion 2 of LK of *xysA* (forward): v2
LKM3	ATTATTCTCGAGGGCGCCTGCACGGCGACGTAC	Deletion 3 of LK of *xysA* (forward): v3
LKM4	ATTATTCTCGAGTGCACGGCGACGTACACCCGGAC	Deletion 4 of LK + CDB of *xysA* (forward): v4
LKM5	ATTATTCTCGAGTCGACGTGGAACGGCGGATACAACG	Deletion 5 of LK + CDB of *xysA* (forward): v5
MRG20	TTTTTTCTCGAGGGCGACCCCGACCCGGAGCCCG	Amplification of the XBD module of Xyl30 without linker. Forward (includes an XhoI site, underlined)
MRG21	TTTTTTCTCGAGAACGCCGGCGACGGCGGCGGTGG	Amplification of XBD module of Xyl30. Forward (includes an XhoI site, underlined)
MRG22	ATTATTTCTAGAGTCAGACGCCACTTCTGTTGTCGCCAC	Amplification of the XBD module of Xyl30. Reverse (includes an XbaI site, underlined)
MRG24	TTTTTTCATATGGGCTTTCACGCCCTCCCCAGATC	Amplification of the CD module of Xyl30. Forward (includes an NdeI site, underlined)
MRG25	ATTATTCTCGAGGAGGGCGTTCAGCACGGCGTTGTAC	Amplification of the CD module of Xyl30. Reverse (includes a XhoI site, underlined)

Table II - Plasmids used in this work

Vector	Characteristics	Reference
pXHis1	pIJ2925 derivative containing the *xysA* gene and promotor	[1]
pSK+	*E. coli* plasmid Amp resistance, *lac* promoter, bla	Stratagene
pSHA2v0	pSK+ derivative containing the *xysA* gene v0 (wt: full linker sequence)	This work
pSHA2v1	pSK+ derivative containing the *xysA* gene v1 (deletion 1 of linker sequence)	This work
pSHA2v2	pSK+ derivative containing the *xysA* gene v2 (deletion 2 of linker sequence)	This work
pSHA2v3	pSK+ derivative containing the *xysA* gene v3 (deletion 3 of linker sequence)	This work
pSHA2v4	pSK+ derivative containing the *xysA* gene v4 (deletion 4 of the linker sequence + 2 aa of CBD)	This work

pSHA2v5	pSK+ derivative containing the *xysA* gene v5 (deletion 5 of linker sequence + 10 aa of CBD including cysteine 365)	This work
pN702GEM3	*E. coli–Streptomyces* shuttle vector; Neo/Kan resistance	[10]
pVR055	pN702GEM3 derivative containing the *xysA* gene v0 (wt: full linker sequence)	This work
pVR056	pN702GEM3 derivative containing the *xysA* gene v1 (deletion 1 of linker sequence)	This work
pVR057	pN702GEM3 derivative containing the *xysA* gene v2 (deletion 2 of linker sequence)	This work
pVR058	pN702GEM3 derivative containing the *xysA* gene v3 (deletion 3 of linker sequence)	This work
pVR059	pN702GEM3 derivative containing the *xysA* gene v4 (deletion 4 of linker sequence + 2 aas of CBD)	This work
pVR060	pN702GEM3 derivative containing the *xysA* gene v5 (deletion 5 of linker sequence + 10 aas of CBD including the first cysteine)	This work
pNX1/X30-SalI	pN702GEM3 derivative containing fusion of the *xysA* and *xyl30* catalytic domains and XBD (using SalI restriction site) → F1	This work
pNX30/X1-SalI	pN702GEM3 derivative containing fusion of *xyl30* and *xysA* catalytic domains and XBD (using SalI restriction site) → F2	This work
pNX1/X30-A	pN702GEM3 derivative containing the fusion of the *xysA* catalytic domain and the *xyl30* linker sequence and XBD → F3-A	This work
pNX1/X30-B	pN702GEM3 derivative containing the fusion the of *xysA* catalytic domain and *xyl30* XBD (without linker sequence) → F3-B	This work
pNX30/X1-A	pN702GEM3 derivative containing fusion of *xyl30* catalytic domain and *xysA* v0 linker sequence and CBD → F4-A	This work
pNX30/X1-B	pN702GEM3 derivative containing the fusion of the *xyl30* catalytic domain and *xysA* CBD v4 (deletion 4 of linker sequence) → F4-B	This work

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Figure 1

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Figure 1

Figure 2

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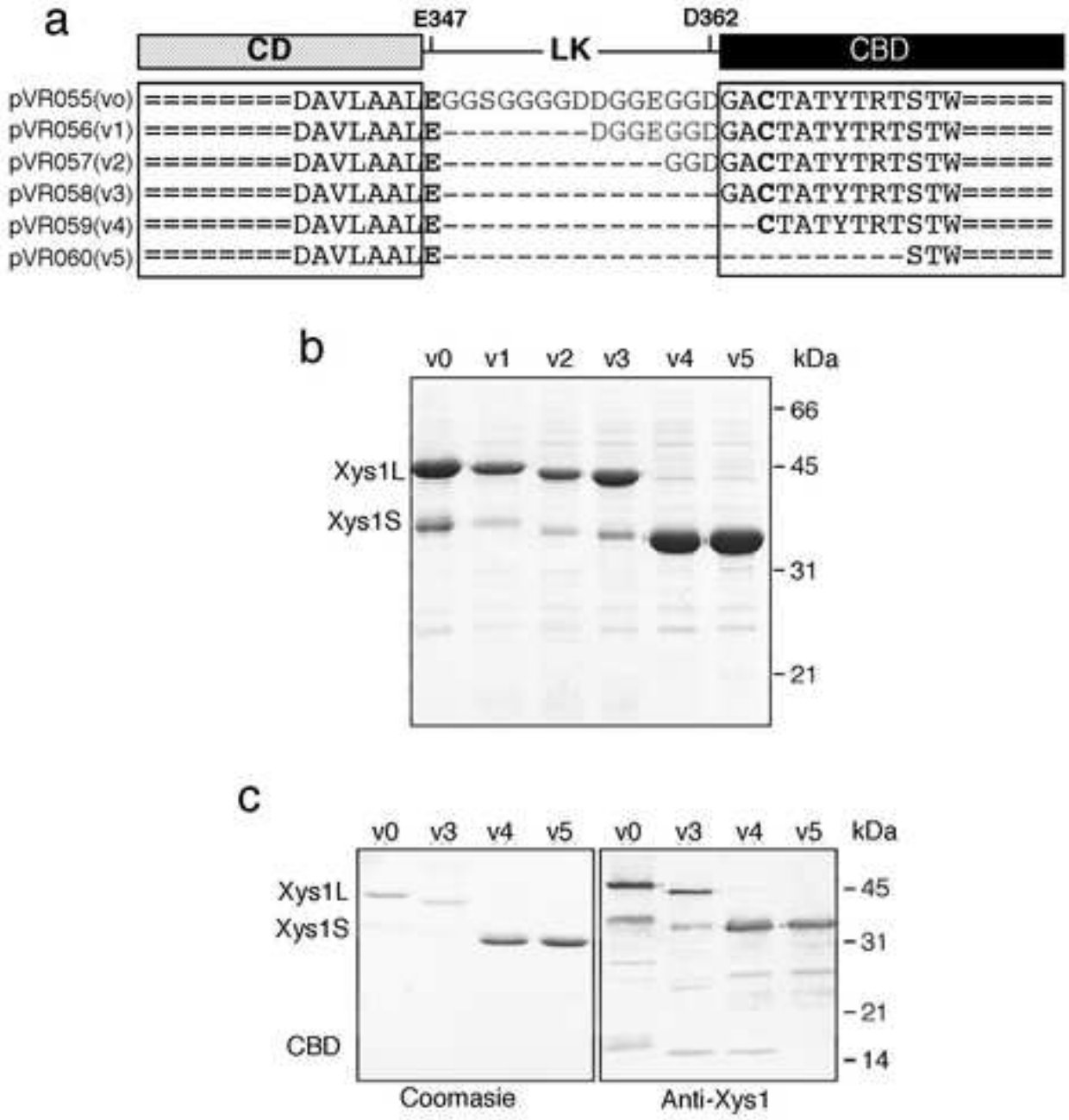

Figure 2

Figure 3

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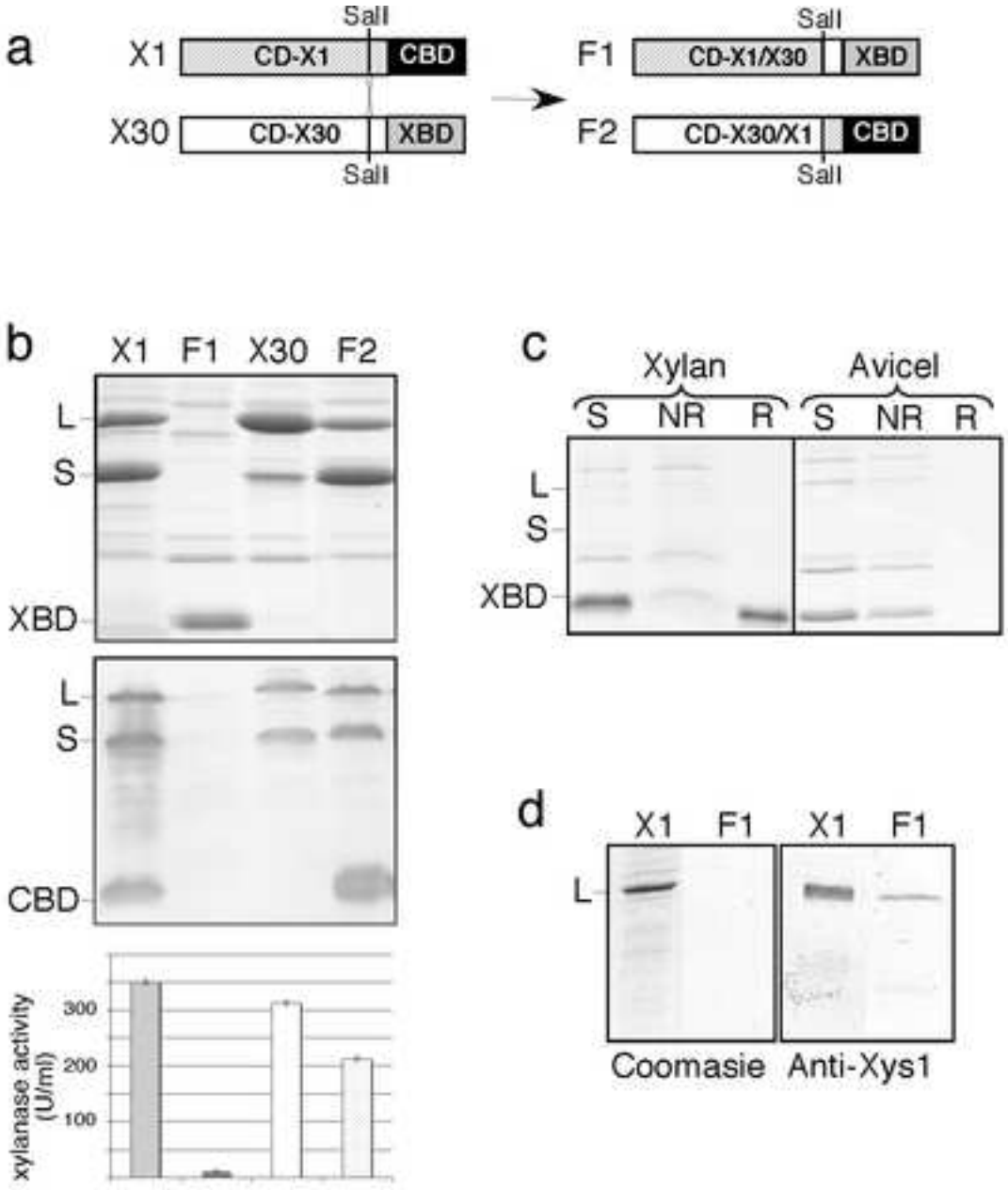

Figure 3

Figure 4

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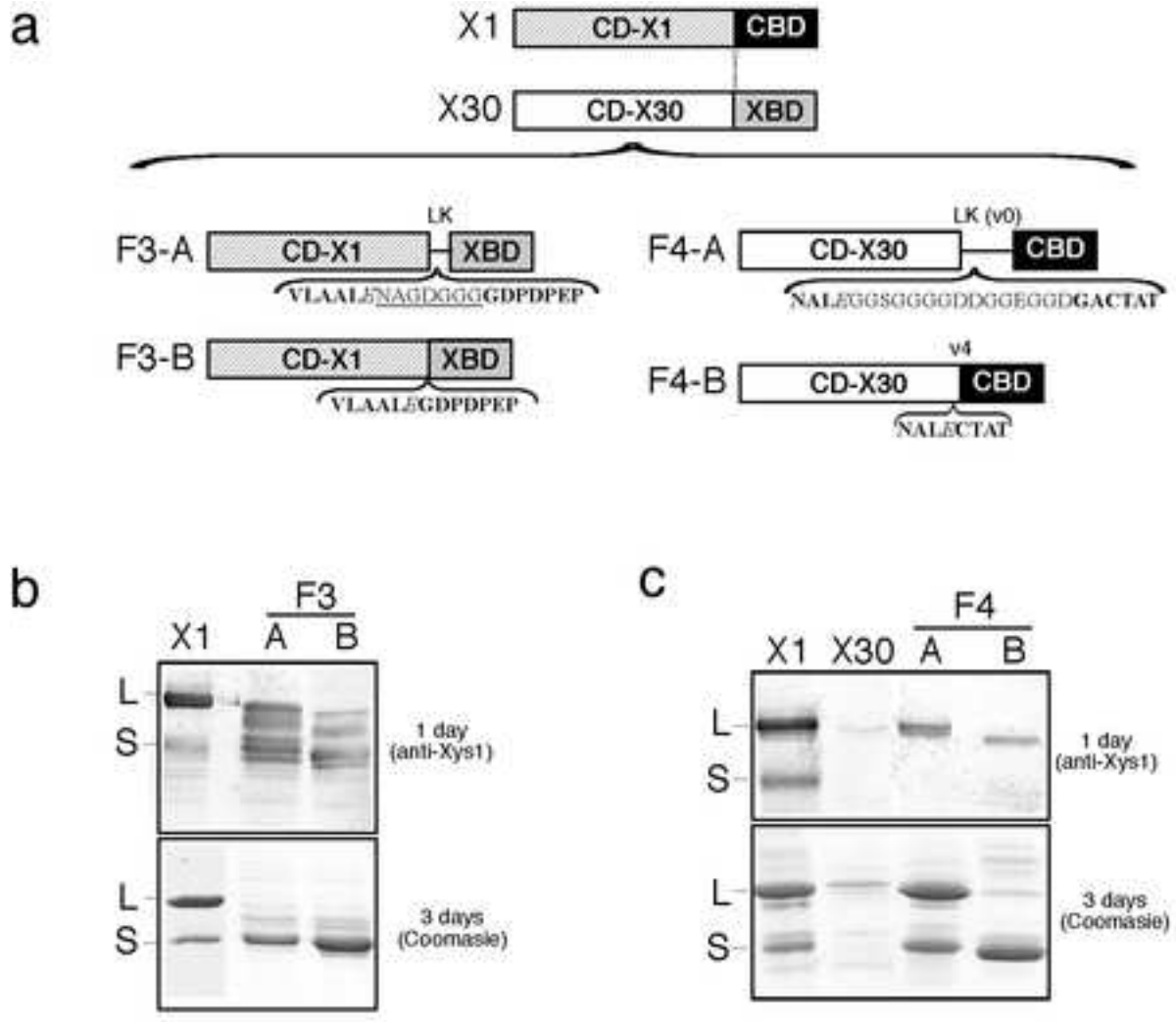

Figure 4

Figure 5

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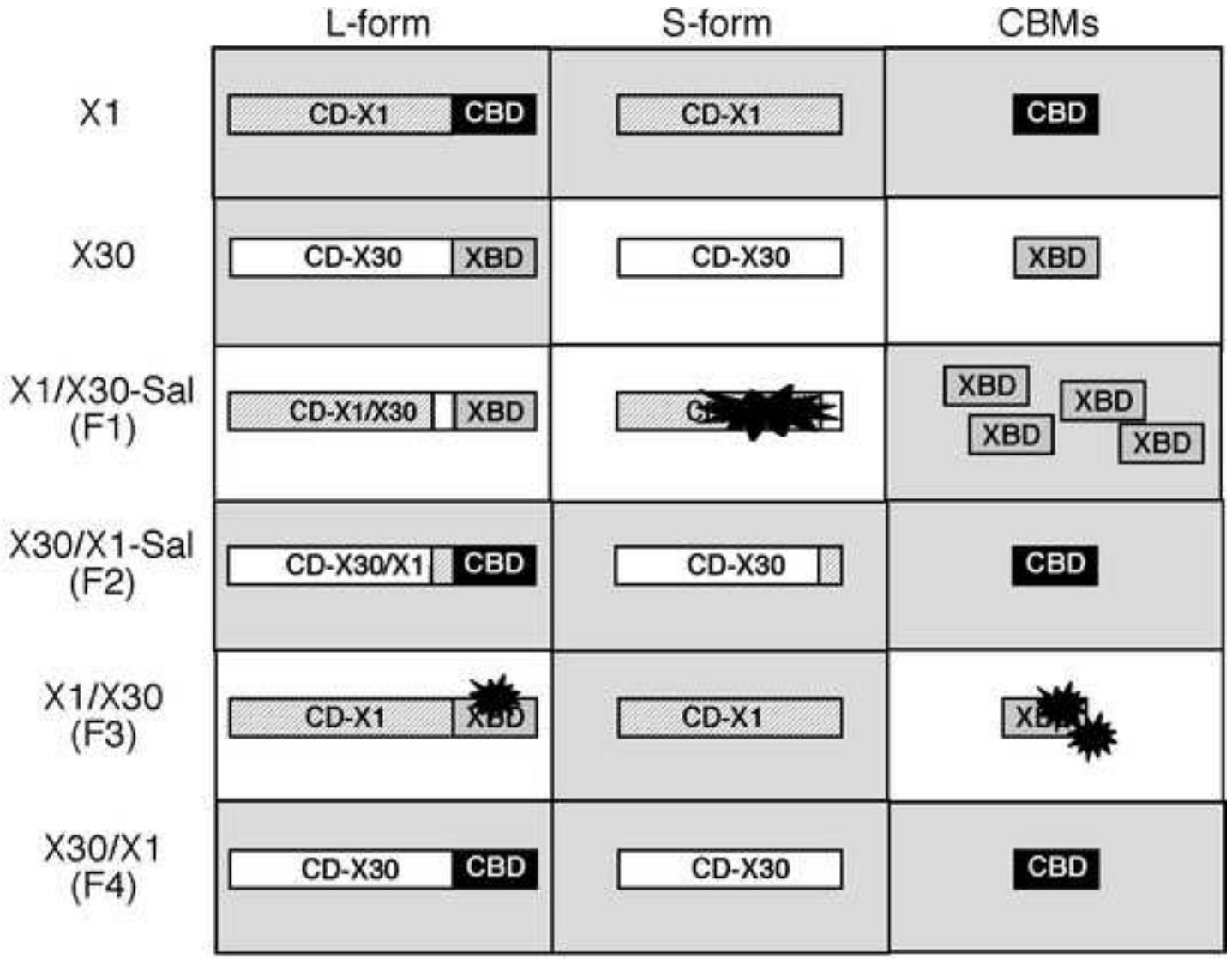

Figure 5