Pseudomonas cellulosa expresses a single membrane-bound glycoside hydrolase family 51 arabinofuranosidase

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In the accompanying paper [Beylot, McKie, Voragen, Doeswijk-Voragen and Gilbert (2001) Biochem. J. **358**, 607–614] the chromosome of *Pseudomonas cellulosa* was shown to contain two genes, *abf51A* and *abf62A*, that encode arabinofuranosidases belonging to glycoside hydrolase families 51 and 62, respectively. In this report we show that expression of Abf51A is induced by arabinose and arabinose-containing polysaccharides. Northern-blot analysis showed that *abf51A* was efficiently transcribed, whereas no transcript derived from *abf62A* was detected in the presence of arabinose-containing polysaccharides. Zymogram and Western-blot analyses revealed that Abf51A was located on the outer membrane of *P. cellulosa*. To investigate the importance of Abf51A in the release of arabinose from poly- and oligo-saccharides, transposon mutagenesis was used to construct an *abf51A*-inactive mutant of *P. cellulosa* (Δ*abf51A*). The mutant did not grow on linear arabinan or sugar beet arabinan, and utilized arabinoxylan much more slowly than the wild-type bacterium. Arabinofuranosidase activity in Δ*abf51A* against aryl-α-arabinofuranosides, arabinan and α1,5-linked arabino-oligosaccharides was approx. 1% of the wild-type bacterium. The mutant bacterium did not exhibit arabinofuranosidase activity against arabinoxylan, supporting the view that *abf62A* is not expressed in *P. cellulosa*. These data indicate that *P. cellulosa* expresses a membrane-bound glycoside hydrolase family 51 arabinofuranosidase that plays a pivotal role in releasing arabinose from polysaccharides and arabino-oligosaccharides.

Key words: arabinan, arabinose, gene inactivation.

INTRODUCTION

The plant cell wall is a highly complex structure comprised primarily of polysaccharides. Micro-organisms that are capable of degrading the plant cell wall synthesize a repertoire of glycoside hydrolases, lyases and esterases [1]. These microbes generally express isoenzymic forms of the major glycoside hydrolases, which are encoded by extensive multigene families [1]. For example, *Clostridium thermocellum* expresses 15 different endo-glucansaes and at least five different xylanases [see P. M. Coutinho and B. Henrissat (2000) Carbohydrate-Active Enzymes Server at http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html]. The functional significance of the multigene families encoding plant-cell-wall hydrolases is currently unclear. There is some evidence that different isoforms of these enzymes recognize different substrates. For example, xylanases belonging to glycoside hydrolase family (GH) 11 exhibit high activity against linear xylan, whereas GH10 xylanases, although less active, will attack xylans that are to some extent substituted [2]. Similarly, some endo-β1,4-glucanases preferentially hydrolyse soluble substrates, whereas others are capable of attacking insoluble cellulose. As discussed in the accompanying paper [3], arabino-furanosidases play a critical role in plant-cell-wall degradation by making arabinoxylans and arabinans accessible to the main-chain-cleaving enzymes. As a result of the complex nature of arabinose substitutions, a variety of different arabino-furanosidases are synthesized by plant-cell-wall-degrading microbes. Many arabinofuranosidases hydrolyse α1,2- and α1,3-arabinofuranosyl linkages [4]. Whereas the nature of the aglycone sugar can influence the catalytic activity of some of these enzymes, other arabinofuranosidases do not distinguish between the saccharides linked to the arabinofuranose moiety and thus exhibit wide substrate specificity [4]. For example, *Pseudomonas cellulosa* contains two genes encoding arabinofuranosidases belonging to GH51 (Abf51A) and GH62 (Abf62A) [3,5]. Abf51A can cleave arabinofuranosyl α1,2 and α1,3 linkages in arabinan and arabino-xylan [3], whereas Abf62A is specific for substituted xylan [5]. In addition to differences in substrate specificity, the cellular location of these enzymes will also influence their biological role. For example, arabinofuranosidases that are secreted into the culture media are likely to attack polysaccharides [6], while the targets for arabinofuranosidases that lack signal peptides and are retained within the cytoplasm [7,8] are presumably oligo-saccharides.

The most informative approach to studying the biological significance of glycoside hydrolases is to investigate the physiological and biochemical effects of inactivating specific genes. This approach has been used successfully to study the importance of the different *Erwinia* pectinases; both polygalacturonases and pectate lyases [9]. Although the complex array of plant-cell-wall hydrolases synthesized by prokaryotes such as *C. thermocellum*, *Cellulomonas fimi* and *P. cellulosa* has been rigorously analysed [10–12], the lack of a genetic system for these organisms has precluded a detailed analysis of the biological significance of the different members of the multigene families encoding these enzymes.

To initiate studies designed to investigate the biological significance of the different plant-cell-wall hydrolases expressed by *P. cellulosa*, this article describes the development of a genetic system for *P. cellulosa*, and its exploitation in elucidating the functional significance of the different arabinofuranosidases encoded by the bacterium's genome. The data showed that inactivation of the *abf51A* gene prevented the organism from growing on sugar beet arabinan and reduced its capacity to utilize arabinoxylan as a carbon source. The results also showed that Abf62A did not appear to be expressed by the pseudomonad,

Abbreviations used: GH, glycoside hydrolase family; Kan, kanamycin; MUA, 4-methylumbellerfyl-α-L-arabinofuranoside; MUAase, 4-methyl-umbelliferyl-α-L-arabinofuranosidase; PNPAase, 4-nitrophenyl-α-L-arabinofuranosidase; Rif, rifampicin; LB, Luria–Bertani broth; HPAEC, high-performance anion-exchange chromatography.

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indicating that Abf51A plays a pivotal role in removing arabinose side chains from plant structural polysaccharides.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

The *Escherichia coli* strains used in this study were CH545 and XL1-Blue, harbouring the pJB4JI [13] and pMB1 [3] plasmids respectively. All *E. coli* strains containing recombinant plasmids were cultured in Luria–Bertani broth (LB) supplemented with 50 μg/ml ampicillin or kanamycin (Kan), as appropriate, at 37 °C unless otherwise stated. To grow *P. cellulosa*, a rifampicin (Rif)-resistant (Rif^r) mutant of the bacterium was cultured in either LB or minimal medium, which consists of M9 salts and the appropriate carbon source, either monosaccharide or polysaccharide, at a final concentration of 0.2 %. The pseudomonad was grown either in liquid culture at 37 °C with high aeration (200 rev./min) for up to 36 h in medium comprising < 10 % (v/v) of the incubator vessel (conical flask), or on solid media at 28 °C for up to 2 weeks.

Transposon mutagenesis

To insert the transposon Tn5 into the *P. cellulosa* genome, the Rif^r pseudomonad and *E. coli* strain CH545, harbouring pJB4JI (a self-transmissible plasmid containing Tn5, which does not replicate in Gram-negative bacteria other than *E. coli* [14]), were cultured in LB to stationary phase. The two cultures were then centrifuged at low speed (1300 ***g***) and the cell pellets were washed in PBS. Approx. 10^8 *E. coli* cells were mixed with an equal number of *P. cellulosa* cells in a total volume of 5 ml, and the mixture was forced through a Type GS 0.22 μm nitrocellulose filter (diameter 25 mm; Millipore), which was then incubated on an LB agar plate for 16 h at 25 °C. The bacteria were then washed off the filter with 5 ml of LB, plated out on LB agar containing 50 μg/ml Rif and Kan, and incubated at 28 °C for 5 days, after which Rif^r/Kan^r (Kan-resistant) pseudomonad transconjugant colonies had appeared. To screen the transconjugants for arabinofuranosidase activity, the plates containing these colonies were overlayed with 3.5 ml of 50 mM sodium phosphate buffer, pH 7.0, containing 4 mM 4-methylumbellerfyl-α-L-arabinofuranoside (MUA). After 15 min the plates were inspected over a UV transilluminator and cells that did not fluoresce were selected as arabinofuranosidase-minus based on the criterion that they did not hydrolyse MUA.

Recombinant DNA techniques

Standard recombinant DNA techniques such as Southern and Northern hybridization, bacterial transformation, agarose gel electrophoresis, plasmid DNA preparation, restriction digestion and ligation were as described previously [14]. To isolate RNA from *P. cellulosa*, cells were isolated from different stages of the growth phase and RNA was extracted using RNAZOL™ B reagent (Biogenesis, Poole, Dorset, U.K.). Approx. 5 μg of RNA were electrophoresed under denaturing conditions on a 1.2 % agarose/formaldehyde gel [14], which was then subjected to standard Northern-blot hybridization.

Immunological methods

The purified arabinofuranosidase was injected into Dutch white rabbits using the regime described previously [15]. The polyclonal antibodies were used in the Western-blot analysis of *P. cellulosa* proteins as described previously [16]. The antibodies were also used in the immunohistochemical analysis of *P. cellulosa* cells essentially as described by Van Den Bosch et al. [17]. Briefly, cells from 1 ml of an overnight culture of *P. cellulosa* grown on wheat arabinoxylan-containing media were collected by centrifugation, and resuspended in 4 % (v/v) formaldehyde in 50 mM Pipes buffer for 30 min. The cells were then washed twice in PBS, and 10 μl were pipetted on to a multi-well microscope slide, left to air-dry for 1 h at 37 °C and then re-hydrated in PBS containing 1 % BSA. The cells were then incubated with 10 μl of a 1/300 dilution of the primary anti-Abf51A antibody and, after removing this antibody, were incubated with goat anti-rabbit IgG antibody conjugated with FITC (Sigma) in PBS containing 1 % BSA. After washing away the secondary antibody the cells were viewed under a fluorescence microscope.

Fractionation of *P. cellulosa*

Stationary cells of *P. cellulosa* were harvested by centrifugation at 6000 ***g*** for 10 min from a 400 ml culture comprising minimal medium in which wheat arabinoxylan was the carbon source. The pelleted cells were resuspended in 10 ml of 50 mM sodium phosphate/12 mM citric acid (PC) buffer, pH 6.5, and sonicated. The cell membranes were then pelleted by centrifugation at 100000 ***g*** at 4 °C for 1 h and the supernatant, consisting of the cytoplasm and the periplasm, referred to as the cell-free extract, was retained for further use. The pelleted membrane fraction was then resuspended in 15 ml of PC buffer. The inner membrane was solubilized selectively using *N*-lauroyl sarcosine, and separated from the outer membrane by centrifugation at 100000 ***g*** at 4 °C for 1 h as described previously [18]. The purity of the different fractions was assessed by measuring the activity of the marker enzymes malate dehydrogenase (cell-free extract) and NADH oxidoreductase (membrane fraction and purified inner-membrane fraction) as described previously [19].

Assays

Protein was measured by the dye-binding method of Sedmak and Grossberg [20] using BSA as the standard. Arabinofuranosidase activity was measured as described in the accompanying paper [3]. To evaluate the capacity of *P. cellulosa* cells to hydrolyse plant structural polysaccharides, two assays were performed. In the first assay the bacterium was cultured in LB supplemented with wheat arabinoxylan (0.2 %) for up to 36 h. At regular time intervals a 1 ml aliquot was removed, the cells were pelleted by centrifugation and the supernatant comprising the culture medium was boiled for 10 min to inactivate the extracellular enzymes. Precipitated material was removed by filtration and the composition of the sugars in the culture medium was analysed by high-performance anion-exchange chromatography (HPAEC). In the second assay, 50 ml cultures of *P. cellulosa*, in which the medium comprised LB supplemented with 0.2 % arabinoxylan and sugar beet arabinan, were grown for 36 h. The cells were pelleted, washed once in 50 mM sodium phosphate buffer, pH 7.0, resuspended in 5 ml of the phosphate buffer and then disrupted by ultrasonication. The cell-free extract (100 μl) and culture supernatant were incubated with 0.2 % sugar beet arabinan or wheat arabinoxylan, in a final reaction volume of 2 ml for up to 4 h at 37 °C. At regular time intervals, 100 μl aliquots were removed and the appearance of arabinose was quantified by HPAEC.

RESULTS

Expression of Abf51A in *P. cellulosa*

To investigate the expression of Abf51A in *P. cellulosa*, the bacterium was grown in different media and the 4-nitrophenyl-α-

Table 1 Expression of arabinofuranosidase activity in *P. cellulosa*

The bacterial strains were cultured in LB supplemented with polysaccharide at a final concentration of 0.1% and/or monosaccharide at 0.05%. When the cultures had reached mid-exponential phase the cells were harvested, disrupted by ultrasonication and assayed for PNPAase activity as detailed in the Materials and methods section. NA, no activity.

Carbohydrate in media	*P. cellulosa* strain	Arabinofuranosidase activity (units · mg^{-1})
None	Wild type	NA
Sugar beet arabinan	Wild type	1.6 ± 0.21
Wheat arabinoxylan	Wild type	2.5 ± 0.32
Arabinose	Wild type	0.8 ± 0.13
Wheat arabinoxylan and glucose	Wild type	NA
Carboxymethyl cellulose	Wild type	NA
Potato β1,4-galactan	Wild type	0.001 ± 0.0005
Carob galactomannan	Wild type	0.005 ± 0.0012
Wheat arabinoxylan	Δ*abf51A*	0.015 ± 0.004

Table 2 Cellular localization of arabinofuranosidase activity

Wild-type *P. cellulosa* was grown to late-exponential phase and fractionated into the different cellular components as described in the Materials and methods section. The enzymes malate dehydrogenase and NADH oxidoreductase were markers for the cell-free extract (combined cytoplasm and periplasm) and membrane fractions, respectively. ND, not determined. For outer- and inner-membrane fractions, the percentage activity is with respect to total membrane activity.

	Activity (%)		
Fraction	Malate dehydrogenase	NADH oxidoreductase	PNPAase
Cell-free extract	98	25	8
Culture medium	0	0	1
Total membrane	2	75	91
Outer membrane	ND	4	95
Inner membrane	ND	96	5

L-arabinofuranosidase (PNPAase) activity in the pseudomonad was determined. The data, presented in Table 1, showed that maximal PNPAase activity was detected in *Pseudomonas* cultured on wheat arabinoxylan, although other arabinose-containing polysaccharides, such as sugar beet arabinan, also induced PNPAase expression. Media containing low levels of arabinose also induced PNPAase activity. Polysaccharides such as cellulose, galactan and galactomannans, which lack arabinose moieties, did not induce a functional pseudomonad arabinofuranosidase, while glucose repressed the expression of this enzyme activity. To investigate the location of the PNPAase activity, *P. cellulosa* grown on wheat arabinoxylan was fractionated into the culture supernatant, outer-membrane, inner-membrane and combined cytoplasmic/periplasmic fractions. The integrity of the different fractions was verified by measuring marker enzyme activity associated with each region of the bacterium. The data, presented in Table 2, showed that *P. cellulosa* had been fractionated correctly, and it was apparent that the PNPAase activity was primarily associated with the outer-membrane fraction. To investigate whether Abf51A was at least a component of this membrane-bound arabinofuranosidase activity, the subcellular fractions of *P. cellulosa* were subjected to Western analysis using anti-Abf51A antibodies. The data, presented in Figure 1, showed that the antibodies cross-reacted with a 55 kDa protein (similar to the deduced molecular mass of mature Abf51A [4]), that is located primarily in the outer membrane of the pseudomonad. In addition, zymogram analysis revealed that the vast majority of 4-

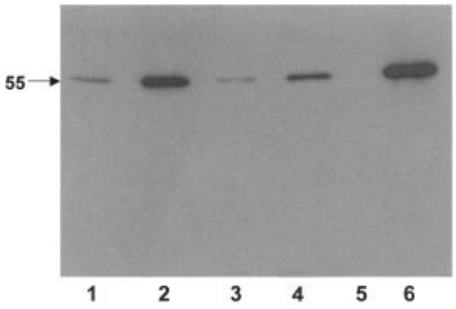

Figure 1 Immunological detection of Abf51A in *P. celllulosa*

Wild-type *P. cellulosa* (lanes 1–4) and the Δ*abf51A* mutant strain (lane 5) were cultured on media containing wheat arabinoxylan and arabinan and were subjected to Western-blot analysis using anti-Abf51A as the primary antibody. Cell-free extract comprising the cytoplasm and periplasm (lane 1); complete membrane fraction (lane 2); inner-membrane fraction (lane 3); outer-membrane fraction (lane 4); combined cell-free extract and membrane fraction (lane 5); purified Abf51A (lane 6). The size of the cross-reacting protein is given in kDa.

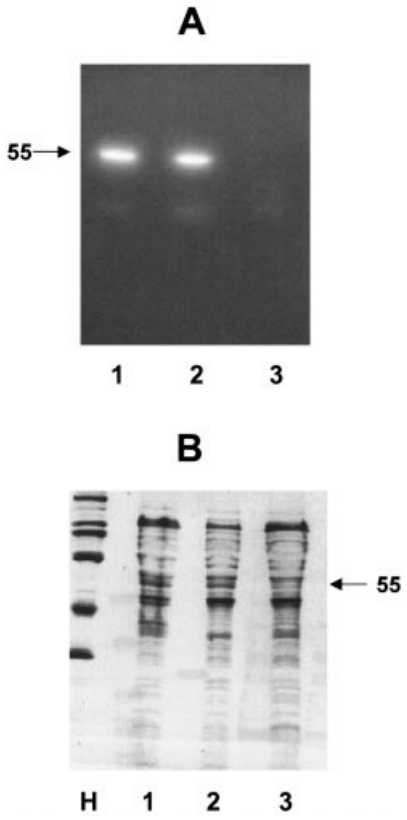

Figure 2 Detection of Abf51A activity by zymogram analysis

Zymogram analysis was carried out as described in the Materials and methods section. The samples used were as follows: the whole-cell fraction (membrane, periplasm and cytoplasm combined) of wild-type *P. cellulosa* cultured on media containing arabinan (lanes 1) and wheat arabinoxylan (lanes 2); the whole-cell fractionation of Δ*abf51A* mutant cultured on wheat arabinoxylan (lanes 3). Lane H contains the high molecular-mass markers from Sigma. (**A**) Zymogram; (**B**) a parallel SDS/PAGE gel stained with Coomassie Brilliant Blue. The size of the major MUAase species is shown in kDa.

methylumbellerfyl-α-L-arabinofuranosidase (MUAase) activity comprises a 55 kDa polypeptide (Figure 2). Immunofluorescence studies of whole cells of the pseudomonad showed that anti-Abf51A antibodies cross-reacted with proteins on the surface of

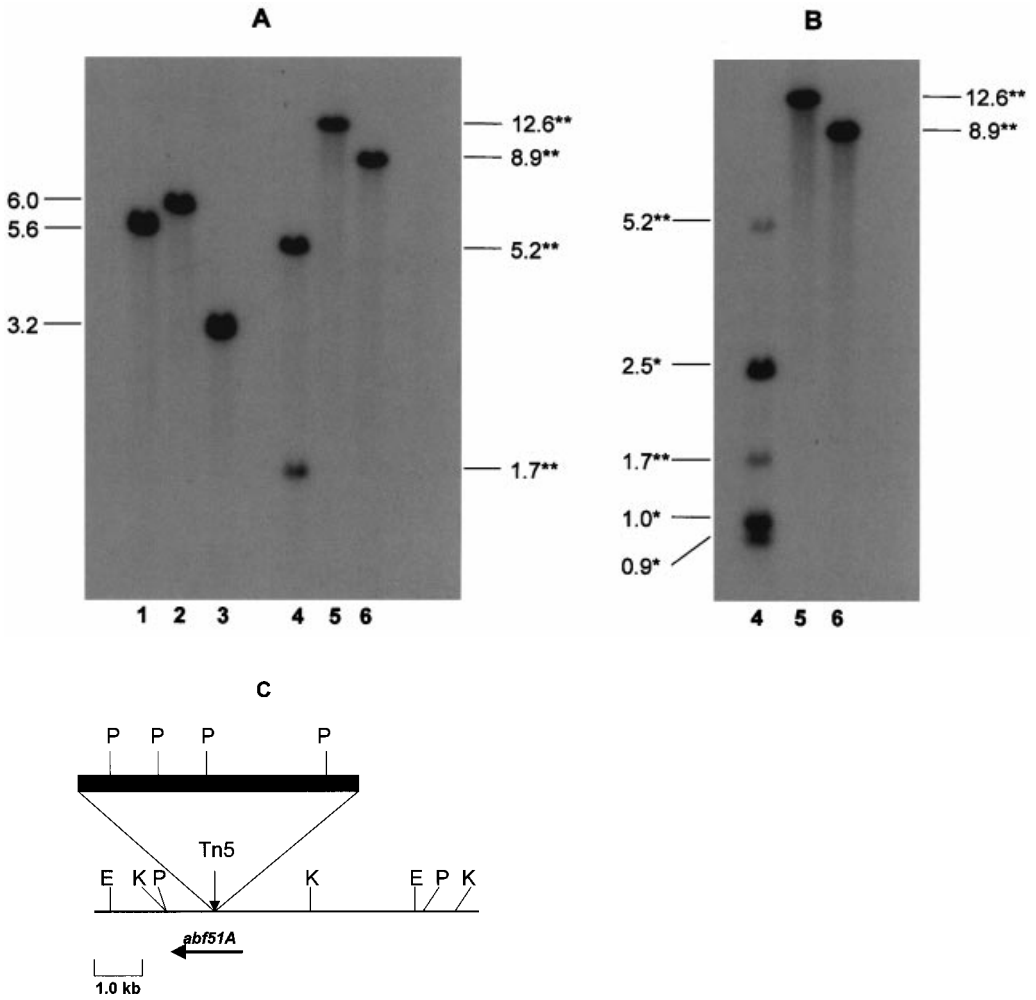

Figure 3 Southern-blot hybridization of *P. cellulosa* genomic DNA with Tn5 and *abf51A*

P. cellulosa genomic DNA was subjected to Southern-blot hybridization using *abf51A* (**A**) and Tn5 (**B**) as the probes. Lanes 1–3 contain DNA from wild-type *P. cellulosa* and lanes 4–6 contain DNA from the Δ*abf51A* mutant. The DNA was digested with the following restriction enzymes: lanes 1 and 4, *Pst*I; lanes 2 and 5, *Eco*RV; lanes 3 and 6, *Kpn*I. The sizes of the hybridizing restriction fragments are shown in kb. DNA bands denoted * were comprised entirely of Tn5 sequences, while restriction fragements denoted ** contained both Tn5 and *P. cellulosa* DNA. (**C**) A simplified physical map of the *Pseudomonas* DNA containing Tn5 (black bar) inserted into *abf51A*. The site of insertion, determined from the Southern-hybridization data presented in (**A**) and (**B**), is indicated by a vertical arrow. Selected restriction sites: E, *Eco*RV; K, *Kpn*I; P, *Pst*I.

Table 3 Activity of the wild-type and Δ*abf51A* strain of *P. cellulosa* against arabinose-containing substrates

The two strains were grown on wheat arabinoxylan to late-exponential phase and then fractionated into disrupted (sonicated) whole cells and culture supernatant. ND, no detectable activity. PNPA, 4-nitrophenyl-α-L-arabinofuranoside.

Strain	Cell fraction	Substrate	Activity (units/mg of total protein)
Wild type	Culture supernatant	PNPA	$2.4 \times 10^{-2} \pm 1.3 \times 10^{-3}$
Wild type	Whole cells	PNPA	2.5 ± 0.3
Δ*abf51A*	Culture supernatant	PNPA	ND
Δ*abf51A*	Whole cells	PNPA	$1.5 \times 10^{-2} \pm 2.3 \times 10^{-3}$
Wild type	Culture supernatant	Arabinan	ND
Wild type	Whole cells	Arabinan	$(7.1 \pm 1.1) \times 10^{-1}$
Δ*abf51A*	Culture supernatant	Arabinan	ND
Δ*abf51A*	Whole cells	Arabinan	$3.6 \times 10^{-3} \pm 7.5 \times 10^{-4}$
Wild type	Whole cells	Arabinotriose	$1.5 \times 10^{-3} \pm 4.2 \times 10^{-4}$
Δ*abf51A*	Whole cells	Arabinotriose	$(9.1 \pm 3.1) \times 10^{-6}$
Wild type	Culture supernatant	Wheat arabinoxylan	ND
Wild type	Whole cells	Wheat arabinoxylan	1.6 ± 0.2
Δ*abf51A*	Culture supernatant	Wheat arabinoxylan	ND
Δ*abf51A*	Whole cells	Wheat arabinoxylan	ND

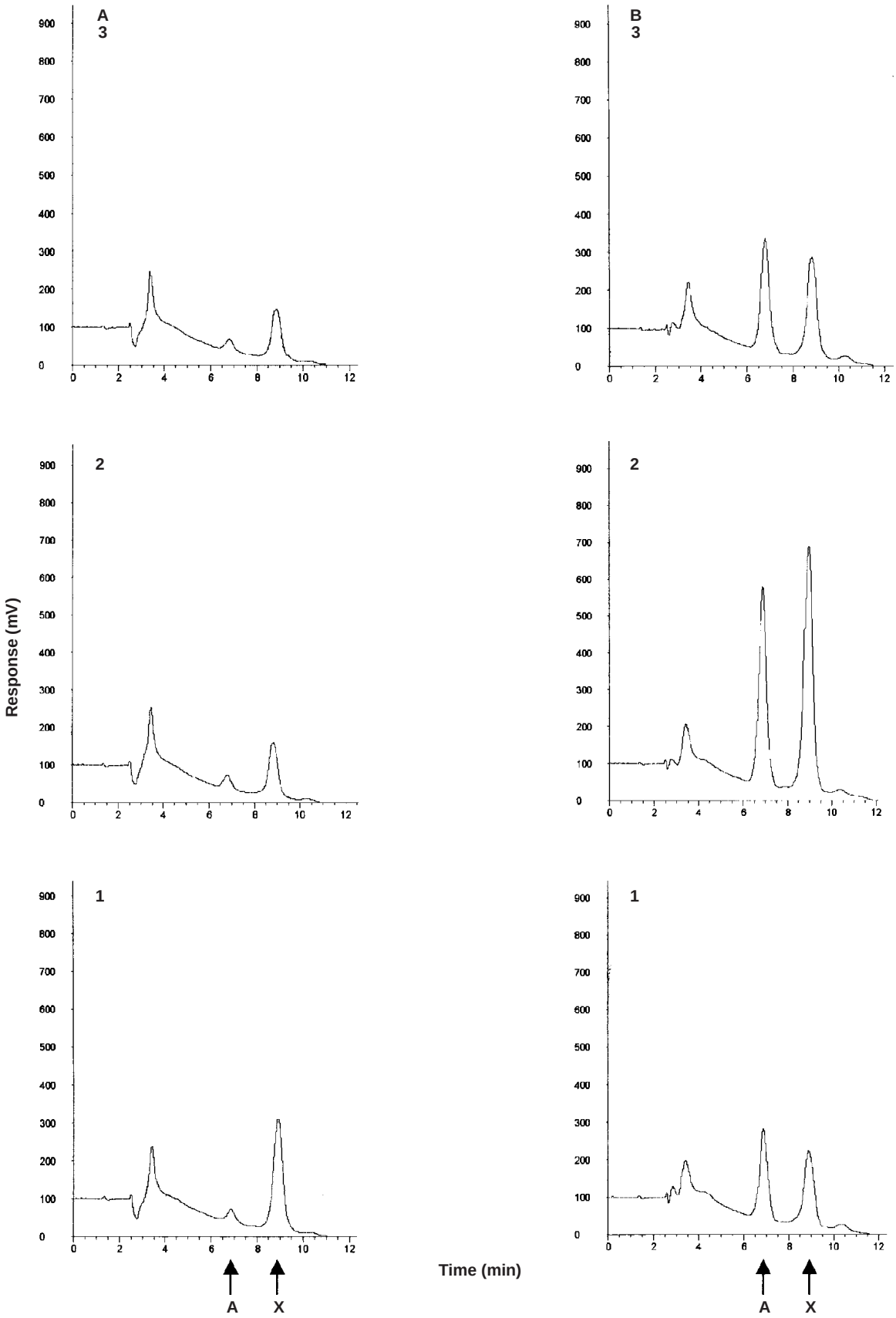

Figure 4 Release of sugars by *P. cellulosa* cultured on wheat arabinoxylan

Δ*abf51A* (panel **A**) and wild-type (panel **B**) *P. cellulosa* were cultured on wheat arabinoxylan and at 18 h (**A1**, **B1**), 24 h (**A2**, **B2**) and 36 h (**A3**, **B3**) the culture supernatant was subjected to HPAEC. The elution times of arabinose (A) and xylose (X) from the HPLC column are indicated with vertical arrows.

the bacterium (results not shown). These data indicate that Abf51A is located on the surface of *P. cellulosa* and, unlike other plant-cell-wall hydrolases expressed by the bacterium [21], does not appear to be glycosylated.

Inactivation of *abf51A*

The data presented above indicate that Abf51A plays a role in the release of arabinose units from pectin and hemicellulose. To evaluate the importance of this enzyme in plant-cell-wall degradation, we used a transposon-mutagenesis strategy to inactivate *abf51A*. *E. coli* strain CH545, which contains Tn5 on the self-transmissible plasmid pJB4JI, was filter-mated with Rif^r *P. cellulosa*, and Rif^r/Kan^r bacteria were selected. The transmissible plasmid does not replicate in Gram-negative bacteria, apart from *E. coli*, and thus any Rif^r/Kan^r bacteria should comprise *P. cellulosa* containing Tn5 inserted into the genome. The filter-mating experiments generated 25000 Rif^r/Kan^r bacteria, 99.9 % of which were shown to be *P. cellulosa* by their capacity to degrade different plant structural polysaccharides (results not shown). To investigate whether Tn5 had randomly integrated into the pseudomonad genome, 15 Rif^r/Kan^r bacteria were subjected to Southern-blot hybridization using Tn5 as the probe. The data (not shown) revealed that in 12 of the transconjugants the transposon had integrated at the same locus, suggesting that the bacterium contains a 'hot spot' for Tn5 integration. Nevertheless, it is likely that Tn5 had integrated randomly in 5000 out of the 25000 transconjugants. Thus, the 25000 Rif^r/Kan^r bacteria were screened for MUAase activity. One transconjugant did not express any apparent MUAase activity. This bacterium was subjected to Southern hybridization using *abf51A* and Tn5 as probes. The data, presented in Figure 3, showed that the MUAase-minus pseudomonad contained a single Tn5 that had integrated into the *abf51A* gene. The absence of Abf51A expression was confirmed by Western and Northern analysis of the mutant, which showed that the pseduomonad did not synthesize Abf51A protein (Figure 1) or *abf51A* mRNA (results not shown). In addition, zymogram analysis showed that the mutant bacterium did not synthesize the 55 kDa MUAase, which was present in the wild-type strain (Figure 2). These data show that the MUAase-minus strain of *P. cellulosa*, designated *P. cellulosa* Δ*abf51A*, contains an inactive *abf51A* gene, and thus does not express Abf51A.

Characterization of *P. cellulosa* Δ*abf51A*

To investigate the importance of Abf51A in the cleavage of arabinose-containing polysaccharides, wild-type and Δ*abf51A* *P. cellulosa* were plated out on media containing sugar beet arabinan, wheat arabinoxylan and linear arabinan as the respective carbon sources. Both strains grew on media containing wheat arabinoxylan, although the growth rate and final yield of bacterial biomass of the Δ*abf51A* strain was approx. 4-fold lower than the wild-type bacterium (results not shown). Only the wild-type pseudomonad could be cultured on media containing arabinan and linear arabinan. These data show that Arb51A plays a critical role in the metabolism of sugar beet and linear arabinan.

To investigate the catalytic activities of wild-type *P. cellulosa* and the Δ*abf51A* mutant, the bacterial strains were grown on wheat arabinoxylan, and the capacity of whole cells and cell-free extracts to hydrolyse different polysaccharides and aryl-glycosides were evaluated. The data, presented in Table 3, showed that the activity of Δ*abf51A* against 4-nitrophenyl-α-L-arabinofuranoside was approx. 1 % of the wild-type bacterium. The mutant strain hydrolysed sugar beet arabinan and arabinotriose at a rate $< 1\,\%$ of the unmodified strain (results not shown). When Δ*abf51A* was grown on wheat arabinoxylan very low levels of arabinose were detected in the culture supernatant, which did not increase with time. In contrast, arabinose and xylose did accumulate in the culture supernatant of wild-type *P. cellulosa* (Figure 4). Finally, no xylan-specific arabinofuranosidase activity was detected in the culture supernatant of the wild-type pseudomonad, or in the whole cells and culture supernatant of Δ*abf51A* (results not shown). These data show that Abf51A is the primary enzyme involved in the release of arabinose from both arabinan and arabinoxylan, and in the metabolism of arabinotriose. Surprisingly, no arabinoxylan-specific arabinofuranosidase activity was detected in Δ*abf51A*, suggesting that Abf62A is not expressed in *P. cellulosa*.

DISCUSSION

The location of Abf51A on the outer membrane of *P. cellulosa* is in contrast to arabinofuranosidases from other aerobic micro-organisms, which are either in the cytoplasm [7,8] or in the culture supernatant [6]. It is believed that the primary target of intracellular and extracellular arabinofuranosidases are oligosaccharides and polysaccharides, respectively. The location of Abf51A suggests that the target substrates for this enzyme are arabinose-containing polysaccharides and oligosaccharides. The membrane location for such an enzyme ensures that the release of arabinose by Abf51A would be in close proximity to *P. cellulosa*, thus the bacterium would be able to utilize the saccharide, rather than the competing micro-organisms within its ecosystem. It is interesting to note that *P. cellulosa* also expresses a glucan glucohydrolase on its outer membrane, an enzyme that releases glucose from cellulo-oligosaccharides [15]. Thus it would appear that the bacterium cleaves polysaccharides via endo-acting enzymes that are released into the environment, and the oligosaccharides generated are hydrolysed to their constituent sugars on the surface of the bacterium, ensuring the preferential uptake of these saccharide units. It should be noted that the spatial organization of degradative enzymes in *P. cellulosa* is similar to that in the mammalian gastrointestinal tract, where the major endo-acting enzymes, such as proteinases, lipases and amylases, are secreted into the intestinal lumen, while the N-terminal peptidases and disaccharidases are on the surface of the epithelium.

The observations that the Δ*abf51A* mutant did not synthesize significant arabinofuranosidase activity, could not grow on linear or sugar beet arabinan, and grew slowly on wheat arabinoxylan, point to the pivotal role that Abf51A plays in removing arabinose units from both polysaccharides and oligosaccharides. Thus *P. cellulosa* appears to express a single membrane-bound arabinofuranosidase that is critical in supplying the bacterium with arabinose derived from sugar polymers. The importance of Abf51A is surprising when one considers the relatively poor activity of the enzyme against α1,5-linked arabino-oligosaccharides. Other microbes such as *Streptomyces chartreusis* synthesize two different arabinofuranosidases that attack arabinan. The GH51 enzyme, similar to Abf51A, removes the 2′ and 3′ arabinose side chains, while the GH43 glycoside hydrolase preferentially cleaves α1,5-linked arabino-oligosaccharides [6]. It is not obvious why *P. cellulosa* only synthesizes a single arabinofuranosidase. It is possible that the very high expression of Abf51A and its membrane location ensure that sufficient arabinose is available for the bacterium. Alternatively, it is possible that the nutrients derived from arabinan are not critical for the survival of the pseudomonad. Rather, the processive endo-α1,5-

arabinanase in conjugation with Abf51A convert arabinans into soluble oligosaccharides, and the degradation of these molecules makes the other plant-cell-wall polysaccharides, primarily cellulose and hemicellulose, more available to enzyme attack. This view is supported by studies which have shown that other prokaryotes, such as *C. thermocellum*, express numerous hemicellulases but do not utilize the sugars (xylose and mannose) derived from their target substrates as carbon or energy sources [22,23].

It is interesting to note that *P. cellulosa* contains a gene (*abf62A*) that encodes a functional xylan-specific arabinofuranosidase (Abf62A). However, this gene is not expressed when the bacterium is cultured on linear arabinan, wheat arabinoxylan or sugar beet arabinan. Thus Abf51A appears to play a key role in the degradation of arabinoxylans, while Abf62A is not critical for the efficient hydrolysis of this substrate. The capacity of Abf51A to remove arabinose from both arabinans and arabinoxylans is not a universal property of GH51 enzymes. As discussed above, *S. chartreusis* α-L-arabinofuranosidase I does not attack arabinoxylans, only arabinans. Thus, it is possible that a primitive form of *P. cellulosa* contained *abf51A* which underwent mutations that gave rise to an arabinofuranosidase with wide substrate specificity, such that it could attack arabinan and arabinoxylan. The later acquisition of *abf62A* by lateral gene transfer did not confer a significant advantage to the bacterium, hence there was no selection pressure for the gene to be expressed. However, if this scenario was correct, one would predict that *abf62A* would also accumulate mutations such that the encoded enzyme would not be active. It is possible that *abf62A* was only recently acquired by *P. cellulosa*, and that there has been insufficient time for the structural gene to accumulate mutations leading to the inactivation of the enzyme. This view is supported by the observation that the 5′ sequence of *abf62A* is identical with those of *xynB* and *xynD*, suggesting that recombination events leading to a common 5′ region in these genes were very recent. Alternatively, it is possible that *abf62A* is expressed by *P. cellulosa*, but only in complex environments, and is not simply induced by the presence of the substrate. This is consistent with a recent study [24] which showed that an *Aspergillus* GH10 xylanase was not induced by its target substrate in monoculture, but was expressed when the fungus was inoculated into compost, a highly complex environment both in terms of the nutrients present and the structure of its microbial community. Nevertheless, the possibility that *abf62A* is not expressed in *P. cellulosa* requires that we re-evaluate the general assumption that cloned genes encoding functional plant-cell-wall hydrolases, esterases and lyases are functional in their target host. Thus to evaluate the possible role of these recombinant enzymes it is important that their expression in the endogenous micro-organism is demonstrated.

Conclusions

This report shows that *P. cellulosa* expresses a single membrane-bound arabinofuranosidase which attacks both arabinan and arabinoxylans. The wide substrate specificity of Abf51A has made the bacterium's xylan-specific arabinofuranosidase redundant, hence the protein does not appear to be expressed. The central importance of a single arabinofuranosidase in *P. cellulosa* is in stark contrast to other micro-organisms, both eukaryotes and prokaryotes, which mediate the release of arabinose from different sugar polymers by synthesizing a range of arabinofuranosidases with diverse substrate specificity.

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