**Structural analysis of the reducing-end xylose-releasing exo-
oligoxylanase Rex8A from *Paenibacillus barcinonensis* BP-23
deciphers its molecular specificity**

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Abbreviations: The abbreviations used are: GH, glycosyl hydrolase; Rex8A, *Paenibacillus barcinonensis* BP-23 reducing-end xylose-releasing exo-oligoxylanase; CtCelA, *Clostridium*

thermocellum β -glucanase; *PhXyl*, *Pseudoalteromonas haloplanktis* xylanase; *TtXyl*, *Terenidibacter turnerae* xylanase; *BhRex*, *Bacillus halodurans* C-125 Rex; *BiRex*, *Bacteroides intestinalis* Rex8; *BaRex*, *Bifidobacterium adolescentis* RexA; *PbRex*, *Paenibacillus barengontzii* Rex; *PhXyn*, *Pseudoalteromonas haloplanktis* Xyn8; *BsXyn*, *Bacillus* sp. KK-1 XynY; *BiXyn*, *Bacteroides intestinalis* Xyn8A; UXOS , methylglucuronic acid-branched xylooligosaccharides; AXOS, arabinose-decorated xylooligosaccharides; AX3, 2³- α -L-arabinofuranosyl-xylotriose (Ara³Xyl₃); AX4, 3³- α -L-Arabinofuranosyl-xylotetraose (Ara³Xyl₄).

Database: structural data are available in PDB database under the accession numbers 6SRD (native form), 6TPP (E70A mutant in complex with EDO), 6TOW (E70A in complex with Xyl4), 6SUD (L320A mutant in complex with xylose), 6SHY (L320A/H321S double mutant in complex with EDO), 6TO0 (E70A in complex with AX3) and 6TRH (E70A in complex with AX4).

Keywords: Xylanase structure; GH8 specificity; reducing-end xylose-releasing exo-oligoxylanase; decorated xylan

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ABSTRACT

Reducing-end xylose-releasing exo-oligoxylenases (Rex) are GH8 enzymes that depolymerize xylooligosaccharides complementing xylan degradation by endoxylenases in an exo manner. We have studied *Paenibacillus barcinonensis* Rex8A and showed the release of xylose from xylooligomers decorated with methyl-glucuronic acid (UXOS) or with arabinose (AXOS). This gives the enzyme a distinctive trait among known Rex, which show activity only on linear xylooligosaccharides. The structure of the enzyme has been solved by X-Ray crystallography showing a $(\alpha/\alpha)_6$ folding common to GH8 enzymes. Analysis of inactivated Rex8A-E70A complexed with xylotetraose revealed the existence of at least four binding subsites in Rex8A, with the oligosaccharide occupying subsites -3 to +1. The enzyme shows an extended Leu320-His321-Pro322 loop, common to other Rex, which blocks the binding of longer substrates to positive subsites further than +1 and seems responsible for the lack or diminished activity of Rex enzymes on xylan. Mutants with smaller residues in this loop failed to increase Rex8A activity on the polymer. Analysis of the complexes with AXOS showed the accommodation of arabinose at subsite -2, which cannot be allocated at subsite -1. Arabinose substitutions at the xylose O2 or O3 are accommodated by hydrophobic interaction and seem tolerated rather than recognized by Rex8A. A strained binding of the branch is facilitated by the lack of direct polar interactions of the xylose occupying this subsite, its water-mediated links allowing some conformational flexibility of the sugar. The plasticity of Rex8A is a notable property of the enzyme for its application in xylan deconstruction and upgrading.

INTRODUCTION

Nowadays, biomass and its derivatives are emerging as a promising raw material to replace petrol based products for the development of a sustainable circular economy [1]. One of the central components of biomass is xylan, an heteropolysaccharide composed of chains of xylose that are ramified to a different extent and with different substituents depending on the source of the polymer [2]. The enzymatic decomposition of xylan involves the catalysis of a complex pool of hydrolytic enzymes to debranch and depolymerize the main backbone of the polysaccharide [3]. Endo-xylanases (EC 3.2.1.8) are the key enzymes in the depolymerization process since they randomly cleave the xylan backbone to soluble xylooligosaccharides. Based on sequence and structure similarities of their catalytic domain, xylanases have been classified into glycosyl hydrolase (GH) families 10, 11 and 30 in CAZy database (<http://www.cazy.org>), but xylanase activity is also found in GH families 5, 8, and 43 [4]. Complementing the xylanase activity, GH8 family contains the few members of the reducing-end xylose-releasing exo-oligoxylanases (Rex) (EC 3.2.1.156) described to date [5-9]. Rex enzymes display the hydrolysis in an exo-splitting manner, progressively removing xylopyranosyl units from the reducing ends of the substrates. The attributed role for Rex enzymes lies in the intracellular depolymerization of xylooligosaccharides since four of the five enzymes described until the date have no signal peptide [8].

GH8 enzymes present an inverting single-displacement reaction mechanism and fold into a $(\alpha/\alpha)_6$ barrel common to other inverting glycosidases. They have been divided into three subfamilies depending on the position of the residue acting as the base catalyst [10]. Rex8A belongs to subfamily GH8a, which has the aspartate at the N-terminus of helix α_8 of the barrel. The three-dimensional **structures** of three GH8a members, all of them endo-enzymes, have been reported, i.e. the β -glucanase from *Clostridium thermocellum*, CtCelA [11] and the xylanases from *Pseudoalteromonas haloplanktis*, PhXyl [12] and *Terenidibacter turnerae*, TtXyl

[13]. Additionally, the molecular basis of the exo-activity observed in Rex enzymes [14] has been attributed to a kink in the loop preceding α_{10} , by crystallographic analysis of *Bacillus halodurans* C-125 Rex, BhRex [5].

Rex8A from *Paenibacillus barcinonensis* is one of the few examples of Rex enzymes described so far [8]. It belongs to the complex xylanolytic system of this bacterium, **including** several enzymes with industrial potential [15] [16] [17] [18]. As the other characterized Rex enzymes, Rex8A shows **an** efficient removal of xylose from linear xylooligosaccharides but only shows minor activity on xylan. However, contrary to known Rex, it also releases xylose from decorated xylooligosaccharides (methylglucuronic acid branched xylooligosaccharides, UXOS), **which** gives RexA a distinctive trait among other Rex enzymes [8]. In this work we have studied the structural traits of Rex8A that could give insight into the unique properties of the enzyme. We have solved the three-dimensional structure of Rex8A and several mutants that have been constructed, including those in the loop blocking the access to polymeric substrates, and analyzed their binding abilities to xylooligomers by soaking and cocrystallization experiments. We have also shown that the activity of Rex8A on decorated oligomers **extends** to xylooligosaccharides with arabinose decorations (AXOS), and depicted their interaction within the binding subsites by crystallographic analysis of their complexes. The results obtained contribute to deciphering the function of Rex8A in xylan depolymerization. Further research needs to be done to understand the role of Rex enzymes in biomass deconstruction and their potential in the upgrading of lignocellulosic residues to added value products.

RESULTS AND DISCUSSION

Crystal structure of Rex8A

To elucidate the molecular basis of its enzymatic activity, the crystal structure of native Rex8A was solved at 1.9 Å resolution (Fig. 1). Experimental details and structure determination procedures are given in the Materials and Methods section and in Table 1. The crystals belong

to the P1 space group, with two molecules in the asymmetric unit and 50 % solvent content within the cell. A xylose molecule from the cryoprotectant solution used prior to data collection has been captured at the active site (WT-xylose). The structure was determined by means of Molecular Replacement using as the search model the coordinates from the reducing-end xylose-releasing exooligoxylanase from *B. halodurans* C-125, *BhRex* (PDB entry 1wu4, [5]). Inactivated mutant Rex8A-E70A was also crystallized and used for soaking experiments with xylotetraose and xylohexaose, however, only two molecules of **ethyleneglycol**, used as additive in the precipitant solution, were found bound at the active site (E70A-EDO), which is explained in terms of the occlusion of part of the active site by a proximal protein molecule located next to the cavity, as explained below.

Rex8A is folded into the expected $(\alpha/\alpha)_6$ folding common to GH8 members, with long loops surrounding one axis of the barrel, and the C and N-terminal segments tightly packed against the opposite face (Fig. 1). Besides the known structure of *BhRex*, having 64 % sequence identity with Rex8A, the coordinates of the *Paenibacillus barengoltzii* G22 *PbRex*, with 68 % identity, were recently released (PDB code 5YXT). Structural comparison shows that the folding of the three enzymes is very well conserved, as reflected in the very low r.m.s.d. of 0.74 and 0.76 Å of the two enzymes vs. Rex8A, for 374 and 370 residues (Q scores of 0.92 and 0.91), respectively. The most significant difference is the Pro250-Arg260 loop highlighted in Fig. 1, which is the most variable region and clearly longer in Rex8A with respect to the other Rexs. A xylobiose molecule bound in the *BhRex* crystal at the -2, -1 subsites suggested the importance of this loop in determining substrate binding. Curiously, this is the loop impeding active site cleft accessibility in Rex8A by being inserted into the distal subsites of the adjacent molecule (Fig. 1, inset), which reinforces its role in binding affinity. Therefore, additional experiments were undertaken to find new crystallization conditions that would allow the formation of complexes with oligosaccharides.

The structure of the Rex8A-Xyl4 complex depicts the active site

The inactivated mutant Rex8A-E70A was incubated with xylotetraose prior to the setting of new crystallization screenings, leading to a new crystal form with different habit and a new trigonal symmetry (E70A-Xyl4). From these crystals, the structure of the Rex8A-Xyl4 complex was determined at 2.05 Å resolution, the electron density maps showing the oligosaccharide occupying subsites -3 to +1, as seen in Fig. 2A. The clear electron density observed in all the subsites could **indicate** a strong interaction and, consequently, the existence of at least four binding subsites in Rex8A.

The overall structure of the complex is very similar to that of the unliganded crystals, and only minor rearrangement of the Pro250-Arg260 loop, which approximates by 0.5 Å to the ligand, are observed upon sugar binding (Fig. 2B). The movement towards the substrate at the main chain of two regions near subsite +1 previously reported in *BhRex* [5] and corresponding to Thr62-Asn64 and Lys355-Arg358 in Rex8A, are not observed in our complex, although a different orientation of the Lys357 and Arg358 side-chains is evident in the crystal. The relevance that this observation may have in substrate binding is discussed later.

Xyl4 is occupying the narrow cleft in an extended conformation with hydrophobic stacking interactions of the xyloses at subsites -2 and +1 to Trp112 and Tyr361 side chains, respectively (Fig. 2B). In addition, the oligosaccharide is accommodated by many polar interactions with the residues at the active site, including several well-ordered water molecules.

As has been described **for** the other Rex enzymes, subsite -3 seems quite open to the solvent, the xylose ring making only hydrophobic contacts to the conserved Ile187. A higher K_m for xylooligosaccharides longer than xylotetraose has been reported in *BhRex8A* [14] although no structural rationale supported the negative effect of binding at this subsite [5]. In Rex8A the cleft is narrowed by the longer Pro250-Arg260 loop (Fig. 1A), which situates the C5-O5 moiety within 5 Å from His256 ring **and making** hydrophobic contact to its side-chain. In addition, O2 is linked to the Pro125 main-chain through a water molecule, which may contribute to fix the xylose bound at this subsite. A subsite -3 has been previously reported **to be present** in the

endoglucanase *CtCelA* [11] and the endo-xylanase *TtXyl*, with 26 and 38 % sequence identity, respectively, to Rex8A, [13], **but not in *PhXyl* [12]**), with 30 % identity. We reported previously that Rex8A shows maximum activity vs. xylotriose, with lower activity vs. xylohexaose, which is consistent with the low number of interactions accommodating xylose bound at this subsite. Therefore, despite that clear electron density seems to indicate a **fixed** position for xylose, the contribution of this subsite **to** substrate affinity might be moderate. On the other hand, considering its position and orientation, decoration of xylose at this subsite could be **allowed** only at O3 that is open to the solvent.

The xylose located at subsite -2 is sandwiched between Trp112 and Phe186 (this last through a T-interaction pattern) in a way conserved in all known GH8 **enzymes** degrading xylose-based oligosaccharides. In addition, most of its O atoms are involved in water-mediated polar interactions. Thus, O2 and O5 are linked through water molecules to Tyr197 hydroxyl, and to Tyr243 (O2) and Phe186 main-chain (O5). Largest differences in the active site with respect to the known Rexs are found in this subsite -2 due to the presence of its extended Pro250-Arg260 loop, **containing** His256, and the substitution of a Tyr by Phe259 in Rex8A. This makes a narrowed cavity at O3, and a wider cleft at O2 filled by a well-ordered net of water molecules that fixes O2 from xylose rings at subsites -2, and -1, and the O glycoside link. Therefore, decorations could appear, in principle, impeded at O3, but possible at O2, but this issue was further investigated, as explained later.

Distortion of the sugar ring located at subsite -1 is a common requirement in GH enzymes for the hydrolysis to proceed. In the case of GH8 enzymes, a ^{2,5}B-like transition state was proposed in the *CtCelA* as this conformation had been observed in the reported complex of this enzyme with cellopentaose [11]. However, in the two other reported complexes with xylooligosaccharides spanning subsites -1, +1 of GH8 endoxylanases from *TtXyl*-Xyl6 [13] and *PhXyl*-Xyl5 [12], this boat conformation has not been trapped, the xylose located at subsite -1 being in a ⁴C₁ chair non-catalytically relevant conformation. In the particular case of the *TtXyl*-

Xyl6 complex, xylose at subsite -1 is in a very peculiar ring-flipped, southern hemisphere 1C_4 conformation. Furthermore, by *in silico* docking analysis, a conformational change from the chair ground state to a pretransition state local minimum in a 2S_0 conformation was proposed [12]. In our Rex8A-Xyl4 complex, the xylose is in a distorted 4C_1 conformation with C1, C2, C3, C4 and C5 being rather flat. Thus, the O2 and O3 atoms keep polar interactions with Asp128 (O2, O3) and Arg268 (O2) similarly to that observed in the CtCelA complex, but the cyclic O5 atom is puckered and makes a polar interaction with **the** Arg68 side-chain, absent in the CtCelA enzyme. The relevance of this conformation is uncertain, considering that inactivation of the enzyme involves removal of the proton-donor side-chain in the Glu70Ala replacement, which might introduce artificial biases at this subsite. The same biases could have happened in the previously reported GH8 complexes, in which the proton donor (CtCelA-E95Q), the base (TtXyl-D281N) or a third catalytically relevant aspartate (PhXyl-D144A) have been altered (Fig. 2C). Consequently, the specific nature of the catalytically relevant enzyme-substrate complex and the conformational itinerary of these class of enzymes remains to be confirmed. Nevertheless, and despite the different conformations captured at subsite -1, a remarkable similarity in the position and ring orientation of the Glu/Xyl bound from -2 to +1 subsites is evident in Fig. 2C. This feature, together with a similar chain bending at the scissile bond, reveals a very conserved catalytic machinery within both, endo and exo GH8 enzymes.

The xylose located at subsite +1 is stacked to Tyr361, conserved in all the GH8 enzymes, and is tightly fixed by many polar interactions to residues unique to the Rex enzymes. Thus, O1, O2 and O3 are making direct polar links to His321, Asp61, and Arg68 side-chains. In addition, O3 and O5 are making water mediated interaction to Tyr360 and Ser264 side-chains.

Interestingly, O2 is pointing to a polar cavity occupied by well-ordered water molecules making a net of hydrogen links connecting the sugar to Asp61 carboxylate and to the main-chain of Arg358, Tyr360 and Tyr361. This network of polar links seems essential for keeping the integrity of the subsite +1 architecture, as will be illustrated below. On the other hand, the most important difference found in the Rex subfamily with respect to the other GH8 enzymes is a kink in the

loop Leu320-Pro322 that blocks the active site at the reducing-end and impedes bindings of longer substrates [5]. Thus, exo-GH8 enzymes may have evolved from their endo-partners by just a few changes in this section of the active site, which may suggest that the reversal of this endo-character could be possibly accomplished, in principle, by mutating selected key residues, an issue that is explained below.

Molecular basis of the exo/endo activity

As mentioned before Rex8A does not show positive subsites further than +1 because it is blocked by loop Leu320-His321-Pro322 that impedes **binding** of longer substrates. This loop is conserved among the characterized Rex enzymes while it is not found in known GH8 xylanases. As an exception, the sequence LHP is not found in the Rex from *Bacteroides intestinalis* (BiRex), although it has an extra span of amino acids in the corresponding region of the enzyme (Fig. 3). This fact should preclude the endo activity of the Rex enzymes, that show only a very minor activity on xylans probably resulting from its reducing-end exo activity. To analyze the contribution of the loop to the exo vs. endo activity of Rex8A a series of mutants were constructed in which the residues Leu and His were replaced by smaller Ala residues. The activity of the two single mutants constructed, L320A and H321A, were assayed on different types of xylans (Table 2). Wild type enzyme showed higher activity on arabinoxylans from wheat and oat spelt than on beechwood glucuronoxylan, although all the activity values detected were very small. The activity on xylans was also confirmed by TLC analysis which showed the release of xylose from the polymers (data not shown). L320A showed a similar low activity on arabinoxylans while no activity was found on glucuronoxylan. A double mutant L320A/H321S constructed showed similar behavior on xylans than L320A. **On the other hand**, H321A mutant did not show activity on the xylans tested.

Then, the activity of wild type Rex8A and its mutant derivatives was analyzed on linear xylooligosaccharides. (Fig. 4A). Rex8A showed activity on the substrates tested, xylotriose, xylotetraose and xylohexaose, releasing xylose and xylobiose as main hydrolysis products, in accordance with its exo mode of action. Null mutant E70A **did not show** activity on these

oligomers, while L320A showed similar activity on xylooligosaccharides **to that observed with** the wild type enzyme. On the contrary H321A and L320A/H321S showed a diminished activity on these substrates, this decrease **being more apparent in the double mutant tested against xylohexaose.**

We investigate the molecular basis of these results performing crystallization experiments, which yielded crystals from the single L320A and double L320A/H321S mutants. The structure of the L320A mutant with a trapped xylose (L320A-Xyl in Table 1) is identical to that from the native-xylose complex shown in Fig. 1, which reveals an equivalent binding mode at subsite +1 consistent with the similar behaviour observed in the activity of this mutant vs. xylooligosaccharides and xylan. Thus, Leu320 does not appear critical for Rex8A exo-activity. **In contrast**, the L320A/H321S double mutant (L320A/H321S-EDO) shows structural rearrangement of some regions delineating the active site, particularly at the loops Leu60-Asp65 and Arg354-Tyr360, at the end of helices $\alpha 1$ and $\alpha 8$ of the barrel, which shift by 1 Å probably as a consequence of the loss of polar links associated **with** the removal of the His321 side-chain. As it is shown in Fig. 4B the His321 side-chain interacts with Asp362 that is linked to Arg354, which, in turn, interacts with Arg358 main-chain. Therefore, removal of His321-Asp362 link might disrupt the polar network building this part of the active site with the observed shift in the loops, what can be detrimental for substrate binding. Most important seems the loss of the His321-xylose interaction, as the NH-O1 hydrogen bond has been reported to be critical for discrimination of the α/β xylose isomer [5]. As it is observed in Fig. 4C and 4D, removal of His321 makes a wider cavity in the mutant, as compared to native, which **allows to bind** an EDO molecule linked to the introduced Ser321-OH. However, the fact that soaking crystals with xylose did not trap xylose at the active site points to an important decrease in affinity, which agrees with the extremely reduced activity observed in this mutant. Therefore, the elongation of the active site does not induce an endo-mode of action but, rather, disturbs the active site for proper positioning of the xylose at +1 subsite, with a detrimental effect in activity.

Analysis of the specificity vs decorated substrates

As mentioned before, previous works showed the activity of Rex8A on methylglucuronic acid decorated xylooligosaccharides (UXOS). The substrates reported, aldotetraauronic ($\text{MeGlcA}^3\text{Xyl}_3$) and aldohexauronic ($\text{MeGlcA}^3\text{Xyl}_5$), showing the decoration in the third xylose from the reducing-end, were shortened in one xylose residue but were not further hydrolysed [8]. These results, together with docking analysis suggested the accommodation of the methylglucuronic moiety in -2 subsite of the enzyme, while the -1 subsite would not allow decorations [8]. This type of substrate accommodation resembled to that described for GH30 bacterial xylanases, which require accommodation in -2 subsite for activity on glucuronoxylans and UXOS [19-22]. We compared hydrolysis products released by a GH30 xylanase, *P. barcinonensis* Xyn30D, or by Rex8A from aldotetraauronic and aldohexauronic acids by TLC analysis. The products released by the enzymes showed identical mobility, clearly indicating the accommodation of decorations in -2 subsite of Rex8A (Fig. 5A).

In addition to the ability of Rex8A to hydrolyse methylglucuronic acid-decorated xylooligosaccharides, our present work reveals that Rex8A exhibits moderate activity on the two tested arabinoxylans (Table 2). Furthermore, from inspection of the crystal structure reported here, we can expect that the cavity observed at -2 subsite could also accommodate arabinose substituents of xylan oligomers. Thus, we tested Rex8A activity on two different arabinose-decorated xylooligosaccharides (AXOS), 2³- α -L-Arabinofuranosyl-xylotriose (Ara^3Xyl_3 , AX3) and 3³- α -L-Arabinofuranosyl-xylotetraose (Ara^3Xyl_4 , AX4), which show the arabinose decoration in the third xylose from the reducing-end, but differ, besides length, in the xylose carbon atom to which the arabinose moiety is linked, C2 or C3, respectively. Considering the shape of the cavity observed in Rex8A at subsite -2, only decorations at O2 would have been expected, as O3 is pointing to a more constricted, narrower pocket, as commented before. Surprisingly, Rex8A cleaved both AXOS, which were shortened in a xylose residue that was released (Fig. 5B). The shortened AXOS were not further cleaved by Rex8A, similarly to the results shown for UXOS. These results confirm the accommodation of decorations in -2 subsite while the -1 subsite would not allow main chain substituents. On the other hand, the activity of mutants on

these substrates follows the same pattern to that observed on xylan and linear xylooligosaccharides (Table 2 and Fig. 5B).

To investigate the binding mode of branched xylooligosaccharides and the specific recognition of the corresponding substitutions, the E70A inactivated mutant was incubated with both substrates and new crystallization conditions were tested. Crystals were grown from the Rex8A-AX3 and Rex8A-AX4 complexes, and their structures were solved leading to the final data given in Table 1. The analysis of both complexes reveals that both AXOS are able to accommodate within the narrow slot similarly to the unsubstituted Xyl4, showing no significant changes in the residues delineating the active site (Fig. 6A). However, some differences are observed in the binding mode and in the conformation adopted by each substrate. First, the AX3 is bound with the three xylose units in a chair conformation and keeping the same interactions pattern described in the Rex8A-Xyl4 complex (Fig. 6B). At subsite -2, the introduced arabinose is oriented to the hydrophilic cavity surrounding the catalyst Asp265 and filled with water molecules, in a way that its **ring** oxygen is making the same hydrogen bonds presented by the unsubstituted O2 of Xyl4. This seems the only polar interaction stabilising the decoration. In the case of the Rex8A-AX4 complex, binding of the substituted tetrasaccharide involves a distortion of the xylose at subsite -3, which is stabilized by a new water-mediated polar interaction to Gly124, while the arabinose at subsite -2 is directed out from the cavity showing no interactions with the enzyme. (Fig. 6C). Apart from this, the hydrogen bond pattern at the other subsites is essentially similar to the other two complexes.

Therefore, it seems that Rex8A is not specifically recognising the arabinose substitutions of these substrates but, rather, it would be tolerating the existence of these decorations at its active site. The structural rationale for this **hypothesis** is made clear from the complexes here reported. The analysis of the binding mode of the tested xylooligosaccharides reveals that the -2 and -3 subsites lack direct polar links between the protein and the substrate, which might confer more plasticity to them. At subsite -2, the xylose unit is fixed by stacking to Trp112 and hydrophobic T-interaction to Phe186. Moreover, the Rex8A QLH motif makes a more prominent

loop that narrows the slot, placing the decorations in a very constrained position. Thus, in the case of substitutions at O3, some movement of the flexible loop could have been expected to open the slot making room for the branch at O3. On the contrary, fitting of the decorated substrate leads to slight distortions at the adjacent subsite -3 that seem easily tolerated by Rex8A.

CONCLUSIONS

The reducing-end specificity of Rex8 enzymes has been attributed to a kink in the loop Leu320-His321-Pro322 blocking subsite +2. According to this, a limited number of mutations have been proposed to mediate the evolution from the general endo-activity of GH8 to the particular exo-xylooligosaccharide activity of Rexs. In an attempt to reverse this evolution, we have investigated the effect of changes in the motif LH and correlated the results with the crystal analysis. While mutants at Leu320 do not introduce significant changes, removal of **His321** impedes a proper positioning of the xylose at +1 subsite, explaining the detrimental effect observed in activity. Thus, construction of a more extended active site does not induce the expected exo-activity but, rather, introduces slight rearrangement of some loops at the active site that result in decreased activity.

Furthermore, we have expanded our previous work on the unique activity of Rex8A on branched glucurono-xylooligosaccharides, by elucidating its ability to degrade arabino-substituted substrates. Moreover, detailed structural analysis of complexes with linear and decorated oligomers reveals the molecular basis of its specificity, and a unique loop at -2 subsite that delineates a narrow pocket determining a constrained binding mode of the decorations at this subsite. Arabinose substitutions at the xylose O2 or O3 are accommodated by hydrophobic interaction and seem “tolerated” rather than recognized by Rex8A. This strained binding of the branch is facilitated by the lack of direct polar interactions of the xylose occupying this subsite, its water-mediated links allowing some conformational flexibility of the sugar.

Rex8 are very interesting enzymes for deconstruction of xylan. Its molecular analysis could be of great value in the design of efficient xylanases to transform lignocellulose and cell-wall materials into bio-fuel or added-value products, with the aim of achieving more sustainable industrial processes.

MATERIALS AND METHODS

Cloning and mutagenesis of Rex8A

Construction of Rex8A and Rex8A-E70A expression vectors were previously reported [8]. Rex8A-L320A, Rex8A-H321A and Rex8A-L320A/H321S were generated by site-directed mutagenesis using the same strategy as with Rex8A-E70A [8]. The following mutagenic oligonucleotide primers were used in the PCR: Rex8A-L320A-fw (5-aacgagcccgcGCgcaccccgtaggcctgctggccaccaat-3) and Rex8A-L320A-rv (5-gcctacgggggtgcGCtgcgggctcgttgaacgggtcaccctc-3); Rex8A-H321A-fw (5-gagcccgcatgGCccccgtaggcctgctggccaccaatgct-3) and Rex8A-H321A-rv (5-caggcctacggggGCcaatgcgggctcgttgaacgggtcacc-3); Rex8A-L320A/H321S-fw (5-aacgagcccgcGCgAGccccgtaggcctgctggccaccaat-3) and Rex8A-L320A/H321S-rv (5-attggtggccagcaggcctacggggCTcGCtgcgggctcgtt-3) (mismatched codons are underlined, changed bases are in capital).

Expression and purification of Rex8A and mutants

Recombinant proteins were purified from *E. coli* BL21 Star (DE3) clones containing the respective plasmid as described elsewhere [8]. Briefly, exponential phase cultures were induced with 0.5 M IPTG at 16° for 18 h and cells were lysed by a Homogenizer PandaPLUS 2000 (GEA). Recombinant His-Tag proteins were purified from cleared cell extracts by immobilized metal affinity chromatography (IMAC) using HisTrap HP columns of 1 ml (GE Healthcare) on a fast protein liquid chromatography system (ÄKTATM FPLC TM, GE Healthcare). Samples were then desalted with PD-10 columns (GE Healthcare) and finally eluted in 20 mM Tris-Cl buffer, pH 7.

Proteins concentration was performed in Centricon Centrifugal Filter Units of 30 kDa MWCO (Millipore). The purity of the protein was verified by SDS-PAGE (sodium dodecyl sulfatepolyacrylamide gel electrophoresis) and the concentration of the enzymes were measured with NanoDrop® ND-1000 (NanoDrop Technologies, Inc), using an extinction coefficient (ϵ) at 280 nm of 99950 for all the variants.

Crystallization and data collection

Initial crystallization conditions for Rex8A (5 mg ml⁻¹ in 20 mM Tris–HCl pH 7) were explored by high-throughput techniques with a NanoDrop robot (Innovadyne Technologies Inc.), using six different commercially screens: PACT and JCSG+ Suites from Qiagen; JBScreen Classic 1-4 from Jena Bioscience; and Index, Crystal Screen and SaltRx packages from Hampton Research. These assays were carried out using the sitting-drop vapour-diffusion method in MRC 96 well crystallization plates (Molecular Dimensions). Rod-shaped crystals diffracting at moderate resolution grew in 22–25% (w/v) PEG 6K, 200 mM NaCl and 100 mM Tris pH 8. Additional optimization experiments led to good-quality crystals with 10 mM of the additive Co[NH₃]₆Cl₃. For data collection, crystals were transferred to cryoprotectant solutions consisting of mother liquor plus 25 % (v/v) glycerol before being cooled in liquid nitrogen.

Additional optimization experiments were performed with the E70A, L320A and L320A/H321S mutants, leading to the final conditions given in Table 1. For getting the E70A-Xyl4 complex, new conditions were investigated with the commercial screens by incubating the inactivated enzyme with the sugar, previously to setting the experiments. This produced a new crystal form allowing occupancy of the active site by the substrate. The complexes of E70A with the AXOS were obtained by cocrystallization and optimizing these conditions, which in the case of AX4 led to a new crystal form. Final cryoprotectant solutions used with the different crystals are summarized in Table 1.

Diffraction data were collected using synchrotron radiation at the XALOC beamline at ALBA (Cerdanyola del Vallès, Spain). Diffraction images were processed with XDS [23] and merged

using AIMLESS from the CCP4 package [24]. A summary of data collection and data reduction statistics for all the crystals is shown in Table 1.

Structure Solution and Refinement

The structure of Rex8A was solved by molecular replacement using the MOLREP program [25]. The search model was the *B. halodurans* exooligoxylanase (PDB entry 1wu4, [5], from which a template was prepared using the program Chainsaw [26] and a protein sequence alignment of Rex8A onto the template. A solution containing two molecules in the asymmetric unit was found using reflections up to 1.93 Å resolution range. Crystallographic refinement was performed using the program REFMAC [27] within the CCP4 suite with flat bulk-solvent correction, maximum likelihood target features. Free *R*-factor was calculated using a subset of 5% randomly selected structure-factor amplitudes that were excluded from automated refinement. Extensive model building using the program COOT [28] was combined with several rounds of refinement leading to a model showing a continuous density for the whole polypeptide chain. At the later stages, water molecules were included in the model, combined with more rounds of restrained refinement that led to a final *R*-factor of 18.9 (*R*_{free} 22.9). Refinement parameters are reported in Table 1. The structure of E70A, L320A and L320/H321S mutants were solved by difference Fourier synthesis using the refined coordinates of the native. The structure of the E70-Xyl4, E70-AX3 and E70-AX4 complexes, showing a different space group, was solved by molecular replacement using MOLREP and the coordinates of native Rex8A as the search model. Refinement for all structures was performed using REFMAC, combined with model building with COOT, and coordinates for the ligands were taken from the Protein Data Bank and manually built into the electron density maps and refined, to reach the *R*-factors listed in Table 1.

Stereochemistry of the models was checked with PROCHECK [29] and the **figures** were generated with PyMOL [30]. Root mean square deviation analysis was made using the program SUPERPOSE within the CCP4 package [24].

Rex8A activity measurements

The catalytic activity of Rex8A and mutants on xylan substrates was analyzed by measuring the amount of reducing sugars released from their hydrolysis using the dinitrosalicylic (DNS) reagent method as assayed by [31] (*doi:10.1016/j.ijbiomac.2019.11.073*) with some modifications. The standard assay was performed in 100 μ l reaction volume using 1.5% of substrate (w/v) in 50 mM Tris-Cl buffer, pH 7, at 40°C and 300 rpm. The enzymatic reactions were carried out for 18 h unless otherwise stated. The optical absorbance was measured at 540 nm and xylanase activity was measured in terms of international units (IU). One unit of xylanase activity was defined as the amount of enzyme that releases 1 μ mol of xylose reducing sugar equivalent per min under the assay conditions described. **The tested xylans** were beechwood xylan from Carl Roth, oat spelt xylan from Sigma-Aldrich and wheat arabinoxylan from Megazyme.

Analysis of hydrolysis products

Xylan and xylooligosaccharides hydrolysis products were analyzed by thin-layer chromatography (TLC) performed on silica gel plates (Macherey-Nagel). Typical assays were performed in Eppendorf tubes using final concentrations of 500 μ g/ml of enzyme, 10 mg/ml of xylooligosaccharide or 1.5% of xylan in 20 mM Tris-HCl buffer pH 7. Reactions were incubated at 40°C and 300 rpm for 18 h and supernatants were concentrated by evaporation before loading in silica plates. Xylobiose, xylotriose, xylotetraose and xylopentaose were purchased from Megazyme. α -L-arabinofuranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl-(1 $\rightarrow$ 4)- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-D-xylose 2³- α -L-Arabinofuranosyl-xylotriose, (Ara³Xyl₃, AX3), and β -D-xylopyranosyl-(1 $\rightarrow$ 4)- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-[α -L-arabinofuranosyl-(1 $\rightarrow$ 2)]- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-D-xylose 3³- α -L-Arabinofuranosyl-xylotetraose (Ara³Xyl₄, AX4) were purchased from Megazyme

Mixtures containing the aldouronic acids 4-O-methyl- α -D-glucuronosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl-(1 $\rightarrow$ 4)- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-D-xylose, (MeGlcA³Xyl₃) (aldotetraouronic acid), and β -D-xylopyranosyl-(1 $\rightarrow$ 4)- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-[4-O-methyl- α -D-glucuronosyl-(1 $\rightarrow$ 2)]- β -D-xylopyranosyl-(1 $\rightarrow$ 4)- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-D-xylose, (MeGlcA³Xyl₅) (aldohexaouronic

acid), were prepared from beechwood 4-O-methyl-D-glucuronoxylan by xylanase treatment as described before [8]. The solvent used was chloroform/acetic acid/water (30:60:10) and the hydrolysis products were detected by spraying ethanol/sulfuric acid mixture (95:5) on the plate and revealing with a heat gun until spots become visible.

AUTHOR CONTRIBUTIONS

F.J.P. and J.S.-A. conceived and designed the experiments. E.J.-O., M.R.-E. and S.V., carried out the experiments and analysed data. F.J.P. and J.S.-A. analysed data and wrote the manuscript. All the authors contributed to critically discuss the results and reviewed and approved the manuscript.

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TABLE 1. Crystallographic statistics (Values in parentheses are for the high resolution shell)							
	WT-xyz	E70A-EDO	E70A-Xyl4	L320A-Xyl	L320A/H321S-EDO	E70A-AX3	E70A-AX4
Crystallization, soaking and cryoprotecting conditions	23 % PEG6K 100 mM Tris, pH8 200 mM NaCl 10 mM [Co(NH ₃) ₆]Cl ₃ 25 % glycerol 50 mM xylose	20 % PEG6K 100 mM Hepes pH7 200 mM MgCl ₂ 10 mM [Co(NH ₃) ₆]Cl ₃ 20 % ethylene glycol 6 mM xylohexaose	18 % PEG3350K 100 mM BisTris propane pH7.5 200 mM KSCN 20 % PEG400 50 mM xylotetraose	22 % PEG6K 100 mM Hepes pH7 200 mM MgCl ₂ 10 mM [Co(NH ₃) ₆]Cl ₃ 6 % glycerol 20 % ethylene glycol 50 mM xylose	22 % PEG6K 100 mM Hepes pH7 200 mM MgCl ₂ 10 mM [Co(NH ₃) ₆]Cl ₃ 6 % glycerol 20 % ethylene glycol 50 mM xylose	16 % PEG3350K 100 mM BisTris propane pH7.5 200 mM KSCN 20 % glycerol 15 mM 2 ³ -α-L-arabinofuranosyl-xylotriose	20% PEG3350 100mM BisTris propane pH7.5 200 mM KSCN 25% glycerol. 5 mM 3 ³ -alpha-L-arabinofuranosyl-xylotetraose
Space group	<i>P</i> 1	<i>P</i> 1	<i>P</i> ₃ 12	<i>P</i> 1	<i>P</i> 1	<i>P</i> ₃ 12	<i>C</i> 2 2 2 ₁
Unit cell parameters							
a, b, c (Å)	51.6, 59.2, 79.5	51.7, 58.4, 79.3	84.6, 84.6, 274.6	51.5, 59.2, 79.4	51.3, 58.6, 78.7	87.9, 87.9, 274.2	82.3, 82.7, 465.3
α, β, γ (°)	87.8, 78.0, 74.3	87.3, 78.2, 73.5	90, 90, 120	87.3, 78.4, 74.6	87.5, 78.1, 74.3	90, 90, 120	90, 90, 90
Data collection							
Beamline	XALOC (ALBA)	XALOC (ALBA)	XALOC (ALBA)	XALOC (ALBA)	XALOC (ALBA)	XALOC (ALBA)	XALOC (ALBA)
Temperature (K)	100	100	100	100	100	100	100
Wavelength (Å)	1.04191	0.979490	0.979260	0.979490	0.979250	0.979260	0.979260
Resolution (Å)	48.58-1.93 (1.98-1.93)	77.63-2.64 (2.77-2.64)	43.94-2.05 (2.09-2.05)	57.04-1.74 (1.77-1.74)	45.78-1.81 (1.85-1.81)	44.49-1.88 (1.88-1.91)	46.62-1.86 (1.86-1.89)
Data processing							
Total reflections	225331 (13133)	72150 (9770)	481963 (29580)	265064 (13708)	209507 (11875)	660511 (32568)	961039 (17154)
Unique reflections	64154 (4213)	24307 (3296)	72614 (4419)	87761 (4448)	75674 (4471)	100926 (4930)	129847 (5330)
Multiplicity	3.5 (3.1)	3.0 (3.0)	6.6 (6.7)	3.0 (3.1)	2.8 (2.7)	6.5 (6.6)	7.4 (3.2)
Completeness (%)	96.4 (93.2)	94.4 (95.2)	100.0 (100.0)	96.6 (95.0)	96.2 (94.8)	99.9 (99.5)	97.2 (80.7)
Mean I / σ (I)	7.2 (2.0)	5.4 (1.8)	12.2 (2.9)	7.1 (2.1)	5.6 (2.0)	13.8 (2.7)	7.4 (3.2)
<i>R</i> _{merge} ^a (%)	12.5 (72.3)	16.4 (68.2)	9.3 (64.2)	9.8 (62.8)	11.1 (64.8)	7.8 (60.9)	15.3 (65.8)
<i>R</i> _{pim} ^b (%)	7.8 (48.9)	10.9 (45.5)	3.8 (26.4)	6.7 (42.3)	7.7 (46.3)	3.2 (25.3)	5.2 (37.7)
CC1/2 (%)	99.2 (66.3)	97.1 (56.3)	99.8 (90.4)	98.7 (78.7)	98.4 (62.2)	99.9 (99.5)	99.2 (56.8)
Molecules per ASU	2	2	2	2	2	2	4
Refinement							
<i>R</i> _{work} / <i>R</i> _{free} ^c (%)	18.9/22.9	19.0/23.7	23.6/26.5	24.2/27.7	17.6/20.6	23.4/25.7	23.8/26.3
N° of atoms/average B (Å²)							
Protein	6226/16.90	6218/29.20	6259/34.33	6254/14.99	6230/20.61	6248/29.96	12557/35.88
Ligand	44/23.48	16/33.57	74/45.61	102/23.31	72/36.50	86/28.17	184/33.96
Water Molecules	606/28.25	227/25.21	311/36.36	597/24.79	487/30.74	423/37.54	194/32.81
All atoms	6876/17.94	6461/29.08	6644/34.55	6953/15.95	6789/21.50	6757/30.42	12935/35.88
Ramachandran plot (%)							
Favoured	99	97	98	98	98	98	96
Outliers	0	0	0	0	0	0	0
RMS deviations							
Bonds (Å)	0.006	0.006	0.007	0.006	0.006	0.007	0.006
Angles (°)	1.418	1.450	1.447	1.461	1.503	1.474	1.417
PDB accession codes	6SRD	6TPP	6TOW	6SUD	6SHY	6TO0	6TRH

^a $R_{\text{merge}} = \sum_{\text{hkl}} \sum_i |I_i(\text{hkl}) - [I(\text{hkl})]| / \sum_{\text{hkl}} \sum_i I_i(\text{hkl})$, where $I_i(\text{hkl})$ is the i th measurement of reflection hkl and $[I(\text{hkl})]$ is the weighted mean of all measurements.

^b $R_{\text{pim}} = \sum_{\text{hkl}} [1/(N - 1)]^{1/2} \sum_i |I_i(\text{hkl}) - [I(\text{hkl})]| / \sum_{\text{hkl}} \sum_i I_i(\text{hkl})$, where N is the redundancy for the hkl reflection.

^c $R_{\text{work}} / R_{\text{free}} = \sum_{\text{hkl}} |F_o - F_c| / \sum_{\text{hkl}} |F_o|$, where F_c is the calculated and F_o is the observed structure factor amplitude of reflection hkl for the working / free (5%) set, respectively.

Table 2. Activity of Rex8A and derived mutants on xylans (mU/mg^b)

	Oat spelt xylan	Wheat arabinoxylan	Beechwood xylan
Wt	38.3 ± 4	26.6 ± 2	8.1 ± 3
E70A	ND ^a	ND	ND
L320A	42.6 ± 1.6	26.8 ± 4	ND
H321A	ND	ND	ND
L320A7H321S	13.2 ± 1.1	5.1 ± 4	ND

^aNot detected

^bMilliunits per milligram

FIGURE LEGENDS

Fig. 1. Structure of Rex8A. Superimposition of native Rex8A-Xyl complex (prune) onto *BhRex*-Xyl₂ (pale cyan). The position of two ethylene glycol molecules trapped in the E70A crystals is shown in yellow. The different molecules are occupying subsites -2, -1 and +1. The N- and C-terminal residues of Rex8 chain are represented as spheres and highlighted with arrows. The sequence alignment of the Rex8 loop Pro250-Arg260, highlighted in the cartoon, is shown in a box marking positions Leu255-His256. Inset: Crystal packing locates Leu255, from loop 250-260 of an adjacent molecule (beige), blocking Rex8 active site cleft. Model generated using PyMol Molecular Graphics System.

Fig. 2. Rex8A active site. **A)** Molecular surface of Rex8A-Xyl4 complex, showing the sugar as olive sticks and the xylose bound at the native crystals in red. Blue/red are positive/negative Rex8A charged regions. Inset: final 2FoFc electron density map of Xyl4, contoured at 1 σ . **B)** Detail of the atomic interactions in the Rex8A-Xyl4 complex, with polar links shown as dashed lines. The residues that rearrange upon substrate binding are highlighted showing its position in the native crystal as salmon sticks. The catalytic residues are coloured in magenta. **C)** Superimposition of the Rex8-Xyl4 complex (olive) onto the reported GH8 complexes *PhXyl*-Xyl5 [12] (slate) and *CtCelA*-Xyl5 [11] (beige), showing ^{2,5}B and ⁴C₁ conformation, respectively, at the sugar bound at subsite -1. Only the catalytic base, the proton donor and a third relevant aspartate are represented, with the replacement done in each case to inactivate the enzymes. Model generated using PyMol Molecular Graphics System.

Fig. 3. Sequence alignment of reducing-end xylose-releasing exo-oligoxyanases (Rexs). Alignment of the amino acid sequences was performed using the Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo>). The Rex sequences shown are *Bacteroides intestinalis* Rex8A (*BiRex*, GenBank EDV05843.1), *Bifidobacterium adolescentis* RexA (*BaRex*, GenBank AAO67498.1), *Paenibacillus barcinonensis* BP-23 Rex8A (*Rex8A*, GenBank ALP73600.1), *Paenibacillus barengontzii* Rex (*PbRex*, GenBank WP_016314299.1) and *Bacillus*

halodurans C-125 Rex (*BhRex*, GenBank BAB05824.1). GH8 xylanases sequences shown are *Pseudoalteromonas haloplanktis* Xyn8 (*PhXyn*, GenBank CAD20872.1), *Bacillus* sp. KK-1 XynY (*BsXyn*, GenBank AAC27700.1) and *Bacteroides intestinalis* Xyn8A (*BiXyn*, GenBank EDV05067.1). Residues 320- 322 forming the LHP loop are highlighted in a green square.

Fig. 4. Exo/endo-activity of Rex8. **A)** Xylotriose (X3), xylotetraose (X4) and xylohexaose (X6) were incubated with Rex8A wt and derived mutants E70A (E), L320A (L), H321A (H) or L320A/H321S (L/H) and the hydrolysis products were analyzed by thin-layer chromatography. Lanes: c, control sample (no digestion); M, size markers X1, X2, X3, X4, and X5. **B)** Superimposition of native Rex8A (salmon) onto the L320A/H321S double mutant (slate), showing polar interactions of native His320 as black dashed lines, and that of mutated Ser320 as a cyan line. The loops that rearrange in the mutant are labelled. Molecular surface of native **(C)** and double mutant **(D)**, showing the larger cavity created in the mutated protein and the molecules bound at each active site. Xylose position is shown in C) as a reference. Model generated using PyMol Molecular Graphics System.

Fig. 5. Rex8A activity against decorated xylooligosaccharides. **A)** A mixture of aldouronic acids (aldotetraouronic (MeGlcA³Xyl₃) and aldohexaouronic (MeGlcA³Xyl₅)), was incubated with enzymes, and the hydrolysis products were analyzed by thin-layer chromatography. Lanes: 1, control sample (no digestion); 2, sample digested with Rex8A; 3, sample digested with Xyn30D; 4, sample digested with Rex8A and Xyn30D; M, size markers X1, X2, X3, X4, X5, and X6. **B)** Arabinose decorated xylooligosaccharides: 2³-α-L-Arabinofuranosyl-xylotriose (Ara³Xyl₃) and 3³-α-L-Arabinofuranosyl-xylotetraose (Ara³Xyl₄) were incubated with Rex8A wt and derived mutants E70A (E), L320A (L), H321A (H) or L320A/H321S (L/H) and the hydrolysis products were analyzed by thin-layer chromatography. Lanes: c, control sample (no digestion); M, size markers X1, X2, X3, X4, and X5. Each digestion was performed and analysed in three independent experiments to confirm the results.

Fig. 6. Binding mode of decorated substrates to Rex8A. **A).** Superimposition of Xyl4 (green), AX3 (purple) and AX4 (slate) as found at the active site of Rex8A; the arabinose

decorations have been highlighted with thicker sticks. Relevant residues are represented. **B)** and **C)** Binding of AX3 and AX4n to Rex8A showing the polar interactions as dashed lines and relevant water molecules. Final 2FoFc electron density map of AX3 and AX4 are contoured at 1σ . The catalytic residues are highlighted in a lighter tone. Model generated using PyMol Molecular Graphics System.

Figure 1

105x117mm (300 x 300 DPI)

Figure 2

105x157mm (300 x 300 DPI)

<u>PhXyn</u>	GGAQN-----RGCAGEGQKAANAVALL	374
<u>BsXyn</u>	NGTAVK-----KGSPVGLIAANAGGAT	388
<u>BiXyn</u>	DGSAP-----AGNYSTGMIGANAVGAF	370
<u>BiRex</u>	DGTQVKDTLRAGEHKALRHSLGLVATSAVASL	365
<u>BaRex</u>	DGTAVDE-----VVLHPVGFLAATAQGAL	326
<u>Rex8A</u>	EGEPFNE-----PALHPVGLLATNAMASL	334
<u>PbRex</u>	SGEPFEE-----PALHPVGLLATNAMASL	330
<u>BhRex</u>	DGEPFEE-----KSLHPVGLIATNAMGSL	332

Figure 3

80x23mm (600 x 600 DPI)

Figure 4

164x123mm (300 x 300 DPI)

Figure 5

80x117mm (199 x 199 DPI)

Figure 6

105x209mm (300 x 300 DPI)