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The modular xylanase Xyn10A from *Rhodothermus marinus* is cell-attached, and its C-terminal domain has several putative homologues among cell-attached proteins within the phylum Bacteroidetes

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Abstract

Until recently, the function of the fifth domain of the thermostable modular xylanase Xyn10A from *Rhodothermus marinus* was unresolved. A putative homologue to this domain was however identified in a mannanase (Man26A) from the same microorganism which raised questions regarding a common function. An extensive search of all accessible data-bases as well as the partially sequenced genomes of *R. marinus* and *Cytophaga hutchinsonii* showed that homologues of this domain were encoded by multiple genes in microorganisms in the phylum Bacteroidetes. Moreover, the domain occurred invariably at the C-termini of proteins that were predominantly extra-cellular/cell attached. A primary structure motif of three conserved regions including structurally important glycines and a proline was also identified suggesting a conserved 3D fold. This bioinformatic evidence suggested a possible role of this domain in mediating cell attachment. To confirm this theory, *R. marinus* was grown, and activity assays showed that the major part of the xylanase activity was connected to whole cells. Moreover, immunocytochemical detection using a Xyn10A-specific antibody proved presence of Xyn10A on the *R. marinus* cell surface. In the light of this, a revision of experimental data present on both Xyn10A and Man26A was performed, and the results all indicate a cell-anchoring role of the domain, suggesting that this domain represents a novel type of module that mediates cell attachment in proteins originating from members of the phylum Bacteroidetes.

Keywords: Cell attachment; Xylanase; Bacteroidetes

1. Introduction

Glycoside hydrolase (GH) multiplicity is a common theme among microorganisms capable of degrading the complex and recalcitrant polysaccharide composites found in plant and algal cell walls. These enzymes typically have a modular organisation consisting of catalytic modules (CMs) usually, but not always, joined to

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non-catalytic modules (NCMs) by flexible linker sequences [1,2]. The most common types of NCMs are carbohydrate-binding modules (CBMs), but a number of other domains or modules, some of yet unknown function, have been reported and include NCMs involved in cell adhesion, or protein anchoring [3].

Two different types of domains have earlier been suggested to play a role in cell adhesion/anchoring of glycoside hydrolases, these are the so-called fibronectinIII-like domains (Fn3-like domains), and the S-layer homology domains (SLH-domains). The Fn3-like domain, which has a length of approximately 100 residues, is phylogenetically spread and presented in a superfamily of sequences representing receptor proteins or proteins involved in cell-surface binding mainly in eukaryotes. It is also found in some extra-cellular bacterial glycoside hydrolases [4]. These domains are commonly distributed in multiple copies in modular glycoside hydrolases, and are often found between catalytic modules and CBMs. Recently it has however been demonstrated that the Fn3-like domains have a role in hydrolysis of insoluble substrates [5]. The SLH-domain is a domain of about 50–60 residues, found at the N- or C-termini of mature proteins [6] and is believed to be anchored to the peptidoglycan [7], or some other structure in the bacterial cell wall [8]. This domain is almost exclusively bacterial with 63 of the 64 sequences reported to pfam-database (http://pfam.wustl.edu) in the bacterial branch, and most commonly occurring within the *Bacillus*/*Clostridium* group and in related Gram-positive bacteria [6].

The thermophilic marine aerobic bacterium *Rhodothermus marinus* stains Gram-negative and is phylogenetically affiliated to the Bacteroidetes (also known as the Cythophaga/Flexibacter/Bacteroides-group) [9], a phylum with many known degraders of organic matter. This group of bacteria is known to produce a number of cellulose degrading enzymes. Moreover, members of the Cytophaga, one of the better studied genera within this phylum, do not produce soluble extra-cellular cellulose hydrolases, but instead keep their enzymes attached to the cell-envelope [10]. Despite established cell-attachment, only one gene within the phylum Bacteroidetes has been reported to encode a homologue to the SLH-domain (an S-layer protein precursor from Cytophaga sp. Jeang 1995).

Rhodothermus marinus resembles other microorganisms within this phylum, in its ability to produce a number of glycoside hydrolase activities [11–13], as well as in displaying enzyme activities suggested to be cell-attached. Primary structures of some of the *R. marinus* glycoside hydrolases are known, including one family 10 xylanase [14], and one family 26 mannanase [15]. The xylanase (Xyn10A) is a modular enzyme that consists of two N-terminal family 4 CBMs followed by a domain of unknown function, a catalytic module classified as GH10 and a finally a 5th domain (D5) at its C-terminus [14,16]. Alignments of the *Rm* Xyn10A-catalytic module with family 10 xylanases, unmasked D5 as an extended C-terminal sequence [14], preceded by a short stretch of repeated glutamic acid and proline residues, typical for the linker sequences often found joining modules in glycoside hydrolases. To cast more light on the possible function of this domain a search for similar sequences was accomplished using accessible databases as well as available partial genome sequences from *R. marinus* and from the related organism *Cytophaga hutchinsonii*. Based on the findings in this search and combined with experimental evidence the possible role in cell-adhesion of the Xyn10A C-terminal domain and its homologues is discussed.

2. Materials and methods

2.1. Sequence analysis and similarity searches

Bioinformatic tools were used to explore the primary structure of D5 in Xyn10A from *R. marinus*. Similarity searches by BLAST, using D5 of *R. marinus* as template, were performed on the NCBI server (http://www.ncbi.nlm.nih.gov) or locally using BioEdit v. 5.0.6. on available genome sequences of *C. hutchinsonii* (from the KEGG database), or partial genome sequences of *R. marinus* (available via Prokaria Ltd, Reykjavik, Iceland). Location of a putative signal peptide was predicted by SignalP v.1.1. (http://www.cbs.dtu.dk/services/SignalP). Matches with open reading frames of unknown function were subjected to an additional search by BLAST after deletion of the part showing high similarity to D5, to predict the putative function of the remaining part of the ORFs.

The ClustalW tool on the EBI server (http://www.ebi.ac.uk/clustalw) was used to create multiple sequence alignments and phylogenetic trees, displayed using Gene doc 2.6.02 [17], and TreeView 1.5 [18], respectively. Theoretical isoelectric points, and amino acid composition of deduced amino acid sequences were analysed by ProtParam (http://www.expasy.org/tools/protparam.html), and Microsoft Excel.

2.2. Cultivation of R. marinus

Rhodothermus marinus was grown with and without xylan (5 g/L, Birch 7500.1 from Carl Roth, Karlsruhe, Germany) at 65 °C, pH 7.1, with aeration on 5 L/min, in 2.5 L modified M162 medium [11,19] in a 3 L bioreactor inoculated with a 100 ml shake-flask culture ($OD_{620\,nm} = 0.7$). Optical density measurements ($OD_{620\,nm}$) monitored cell growth. Mannanase producing *R. marinus* were grown in 100 ml shake-flask cultures

using the M162 medium including Locust Bean Gum 6 g/L (Sigma–Aldrich, St. Louis, Mo).

Samples for activity analysis and electrophoresis were withdrawn during the early log, mid-log, late log and in the stationary phases and kept at 4 °C until analysis. The culture supernatant and cell-fraction were separated by centrifugation at 25,000*g* for 30 min at 4 °C. The whole cell-fraction was washed with 20 mM sodium phosphate buffer at pH 7.0, recentrifuged and resuspended to the original sample volume in the above buffer.

2.3. Activity analysis and electrophoresis

Xylanase activity was measured in the culture supernatant, and on whole cells using the DNS method as described elsewhere [20] with birch xylan (Carl Roth) as substrate and using individual enzyme blanks. Xylanase production in *R. marinus* was also analysed using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) with 10% separation gels [21], activity stained with Congo-red as previously described [22], except for the following modifications: the over-layer agarose gel contained 0.05% oat spelt xylan (Sigma), the buffer used was 50 mM Tris–HCl, pH 7.5, and incubation time at 65 °C was 60 min.

Mannanase activity was determined by a halo plate assay containing 3.5% (w/v) agar and 0.1% (w/v) Azo-Carob Galactomannan (Megazyme, Bray, Ireland). Samples (80 μL) were loaded into wells and plates were incubated at 65 °C overnight.

2.4. Immunocytochemistry

Drops of cell suspension were dried on SuperFrost microscope slides (Menzel-Gläser, Germany). When completely dry, the cells were fixed for 20 min in Stefanini fixative (2% paraformaldehyde and 15% saturated aqueous picric acid solution in 0.1 M phosphate buffer, pH 7.2), followed by repeated rinsing in sucrose-enriched 10% Tyrodes' solution and finally in phosphate-buffered saline (PBS). The cells were then permeabilized and blocked (in the same step) using a blocking solution containing; 0.25% Triton X-100 and 0.25% BSA (both from Sigma) in PBS. This was followed by incubation in a moist chamber with a rabbit anti-CBM4-2 primary antibody [immunoglobulin fraction (10 mg/ml) from serum drawn from a rabbit immunised with recombinant produced purified carbohydrate binding module (CBM4-2) of Xyn10A] in a 1:100 dilution in blocking solution, over night. The next morning excess antibody was rinsed off and the cells were further incubated for 2 h, with flourescent Rhodamine Red-X-conjugated donkey anti-rabbit secondary antibody (Jackson Laboratories, PA, USA) diluted 1:400 in blocking solution. The slides were then rinsed 2×10 min in blocking solution and once in PBS.

After this procedure flourescent Sytox green (Molecular Probes,WA, USA) was added in a 1:3000 solution of PBS for 10 min, and then rinsed for another 15 min in PBS before mounting.

The immuno-labelled cells were visualized using an Olympus BX-60 microscope connected to an Olympus DP-50 digital camera. Photomicrograps were taken with the viewfinder Lite software.

3. Results and discussion

3.1. Similarity between C-terminal parts of R. marinus xylanase and mannanase

Initially, the only domain among the publicly accessible sequences found to share primary structure similarity with the Xyn10A D5 domain of *R. marinus*, was from another hemicellulose degrading enzyme originating from the same organism (*Rm* Man26A) [15]. The similarity was restricted to the C-terminal part of the two enzymes (33% identity) (Fig. 1). Evaluation of a multiple sequence alignment including the *R. marinus* mannanase and a number of known catalytic modules (CMs) of GH 26 suggested also the C-terminal part (residues 939–1021) of this enzyme to be a separate domain, as it flanks the CM downstream the consensus region, rather than lying within it even though no linker sequence separating it from the catalytic module is distinguishable in this enzyme (data not shown).

It was also noted that D5 of RmXyn10A (residues 913–997, in the Xyn10A-sequence) has a theoretical p*I* value of 11.05, which is strikingly higher than either the full-length xylanase or any isolated module thereof (all with p*I*:s of 4–4.5) but in better agreement with the C-terminal domain of *Rm* Man26A (p*I* 11.65). The high similarity between these two domains and their occurrence at the C-terminus of two modular hemicellulases

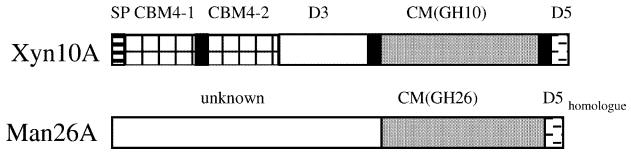

Fig. 1. Domain structure of the xylanase Xyn10A and mannanase Man26A of *Rhodothermus marinus*. The identified domains (D) or unknown regions in the primary sequence are shown as blocks. Regions/domains of unknown function are shown in white, catalytic modules (CM) in grey and carbohydrate binding modules (CBM) in a squared pattern. Identified linker sequences are shown in black, and signal peptides striped. The C-terminal domains of the respective protein are marked by dashes.

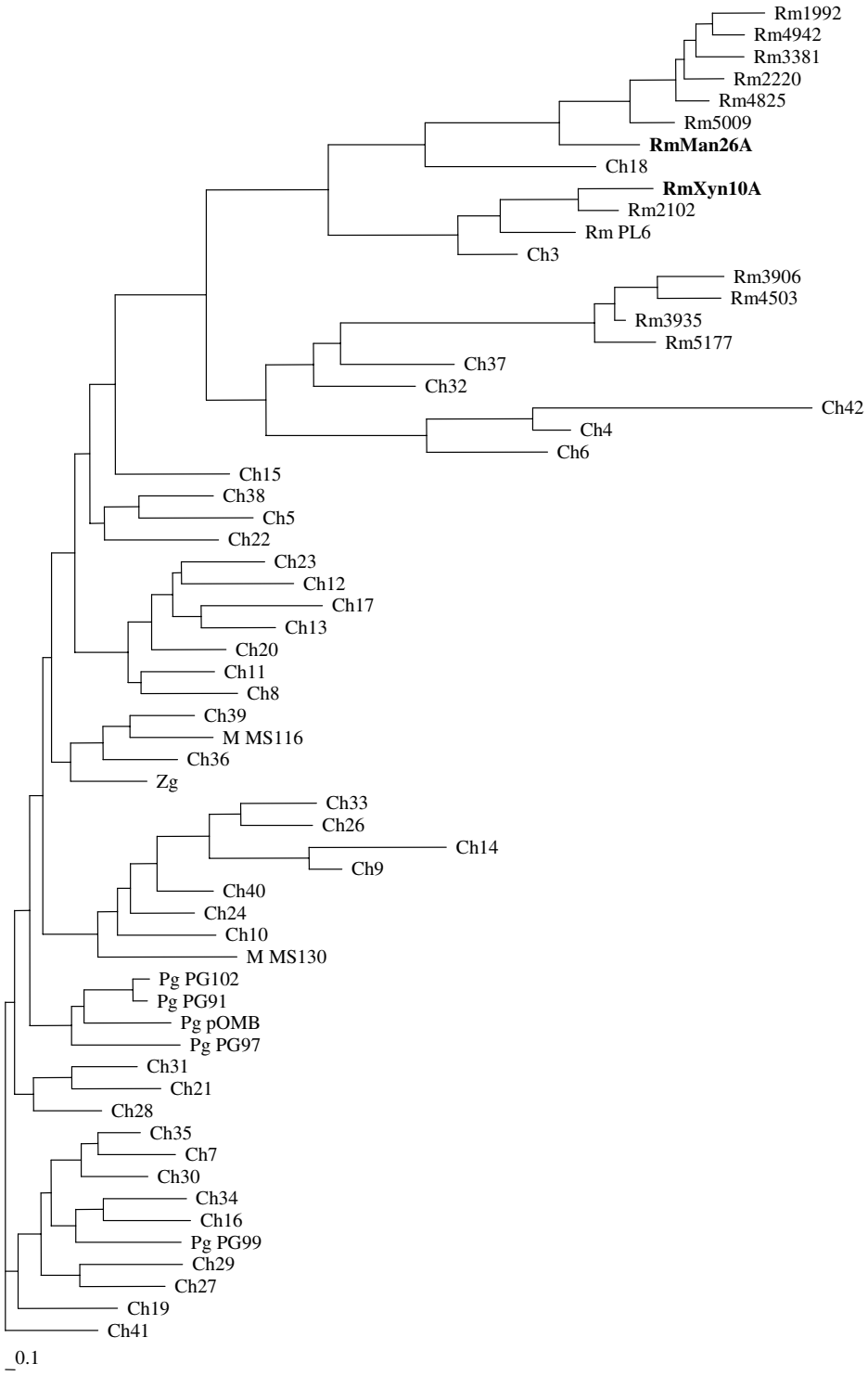

Fig. 2. Phylogram over the putative domains found by BLAST - similarity search using D5 of *Rm* Xyn10A as template. Open reading frames from the respective organism are numbered, and labelled: Ch (*Cythophaga hutchinsonii*), M (*Microscilla* sp.), Pg (*Porphyromonas gingivalis*), Rm (*Rhodothermus marinus*), Zg (*Zobellia galactanivorans*).The phylogram is displayed using TreeView, and created using the EBI-ClustalW-tool with the output in the phylip format, and using default parameters, with correct dist. ''on'', and ignore gaps ''off''.

from the same organisms suggested the possibility of a common function. Affinity electrophoresis analysis in a previous work failed to show any mannan, xylan or glucan-binding capacity of the xylanase domain, ruling out a carbohydrate binding function to substrates related to either of the catalytic modules [23].

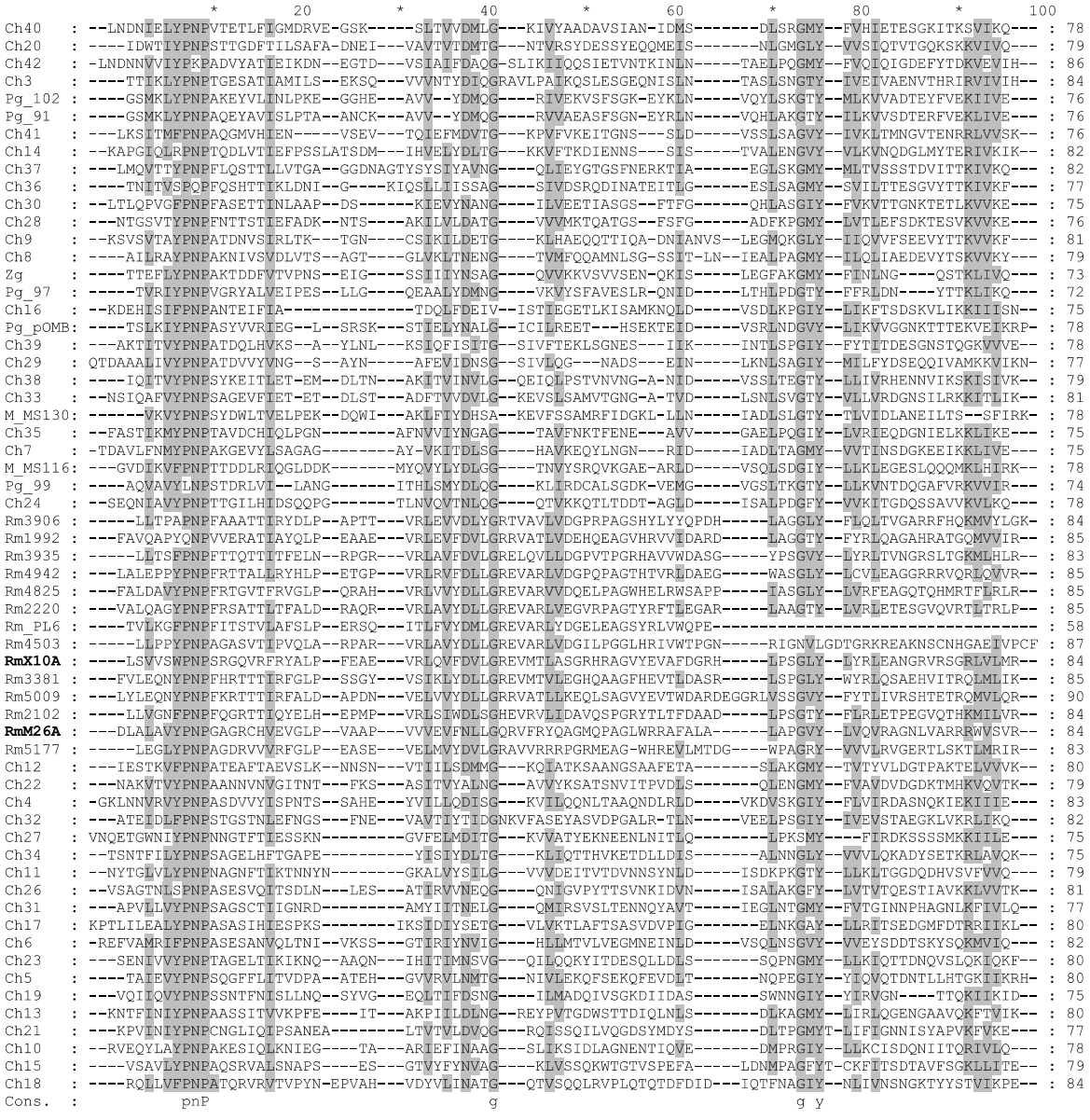

Fig. 3. Multiple sequence alignment of C-terminal domains encoded in the proteins found by BLAST - similarity search using D5 of *Rm* Xyn10A as template. The first consensus region starts at position 7 in the alignment (residue 915, Xyn10A numbering) and spans seven residues with the motif [(I/L/M/V), X, (I/L/M/V), (F/W/Y), P, N, P]. The second consensus region spans positions 37–44 [(I/L/V), X, (I/L/M/V), (I/L/M/T/V/F/W/Y), (D, N), (I/L/M/V), X, G], and mostly involves conserved hydrophobic residues. This is also true for the third region, which is located at position 74–85 in the alignment, and has 1–2 inserted residues in a few of the sequences [(I/L/M/V), X, X, G, (I/L/M/T/V), Y, -, -, (F/I/L/M/V), (I/L/M/V), X, (I/L/M/V)]. The domains originate from five species all affiliated to the Bacteroidetes phylum. Open reading frames from the respective organism are numbered, Ch (*Cythophaga hutchinsonii*), M (*Microscilla* sp.), Pg (*Porphyromonas gingivalis*), Rm (*Rhodothermus marinus*), Zg (*Zobellia galactanivorans*). The alignment is created using the EBI-ClustalW-tool, and default parameters. The resulting alignment was analysed in GeneDoc. Conserved residues are identified [The following residues are grouped, and considered conserved within the group: 1.(D,N); 2.(E,Q); 3.(S,T); 4.(K,R); 5.(F,Y,W); 6.(L,I,V,M).] and shaded if present in more than 60% of the sequences.

3.2. Homologous C-terminal domains are encoded in multiple genes in R. marinus *and related organisms*

The observations presented above prompted an extended search for homologues to D5 of Xyn10A using partial genomic sequence data from *R. marinus* (available via Prokaria Ltd, Iceland), and publicly available sequence databases attempting to unravel the function of this domain. Using this approach, a number of hits in mostly putative open reading frames were found, invariably located at the C-termini in the deduced amino acid sequence of the respective gene. The highest score hits originated from five different microorganisms all classified within the phylum Bacteroidetes. Most of the hits originated from genome sequences of two microorganisms (*R. marinus* and *C. hutchinsonii*), but

Table 1
A summary of average properties for the C-terminal domains found in the five microbial species, in which genes encoding the putative homologue to the C-terminal domain of *Rm* Xyn10A were found

	Ch	M	Pg	Rm	Zg[a]
Number of genes	39	2	5	13[c]	1
Average length (residues)	79 ± 0.5	78 ± 0	75 ± 1	85 ± 0.5	73
Theoretical molecular weight (kDa)	8.64 ± 0.06	8.93 ± 0.04	8.43 ± 0.13	9.54 ± 0.09	7.97
PI	5.6 ± 0.2	5.4 ± 0.3	7.5 ± 0.7	9.4 ± 0.5	6.0
Content of selected residues[b] (% of total number of residues)					
Ala	6.1 ± 0.5	4.5 ± 1.9	5.9 ± 1.4	8.7 ± 1.2	4.1
Arg	1.6 ± 0.2	3.8 ± 1.3	4.5 ± 0.9	11.2 ± 0.9	0
Asn	7.4 ± 0.4	3.2 ± 0.6	4.8 ± 0.3	2.4 ± 0.4	8.2
His	0.9 ± 0.1	1.3 ± 0	1.3 ± 0.02	2.4 ± 0.4	0
Ile	9.5 ± 0.6	7.0 ± 2.0	6.1 ± 1.1	2.0 ± 0.6	9.6
Lys	6.2 ± 0.3	7.7 ± 0	7.7 ± 1.3	1.0 ± 0.3	8.2
Pro	4.1 ± 0.2	3.2 ± 0.6	3.7 ± 0.7	6.6 ± 0.6	4.1
Ser	7.9 ± 0.5	7.0 ± 2.0	6.1 ± 0.8	3.2 ± 0.6	11

The microbial species are: *Cytophaga hutchinsonii* (Ch), *Microscilla* sp. (M), and *Porphyromonas gingivalis* (Pg), *Rhodothermus marinus* (Rm), and *Zobellia galactanivorans* (Zg). Values are given as the average ± SEM.

[a] Only one gene found, no statistics possible.

[b] Values are given for those residues where the domains from enzymes of thermophilic *R. marinus* differ significantly from the domains from the mesophilic microorganisms.

[c] The ORF encoding a putative family 6 pectate lyase was excluded, due to a short C-terminal domain (58 residues) indicating a frameshift possibly caused by a sequencing error.

additional hits were also found within three other species, from the same phylum. In addition, three of these five microorganisms (*R. marinus*, *C. hutchinsonii*, and a *Microscilla* sp.), were classified into the same class and order (Sphingobacteria; Sphingobacteriales), and both *R. marinus* and *C. hutchinsonii* to the same family (Chrenotrichaceae) within the bacterial lineage. The remaining two microorganisms (*Porphyromonas gingivalis* and *Zobellia galactanivorans*), belong to separate classes of Bacteroidetes: Bacteroides and Flavobacteria, respectively. In the phylogram analysis, most of the sequences originating from *R. marinus* ($n = 14$) clustered together, and had the shortest distance to some of the sequences originating from its closest relative, *C. hutchinsonii* ($n = 39$). Sequences from the latter were, however, distributed throughout the phylogram (Fig. 2). Presence of only two genes from *Microscilla* sp., and one from *Z. galactanivorans*, rendered discussion of evolutionary relationships premature. In contrast, four of the five sequences from *P. gingivalis* ($n = 5$) clustered together in the phylogram, in a position relatively distant to the *R. marinus* sequences.

A multiple sequence alignment including all the putative domains revealed three consensus regions of which the first was most conserved (Fig. 3). Some physico-chemical properties of respective domain were also analysed, revealing that most of the *R. marinus* sequences, which are on average a few residues longer, have a relatively high p*I* (Table 1). It was also noted that the content of a number of amino acid residues in the *R. marinus* domains differed significantly from the domains of the other species, probably reflecting the thermophilicity of this organism. There is for instance a dramatic increase in the Arg/Lys-ratio, as previously reported for many thermophilic proteins [24], a decrease of residues prone to deamidation (in this case seen as a decrease in Asn content), and an increase in Pro (which could increase rigidity in the structure).

Most of the genes (except Xyn10A and Man26A) found during the search were uncharacterised ORFs, which motivated analysis of the deduced polypeptide regions upstream the D5 homologues. Analysis of the full-length genes revealed that all had a potential N-terminal signal peptide. Furthermore, the best matches encoded proteins predominantly implicated in extra-cellular functions including glycoside hydrolases, and various other cell attached proteins (Table 2), while regions encoding polypeptides of clear intracellular function were absent. The relatively ubiquitous occurrence of this domain within taxonomic subdivisions of a single phylum, its conserved location at the C-termini, and the identification of a conserved motif within its primary structure (which is likely to reflect a structural common fold or motif), along with the common extra-cellular nature of the proteins or activities the domain is associated to, strongly suggest that this is a novel type of module conserved among members of this particular phylum. This along with a revision of the experimental evidence on the cellular location of the *R. marinus* enzymes (see below) makes it extremely tempting to postulate that the cell-attachment of glycoside hydrolases and of other enzymes could be mediated by this domain within the addressed taxonomic group.

Table 2
Putative function of ORFs (in the cases where similarity could be identified) encoding modular proteins that contain a putative homologue to the C-terminal domain of Xyn10A from *Rhodothermus marinus*

Bacterial lineage	Gene or ORF	N-terminal function	Identification
Bacteroidetes; Sphingobacteria; Sphingobacteriales; Crenotrichacae; *Cytophaga hutchinsonii*	ORF 4, 10, 19, 27, 42	Cell wall surface anchor protein/cell surface protein	By sequence similarity (Q97P71); (Q8TJE3); (Q8TI59); (Q8TJS8); (Q97P71)
	ORF5, 23, 31	RCC1 repeats protein	By sequence similarity (Q97FL4); (Q97FL4); (Q97FL4)
	ORF6	Surface antigen	By sequence similarity (Q8TTC5)
	ORF7	Putative signal peptide protein	By sequence similarity (Q8XQF7)
	ORF 8, 9, 15	Hemagglutinin/hemolysin related protein	By sequence similarity (Q8XQP2); (Q8Y366); (Q8XPU1)
	ORF11	Putative β-agarase	By sequence similarity (Q934I7)
	ORF13, 14	GH 9	By sequence similarity (Q9RBJ4); (P71140)
	ORF16	Subtilase fam. protein	By sequence similarity (Q8YWJ8)
	ORF17	Secreted metal-binding prot. (plastocyanin/azurin fam.)	By sequence similarity (Q8TR26)
	ORF18, 33, 38	GH 10	By sequence similarity (AAM21605); (Q9F1V3); (BAC16332)
	ORF20	Putative autotransporter protein	By sequence similarity (Q8PKM0)
	ORF22	Putative RTX-family exoprotein	By sequence similarity (Q8X4H5)
	ORF24	Kelch-like protein	By sequence similarity (Q8P3F9)
	ORF26	Secreted metalloprotease	By sequence similarity (Q97TN3)
	ORF28, 41	GH 18	By sequence similarity (O30678); (Q9C105)
	ORF29	Integrin like repeats	By sequence similarity (Q97F11)
	ORF30	predicted secreted metalloprotease and GH 18	By sequence similarity (Q97TN3 and Q9KQP6)
	ORF32	GH 8 (and 18)	By sequence similarity (P37701 and Q9C105)
	ORF35	GH 26	By sequence similarity (O30654)
	ORF36, 40	GH 5	By sequence similarity (Q9AQH0); (Q95YN1)
Bacteroidetes; Sphingobacteria; Sphingobacteriales; Flexibacteraceae; *Microscilla* sp.	ORF1, 2	MS130 and MS116, putative β-agarase	Annotated (Q93PA1); (Q934I6)
Bacteroidetes; Sphingobacteria; Sphingobacteriales; Crenotrichaceae; *Rhodothermus marinus*	ORF4825	Extracellular serine protease	By sequence similarity (Q8YDM7)
	ORF3935	GGDEF family protein	By sequence similarity (Q8P9F2)
	ORF PL6	Polysaccharide lyase family 6	By sequence similarity (Q9RKE2)
Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae: *Zobellia galactanivorans*	ORF1	β-Agarase A precursor	Annotated (Q9RGX9)

3.3. Experimental data support cell-attachment

A series of batch-cultivations of *R. marinus* were performed in order to induce expression of the native xylanases and monitor their cellular location as to provide experimental evidence for the hypothesis presented above. The gene encoding *Rm* Xyn10A encodes an N-terminal putative signal peptide [14], ruling out an intracellular location. This finds support in a previous investigation in which it was demonstrated that xylanase activity was not located in the intra-cellular protein fraction [11].

In the presence of xylan, *R. marinus* had a specific growth rate of 0.35 h^{-1}, and grew to a final $OD_{620\,nm}$ of approximately 5. Without xylan, the final $OD_{620\,nm}$ was lower, and xylanase activity not detectable. Xylanase activity was hence measured in both *R. marinus* cell slurries (cell attached activity), and in the cultivation medium, of the culture grown in presence of xylan. In both fractions, the activity was increasing throughout the cultivation and the cell associated activity was always higher than that of the cultivation medium (Fig. 4(a)), while intracellularly, it was below the detection limit of the assay as determined by protein fractionation [11]. Evidence that the cell-bound activity originated from Xyn10A emerged from two techniques: activity staining and immunocytochemistry. Activity staining after electrophoresis of samples (identical to those analysed for activity) showed a single band of xylanohydrolytic activity of the expected molecular mass ($M_r \approx 110$ kDa) in the cell fraction (showing intracellular and cell-attached proteins, Fig. 4(b)), excluding the possibility that the cell-bound activity arose from additional cell-attached xylanases produced by the organism. Definite proof of the attachment of Xyn10A to *R. marinus* cells was collected after immunocytochemical analysis, in which primary antibodies against the carbohydrate binding module of Xyn10A (produced as a recombinant protein in *Escherichia coli*) were effectively bound to the cells (Fig. 5).

Two activity bands were observed in samples from the cultivation medium, one with high (≈100 kDa) and one with low (<25 kDa) apparent molecular mass (Fig. 4(b)). The low molecular mass activity band proved presence of an additional secreted xylanase in *R. marinus*. Interestingly the M_r differences between the higher molecular mass (100 kDa) activity-band and that of Xyn10A conformed well to the M_r of D5, suggesting the possibility of release after (proteolytic) cleavage at the linker preceding D5. This would explain the previously observed slow release of xylanase to the growth medium during the stationary phase [11]. An alternative explanation for the release would be cell lysis, but the apparent decrease in molecular mass in our current results supports the former. Further support for proteolytic cleavage was collected from production patterns of recombinant *Rm* Xyn10A and truncated variants in *E. coli*, which showed two linker positions in the heterologous full-

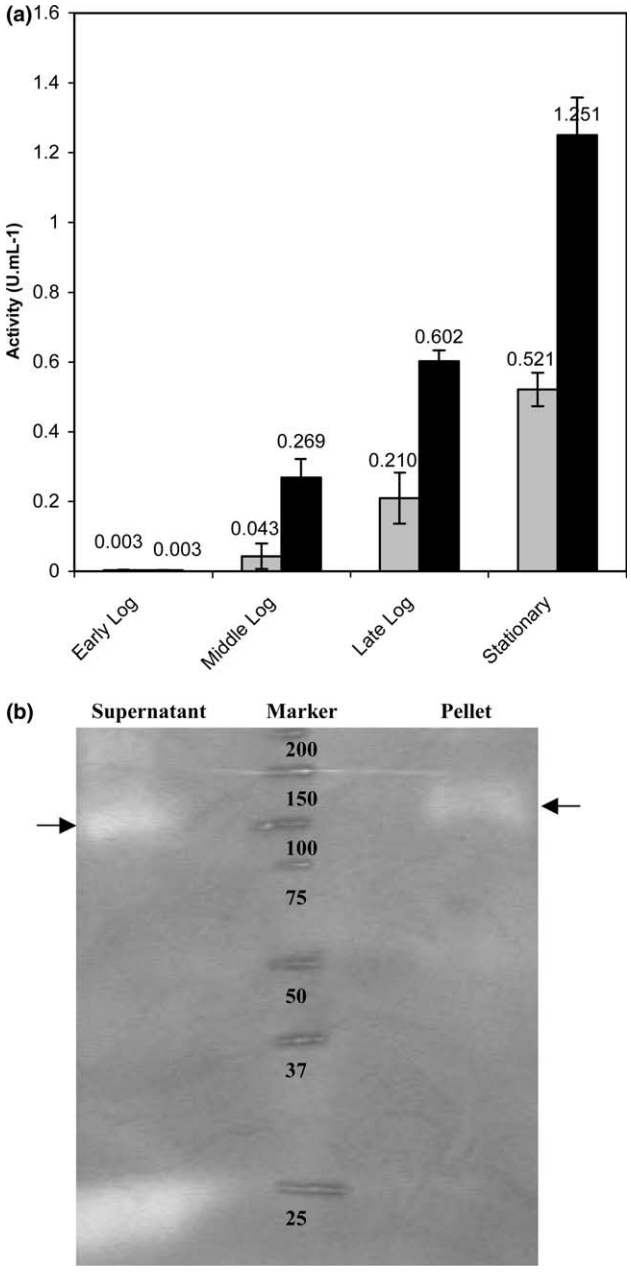

Fig. 4. Xylanase activity in the culture supernatant and on *R. marinus* whole cells. Activity was analysed by the DNS-assay for reducing sugars (a) using birch xylan as the substrate, and by activity staining after electrophoretic separation (by SDS–PAGE) and in-gel refolding (b), using Congo-red staining of a xylan-containing overlayer-gel. Samples for activity analysis (a), were taken at times corresponding to different phases of the growth-curve, after 3 h (early log phase), 9 h (mid-log phase), 13 h (late log-phase) and 16 h (stationary phase) and the activity of the supernatant (grey) and the whole cells (black) was analysed. The positions of the standards in the prestained molecular mass ladder (Biorad) after separation is indicated by a scalpel in the overlayer gel, and activity bands from the mid-log sample (9 h) of the supernatant (left) and cell pellet (right) of a size corresponding to Xyn10A are indicated by arrows (b).

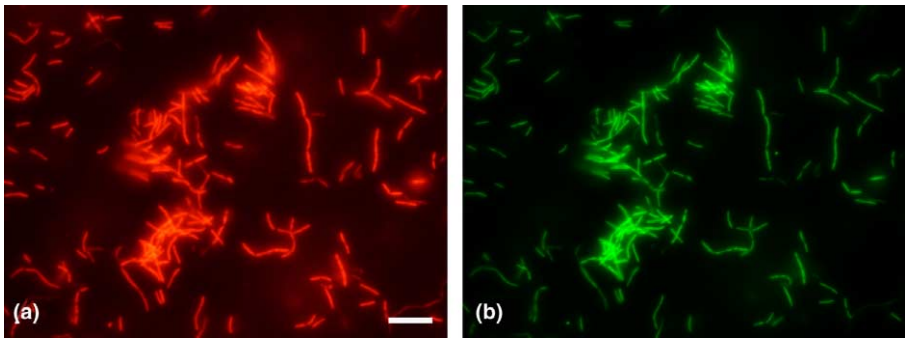

Fig. 5. Fluorescence microscopy image after immunocytochemical staining of *R. marinus* cells from the late growth phase grown in the presence of xylan. Binding of the primary xylanase-antibody to the cells is visualised after incubation with secondary antibody conjugated to the fluorophore Rhodamine Red-X (a). Subsequent staining of all cells with fluorescent Sytox Green nucleic acid stain (b) demonstrated that the xylanase was expressed on the surface of all cells. Bar represents 10 μm.

length enzyme to be susceptible to proteolytic cleavage, one of them being the linker preceding D5 [23]. Since the functions of the other modules in this enzyme are known except for the third domain (D3), cell attachment could theoretically be mediated by either domain. The third domain, however, has no homologues in this microorganism or in related ones (an does not so far show significant sequence similarities to any deposited sequence), which practically discounts it from any common function such as cell attachment, and leaves D5 as the most credible site of cell anchoring.

Cultivations in presence of galactomannan (locust bean gum) in our laboratory showed also the major part of the mannanase activity associated with the cell slurry (data not shown). This finding together with the facts that the only shared similarity between the two *R. marinus* enzymes is in the C-terminal domain, and the data from Politz and coworkers [15] promote the cell-attachment hypothesis. Politz and co-workers found cell-bound (and no intracellular) mannanase activity after protein fractionation of *R. marinus* cells. No N-terminal signal peptide was recognised in Man26A by Politz and coworkers, but as the collected data on the location of the enzyme proved export of the enzyme at least to the periplasmic space [15] we have (using the program SignalP) recognised a likely location of a signal peptide on the N-terminal side of the catalytic module (comprising the sequence MTLLLVWLIFTGVA). In accordance with the xylanase experiments and with the activity graphs published by Gomes and Steiner [13], some mannanase activity was also released to the culture medium during the later growth phase and the stationary phase.

3.4. How are the domains attached to the cells?

Currently we can only speculate on how Xyn10A is attached to the *R. marinus* cell. The domains found in this search did not share significant sequence similarities with SLH-domains (proposed to anchor GH to many G-positive microorganisms by noncovalent interactions with secondary cell wall polymers, in turn covalently linked to peptidoglucan [7,8]) so no conclusion can be made based on their mechanism. Another difference is that *R. marinus* is a G-negative bacterium [25]. Based on the observation of the three conserved regions (with a number of hydrophobic residues) in the domain, attachment could be mediated by hydrophobic stretches (specific or unspecific), but a protein-carbohydrate interaction (e.g., via lipopolysaccharides (LPS), found to often be mediated by Ca^{2+}) [6] is also possible. At this point further discussion will have to await isolation and/or characterisation of *R. marinus* cell wall polymers, in order to study possible alternatives.

4. Conclusion

Bioinformatic and experimental lines of evidence are analysed in this study to elucidate the function of the fifth domain (D5) in Xyn10A. Our results clearly demonstrate that this domain is relatively wide-spread within subdivisions of the Bacteroidetes taxonomic group and that it displays a common primary structure motif and location at the C-termini which reflects its common function and evolutionary history. Moreover, experimental evidence from *R. marinus* cells in combination with evidence gathered from previous studies leads to the suggestion that this domain type mediates cell-attachment in proteins produced by members of the Bacteroidetes. Additional studies are motivated to highlight the mechanism of cell-attachment and the structural basis of this function. Finally, identification of a potential cell receptor for this module (if any) opens the door for use of the receptor/module interaction in biochemical studies, and for biotechnological applications.

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