Medium- to large-sized xylo-oligosaccharides are responsible for xylanase induction in *Prevotella bryantii* B₁₄

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Experiments were done to define the nature of the xylan-derived induction signal for xylanase activity, and evaluate which xylanase genes among the three known ones (*xynA*, *xynB* and *xynC*) are induced by the presence of xylan in *Prevotella bryantii* B₁₄. During the later stages of exponential growth on glucose, addition of 0.05 % water-soluble xylan (WS-X) stimulated xylanase formation within 30 min. Xylose, xylobiose, xylotriose, xylotetraose, xypentaose, arabinose and glucuronic acid all failed to induce the xylanase activity. An acid-ethanol-soluble fraction of WS-X (approximate degree of polymerization 30) enhanced the activity significantly, whereas the acid-ethanol-insoluble fraction had no effect, unless first digested by the cloned *P. bryantii* XynC xylanase. These results indicate that medium- to large-sized xylo-oligosaccharides are responsible for induction. The transcription of all three known xylanase genes from *P. bryantii* was upregulated coordinately by addition of WS-X. There have been relatively few investigations into the regulation of xylanase activity in bacteria, and it appears to be unique that medium- to large-sized xylo-oligosaccharides are responsible for induction.

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INTRODUCTION

Prevotella spp. are among the predominant hemicellulolytic bacteria in the rumen. *P. bryantii* B₁₄ is actively xylanolytic and can grow on xylan as sole carbon source by utilizing around 80 % of the total pentose present in water-soluble xylans in pure culture (Miyazaki *et al.*, 1997). Gene cloning has identified two family 10 endoxylanases (XynA and XynC) and a family 43 exoxylanase/xylosidase (Gasparic *et al.*, 1995; Flint *et al.*, 1997) in *P. bryantii* B₁₄, in addition to an endoglucanase (Gardner *et al.*, 1995). Comparison of the cloned XynA and XynC enzymes shows that they differ in their substrate specificities (Flint *et al.*, 1998). XynC is almost inactive against straight-chain xylo-oligosaccharide substrates smaller than X₇ and does not release significant amounts of X₂, whereas XynA attacks X₅, releasing X₂ and X₃.

Previous work demonstrated that the *xynA* and *xynB* genes are located in the same operon, together with *xynD*, which encodes a putative sodium-symporter that may be involved in xylo-oligosaccharide uptake (Miyazaki *et al.*, 2003). Xylanase activity is induced in *P. bryantii* during growth on xylans, but is not induced by xylose (Gardner *et al.*, 1995;

Miyazaki *et al.*, 1997). This induction is partly due to increased transcription of the *xynA* and *xynB* genes during growth on xylan, mediated by the product of the linked *xynR* regulatory gene (Miyazaki *et al.*, 2003). The cloned XynR product has been shown to bind just upstream of the *xynABD* operon and to stimulate transcription (Miyazaki *et al.*, 2003). XynR shows homology with two-component regulators (Miyazaki *et al.*, 2003) and is one of the first such regulators shown to govern expression of polysaccharide-degrading enzymes in bacteria. Little is known, however, about the nature of the inducer that stimulates xylanase gene expression in *P. bryantii*, or about the sensing mechanism that responds to it. This study reports investigations into the regulation of xylanase activity of *P. bryantii* B₁₄ that demonstrate repression by glucose, and better define the nature of the xylan-derived induction signal. We report that in addition to *xynA* and *xynB*, the unlinked *xynC* gene is also subject to regulation by xylan.

METHODS

Organism and growth conditions. *P. bryantii* B₁₄ was grown anaerobically in RG medium or WS-X medium. RG medium contained (per litre): rumen fluid, 150 ml; K₂HPO₄, 0.9 g; KH₂PO₄, 0.9 g; (NH₄)₂SO₄, 1.8 g; NaCl, 1.8 g; CaCl₂·2H₂O, 0.24 g; MgSO₄·7H₂O, 0.38 g; cysteine.HCl, 0.5 g; yeast extract (Difco), 0.5 g; Trypticase (BBL), 1 g; glucose, 1 g. WS-X medium contained 0.05 % water-soluble xylan (WS-X), prepared as described below, instead of

Abbreviations: DP, degree of polymerization; EtOH, ethanol; EtOH-insol, EtOH-sol, acid-ethanol-insoluble and -soluble fractions; WS-X, water-soluble xylan.

glucose. Incubations were performed in triplicate at 39 °C in 10 ml Hungate tubes. Cell growth was measured by using a Spectronic 20 spectrophotometer (Sigma) at 600 nm (path length 15 mm).

Preparation of xylan fraction. WS-X, and the acid-EtOH-insoluble (EtOH-insol) and -soluble (EtOH-sol) fractions from WS-X were prepared as described previously (Miyazaki *et al.*, 1997). Ten grams of oat spelt xylan (Sigma) was added to 90 ml distilled water, and the mixture was shaken continuously at 39 °C for 2 h. After centrifugation (5000 g, 30 min, 4 °C), its supernatant was taken as WS-X. Three volumes of a cold ethanol/acetic acid solution (95% ethanol, 5% glacial acetic acid) was added to the WS-X, and the mixture was kept on ice for 30 min. This mixture was then centrifuged (16 000 g, 4 °C, 10 min), and the insoluble xylan pellet was dissolved in 50 ml sodium phosphate buffer (50 mM, pH 6.5) containing 2 mM DTT (EtOH-insol). The supernatant fluid was lyophilized, and dissolved in the same buffer (EtOH-sol). Each fraction was adjusted to 2% (w/v) total pentose content.

Predigestion of EtOH-insol with cloned XynC. Cloned XynC from *P. bryantii* B₁₄ was prepared as previously described (Zhang & Flint, 1992; Garcia-Campayo *et al.*, 1993); 0.3 U of this enzyme preparation was added to 10 ml 2% (w/v) EtOH-insol, and incubated at 37 °C for 4 h. A further 0.3 U of enzyme was then added to the digest and incubated for a further 4 h. After incubation, this digest was transferred to a 50 ml serum bottle, and the atmosphere of the bottle was replaced by 100% CO₂ before sealing. Such samples were designated XynC-digest.

Average degree of polymerization (DP) of xylan fraction. The xylose equivalent of each xylan fraction was determined by using the orcinol reagent with xylose as the standard (Schneider, 1957). The reducing sugar content was measured by using the method of Nelson and Somogyi with xylose as the standard (Nelson, 1944). Average DP was determined from the ratio of xylose equivalent to reducing sugar content.

Measurement of xylanase activity. After incubation, the cell culture was kept on ice for 5 min. The culture was centrifuged at 10 000 g at 4 °C for 5 min, and the cell pellet was washed twice with sodium phosphate buffer (pH 6.8) containing 2 mM DTT. After dissolving this pellet in 1 ml of the same buffer, the cells were disrupted with an ultrasonicator (UD-201, Tomy). This mixture was kept at -20 °C until measurement. Xylanase activity was determined as described previously (Flint *et al.*, 1991).

Total RNA extraction. At 30 min after adding 0.05% WS-X to mid-exponential-phase cells grown in RG medium, total RNA was extracted from the culture by Sepazol-RNA 1 super (Nacalai Tesque) according to the supplier's instructions. Total RNA was aliquoted and stored at -80 °C until required for analysis.

Dot blot analysis. Total RNA samples of 0.25–2.0 µg were used for dot blot analysis. Total RNA was transferred to positively charged nylon membrane (Hybond-N⁺, Amersham Pharmacia Biotech) by using a dot blotter (Multimicro filter FLE348, Advantec MFS) according to the supplier's instructions. Nucleotides were fixed to the membrane by UV irradiation.

Probes. For the detection of the target mRNA, digoxigenin (DIG)-labelled probes were prepared by PCR using the DIG Probe Synthesis Kit (Roche Diagnostics). The primers used were XA-F (5'-CAGCCTACGATGAAGGATG-3') and XA-R (5'-CCTCGTTAACGACATCCC-3') for the *xynA* probe, XB-F (5'-GAACAGGATGCCAAGGACTG-3') and XB-R (5'-GGAGCCTCAGCAAAGTGC-3') for the *xynB* probe, and XC-F (5'-GATGAGCCTGCTGAGGTTATC-3') and XC-R (5'-CTCAGATCCCAGTTGTTGG-3') for the *xynC* probe, corresponding to a 403 bp, a 416 bp and a 420 bp fragment, respectively.

Detection of DIG-labelled probes. The membrane was washed with 0.1% SDS/2 × SSC for 15 min at room temperature, followed by 0.1% SDS/0.2 × SSC for 15 min at 50 °C. Additionally, it was shaken for 30 min in skim milk solution [3 g skim milk per 100 ml TBS (pH 7.4); TBS contained 137 mM NaCl, 2.68 mM KCl, 25 mM Tris/HCl]. After washing three times with TBS, the membrane was incubated at 37 °C for 1 h in anti-DIG solution [1 µl anti-digoxigenin-AP (Boehringer Mannheim) in 3 ml TBS]. The membrane was shaken vigorously twice for 15 min in T-TBS solution (0.05% Tween 20 in TBS). After soaking it for 5 min in AP 9.5 solution [100 mM Tris buffer (pH 9.5), 100 mM NaCl, 5 mM MgCl₂], the membrane was incubated for 5 min with CDP-Star (Amersham Life Science). Chemiluminescent signals were detected by exposure to X-ray film (Medical X-ray Film MXJB-1, Kodak). Non-specific hybridization of each probe was checked by using yeast RNA (Ambion). Cross-hybridization experiments between the three xylanase genes were performed by applying plasmid DNA of each xylanase gene. Neither non-specificity nor cross-hybridization was observed.

RESULTS

Catabolite repression

P. bryantii was grown in WS-X medium containing 0.025–0.2% (w/v) glucose to investigate the phenomenon of catabolite repression. Cell growth was faster with increasing amounts of glucose (Fig. 1a). No xylanase activity was detected until after 8 h in the medium containing 0.2% glucose and until after 7 h in the case of 0.1% glucose, whereas activity was detected at 7 h for glucose concentrations of 0.075% or less (Fig. 1b). These results indicate that catabolite repression, or perhaps inducer exclusion, resulted in delayed xylanase expression in the presence of glucose above 0.1% (w/v), although diauxic growth was not apparent (Fig. 1a).

Fig. 1. Effect of glucose on xylanase induction in *P. bryantii* B₁₄. Cells were grown in 0.05% WS-X medium containing 0.025% (Δ), 0.05% (○), 0.075% (□), 0.1% (▲) or 0.2% (●) glucose. (a) Cell growth; (b) xylanase activity.

Xylanase induction by different xylan fractions

Xylanase induction was not detected when WS-X (0.05 %) was added to cultures grown with 0.1 % glucose in early exponential phase (OD_{600} 0.4), consistent with a repressive effect of the residual glucose. When 0.1 % WS-X was added in mid-exponential phase (OD_{600} 0.6), however, significant induction was detected within 30 min (Fig. 2). This culture density and induction period were used in the subsequent tests, described below.

WS-X was fractionated as described in Methods, and each fraction examined for its ability to induce xylanase activity (Fig. 3). The EtOH-sol fraction induced xylanase activity 7 times more effectively than the EtOH-insol fraction, which was scarcely more effective than glucose (Fig. 3). Solubility in acid-EtOH depends principally on the molecular size of xyans, and the average DP of EtOH-sol was much less than that of EtOH-insol (Fig. 3b). These results therefore indicate that a relatively small molecule derived from xylan induces xylanase activity. Although the EtOH-insol fraction failed to induce xylanase activity, this fraction did result in significant induction when it was first digested by the cloned XynC enzyme (XynC-digest, Fig. 3a). The average DP of EtOH-insol was reduced to the same level as that of EtOH-sol after digestion by cloned XynC (Fig. 3b). As XynC is an endoxylanase and cannot act on xylo-oligosaccharides smaller than X_7 (Flint *et al.*, 1991), the digests are expected to contain mainly xylo-oligosaccharides larger than X_8 .

Purified, straight-chain xylo-oligosaccharides and xylose were examined for their potential roles as inducers (Fig. 4). Xylose and xylobiose (X_2) did not enhance the activity compared to glucose. X_3 – X_5 , and arabinose or glucuronic acid, which are constituents of xylan, increased the activity only slightly relative to glucose, but did not approach the effect of WS-X (Fig. 4a, b). These results indicate that straight-chain xylo-oligosaccharides larger than X_6 , or perhaps a specific substituted oligosaccharide, could be the inducer. Treatment of WS-X with phosphoric acid, which removes arabinose side chains (Cotta, 1993), did not

Fig. 2. Xylanase induction after addition of 0.05 % glucose or WS-X. *P. bryantii* B₁4 was grown in 0.1 % glucose medium to mid-exponential phase, then 0.05 % glucose (○) or WS-X (●) was added at the time indicated by the arrow.

Fig. 3. Effect of the xylan fractions on the xylanase activity (a), and average DP of the fractions (b). *P. bryantii* B₁4 was grown in the 0.1 % glucose medium to mid-exponential phase, then 0.05 % glucose (G), WS-X, EtOH-insol, EtOH-sol or XynC-digest was added. After 30 min further incubation, xylanase activities were measured. The xylanase activity after addition of glucose is expressed as 100 %.

Fig. 4. Effect of xylo-oligosaccharides (a) and the constituents of xylan (b) on xylanase induction. *P. bryantii* B₁4 was grown in 0.1 % glucose medium to mid-exponential phase, then 0.05 % glucose (G), WS-X, xylose (X_1), xylo-oligosaccharides (X_2 – X_5), arabinose (Ara) or glucuronate (Glu) was added. After 30 min further incubation, xylanase activities were measured. The xylanase activity after addition of glucose is expressed as 100 %.

abolish its ability to induce activity (results not shown), indicating that arabinose substituents are not essential for induction. No difference in the composition of side residues was observed between EtOH-sol and EtOH-insol (results not shown).

Xylanase gene expression

It was shown recently that the expression of the *xynA* and *xynB* genes was regulated by a regulatory protein, XynR, at the transcriptional level (Miyazaki *et al.*, 2003). Thirty minutes after addition of WS-X, transcription of the *xynC* gene as well as that of *xynA* and *xynB* was enhanced (Fig. 5). This result suggests that the transcription of these xylanase genes may be regulated coordinately.

DISCUSSION

Regulation of xylanase activity has been studied previously mainly in filamentous fungi, where xylanases are induced by the presence of xylose (Gouka *et al.*, 1996; Mach *et al.*, 1996; Zeilinger *et al.*, 1996; Orejas *et al.*, 1999) or xylobiose (Biely *et al.*, 1980). There have been relatively few investigations into the regulation of xylanase activity in bacteria. In *P. bryantii* we found no evidence that xylose or xylobiose could induce xylanase production. Rather, the most effective inducer was an ethanol-soluble fraction (EtOH-sol) prepared from water-soluble oat spelt xylan, with a mean DP of approximately 30. Also effective was a xylan hydrolysate produced by the enzyme XynC, with a DP around 35. Neither the monosaccharides arabinose or glucuronic acid, nor the small oligosaccharides xylotriose, xylo-tetraose or xylopentaose, gave any major degree of induction when tested individually. A comparatively large amount of arabinose is contained in oat spelt xylan but little glucuronic

acid, and vice versa in birch wood xylan. Nevertheless, both xylan preparations induced xylanase activity notably (Miyazaki *et al.*, 1997), suggesting that the presence of side chain residues on the xylose backbone is not essential for the inducers. Moreover, a partially hydrolysed oat spelt xylan produced by phosphoric acid, in which most of side groups were removed (Cotta, 1993), showed a significant inducing effect (data not shown). From these observations it can be concluded that the inducers are not xylans with side group residues, but medium- to large-sized water-soluble xylo-oligosaccharides.

The inability to induce xylanase activity in *P. bryantii* by addition of WS-xylan during early growth stages in the presence of glucose is strongly suggestive of catabolite repression, or inducer exclusion. In *Escherichia coli* and *Bacillus* sp. catabolite repression acts through well-established mechanisms involving binding of CRP (Pedersen *et al.*, 1995; Kristensen *et al.*, 1997) or CcpA (Heuck *et al.*, 1995; Henkin, 1996; Monedero *et al.*, 1997) repressor proteins to operator sites in response to cAMP levels. Another well-known mechanism is inhibition of uptake of sugars other than glucose by the glucose-specific components of phosphotransferase systems, which can result in inducer exclusion (Postma *et al.*, 1993). Because no phosphotransferase system activity (Martin & Russell, 1986) and a very low concentration of cAMP (Cotta *et al.*, 1994) were detected in *P. bryantii* B14, it seemed that previously defined catabolite regulatory mechanisms could not explain the regulation of xylanase, as Fields & Russell (2001) suggested for β -glucanase induction.

The *P. bryantii* *xynA* and *xynB* xylanase genes, together with the *xynD* putative oligosaccharide transporter, were previously shown to be positively regulated at the transcriptional level by the multidomain regulatory protein XynR, which has homology to bacterial two-component regulators (Miyazaki *et al.*, 2003). In addition, *xynR* mRNA expression was itself regulated in response to xylan (Miyazaki *et al.*, 2003). There must be a mechanism, presumably involving a sensor protein domain, mediating the response to xylo-oligosaccharides, most likely present in the periplasmic space, or outside the cell. These oligosaccharides will be generated by the initial hydrolysis of the xylan polymer, probably by the enzyme XynC, which seems to be located on the cell envelope and has a preference for substrates with a chain length greater than 8. The induction signal must then be communicated to XynR, probably through its unique N-terminal input domain, resulting in positive control of xylanase gene transcription via the C-terminal DNA-binding domain of XynR. Direct recognition of xylo-oligosaccharides by the XynR N-terminal domain cannot be ruled out, but seems less likely, as this would imply that one end of the regulatory protein could be in contact with large oligosaccharides at the same time as the other end is binding to DNA.

The present results confirm that not only *xynA* and *xynB* transcription, but also transcription of the unlinked *xynC* gene, is induced by WS-X. It is not known whether *xynC*

Fig. 5. Expression of xylanase genes (dot blot). *P. bryantii* B₁₄ was grown in 0.1% glucose medium to mid-exponential phase, then 0.05% glucose (G) or WS-X was added. After 30 min further incubation, total RNA was extracted from each culture and applied to the membrane.

is also under control of the *xynR* regulator, or of another regulator that responds to the same induction signal.

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