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Two distinct arabinofuranosidases contribute to arabino-oligosaccharide degradation in *Bacillus subtilis*

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Bacillus subtilis produces α-L-arabinofuranosidases (EC 3.2.1.55; AFs) capable of releasing arabinosyl oligomers and L-arabinose from plant cell walls. Here, we show by insertion-deletion mutational analysis that genes *abfA* and *xsa*(*asd*), herein renamed *abf2*, encode AFs responsible for the majority of the intracellular AF activity in *B. subtilis*. Both enzyme activities were shown to be cytosolic and functional studies indicated that arabino-oligomers are natural substrates for the AFs. The products of the two genes were overproduced in *Escherichia coli*, purified and characterized. The molecular mass of the purified AbfA and Abf2 was about 58 kDa and 57 kDa, respectively. However, native PAGE gradient gel analysis and cross-linking assays detected higher-order structures (>250 kDa), suggesting a multimeric organization of both enzymes. Kinetic experiments at 37 °C, with *p*-nitrophenyl-α-L-arabinofuranoside as substrate, gave an apparent K_m of 0.498 mM and 0.421 mM, and V_{max} of 317 U mg^{-1} and 311 U mg^{-1} for AbfA and Abf2, respectively. The two enzymes displayed maximum activity at 50 °C and 60 °C, respectively, and both proteins were most active at pH 8.0. AbfA and Abf2 both belong to family 51 of the glycoside hydrolases but have different substrate specificity. AbfA acts preferentially on (1→5) linkages of linear α-1,5-L-arabinan and α-1,5-linked arabino-oligomers, and is much less effective on branched sugar beet arabinan and arabinoxylan and arabinogalactan. In contrast, Abf2 is most active on (1→2) and (1→3) linkages of branched arabinan and arabinoxylan, suggesting a concerted contribution of these enzymes to optimal utilization of arabinose-containing polysaccharides by *B. subtilis*.

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INTRODUCTION

α-L-Arabinofuranosidases (α-L-arabinofuranoside arabinofuranohydrolases, EC 3.2.1.55; AFs) catalyse the hydrolysis of terminal α-1,2-, α-1,3- or α-1,5-L-arabinofuranoside bonds in different hemicellulosic homopolysaccharides (branched and debranched arabinans) and heteropolysaccharides (arabinoxylans, arabinogalactans, etc.). The complete degradation of hemicellulose and arabinose-containing polysaccharides requires the concerted action of many different enzymes, and AFs play a key role in this synergistic process. To hydrolyse arabinan, in addition to AFs that act in an exo- fashion and cleave arabinose side chains, α-1,5-arabinanases (EC 3.2.1.99; ABNs) act in an endo- fashion, i.e. they attack the α-1,5-linked L-arabinofuranose backbone of the homopolysaccharide (Beldman *et al.*, 1997). Arabinan-degrading enzymes have several biotechnological applications: improvement of wine flavours, pulp treatment, juice clarification, quality of animal feedstock, production of important medicinal compounds, and production of bioethanol (Numan & Bhosle, 2006; Saha, 2000; Shallom & Shoham, 2003).

Bacillus subtilis, an aerobic, mesophilic, endospore-forming bacterium, produces a vast number of polysaccharolytic enzymes, including ABNs and AFs, capable of releasing arabinosyl oligomers and L-arabinose from plant cell walls (Kaji & Saheki, 1975; Kaneko *et al.*, 1994; Leal & Sá-Nogueira, 2004; Sakai & Sakamoto, 1990; Weinstein & Albersheim, 1979). In a previous study, we characterized the transcriptional regulation of three *B. subtilis* genes involved in arabinan degradation (*abnA*, *xsa*, and *abfA*), which respond to arabinose (Raposo *et al.*, 2004). The *abnA* gene was shown to encode an extracellular endo-ABN

Abbreviations: ABN, α-1,5-arabinanase; AF, α-L-arabinofuranosidase; GH 51, glycoside hydrolase family 51; *p*NPA*f*, *p*-nitrophenyl-α-L-arabinofuranoside; *p*NPA*p*, *p*-nitrophenyl-α-L-arabinopyranoside; *p*NPX*p*, *p*-nitrophenyl-β-D-xylopyranoside.

The GenBank/EMBL/DDBJ accession number for the nucleotide sequence of the *abf2* gene reported in this paper is EU073712.

that hydrolyses sugar beet arabinan and linear α-1,5-L-arabinan (Leal & Sá-Nogueira, 2004; Raposo *et al.*, 2004). To further characterize this *B. subtilis* hemicellulolytic system we have now analysed the function of the *abfA* and *xsa* genes by mutagenic studies and determination of the capacity of the different mutants to utilize either sugar beet arabinan or α-1,5-linked arabino-oligosaccharides. The determination of the subcellular localization of their products indicates that the two enzymes are scattered throughout the cytosol and do not localize in particular foci. The two potential AFs were expressed in *Escherichia coli* and the biochemical properties of the recombinant proteins determined. Both enzymes exhibited AF activity; however, they displayed different substrate specificity. Since the product of *xsa* does not show *β*-xylosidase activity we propose to rename this gene *abf2*.

METHODS

Substrates. Sugar beet arabinan, debranched arabinan (linear α-1,5-L-arabinan, purity 95 %), α-1,5-linked arabino-oligosaccharides (arabinobiose, arabinotriose, arabinotetraose, purity 95 %), and wheat arabinoxylan were purchased from Megazyme International Ireland, and larch wood arabinogalactan, *p*-nitrophenyl-α-L-arabinofuranoside (*p*NPA*f*), *p*-nitrophenyl-α-L-arabinopyranoside (*p*NPA*p*), and *p*-nitrophenyl-*β*-D-xylopyranoside (*p*NPX*p*) from Sigma.

Bacterial strains and growth conditions. The *B. subtilis* strains used in this study are listed in Table 1. *E. coli* DH5α (Gibco-BRL) was used for routine molecular cloning work and *E. coli* BL21(DE3) pLysS (Studier *et al.*, 1990) as the host for production of native and recombinant AbfA and Abf2. *E. coli* strains were grown in Luria–Bertani (Miller, 1972) medium and kanamycin (20 μg ml^{-1}), chloramphenicol (25 μg ml^{-1}) or IPTG were added as appropriate. For growth kinetics experiments, overnight cultures of the different *B. subtilis* strains, grown in minimal medium C (Pascal *et al.*, 1971) supplemented with L-tryptophan (100 μg ml^{-1}), potassium glutamate (8 g l^{-1}) and potassium succinate (6 g l^{-1}) (CSK medium; Débarbouillé *et al.*, 1990), were washed and resuspended in 200 μl (to an OD_{600} of 0.05) of the same medium without potassium succinate (CE-minimal medium), supplemented with one of the various carbon sources: arabinose (26 mM), glucose (22 mM), arabinobiose (13 mM), arabinotriose (8.6 mM), arabinotetraose (6.5 mM) and sugar beet arabinan (0.26 mM). The cultures were incubated in Multiple Well Plate 96-Well Round Bottom with Lid plates (Sarstedt), at 37 °C and 180 r.p.m., and growth was followed by periodic reading in a Molecular Devices Spectra max Plus Microplate Spectrophotometer at 600 nm. Transformation of *E. coli* and *B. subtilis* strains was performed as previously described (Inácio *et al.*, 2003).

DNA manipulation and sequencing. DNA manipulations were carried out as described by Sambrook *et al.* (1989). Restriction enzymes were purchased from Fermentas and used according to the manufacturer's instructions. DNA was eluted from agarose gels with the GFX gel band purification kit (GE Healthcare). DNA sequencing was performed with the ABI PRISM BigDye Terminator Ready Reaction Cycle Sequencing kit (Applied Biosystems). PCR amplifications were done using high-fidelity *Pfu* Turbo DNA polymerase (Stratagene), and the resulting products purified with the QIAquick PCR purification kit (Qiagen).

Construction of plasmids and strains. To construct the *B. subtilis* integrative plasmid pTL2, encoding a fusion of the C-terminus of AbfA to a His_6-tag, the *abfA* C-terminal-encoding region was amplified by PCR of chromosomal DNA from *B. subtilis* $168T^+$ using primers ARA124 and ARA234 (Table 1). The resulting 1215 bp DNA fragment was digested with *Kpn*I and *Xho*I and cloned into pET30a(+) (Novagen), yielding pTL1. This plasmid was then digested with *Xba*I and *Bgl*II, and a chloramphenicol resistance (Cm^R) cassette from pMS38 (Zilhão *et al.*, 2004) was inserted between those sites, generating pTL2. For the construction of pTL4, encoding a fusion of the C terminus of Abf2 to a His_6-tag, the C-terminal-encoding region of the *abf2* gene was amplified by PCR of chromosomal DNA using primers ARA112 and ARA233 (Table 1), and the resulting 1380 bp PCR product digested with *Eco*RI and *Xho*I was cloned into pET30a(+), yielding pTL3. To generate pTL4, the pMS38 (Zilhão *et al.*, 2004) *Xba*I–*Eco*RI DNA fragment, containing the Cm^R cassette, was subcloned into pTL3. Plasmids pTL2 and pTL4 were integrated into the host chromosome by means of a single-crossover (Campbell-type) recombinational event that occurred in the region of homology (Table 1).

Plasmids pTL11 and pTL12 are pET30a(+) derivatives encoding recombinant AbfA-His_6 and Abf2-His_6, respectively, under the control of a T7 inducible promoter. To construct pTL11, the coding sequence of *abfA* was amplified by PCR with primers ARA121 and ARA226, which introduced a unique *Xba*I site in the 5′-end region of the gene (Table 1). The resulting 1479 bp DNA fragment digested with *Xba*I/*Kpn*I was cloned into pTL1. The same strategy was used in the construction of pTL12: the coding sequence of the *abf2* gene was amplified by PCR of chromosomal DNA using primers ARA220 and ARA227, which created a unique *Nde*I site at the start of the codon. The resulting 994 bp PCR product digested with *Nde*I/*Eco*RI was subcloned in pTL3.

Linearized plasmids pMPR7 and pZI12 were used separately to delete the *abfA* gene and the *abf2* gene, respectively, in the wild-type *B. subtilis* $168T^+$ chromosome. Plasmid pMPR7 was obtained by subcloning the 1441 bp *Ssp*I DNA fragment from pTN13 (Sá-Nogueira *et al.*, 1997) into pSN21 (Sá-Nogueira & Mota, 1997) digested with *Sma*I. The construction of pZI12 was achieved by subcloning an 1106 bp *Nsi*I–*Bam*HI DNA fragment (containing the Cm^R cassette) from pMS38 (Zilhão *et al.*, 2004) into pMPR5 digested with *Nsi*I and *Bam*HI. This latter plasmid, pMPR5, was constructed by subcloning a 1700 bp DNA fragment amplified by PCR of chromosomal DNA using primers ARA87 and ARA91 (Table 1) into pBluescript II KS(−) (Stratagene) digested with *Sma*I.

Construction of green fluorescent protein (GFP) fusions. GFPmut2 fused to the N-terminus of Abf2 under the control of the *abf2* promoter was engineered in three successive steps using the splicing by overlay extension (SOE) technique (Horton *et al.*, 1989). First, the coding region of the *abf2* gene was amplified by PCR, with primers ARA350 and ARA270 (Table 1), using chromosomal DNA of wild-type strain *B. subtilis* $168T^+$ as template. A linker encoding four asparagines was engineered in the primer ARA350 (Glaser *et al.*, 1997). Second, a 726 bp fragment comprising the coding region of the *gfpmut2* gene was PCR amplified from plasmid pEA18 (Quisel *et al.*, 1999) by using the primers ARA349 and *gfp*30D (Table 1). Third, the promoter region of *abf2* was amplified by PCR, with primers ARA87 and ARA348 (Table 1), using chromosomal DNA. P_{abf2}-*gfpmut2-abf2* was amplified with primers ARA87 and ARA270 (Table 1), using an equimolar mix of *abf2*, *gfpmut2* and *abf2* promoter region PCR products as templates. The resulting fragment was inserted into pGEM-TEasy vector (Promega), generating pZI51. The same strategy was used to obtain an N-terminal fusion of AbfA to GFPmut2 under the control of the *araABDLMNPQ-abfA* promoter. First, the coding region of *abfA* was amplified with primers ARA347 and ARA269 (Table 1). A linker in the primer ARA347 was engineered as described above. Second, the *araQ* C-terminal coding region was amplified

Table 1. Plasmids, *B. subtilis* strains, and oligonucleotides used in this study

Plasmid, strain, or oligonucleotide	Relevant construction, genotype, or sequence (5′–3′)*	Source or reference†
Plasmids		
pTL2	Integrative plasmid encoding a fusion of the C terminus of AbfA to His_6-tag, *cat*	This work
pTL4	Integrative plasmid encoding a fusion of the C terminus of Abf2 to His_6-tag, *cat*	This work
pET30a(+)	Expression vector, allowing N- or C-terminal His_6-tag insertion, T7 promoter, *kan*	Novagen
pTL11	pET30a(+) containing the *afbA*-encoding region in the MCS	This work
pTL12	pET30a(+) containing the *abf2*(*xsa*)-encoding region in the MCS	This work
pMPR7	pSN21 (Sá-Nogueira & Mota, 1997) derivative containing the *spc* gene inserted into the *abfA* gene	This work
pZI12	$pBKS^-$ (Stratagene) derivative containing the *cat* gene inserted into the *abf2*(*xsa*) gene	This work
pGEM-Teasy	Vector for cloning of PCR products, MCS, *bla*	Promega
pZI50	pGEM-Teasy containing the $P_{abf2(xsa)}$-*gfpmut2-abf2*(*xsa*) fusion in the MCS	This work
pZI51	pGEM-Teasy containing the *araQ-gfpmut2-abfA* fusion in the MCS	This work
pMLK83	Promoterless *gusA* preceded by a RBS efficiently used in *B. subtilis* and MCS, *neo*, flanked by *amyE*-5′ and *amyE*-3′, *bla*	Karow & Piggot (1995)
***B. subtilis* strains**		
$168T^+$	Prototroph	Raposo *et al.* (2004)
IQB206	Δ*araL-abfA*::*spc*	Sá-Nogueira *et al.* (1997)
IQB419	Δ*abfA*::*spc*	pMPR7→$168T^+$‡
IQB460	Δ*abf2*(*xsa*)::*cat*	pZI12→$168T^+$‡
IQB462	Δ*abfA*::*spc* Δ*abf2*(*xsa*)::*cat*	pZI12→IQB419‡
IQB493	*amyE*::[*gusA neo*] $P_{abf2(xsa)}$-*gfpmut2-abf2*	pZI51 and pMLK83→ IQB460‡
IBQ494	*amyE*::[*gusA neo*] *araQ-gfpmut2-abfA*	pZI50 and pMLK83→ IQB419‡
IQB600	*abf2*(*xsa*)::pTL4	pTL4→$168T^+$
IQB602	*abfA*::pTL2	pTL2→$168T^+$
Oligonucleotides§		
ARA87	$^{-207}$AAAATAGCGGATTACGGCATCG^{-186}	
ARA91	$^{+1509}$GTATTGTCTGCAGGATTCCGG^{+1489}	
ARA112	$^{+213}$GGTCGACACGGACTCAGACATCCC^{+236}	
ARA121	$^{+11744}$GACAGACGATGATCCGTTGG^{+11725}	
ARA124	$^{+10608}$ACGACAGAAACCAATGAAGTG^{+10628}	
ARA220	$^{+1084}$TGTGGAAATGAGAAGCCGCC^{+1065}	
ARA226	$^{+10270}$CCTGATCTAGAGGGAGTCAAAGG^{+10291}	
ARA227	$^{+91}$GATCAGACATATGTCTGAACATC^{+112}	
ARA233	$^{+1593}$GCGCTCGAGAGAATCAGCACGCAGC^{+1569}	
ARA234	$^{+11823}$GCTACTCGAGTGACTGTTTTTTCAGGCGG^{+11795}	
ARA269	$^{+12100}$CCTCAAGCGCTCGAGCAGGTGTTGG^{+12076}	
ARA270	$^{+1750}$GATAATGGCCTCGAGCACTACAATTGC^{+1724}	
ARA345	$^{+9934}$TCAGGCAATATGCTCTTGG^{+9952}	
ARA346	AGTGAAAAGTTCTTCTCCTTTACTCAT^{+10313}CACACGTTTCCTCCTTTCATTTAACCTTT-GACTCC^{+10281}	
ARA347	GGCATGGATGAACTATACAAAAATAATAATAAT^{+10314}ATGAAAAAAGCGCGAATGATTG-TAGAC^{+10340}	
ARA348	AGTGAAAAGTTCTTCTCCTTTACTCAT^{+99}GCCTCTGATCATTCCTTTCATACGG^{+75}	
ARA349	TTTGTATAGTTCATCCATGCC	
ARA350	GGCATGGATGAACTATACAAAAATAATAATAAT^{+100}ATGTCTGAACATCAAGCAGTG^{+114}	
*gfp*30D	AGTAAAGGAGAAGAACTTTTCACTGGAG	

*MCS, multiple cloning site.

†The arrows indicate transformation and point from donor DNA to the recipient strain.

‡Transformation was carried out with linearized plasmid DNA

§The numbers within the primers refer to the position of the sequence relative to the transcription start site of the *abf2(xsa)* gene or the *araABDLMNPQ-abfA* operon. Restriction sites used are underlined.

using primers ARA345 and ARA346 (Table 1). *araQ-gfpmut2-abfA* was amplified with primers ARA345 and ARA269 (Table 1) from the three PCR fragments, as described above, and inserted into pGEM-TEasy, yielding pZI50.

The P_{abf2}-*gfpmut2-abf2* and *araQ-gfpmut2-abfA* fusions were integrated, in separate experiments, into the *B. subtilis* chromosome of the Δ*abf2* and Δ*abfA* null mutants (IQB460 and IQB419, Table 1) by congression, using linearized pZI51 and pZI50, respectively, together with linearized pMLK83 (Karow & Piggot, 1995). Selection was made for the neomycin-resistance cassette (from pLMK83), integrated at the *amyE* locus by a double recombination event. The transformants were then screened for sensitivity to chloramphenicol or sensitivity to spectinomycin, indicative of the integration of P_{abf2}-*gfpmut2-abf2* or *araQ-gfpmut2-abfA* fusions at the *abf2* or *abfA* locus, respectively, of the recipient strains IQB460 and IQB419. The correct insertion of the fusions in the resulting strains, IQB493 and IQB494 (Table 1), respectively, was confirmed by PCR.

AF activity assays in *B. subtilis*. To measure the AF activity in *B. subtilis*, the strains were grown on minimal medium C (Pascal *et al.*, 1971) supplemented with 1 % (w/v) casein hydrolysate. During the early exponential phase (OD_{600} 0.11–0.15), L-arabinose at a final concentration of 0.4 % (w/v) was added. Samples were collected 2 h after induction. The harvested cells were suspended in 1 ml Z buffer (Miller, 1972), and two drops of chloroform and one drop of 0.1 % (w/v) SDS were added and mixed vigorously for 10 s on a tabletop vortex apparatus. The enzyme reaction was started by adding 200 µl *p*NP*A*f [4 mg ml^{-1} in P buffer (Miller, 1972)], and incubated for 20 min at 28 °C. Adding 250 µl of 2 M Na_2CO_3 then stopped the reaction and the A_{400} was measured after a 5 min centrifugation to pellet cell debris. The level of accumulated AF activity was calculated as described by Miller (1972).

Fluorescence microscopy. *B. subtilis* strains harbouring GFP fusions, and the wild-type strain $168T^+$ (used as negative control), were grown as described above for the AF assays. Two hours after induction, 0.5 ml aliquots were collected, harvested and washed three times with PBS, to eliminate possible traces of tryptophan present in the culture medium, and resuspended in 0.1 ml of the same buffer. Samples were then applied to agarose-coated microscope slides, and images were acquired under a Leica DMRA2 microscope coupled with a CoolSNAP HQ Photometrics camera (Roper Scientific).

Production and purification of recombinant AFs. *E. coli* BL21(DE3) pLysS cells harbouring pTL11 or pTL12 were grown at 37 °C and 160 r.p.m. in 1 litre of LB with appropriate antibiotic selection. When the OD_{600} reached 0.6 the expression of AbfA or Abf2 was induced by the addition of 1 mM IPTG. The culture was grown for an additional 3 h at 37 °C and 160 r.p.m. Cells were harvested by centrifugation at 4 °C, 8000 ***g***, 10 min. All subsequent steps were carried out at 4 °C. The harvested cells were resuspended in Start Buffer [20 mM sodium phosphate, pH 7.4, 10 mM imidazole, 50 mM NaCl and 1 mM PMSF] and lysed by passing twice through a French pressure cell. The lysate was centrifuged for 30 min at 15 000 ***g*** and the proteins from the supernatant were loaded onto a 1 ml Histrap column (Amersham Phamarcia Biotech). The bound proteins were eluted with a discontinuous imidazole gradient and the fractions containing AbfA or Abf2 that were more than 95 % pure were dialysed overnight against storage buffer (20 mM sodium phosphate, pH 7.4, 50 mM NaCl, 10 % glycerol) and then frozen in liquid nitrogen and kept at –80 °C until further use.

To prepare the cell-free extracts, for small-scale analysis, the cells were resuspended in lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole) and disrupted in the presence of lysozyme (1 mg ml^{-1}) by three cycles of freezing in liquid nitrogen and thawing for 5 min at 37 °C, followed by incubation with benzonase (Invitrogen). After 15 min centrifugation at 16 000 ***g*** and 4 °C the soluble and insoluble fractions of the crude extract were obtained.

Protein analysis. The analysis of production, homogeneity and molecular mass of the enzymes was done by SDS-PAGE, using broad-range molecular mass markers (Bio-Rad) as standards. Recombinant AbfA-His_6 and Abf2-His_6 were also analysed on native gradient (5–12.5 %) gels using a high-molecular-mass calibration kit for electrophoresis (Amersham) as standards. The degree of purification was determined by densitometric analysis of Coomassie-blue-stained SDS-PAGE gels. The protein content was determined using Bradford reagent (Bio-Rad) with BSA as standard.

Cross-linking was performed with purified His_6-tagged proteins. The reaction was initiated by adding glutaraldehyde, freshly prepared from stock solution in distilled water, to a final concentration of 2.5 mM followed by overnight incubation on ice.

Biochemical characterization. For the determination of substrate specificities, various *p*NP-glycosides (*p*NP*A*f, *p*NP*A*p and *p*NP*X*p) and non-chromogenic substrates (arabinobiose, arabinotriose, sugar beet arabinan, linear arabinan, arabinoxylan and arabinogalactan) were used. To measure the activity of the enzymes against arabinose-containing polysaccharides and oligosaccharides, the reducing sugar content after hydrolysis was determined by the Nelson–Somogyi method (Somogyi, 1952), with L-arabinose as standard, as previously described (Leal & Sá-Nogueira, 2004). One unit of activity was defined as the amount of enzyme that produces 1 µmol arabinose equivalents per minute. The enzyme activities towards *p*NP-glycosides were determined using a reaction mixture containing 0.4 mM substrate in PC buffer (72.8 mM sodium phosphate, 13.6 mM citric acid), pH 6.6, and appropriately diluted enzyme. The reaction was incubated at 37 °C and the increase in A_{400} was measured against time. The amount of product generated was calculated using a molar absorption coefficient for *p*-nitrophenol of 10 500 M^{-1} cm^{-1} at 400 nm. All assays were performed in triplicate.

Temperature and pH values for maximum enzymic activity of the AFs were determined by incubation for 5 min using 0.4 mM *p*NP*A*f as substrate in PC buffer, and calculated as described above. Enzymic activity was also determined in the presence of 1 mM EDTA using the same conditions. The effect of temperature was tested in PC buffer, pH 6.6, at temperatures ranging from 30 °C to 80 °C. The effect of pH on the activity was assayed at 37 °C in a series of Britton–Robinson buffers from pH 3.0 to 9.0 (0.1 M boric acid, 0.1 M acetic acid, 0.1 M phosphoric acid, adjusted to the desired pH with NaOH) (Britton & Robinson, 1931). Thermal stability of the enzymes was estimated by incubating the diluted solution of enzymes in PC buffer, pH 6.6, at 55 °C for AbfA and 70 °C for Abf2. Samples were removed after 10–120 min, kept in ice for 5 min, and residual enzyme activity was determined, at pH 6.6 and 37 °C, using 0.4 mM *p*NP*A*f in PC buffer (pH 6.6) as substrate.

Nucleotide sequence accession numbers. The nucleotide sequence of the *abf2* gene reported in this paper has been submitted to GenBank under accession number EU073712. The nucleotide sequence of the *abfA* gene was previously assigned GenBank accession number X89810.

RESULTS AND DISCUSSION

Functional analysis of AbfA and Abf2 in *B. subtilis*

By primary amino acid sequence analysis, the *abfA* and *abf2* genes from *B. subtilis* most probably encode AFs (EC

3.2.1.55) (Raposo *et al.*, 2004; Sá-Nogueira *et al.*, 1997; Wipat *et al.*, 1996). The primary structure of *B. subtilis* AbfA is very similar to that of the characterized AbfA from *Geobacillus stearothermophilus* T-6 [71 % identity (Gilead & Shoham, 1995)], AbfATK4 from *Geobacillus caldoxylolyticus* TK4 [70 % identity (Canakci *et al.*, 2007)] and Araf51 from *Clostridium thermocellum* [63 % identity (Taylor *et al.*, 2006)], and AF activity was reported for the *B. subtilis abfA* gene product (Wipat *et al.*, 1996). The amino acid sequence of Abf2 displays high identity to characterized AFs from *Bacillus pumilus*, ArfA [65 % identity (Degrassi *et al.*, 2003)], *Thermobacillus xylanilyticus*, AbfD3 [64 % identity (Debeche *et al.*, 2000)], *Clostridium cellulovorans*, ArfA [60 % identity (Kosugi *et al.*, 2002)] and *Clostridium stercorarium* ArfB [56 % identity (Zverlov *et al.*, 1998)]. Although AbfA (500 aa) and Abf2 (495 aa) share only 23 % identity, they both belong to family 51 of the glycoside hydrolases (GH 51) from different bacteria (http://www.cazy.org/fam/GH51.html).

To characterize the function of these genes in *B. subtilis* we constructed, by insertion-deletion mutations, single Δ*abfA* and Δ*abf2* mutants (IQB419 and IQB460; Table 1) and the double Δ*abfA* Δ*abf2* mutant (IQB462; Table 1). The wild-type 168T$^+$, and *abf2* and *abfA* null mutant strains were grown on minimal medium C supplemented with 1 % casein hydrolysate in the presence of arabinose. Samples were collected 2 h after induction and the intracellular level of accumulated AF activity was measured, using *p*NPA*f* as substrate. The results showed that the single *abf2* and *abfA* *B. subtilis* null mutants retained 70 % and 38 % of AF activity, respectively, relative to the wild-type (data not shown). In the Δ*abfA* Δ*abf2* double null mutant a complete loss of AF activity was observed (data not shown). In conclusion, both genes encode AFs and are responsible for the majority of the intracellular AF activity in *B. subtilis*. Interestingly, the difference in AF activity observed in the single *abf2* and *abfA* null mutants might reflect the distinct levels of *abfA* and *abf2* gene expression observed previously (Raposo *et al.*, 2004). Since the two enzymes displayed similar kinetic parameters for *p*NPA*f* (see below), the different expression observed at the transcriptional level might lead to dissimilar synthesis of the two enzymes.

The physiological effect of the mutations on the utilization of sugar beet arabinan and α-1,5-linked arabino-oligosaccharides as the sole carbon and energy source was determined; the results are summarized in Table 2. The double mutant was unable to grow on minimal medium with either arabinan or α-1,5-linked arabino-oligosaccharides (arabinobiose, arabinotriose and arabinotetraose), but it utilized arabinose like the wild-type strain, which indicates that the products of arabinan degradation, i.e. arabinose oligomers, are the natural substrates for the two AFs. The inactivation of the *abfA* gene in the single mutant IQB419 has a much more drastic effect on the growth on α-1,5-linked arabino-oligosaccharides as sole carbon source when compared to the *abf2* single mutant, strain IQB460. On the other hand, the *abfA* and *abf2* single mutations had a more similar effect on growth in the presence of sugar beet arabinan (Table 2). These observations strongly suggest a different contribution of the two enzymes to utilization of arabino-polysaccharides (discussed below).

Localization of AbfA and Abf2 activities

Although the majority of microbial AFs are secreted into the culture medium, some are retained within the cytoplasm or are membrane-associated (Beylot *et al.*, 2001a; Shallom & Shoham, 2003). AbfA and Abf2 activity was detected in whole cells and the natural substrates of these enzymes are most probably degradation products or arabinose-containing polysaccharides (see above), suggesting that the two AFs are retained in the cytoplasm. However, using the PSORT program (Nakai & Horton, 1999) AbfA was predicted to have a putative transmembrane motif (residues 342–358); thus we tested the possibility of a potential membrane association of AbfA. To analyse the subcellular localization of the two enzymes we constructed and visualized GFP-AbfA and GFP-Abf2

Table 2. Growth of the *B. subtilis* wild-type and mutant strains in minimal medium supplemented with different carbon sources

Cell growth was monitored in CE-minimal medium with or without the indicated carbon sources as described in Methods. Doubling time was estimated from a semi-logarithmic plot of time vs OD_{600}. –, No growth; it was not possible to determine accurate kinetic parameters.

Carbon source	Doubling time (h)				
	168T$^+$ (wild-type)	IQB462 (Δ*abfA* Δ*abf2*)	IQB419 (Δ*abfA*)	IQB460 (Δ*abf2*)	IQB206 (Δ*araL-abfA*)
No carbon source	–	–	–	–	–
Arabinose	6.2 ± 0.2	5.6 ± 0.9	7.3 ± 0.1	6.9 ± 0.5	10.1 ± 1.6
Glucose	4.4 ± 0.4	4.3 ± 0.5	4.9 ± 0.2	4.4 ± 0.5	5.4 ± 0.7
Arabinobiose	11.4 ± 0.8	–	16.7 ± 0.2	10.2 ± 1.3	–
Arabinotriose	9.7 ± 0.6	–	–	10.1 ± 2.7	–
Arabinotetraose	10.4 ± 2.5	–	–	10.8 ± 1.3	–
Sugar beet arabinan	10.7 ± 2.0	–	15.5 ± 1.6	13.0 ± 0.3	–

fusion proteins by fluorescence microscopy. These GFP-AbfA and GFP-Abf2 fusions were integrated into the *abfA* and *abf2* regions, respectively, of the *abfA* null mutant (IQB419; Table 1) and *abf2* null mutant (IQB460; Table 1), restoring an entire copy of the *abfA* and *abf2* coding region with *gfpmut2* fused to the 5′-end. The resulting strains, IQB493 (GFP-Abf2) and IQB494 (GFP-AbfA) (Table 1), were grown as described above and samples were collected after 2 h in the presence of inducer (arabinose) to measure AF activity and for fluorescence microscopy. The results indicated that the GFP-fused proteins were functional and the level of AF activity was similar to that observed in the wild-type strains (data not shown). Both recombinant AFs were retained in the cytoplasm and the two enzymes were scattered throughout the cytosol and not localized in particular foci (Fig. 1). These data are in agreement with previous cell fractionation studies in which the majority of the AF activity was found in the cytoplasmic fraction and not in the membrane fraction (data not shown).

Overproduction in *E. coli*, purification and characterization of AbfA and Abf2

The full-length of *abfA* and *abf2* coding regions were cloned in the expression vector pET30a(+) (Novagen), which allows the insertion of a His_6-tag at the C-terminus. The resulting plasmids, pTL11 and pTL12, bearing the recombinant *abfA* and *abf2*, respectively, under the control of a T7 promoter, were introduced into *E. coli* BL21(DE3) pLysS (Studier *et al.*, 1990) for the overexpression of the recombinant proteins. The cells were grown in the presence and absence of the inducer IPTG; soluble and insoluble fractions were prepared as described in Methods and analysed by SDS-PAGE. In the soluble fraction of IPTG-induced cells harbouring pTL11 (AbfA-His_6) or pTL12 (Abf2-His_6), proteins of about 58 kDa and 57 kDa were detected, which matched the predicted size for the recombinant proteins, 58.1 kDa and 57.5 kDa, respectively (data not shown). The recombinant AbfA and Abf2 were purified to more than 98 % homogeneity by Ni-NTA agarose affinity chromatography (Fig. 2a and c, respectively). The effect of adding a C-terminal His_6-tag to Abf2 and AbfA was analysed in *B. subtilis*. The AF activity in *B. subtilis* strains IQB600 and IQB602 (Table 1), expressing recombinant Abf2-His_6 and AbfA-His_6, respectively, was measured and the results indicated that the presence of the His_6-tag did not significantly affect the functionality of the recombinant AFs when compared with the native forms (data not shown).

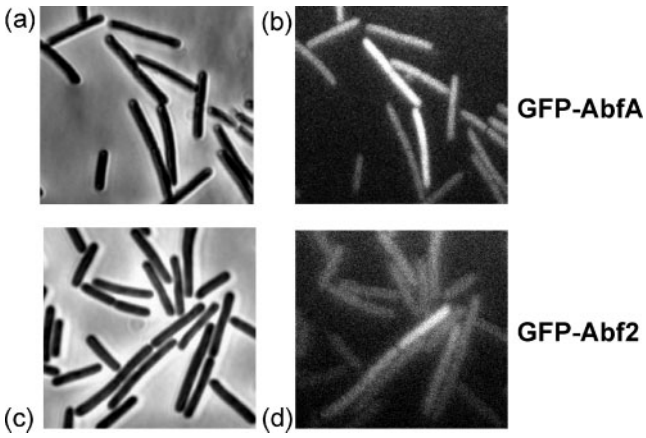

Fig. 1. Fluorescence microscopy of *B. subtilis* expressing GFP-AbfA and GFP-Abf2: phase-contrast (a, c), and GFP localization (b, d) images of cells in exponential phase grown in minimal medium C supplemented with 1 % (w/v) casein hydrolysate in the presence of arabinose (see Methods).

To assess the oligomeric state of the AFs, the purified proteins were analysed by gradient native PAGE. Recombinant AbfA displayed only one major band, with an estimated mass of 296 kDa (Fig. 2b). Abf2 was present in two oligomeric states, a predominant form with an estimated molecular mass of 112 kDa that could correspond to the dimer and an additional oligomeric form of about 276 kDa (Fig. 2d). A comparable pattern was obtained when the recombinant proteins were cross-linked with glutaraldehyde and subjected to SDS-PAGE (Fig. 2e). Abf2 displayed the presumptive dimeric form (115 kDa) and an oligomeric form higher than the tetramer (230 kDa). A similar result was obtained with AbfA: two oligomeric forms were observed, the dimer (116 kDa) and an oligomer with a higher-order structure than the tetramer (232 kDa). Together, these results suggested that the two enzymes exist as oligomers. In the absence of cross-linking agent, both proteins migrated as a single band, implying that SDS can completely dissociate potential oligomeric protein complexes (Fig. 2a, c and e). Although the higher-order oligomeric form detected for both enzymes has an estimated molecular mass close to the pentamer, two lines of evidence suggest that this form corresponds to a hexamer: (i) the crystal structures of AbfA from *G. stearothermophilus* and Araf51 from *C. thermocellum*, which display 71 % and 63 % identity, respectively, to AbfA from *B. subtilis*, showed that they are organized as hexamers, trimers of dimers (Hovel *et al.*, 2003; Taylor *et al.*, 2006); (ii) the cross-linking experiments did not reveal a trimeric form, favouring the oligomerization as dimers and a higher-order structure with a mass greater than 250 kDa.

Enzymic characterization of purified AbfA and Abf2

The biochemical and biophysical properties of purified recombinant AbfA-His_6 and Abf2-His_6 were determined as described in Methods. The ability of the two AFs to hydrolyse different substrates was assayed; the results are summarized in Table 3. AbfA was able to release arabinose from arabinobiose, arabinotriose, linear α-1,5-L-arabinan, sugar beet arabinan (branched), larch wood arabinogalactan

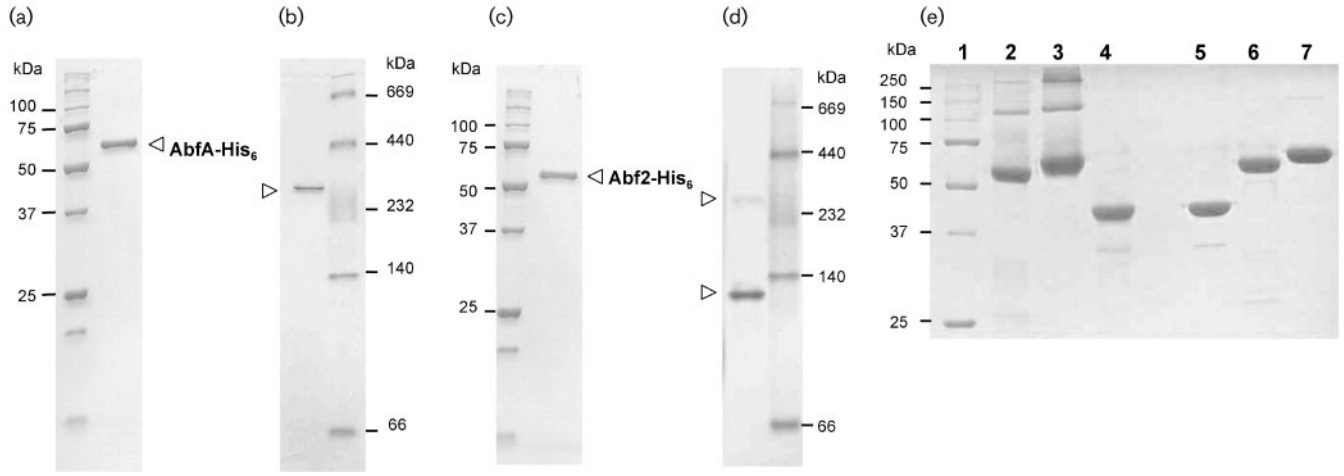

Fig. 2. Purification and characterization of the oligomeric state of AbfA and Abf2. (a, b) Purified recombinant AbfA (a) and Abf2 (c) (1 μg of each protein) were separated by SDS-PAGE on 12.5 % gels and stained with Coomassie blue. The positions of the recombinant proteins are shown by open arrowheads. The sizes of the broad-range molecular mass markers (Bio-Rad) are indicated. (b, d) The analysis of oligomer formation by the recombinant proteins (4 μg of each protein) was performed under native conditions. The proteins were separated on native gradient 5–12.5 % gels and stained with Coomassie blue. The AbfA (b) and Abf2 (d) oligomeric forms are shown by arrowheads. The sizes of the proteins in the high molecular mass calibration kit for electrophoresis (Amersham Biosciences) are indicated. (e) Glutaraldehyde cross-linking of AbfA and Abf2. Proteins (3.8 μg) were separated by SDS-PAGE (12.5 %) after cross-linking with 2.5 mM glutaraldehyde [lane 2, Abf2; lane 3, AbfA; lane 4, negative control (MBP 2* maltose-binding protein; Biolabs)] or in the absence of cross-linker (lane 5, MBP2*; lane 6, Abf2; lane 7, AbfA). The sizes of the broad-range molecular mass markers (Bio-Rad) are indicated (lane 1).

and wheat arabinoxylan but was not active towards *p*NPA*p* or *p*NPX*p*. Although the enzyme cleaved larch wood arabinogalactan and wheat arabinoxylan the determination of the individual kinetic constants was not possible, most probably due to both the high K_m of AbfA and low solubility of the polysaccharides. Abf2 was able to hydrolyse linear α-1,5-L-arabinan, sugar beet arabinan (branched) and wheat arabinoxylan but was not active towards larch wood arabinogalactan, *p*NPA*p* or *p*NPX*p* (data not shown). In addition, Abf2 was able to cleave arabinobiose and arabinotriose; however, most probably due to the high K_m of the enzyme for arabinobiose the precise determination of the kinetic parameters was not possible. In sum, the substrate-specificity assays indicated that AbfA has the

Table 3. Catalytic activity of AbfA and Abf2

Enzyme	Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
AbfA	*p*NPA*f*	306 ± 14	0.49 ± 0.07	614
Abf2	*p*NPA*f*	295 ± 19	0.42 ± 0.06	701
AbfA	Sugar beet arabinan (branched)	8.2 ± 1.0	4.4 ± 0.6	1.8
Abf2	Sugar beet arabinan (branched)	22 ± 0.04	0.35 ± 0.03	64
AbfA	Linear α-1,5-L-arabinan	12 ± 0.4	0.36 ± 0.007	33
Abf2	Linear α-1,5-L-arabinan	0.87 ± 0.02	0.50 ± 0.07	1.7
AbfA	Arabinobiose	32 ± 1.3	0.78 ± 0.11	41
Abf2	Arabinobiose	ND	ND	ND
AbfA	Arabinotriose	88 ± 5.7	1.1 ± 0.08	80
Abf2	Arabinotriose	0.75 ± 0.005	2.5 ± 0.2	0.29
AbfA	Wheat arabinoxylan	ND	ND	ND
Abf2	Wheat arabinoxylan	2.3 ± 0.04	0.23 ± 0.007	9.8
AbfA	Larch wood arabinogalactan	ND	ND	ND
Abf2	Larch wood arabinogalactan	NA	NA	NA

ND, Although enzyme activity was present it was not possible to measure the individual kinetic constants.
NA, No detectable activity.

following decreasing order of reactivity on arabinose-containing polysaccharides: debranched arabinan>sugar beet arabinan>wheat arabinoxylan=larch wood arabinogalactan (weak activity). Recombinant Abf2 was able to hydrolyse linear α-1,5-L-arabinan, sugar beet arabinan and wheat arabinoxylan. Moreover, it displayed higher activity towards branched arabinan, a molecule comprising a backbone of α-1,5-linked L-arabinofuranosyl residues decorated with α-1,2-, and α-1,3-linked L-arabinofuranosyl units, than towards debranched arabinan. In addition, arabinoxylan, which has L-arabinofuranose residues attached to the main chain by α-1,2- and/or α-1,3-glycosidic linkages, is preferred to linear α-1,5-L-arabinan. Taken together, these results highlight the preference of AbfA to hydrolyse (1⟶5) linkages and corroborate the data obtained on the growth kinetics of the wild-type and *abfA* and *abf2* mutant strains on different arabino-polysaccharides as sole carbon sources (see above; Table 2). The activity of the two enzymes towards sugar beet arabinan resembles that of AF I and II, respectively, partially purified from the culture supernatant of *B. subtilis* F-11 (Weinstein & Albersheim, 1979). AbfA's substrate specificity is similar to that of an AF purified from the culture supernatant of *B. subtilis* 3-6 (Kaneko *et al.*, 1994). The kinetic parameters determined by measuring the hydrolysis of the synthetic substrate *p*NP*A*f were very similar for both enzymes (Table 3) and comparable to the values determined for other bacterial AFs (Beldan *et al.*, 1997; Beylot *et al.*, 2001b; Debeche *et al.*, 2000; Degrassi *et al.*, 2003; Gilead & Shoham, 1995; Kosugi *et al.*, 2002; Taylor *et al.*, 2006; Zverlov *et al.*, 1998).

The addition of the chelating agent EDTA did not affect the activity of either AbfA and Abf2, suggesting that no metals are needed for the enzymic reaction. The effect of pH on the activity of the enzymes was determined in seven different buffers ranging between pH 3.0 and pH 9.0. From a plot of relative activity versus pH value both AFs were found to exhibit maximum activity at pH 8.0. The effect of temperature on the enzymes' activity was analysed over a range from 30 to 80 °C. AbfA was most active at 50 °C, while Abf2 had maximum activity at 60 °C. The thermal stability data showed that Abf2 is more thermostable than AbfA: Abf2 had a half-life of about 9.34 min at 70 °C and AbfA had a half-life of about 17.6 min at 55 °C (data not shown).

AbfA and Abf2: two different AFs for concerted action on arabino-oligosaccharides by *B. subtilis*

Although Abf2 and AbfA have similar biochemical and biophysical properties the two enzymes showed different substrate specificity. AbfA acts preferentially on (1⟶5) linkages, and Abf2 on (1⟶2) and (1⟶3) linkages, which suggest a concerted contribution of these enzymes to optimal utilization of arabinose-containing polysaccharides by *B. subtilis* and some other *Bacillus* species. The functional and subcellular localization analyses performed in this work indicated that both AFs are retained within the cytoplasm, where presumably their targets are oligosaccharides with (1⟶5), (1⟶2), and (1⟶3) linkages, such as arabino-oligomers, arabinoxylo-oligosaccharides and arabinogalacto-oligosaccharides. In a previous study we showed that arabinose is transported into the cell mainly by a permease, AraE (Sá-Nogueira & Ramos, 1997). Additionally, the predicted products of the genes *araN*, *araP* and *araQ*, belonging to the metabolic operon *araABDLMNPQ-abfA*, are homologous to components of bacterial binding-protein-dependent transport systems involved in the transport of malto-oligosaccharides and multiple sugars. An insertion-deletion mutation in the region downstream of the *araD* gene caused only a small decrease in doubling time of the mutant strain (IQB206) in a minimal medium with arabinose as the sole carbon source. Therefore a possible role of the AraNPQ proteins in the transport of L-arabinose and/or arabinose oligomers was suggested (Sá-Nogueira *et al.*, 1997). Thus, we determined the physiological effect of this mutation (strain IQB206) on the utilization of arabinan and α-1,5-linked arabino-oligosaccharides as the sole carbon and energy source (Table 2). This mutant strain was unable to grow on arabinan and α-1,5-linked arabino-oligosaccharides (arabinobiose, arabinotriose, and arabinotetraose) as the sole carbon sources (Table 2), which strongly suggests the involvement of the AraNPQ system in the transport of arabinose oligomers into the cell. The slight decrease in the doubling time with arabinose observed in strain IQB206 (Table 2) is consistent with previous results (Sá-Nogueira *et al.*, 1997).

Taken together, these observations lead us to propose the following pathway for the depolymerization of arabinose-containing polysaccharides in *B. subtilis* (Fig. 3). The extracellular degradation of arabinan, arabinoxylan and arabinogalactan, is accomplished by arabinanases, xylanases and galactanases. The resulting arabino-oligosaccharides (arabinose oligomers, arabinoxylo-oligosaccharides and arabinogalacto-oligosaccharides) enter the cell by specific transport systems, namely the AraNPQ ABC-type transporter. Once inside the cell, arabino-oligosaccharides with (1⟶5), (1⟶2) and (1⟶3) linkages are degraded by the concerted action of the two GH 51 AFs, AbfA and Abf2, releasing arabinose. A complete depolymerization to monosaccharides, arabinose, xylose and galactose, is accomplished together with β-xylosidases and β-galactosidases. At the level of gene expression *abfA* and *abf2* are induced by arabinose and repressed by glucose (Raposo *et al.*, 2004). AraR, the key regulator of arabinose utilization in *B. subtilis*, in the absence of the inducer (arabinose) represses and tightly controls the transcription of both *abfA* and *abf2* by binding to two in-phase operators in both the promoter region of the *araABDLMNPQ-abfA* operon and the promoter region of *abf2* (Mota *et al.*, 1999; Raposo *et al.*, 2004). Glucose repression of the expression of the two AF genes is achieved by CcpA, the master regulator of carbon catabolite repression, associated mainly with co-effector HPr(Ser-P) (Inácio & de Sá-Nogueira, 2007). In

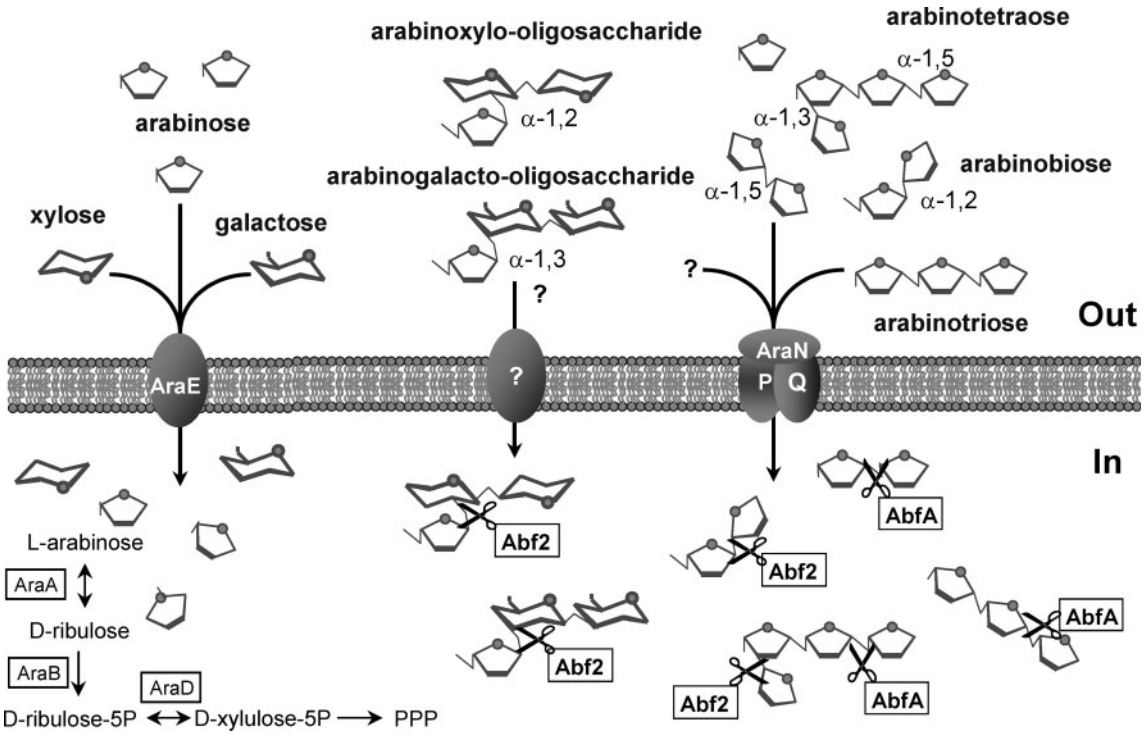

Fig. 3. The non-redundant roles of AbfA and Abf2 in arabino-oligosaccharide degradation in *B. subtilis*. Arabino-oligosaccharides resulting from the action of extracellular enzymes in different hemicellulosic homopolysaccharides (branched and debranched arabinans) and heteropolysaccharides (arabinoxylans, arabinogalactans) are transported into the cell by specific transport systems. Arabinose oligomers enter the cell most probably via AraNPQ, an ABC-type transporter (Sá-Nogueira *et al.*, 1997). Mixed oligomers, arabinoxylo-oligosaccharides and arabinogalacto-oligosaccharides may be transported into the cell also via AraNPQ or by other unidentified transport systems (?). The transport of the monosaccharides, arabinose, xylose and galactose, is accomplished by the same transport system, the AraE permease (Sá-Nogueira & Ramos, 1997; Krispin & Allmansberger, 1998). Once inside the cell, arabino-oligosaccharides with α-1,5, α-1,2, and α-1,3 linkages are degraded by the concerted action of the two GH 51 AFs, AbfA and Abf2. L-Arabinose is then converted, by the action of the products of the *araA*, *araB* and *araD* genes (Sá-Nogueira *et al.*, 1997), to D-xylulose 5-phosphate, which is further catabolized through the pentose phosphate pathway (PPP).

the presence of glucose and arabinose plus glucose CcpA binds to one *cre* element located downstream near the promoter region *abf2* and to two *cre* elements present in the *araABDLMNPQ-abfA* operon, *cre araA* located near the promoter and *cre araB* positioned within the *araB* gene (Inácio *et al.*, 2003; Inácio & de Sá-Nogueira, 2007).

The majority of microbial AFs are secreted into the culture medium. However, *Bacillus* species and other aerobic bacteria secrete a moderate number of enzymes that attack the polysaccharide backbone, releasing quite large oligo-saccharides (Numan & Bhosle, 2006; Shallom & Shoham, 2003). The complete hydrolysis of these products is accomplished by intracellular or membrane-associated enzymes (Beylot *et al.*, 2001a; Shulami *et al.*, 2007). Thus, the cytoplasmic nature of AbfA and Abf2 in *B. subtilis* might represent an adaptive advantage for the bacterium since competing non-hemicellulolytic micro-organisms are unable to utilize the relatively large arabino-oligomers and arabino-oligosaccharides products released by the extracellular enzymes of *B. subtilis* (Shallom & Shoham, 2003). Nonetheless, in particular environmental conditions, the presence of these cytoplasmic enzymes in the extracellular milieu cannot be excluded. A study of the *B. subtilis* extracellular proteome detected a significant number of proteins without a signal peptide in the growth medium, and it was proposed that in addition to the known secretory pathways this bacterium might possess alternative mechanisms to release such proteins to the external environment (Antelmann *et al.*, 2001). Moreover, cell lysis during exponential growth, or during entry into the stationary phase (González-Pastor *et al.*, 2003), may contribute to the presence of these enzymes in the extracellular milieu.

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