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ORIGINAL PAPER

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Characterization of a β-glycosidase from the thermoacidophilic bacterium *Alicyclobacillus acidocaldarius*

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Abstract In cell free extracts of the thermoacidophilic gram-positive bacterium *Alicyclobacillus acidocaldarius* ATCC27009, we have identified β-gluco- and galactosidase activities showing a specific activity of 0.1 and 12 U/mg, respectively. The two enzymatic activities are associated with different polypeptides and we show here the functional cloning, the expression in *Escherichia coli* and the characterisation of the β-glucosidase (Aaβ-gly). The enzyme, which is optimally active and stable at temperatures above 65°C, belongs to glycoside hydrolase family 1 (GH1) and shows wide substrate specificity on different aryl-glycosides and cello-oligosaccharides with k_{cat}/K_M for 4-nitrophenyl-β-D-glucoside and cellobiose of 2,976 and 185 $s^{-1}mM^{-1}$, respectively. Interestingly, upstream to the β-glycosidase gene, we identified a second ORF homologous to the ATPase subunit of the bacterial ABC transporters (*abc1*) that is co-transcribed with the β-glycosidase gene *glyB* and that could be involved in the carbohydrate import. The activity of the enzyme on cello-oligosaccharides of up to five glucose units strongly indicates that the enzyme could be involved in vivo in the degradation of glucans together with endoglucanase enzymes previously described. This, together with the co-expression of the two genes, suggests a role for the *glyB-abc1* cluster in *A. acidocaldarius* in the degradation of cellulose and hemicelluloses.

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Enzymes: EC 3.2.1.21; EC 3.2.1.23; EC 3.2.1.25; EC 3.2.1.38; EC 3.2.1.37

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Abbreviations 2, 4NP-β-Gal: 2-, 4-nitrophenyl-β-D-galactopyranoside · 2, 4NP-β-Glc: 2-, 4-nitrophenyl-β-D-glucopyranoside · Xyl: Xyloside · Fuc: Fucoside · Ara: Arabinoside · Man: Mannoside · X-Gal; -Glc: 5-bromo-4-chloro-3-indolyl-β-D-galacto- and glucopyranoside · IPTG: Isopropyl-β-D-thiogalactopyranoside · GH1: Glycoside hydrolase family 1 · CMC: Carboxy-methyl cellulose · LB: Luria Bertani medium

Introduction

The thermoacidophilic gram-positive bacterium *Alicyclobacillus acidocaldarius* grows optimally in strictly aerobic conditions at 60°C and pH 3-4. This organism, isolated for the first time in Yellowstone National Park USA (Darland and Brock 1971), was considered a member of the *Bacillus* genus; more recently, comparative analysis of the 16S rRNA sequences and cellular fatty acid profiles led to the reassignment to the new genus *Alicyclobacillus*. In fact, one of the peculiarities of these organisms is the presence in their cellular membranes of ω–cyclohexane or ω-cyclopentane fatty acids (Wisotzkey et al. 1992).

Alicyclobacilli have been isolated from different habitats including geothermal sites, submarine hot spring, but also soft drinks and heat-processed foods (Matsubara et al. 2002; Palop et al. 2000; Goto et al. 2002 and references therein). It has been demonstrated that *A. acidocaldarius* can use as carbon energy source several sugars including L-arabinose, ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, ramnose, D-turanose, mannitol, melibiose, cellobiose, lactose, maltose, sucrose, trehalose, tagatose and the polysaccharides cellulose, xylan, starch and glycogen (Goto et al.

2002; Eckert and Schneider 2003). Therefore, it is expected that this organism is an interesting source of glycoside hydrolases (GH); indeed, an α-amylase and a ciclomaltodextrinase have been identified in *A. acidocaldarius* and characterized in detail (Koivula et al. 1993; Matzke et al. 2000; Scheffel et al. 2004). Interestingly, these genes are clustered together with the components of a maltose/maltodextrine transport system, a putative transcriptional regulator, and an α-glucosidase suggesting a coordinated action in the hydrolysis and utilization of starch (Hulsmann et al. 2000).

Recently, two endo-glucanases active on carboxymethyl cellulose (CMC) and xylan have been isolated from *A. acidocaldarius* (Eckert and Schneider 2003; Eckert et al. 2002). Therefore, apparently, *A. acidocaldarius* displays at least two enzymes of the typical microbial cellulosic apparatus: a 1,4-β-D-glucan-4-glucanohydrolase (EC 3.2.1.4) and a cellobiohydrolase (EC 3.2.1.91). The microbial multiple enzyme systems active on cellulose and hemicellulose include also β-glucosidases (EC 3.2.1.21) that hydrolyse soluble cellodextrines and cellobiose to glucose (Lynd et al. 2002). Thus, it is conceivable that *A. acidocaldarius* may have intracellular β-glucosidase activities that allow the exploitation of β-glucans as energy source.

In the framework of our studies on GH from thermophilic organisms, we searched β-gluco- and galactosidase activities in cell free extracts of the *A. acidocaldarius* ATCC27009. We found that the two enzymatic activities are associated with different polypeptides and we show here the cloning and the characterization of the β-glucosidase belonging to GH1 and showing wide substrate specificity on different aryl-glycosides. In particular, the activity of the enzyme on cello-oligosaccharides of up to five glucose units strongly indicates that the enzyme could be involved in vivo in the degradation of glucans together with the endoglucanases previously discovered.

Materials and methods

Reagents and strains

Unless otherwise indicated all commercially available substrates were purchased from Sigma (USA). The synthetic oligonucleotides were from Qiagen (Germany) and Primm (Italy). The *Escherichia coli* strain M5154 (*ΔlacZ89, λ-trpA49 (Am), recA11, relA1, rpsL150 (str^r), spoT1*) was obtained from the *E. coli* Genetic Stock Center (USA); the other *E. coli* strains were from our collection.

Preparation of *A. acidocaldarius* extracts

The *A. acidocaldarius* strain ATCC 27009 was grown for 24 h at 60°C in 1 l culture containing 1.3 g $(NH_4)_3SO_4$; 0.3 g KH_2PO_4; 0.6 g of $MgSO_4*7H_2O$; 0.13 g $CaCl_2*2H_2O$; 1 g yeast extract; 1 g casaminoacids; 2 g sucrose. After centrifugation at 4,200 *g* for 10 min at 4°C, we obtained about 0.4 g of wet pellet. Cells were resuspended in 3 mL/g of cells of 20 mM sodium phosphate buffer pH 7.3; 150 mM NaCl (PBS), supplemented with 1% (v/v) Triton X-100, and were lysed by treatment on a Cell Disruption equipment (Constant Systems Ltd., UK) at 4°C. After centrifugation at 12,000 *g* at 4°C for 30 min, the supernatant was incubated with the Benzonase nuclease enzyme (Novagen, Germany) to remove the genomic DNA and filtered with a 0.45 μm Acrodisc filter (Millipore, Bedford, MA, USA); the final solution was considered the crude extract.

SDS-PAGE activity staining

To identify β-glucosidase and β-galactosidase activities by SDS-PAGE, the samples were directly loaded onto a 8% gel without incubation at high temperature. After the run, the gel was rinsed in pure water to remove SDS and successively in sodium phosphate buffer 50 mM pH 6.5, methanol 10%, and 2 mg/mL of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) or X-Glc at 65°C., The gel was stored at 4°C to stop the reaction when activity bands appeared.

Preparation of the genomic library of *A. acidocaldarius*

The genomic DNA was prepared starting from 50 mL of *A. acidocaldarius* cell culture grown as described above and resuspended in a Tris–HCl buffer 10 mM pH 7.8; LiCl 100 mM; EDTA 10 mM; SDS 1% (v/v). The cellular suspension was diluted with one part of phenol:chloroform:isoamylic alcohol (25:24:1) (2FC) and sand and it was subjected to two cycles of 2 min vortexing and 2 min incubation in ice. The suspension was centrifuged at 4,400 *g* for 10 min at 4°C and the aqueous phase was extracted twice with 2FC. After incubation with 10 μg of RNase for 1 h at room temperature to remove the contaminant total RNA, the genomic DNA was precipitated with ethanol at −20°C and resuspended in water.

The genomic library was prepared by incubating about 15 μg of *A. acidocaldarius* genomic DNA with BamHI enzyme. The ligation of fragments of about 3 kb in the vector ZAP Express (Stratagene, La Jolla, CA, USA) the subsequent packaging in the Gigapack III Gold packaging kit (Stratagene) and the mass excision in vivo of the phagemidic library were performed by following the instructions of the manufacturer. The phage library was used to infect the *E. coli* XL1-Blue MRF' strain and the phage title was measured by using standard molecular cloning techniques (Sambrook et al. 1989).

Screening of the library and cloning of the *glyB* gene

After the mass excision in vivo, the phagemidic library of *A. acidocaldarius* was used to transform the *E. coli* strain M5154. The selection on lactose was performed by

plating the transformed cells on agar plates with minimal medium supplemented with 0.2% (w/v) lactose and 30 μg/mL kanamycin. Positive clones, i.e. colonies expressing β-galactosidase activity, appeared not before 3 days at 37°C.

The screening for β-galactosidase activity was performed by plating the transformed cells on Luria-Bertani (LB) agar plates supplemented with 20 mg/mL (w/v) of X-Gal, 30 μg/mL kanamycin, and isopropyl-β-D-thiogalactopyranoside (IPTG) 1 mM. Plates were incubated overnight at 37°C and blue clones were collected.

The positive clones obtained by both selection and screening procedures were isolated and characterized. In particular, the restriction map of the purified phagemids was determined by using the following restriction enzymes: ApaI, AseI, EcoRI, Hind III, NarI, SacI, SacII, SalI, ScaI, SpeI, XbaI and XhoI. All the isolated clones showed similar restriction pattern. The sequence of the insert of the clone pBKlac6, isolated with the selection procedure, was determined on both strands.

The coding sequence of the *glyB* gene was amplified by PCR by using the Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and the phagemid pBKlac6 as template. The oligonucleotides used for the amplification are the following:

Nde-Agly: 5′-GGGAATTCCCATATGAGGGAGA-TGCGCAAATTTCC-3′
Nco-Agly: 5′-CCTTTGCCATGGTCACGTCCCCT-TCGACATGTG-3′

The amplified product was ligated to the expression vector pCRII TA cloning (Invitrogen) under the control of the T7 RNA polymerase promoter. The recombinant plasmid obtained was named pCRII-Aaβgly and the insert was completely re-sequenced.

Reverse transcriptase-PCR

Alicyclobacillus acidocaldarius cells were grown as described above and the total RNA was extracted using the Rneasy Kit (Qiagen). Contaminating DNA was eliminated by digestion with Dnase RNase free TURBO DNA-free (Ambion, USA) and RT-PCR was performed by using 2 μg of total cellular RNA by using the SuperScript ONE-STEP RT-PCR with Platinum Taq DNA polymerase (Invitrogen). The primers used for the RT-PCR of the *glyB* gene were prepared to produce an amplicon of 790 nucleotides (nt) (positions 351–1,140 from the *glyB* first ATG). To test for the presence of a transcript transversing *abc1* and *glyB* genes, we used primers producing amplicons of 716 nt (positions from −183 to 533) and 1,121 nt (positions from −588 to 533).

Expression and purification of the *glyB* gene

The recombinant β-glycosidase was expressed from the vector pCRII-Aaβgly by using freshly transformed colonies of *E. coli* BL21 (DE3) strain inoculated in 2 l of LB medium supplemented with 50 μg/mL of kanamycin. When the cell culture reach 1.0 optical densities at 600 nm, the expression of the gene is induced by adding 1 mM IPTG and allowing the growth overnight at 37°C. Cells were harvested by centrifugation at 4,200 *g* for 10 min at 4°C and the wet pellet was resuspended in 3 mL of PBS buffer supplemented with Tryton X-100 1% (v/v) for every gram of cell pellet. Cells were lysed by using a French pressure cell press apparatus (American Instruments Company, USA) and then were centrifuged at 11,900 *g* for 30 min at 4°C. The supernatant was incubated for 1 h at 37°C in the presence of 100 units of Benzonase nuclease (Novagen) to remove the genomic DNA. After centrifugation at 11,900 *g* for 30 min at 4°C, the extract was clarified by filtering on a 0.45 μm Acrodisc filter (Millipore) and it was incubated at 60°C for 1 h; the centrifugation at 11,900 *g* for 30 min at 4°C allowed the removal of the denatured proteins. After the addition of 1 M $(NH_4)_3SO_4$, the supernatant was applied onto a Hi-Load Phenyl-sepharose reverse phase column for FPLC (Amersham Pharmacia, Sweden) equilibrated with sodium phosphate buffer 50 mM pH 7.0 and 1 M $(NH_4)_3SO_4$. At these conditions, the enzymes bound to the column and were eluted in water. The β-galactosidase activity was identified by following the standard assay (see below) and the fractions were pooled, concentrated by ultrafiltration by using an Amicon YM-30 membrane (Millipore), and equilibrated with sodium phosphate buffer 20 mM pH 6.5. The sample was then applied to a Superose-O–6 gel filtration column for FPLC (Amersham Biotech, Sweden) equilibrated with the same buffer. The β-galactosidase activity was identified by using the standard assay; active fractions were pooled and concentrated. After this procedure, the enzyme was more than 95% pure by SDS-PAGE and stable for several months when stored in NaN_3 0.02% (v/v) at 4°C. Protein concentrations were determined with the method of Bradford (1976), with bovine serum albumin as standard.

Enzymatic assays

The standard assay for the β-galactosidase activity was performed in sodium acetate buffer 50 mM pH 5.5 and 4NP-β-Gal 5 mM at 65°C. The hydrolysis of the substrate was followed in a thermostated spectrophotometer Cary 100 Scan (Varian, Australia) at 405 nm in a 1 cm cuvette, using a molar extinction coefficient of 4-nitrophenol at these conditions as 1,080 $M^{-1}cm^{-1}$. One unit of enzyme activity was defined as the amount of enzyme catalysing the hydrolysis of 1 μmole of substrate in 1 min at the conditions described.

Enzymatic assays were performed on di-, and oligosaccharides at the temperatures indicated in sodium acetate buffer 50 mM pH 5.5, by incubating the concentrations indicated of substrate and 2 μg of Aaβ-gly in the final volume of 1.0 mL. The enzymatic reaction was

linear for up to 15 min and initial rates of hydrolysis were taken by stopping the reaction in dry ice after 5 min. The amount of glucose produced in the reaction was determined using the glucose oxidase-peroxidase system GOD/POD (Roche Molecular Biochemicals, Switzerland).

Spontaneous hydrolysis of both chromogenic and non-chromogenic substrates was subtracted by using blank mixtures without the enzyme.

Kinetic constants of Aaβ-gly were measured in sodium acetate buffer 50 mM pH 5.5 at 65°C by using 1–5 µg of enzyme in each assay. Concentrations ranging between 0.1 and 25 mM were used for 4NP-β-Gal and 4NP-β-Glc whilst 0.25–37 and 0.1–37 mM concentrations were used for 2NP-β-Gal and 2NP-β-Glc substrates, respectively. The molar extinction coefficient for 2-nitrophenol in 50 mM sodium acetate buffer pH 5.5 and 65°C was 204 $M^{-1}cm^{-1}$. Kinetic constants of non-chromogenic substrates were measured as described above by using 1 µg of enzyme and concentration ranges of 0.01–150 and 2.5–600 mM for cellobiose and lactose, respectively. All kinetic data were calculated as the average of at least two experiments and were plotted and refined with the program GraFit (Leatherbarrow 1992).

Enzyme characterisation

Dependence on pH was measured at 65°C in a 200 μL final volume reaction mixture containing 0.05 µg of enzyme, 75 mM 4NP-β-Glc substrate, and sodium citrate buffer 50 mM in the range 3.3–6.0, sodium malate buffer 50 mM in the range 6.5–7.0, and sodium borate buffer 50 mM in the range 7.6–9.0. The reaction was stopped after 5 min by adding 800 μL of 1 M Na_2CO_3. The enzyme activity was calculated using a molar extinction coefficient of 17,300 $M^{-1}cm^{-1}$.

Dependence of the enzyme activity on metals was measured in standard conditions at 65°C on 4NP-β-Glc substrate by using Mg^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+} at 5 mM concentration each, whilst the effect on the activity of EDTA and β-mercaptoethanol was analysed at 5 and 12.5 mM, respectively. The reaction was stopped after 2 min by adding 800 μL of 1 M Na_2CO_3.

Dependence on temperature was determined by assaying 1–4 µg of Aaβ-gly in buffer sodium acetate 50 mM pH 5.5 and 2 mM 4NP-β-Glc substrate in the temperature range 35–90°C. The molar extinction coefficients for 4-nitrophenol in buffer sodium acetate 50 mM pH 5.5 were accurately measured at each temperature and were in the range 1,324–4,266 $M^{-1}cm^{-1}$.

Thermal stability was tested by incubating pure Aaβ-gly (0.2 mg/mL) in sodium phosphate buffer 50 mM pH 6.5 at the indicated temperatures. At intervals, aliquots were withdrawn from the mixture and assayed at 65°C in buffer sodium acetate 50 mM pH 5.5 on 2 mM 4NP-β-Glc substrate.

Molecular mass of denatured Aaβ-gly was determined on SDS-PAGE 8% in reducing conditions by using as molecular weight markers (Amersham Biotech) phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumine (43,000) and carbonic anhydrase (30,000). Molecular mass of native Aaβ-gly was determined by gel filtration on a Superose-O–6 FPLC column (Amersham Biotech); molecular weight markers were thyroglobulin (660,000), ferritin (490,000), aldolase (158,000) and ribonuclease (13,700).

Results

Identification of β-glycosidase activities in A. acidocaldarius

As a preliminary stage of this work, we searched β-glycosidase activities in protein extracts of the *A. acidocaldarius* strain ATCC27009. Assays were performed by using 2-nitrophenyl-β-D-galacto- and glucopyranoside substrates (2NP-β-Gal and Glc) at the final concentration of 35 mM, in 50 mM sodium phosphate buffer pH 6.5 at 70°C. Crude extracts revealed both β-galactosidase and β-glucosidase activities with a specific activity of 12 and 0.1 U/mg, respectively. Interestingly, activity staining by using X-Gal and X-Glc substrates of the crude extract run on SDS-PAGE and subsequent renaturation revealed that two polypeptides of different molecular weight were responsible of these activities (not shown).

Cloning and sequence analysis of the β-glycosidase

On the basis of the above results, we decided to clone the genes encoding for the two enzymatic activities. To this aim, we prepared a genomic library of *A. acidocaldarius* in the Zap Express vector (see Materials and methods for details) that showed a titer of about 10^8 plaque-forming units (pfu) per ml with inserts of about 3–10 kbp. The analysis of the white : blue ratio of the gene bank was 20:1 indicating that the genomic inserts were cloned efficiently.

In order to perform the functional cloning of the genes encoding for the β-glycosidases, we screened the *E. coli* strain M5154 transformed with the phagemidic gene bank after mass excision in vivo. This bacterial strain lacks the endogenous β-galactosidase gene *lacZ*, but, having a functional permease gene *lacY*, can efficiently import lactose. Therefore, phagemids carrying a functional β-galactosidase gene from *A. acidocaldarius* could complement the *E. coli* M5154 strain allowing to grow on minimal lactose medium. In a complementary approach, positive clones were identified by white/blue screening on the same *E. coli* M5154 strain grown on rich medium supplemented with X-Gal.

By using both these selections, we screened about 10^6 colonies yielding 3 and 8 positive clones on rich medium/X-Gal and minimal medium/lactose, respectively. These

11 clones showed a similar pattern of bands after treatment with several restriction enzymes (see Materials and methods for details), suggesting that they contain the same genomic region. In one of the clones, named pBKlac6, isolated from a colony grown on minimal medium/lactose, the insert was completely sequenced. It was 4,136 bp long and showed a complete Open Reading Frame (ORF) of 1,443 bp encoding for a polypeptide of 471 amino acids for a calculated molecular weight of 54,673 Da. The analysis of the available data bank by using the BLASTP software (http://www.ncbi.nlm.nih.gov/BLAST/) revealed that this ORF showed high similarity with enzymes belonging to the glycoside hydrolase family 1 (GH1) of the carbohydrate-active enzyme classification (http://www.afmb.cnrs-mrs.fr/CAZY/index.html). The highest similarities were found with the β-glycosidases from *Thermoanaerobacter tengcongensis* and *Thermoanaerobacter brockii* (56% identity, 72% similarity), and the β-glucosidases from *Paenibacillus polymixa* and *Clostridium thermocellum* (48% identity, 66% similarity). The multi-alignment of the amino acid sequence of the ORF from *A. acidocaldarius* with these proteins and with the β-glycosidases from the hyperthermophilic bacterium *Thermotoga maritima* and the thermoacidophilic archaeon *Sulfolobus solfataricus* allowed us to identify the putative catalytic residues (Fig. 1). On the basis of these analyses, this ORF was named *glyB*. The GenBank Accession Number of this *A. acidocaldarius* genomic fragment is DQ092439.

The analysis by Southern blot of the genome of *A. acidocaldarius* digested with the BamHI restriction enzyme and hybridized with an internal probe of *glyB* revealed a single band of about 4,000 bp, whose dimensions are in good agreement with those of the insert sequenced from the analysed clone pBKlac6 (data not shown). This indicates that the *glyB* gene is present as a single copy in the *A. acidocaldarius* genome.

Upstream to the *glyB* gene and on the same coding strand, we identified a large fragment of a second ORF whose 5′-end was not complete. The search in the available databank with the program BLASTP by using as query the second ORF revealed its high similarity with members of the superfamily of ATP-binding cassette (ABC) transport system that was named *abc1*.

The analysis of the cloned *A. acidocaldarius* genomic fragment showed that *glyB* and *abc1* are in different reading frames and are separated by 43 bp (Fig. 2a). The program BPROM for the prediction of bacterial promoters (http://www.softberry.com/berry.phtml) revealed only one possible cassette of −10 and −35 sequences located in a region at about 300 bp from the *glyB* start codon and within the coding region of the putative ABC transporter gene. In addition, we found a purine-rich region immediately upstream to the first possible ATG start codon of *glyB* that could act as putative ribosome binding site (RBS) (Fig. 2a). Nevertheless, the close vicinity of the two ORFs and the absence of a terminator-like sequence between them may suggest that *glyB* and *abc1* are co-transcribed; to test this hypothesis, we made a RT-PCR experiment on the total RNA extracted from *A. acidocaldarius* cells (Fig. 2b). We obtained clear bands by using primers annealing in different regions of the *abc1* gene while RNase pre-treatment prevented the amplification reaction indicating that transcripts covering both genes were present in the total RNA fraction of *A. acidocaldarius* (Fig. 2). Unfortunately, no bands could be observed by Northern blot analysis, suggesting that *glyB* gene is expressed at low level.

Expression of the recombinant β-glycosidase from A. acidocaldarius

To determine which of the polypeptides showing β-glucosidase and β-galactosidase activity found in *A. acidocaldarius* corresponded to *glyB*, we over-expressed this gene in the *E. coli* strain BL21 (DE3) under the control of the promoter of the T7 RNA polymerase. The recombinant enzyme, named Aaβ-gly, was purified by using a heating step at 60°C followed by centrifugation that

a

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            >Gly B  LGDLVPMWIT HNEPWCASIL GYG..IGVHA PGLKDWRRAY RAAHHLLLSH
 >T.tencongensisBglB2  LGDVIPLWIT HNEPWCSSIL SYG..IGEHA PGHKDWREAL IAAHHILLSH
       >T.brockiiCglT  LGDAIPLWIT HNEPWCSSIL SYG..IGEHA PGHKNYREAL IAAHHILLSH
   >C.termocellumBGLA  LGDIVPIWFT HNEPGVVSLL GHF..LGIHA PGIKDLRTSL EVSHNLLLSH
      >T.maritimaBGLA  FGDRVKNWIT LNEPWVVAIV GHL..YGVHA PGMRDIYVAF RAVHNLLRAH
      >P.polimixaBGLA  FHGKIQHWLT FNEPWCIAFL SNM..LGVHA PGLTNLQTAI DVGHHLLVAH
 >S.solfataricusBGALS  FDDLVDEYST MNEPNVVGGL GYVGVKSGFP PGYLSFELSR RAMYNIIQAH
            Consensus  fgd.!..w.T .NEPwvv..l gy....g.ha PG......a. ra.hnll.aH
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b

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            >Gly B  IPIYITENGA AFDDRV.QDG GVHDADRVAY LAGHFAAAHR FLEEGGNLRG
 >T.tencongensisBglB2  LPMYITENGV AFKDEVTEDG RVHDYERIEY IKEHLKAIAR FIEEGGNLKG
       >T.brockiiCglT  LPMYITENGA AFKDEVTEDG RVHDDERIEY IKEHLKAAAK FIGEGGNLKG
   >C.termocellumBGLA  PNIVISENGA AFKDEIGSNG KIEDTKRIQY LKDYLTQAHR AIQDGVNLKA
      >T.maritimaBGLA  PEVYITENGA AFDDVVSEDG RVHDQNRIDY LKAHIGQAWK AIQEGVPLKG
      >P.polimixaBGLA  IDIYITENGA CINDEV.VNG KVQDDRRISY MQQHLVQVHR TIHDGLHVKG
 >S.solfataricusBGALS  LYMYVTENGI ADD..... ..ADYQRPYY LVSHVYQVHR AINSGADVRG
            Consensus  ...y!tENGa afdd.v...g .v.D..Ri.Y l..h..q.hr ai..G..lkg
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Fig. 1 Amino acid sequence alignment of *glyB* with GH1 thermophilic β-glycosidases. Sequences were aligned with the program Multalin (Corpet 1988). GeneBank accession numbers are in parentheses: *glyB* (DQ092439); *T. tencongensis* BglB2 (AAM23648.1); *T. brockii* CglT (CAA91220.1); *C. thermocellum* BGLA (CAA42814.1); *T. maritima* BGLA (CAA52276.1); *P. polimixa* BGLA (AAA22263.1); *S. solfataricus* BGALS (AAA72843.1). The NEP consensus sequence containing the glutamic acid residue acting as the acid/base of the reaction is reported in (**a**). The ENG consensus sequence containing the glutamic acid residue acting as the nucleophile of the reaction is reported in (**b**)

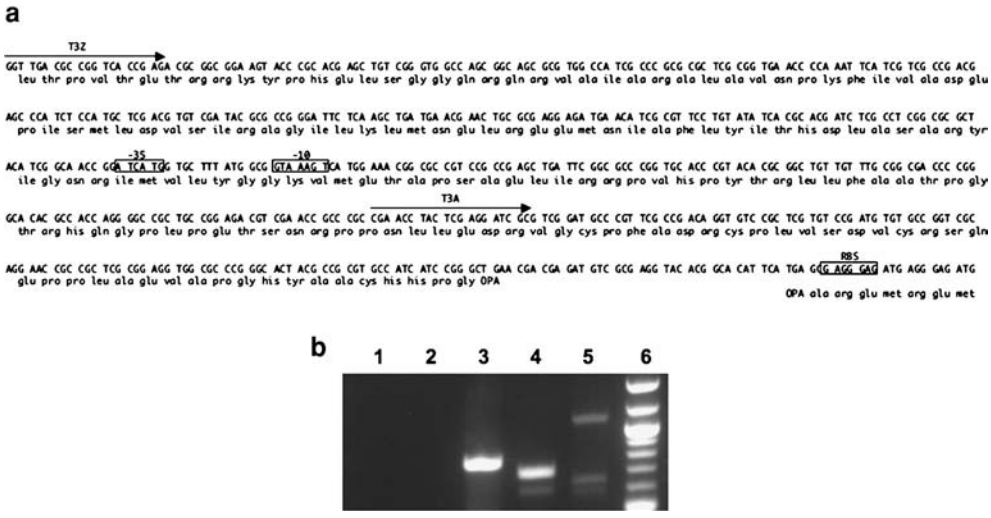

Fig. 2 Transcriptional analysis of the *glyB* locus. **a** *Alicyclobacillus acidocaldarius* genomic DNA fragment with the translated ORFs *abc1* and *glyB* in the +1 and zero frames, respectively. The first eight amino acids of *glyB* are reported. The putative promoter regions −35 and −10 in the *abc1* gene and the putative RBS upstream of *glyB* are boxed; the primers T3Z and T3A used for the RT-PCR experiments are indicated with *arrows*. **b** RT-PCR of total cellular RNA. *Lane* 1, *glyB* gene transcript after treatment of the RNA preparation with RNase; *lane* 2, reaction for the *glyB* gene without reverse transcriptase; *lane* 3 same as *lane* 1 but without RNase treatment; *lane* 4, region transversing *abc1* and *glyB* genes by using the primer T3A; *lane* 5, region transversing *abc1* and *glyB* genes by using the primer T3Z; *lane* 6, molecular weight marker 1 Kb Ladder (1.5, 1.2, 1.0, 0.9, 0.8, 0.7, 0.6. 0.5)

allowed the elimination of about 50% of the host contaminating proteins. Successively, Aaβ-gly was purified to homogeneity by using a hydrophobic interaction chromatography and a gel filtration (see Materials and methods for details). From 2 l of *E. coli* culture we routinely obtain 5 mg of pure Aaβ-gly with a purification of 220-fold, 34% final yield and a specific activity of 73.07 U/mg on 4NP-β-Gal at 65°C in 50 mM sodium phosphate buffer pH 6.5 (Fig. 3a). The molecular weight of the denaturated enzyme is ≈54,000 Da, in good agreement with that calculated from the amino acidic sequence.

To determine to which of the two β-glycosidase activities found in *A. acidocaldarius* corresponded Aaβ-gly, we tried to purify the β-glucosidase activity from *A. acidocaldarius*. Unfortunately, the enzyme was expressed in scarce amounts and we could not sequence the amino-terminal of the protein. Therefore, we run together in a SDS-PAGE the crude extracts of *A. acidocaldarius* and a sample of the recombinant enzyme (Fig. 3b). The gel was stained with X-Gal and X-Glc substrates; interestingly, Aaβ-gly revealed activity on both the chromogenic substrates and showed the identical migration of the polypeptide of higher molecular weight in *A. acidocaldarius* extracts. It is worth noting that this polypeptide in the extracts of *A. acidocaldarius* has low activity on X-Glc and no activity on X-Gal, confirming that the enzyme was expressed at low levels in the thermoacidophilic bacterium (Fig. 3b).

Enzymatic characterization of Aaβ-gly

The molecular weight of Aaβ-gly in native conditions was determined by analytical gel filtration on a Supe-

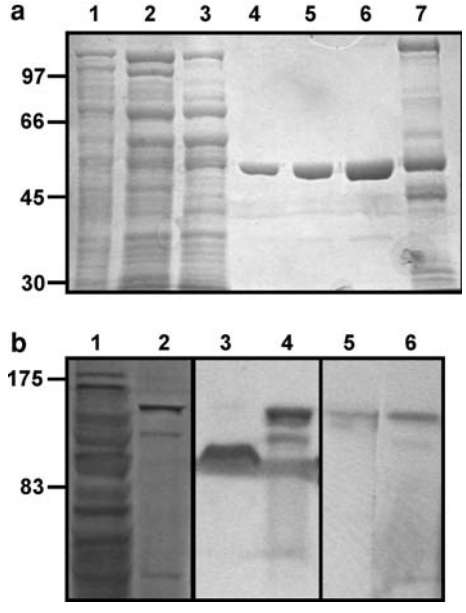

Fig. 3 Electrophoretic analysis of recombinant β-glycosidase. **a** SDS-PAGE of recombinant Aaβ-gly after different purification steps. *Lanes* 1–2, crude extract (30 and 60 μg, respectively); *lane* 3, result of the heat treatment at 60°C (30 μg); *lane* 7, result of the reverse-phase chromatography (10 μg); *lanes* 4–6, result of the gel filtration (2, 4 and 8 μg). **b** Analysis of the β-glucosidase and β-galactosidase activities by SDS-PAGE. *Lanes* 1, 3 and 5 *A. acidocaldarius* crude extract (85, 170 and 500 μg, respectively); *lanes* 2, 4 and 6 recombinant Aaβ-gly (5, 30 and 30 μg, respectively). *Lanes* 1 and 2 are coomassie stained; *lanes* 3 and 4 were activity stained by incubation with X-Gal; *lanes* 5 and 6 were activity stained by incubation with X-Glc. All the samples were not heat denatured before loading

rose-O–6 column (see Materials and methods for details) and resulted of 456,000 ± 31,000 (data not shown). Therefore, considering that the molecular weight of the monomer calculated by SDS-PAGE is 54,000, we deduced that Aaβ-gly is an octamer in native conditions.

The dependence on pH of Aaβ-gly was analysed by assaying the enzyme in the range of pH 3.3–9.0 at 65°C on 5 mM 4NP-β-Glc (Fig. 4). The enzyme produced a bell-shaped curve with similar specific activity in the pH range 4.0–6.0 with a maximum at pH 5.5. This is not surprising, in fact, though *A. acidocaldarius* thrives at pH 3–4, it is known that acidophiles maintain a pH value in their cytoplasm close to neutrality (Hulsmann et al. 2000). In addition, the two limbs of the curve indicate the involvement in activity of two groups with the pK_as of 3.97 ± 0.21 and 6.79 ± 0.18, respectively. Aaβ-gly showed similar specific activity at pH 5.5 in all the buffers used (sodium citrate, phosphate and acetate at 50 mM concentration) indicating that no buffer-effect occurred, therefore, for the following characterization we used sodium acetate buffer 50 mM, pH 5.5.

The effect on catalysis of divalent cations, namely Mg^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+} and Ni^{2+}, at 5 mM concentration each, was tested at 65°C on 2 mM 2NP-β-Glc. None of the metals tested activated Aaβ-gly significantly while Zn^{2+} and Co^{2+} inhibited the enzyme of 33 and 96%, respectively. In addition, EDTA had no effect on catalysis, suggesting that Aaβ-gly promotes the hydrolysis of glycosides without the help of metal co-factors. β-Mercaptoethanol had no effect, demonstrating that reducing conditions did not influence catalysis.

As expected for an enzyme from a thermophilic organism, the activity of Aaβ-gly is strictly dependent on temperature rising to a maximum at 85°C (Fig 5a). The linearity of the Arrhenius plot, shown in the inset of Fig 5a, demonstrated that the activation energy of the catalysed reaction (Ea) is not dependent on the temperature in the range 35–85°C and, showing a value of 37.9 ± 0.9 KJ/mol, is comparable to that of other thermophilic glycosidases from GH1 (Ait et al. 1982; Kengen et al. 1993; Plant et al. 1988). Instead, the drop of activity at 90°C presumably is due to the thermal denaturation of the enzyme at this temperature. In fact, experiments of thermal stability demonstrated that Aaβ-gly is only barely stable at 80°C (8% of residual activity after 10 min) (Fig 5b). However, the enzyme is completely stable for more than 3 h at 65°C; this is fully consistent with the optimal temperature of growth of *A. acidocaldarius* (60°C). On the basis of these results, we performed all the enzymatic characterization of Aaβ-gly at 65°C.

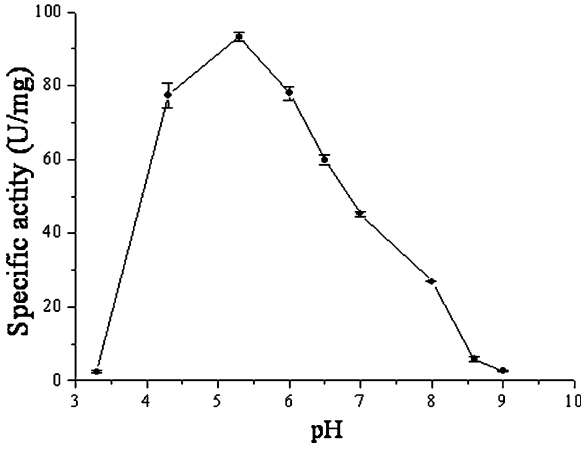

Fig. 4 pH dependence of Aaβ-gly

Substrate specificity of Aaβ-gly

The specific activity of the enzyme on several aryl-glycosides, di- and oligosaccharides is reported in Table 1. To facilitate the comparison, for aryl-glycosides and disaccharides the activity on 2NP-β-Glc is taken as 100% while for cello-oligosaccharides the activity on cellobiose is 100%. Aaβ-gly showed wide substrate specificity being able to hydrolyse efficiently β-D-gluco-, -galacto- and fucosides and recognizing as substrates glucose disaccharides as cellobiose and gentiobiose showing β-1,4 and β-1,6 glucosidic bonds, respectively.

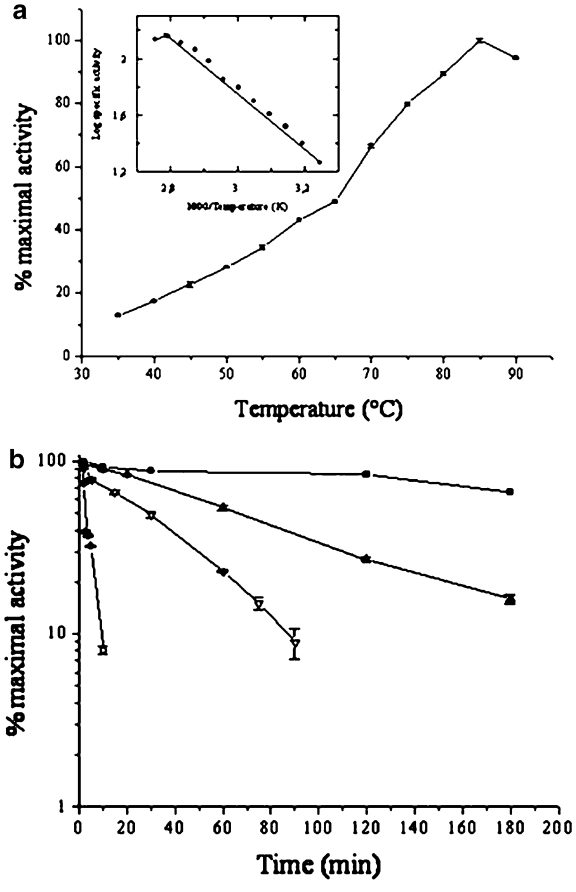

Fig. 5 Effect of temperature on Aaβ-gly. **a** Thermal activity and derived Arrhenius plot (inset) of Aaβ-gly. **b** Thermal stability of Aaβ-gly at 80°C (*open circles*), 75°C (*closed squares*), 70°C (*open triangles*), and 65°C *closed circles*)

Table 1 Specific activity of Aaβ-gly on different substrates[a]

Substrates	U/mg	%
2NP-β-D-Glc (37 mM)	613.23	100
4NP-β-D-Glc (37 mM)	143.02	23
4NP-β-D-Gal (37 mM)	337.68	55
2NP-β-D-Gal (37 mM)	1,211.27	197
4NP-β-D-Xyl (21 mM)	7.85	1
2NP-β-D-Xyl (18 mM)	47.79	8
4NP-β-L-Fuc (25 mM)	10.32	2
2NP-β-D-Fuc (24 mM)	576.72	94
4NP-α-L-Ara (18 mM)	11.18	2
4NP-β-D-Man (37 mM)	2.71	< 1
Lactose (500 mM)	288.08	47
Cellobiose (200 mM)	163.10	27
Gentiobiose (200 mM)	54.32	9
Cellobiose (20 mM)	79.74	100
Cellotriose (20 mM)	97.49	122
Cellotetraose (20 mM)	18.17	23
Cellopentose (20 mM)	16.53	21

[a]Assays were performed in 50 mM sodium acetate buffer, pH 5.5, at 65°C; the concentration of the substrates is indicated in parentheses

On the other hand, the enzyme hydrolyses at low rates aryl-β-D-xylosides and 4NP-β-D-mannoside; the former is lacking the oxydryl at the C6 compared to glucose, while the latter shows an axial oxydryl in the C2 position compared to glucose. Hence, our results suggest that the nature of the oxydryl groups in C6 and C2 is crucial for activity. As expected from the functional cloning approach used for the isolation of the *glyB* gene, Aaβ-gly hydrolysed lactose efficiently.

It is worth noting that the enzyme recognized as substrates linear oligosaccharides of glucose of up to four units, instead, longer oligosaccharides, i.e. cellopentaose, are hydrolysed less efficiently.

The kinetic constants of the enzyme were measured for the best substrates and are reported in Table 2. The enzyme showed an higher affinity for aryl-glycosides compared to disaccharides; however, it is worth noting that the K_M for cellobiose is similar to that of 4NP-β-Gal. Interestingly, in calculating the steady-state kinetic constants, we found that the enzyme produced a not linear Lineweaver–Burk plot with 2NP-β-Glc substrate, showing a bi-phasic behaviour with two different slopes. Therefore, we calculated the kinetic constants in the intervals of substrate concentration at which the plot gave a straight line (Table 2).

Discussion

We have described here the cloning and the characterisation of a β-glycosidase from the acidophilic bacterium *A. acidocaldarius*. The gene was isolated by functional cloning and complementation of the β-galactosidase gene of an *E. coli* mutant strain. It is worth noting that despite the thermophilic nature of the recombinant Aaβ-gly, which is optimally active at 85°C, the enzyme conferred to *E. coli* the ability to grow on lactose. Presumably, the residual activity at 37°C, about 10% of the maximal activity of the enzyme, allowed the hydrolysis of sufficient amounts of lactose that could be used by *E. coli* as the only carbon source.

As expected from the growth conditions of *A. acidocaldarius*, Aaβ-gly is stable for more than 3 h at 65°C, a temperature at which the enzyme shows about 50% of its maximal activity (Fig. 5). The analysis of the amino acid sequence of Aaβ-gly allowed classifying this novel enzyme as a member of GH1. Glycoside hydrolase family 1 in the carbohydrate-active enzyme classification is one of the largest families including enzymes from the three kingdoms of living organisms archaea, bacteria and eukarya. These β-glycosidases have a typical $(\beta/\alpha)_8$ barrel and follow a retaining reaction mechanism in which the configuration of the product is retained if compared to that of the substrate (Kempton and Withers 1992; Barrett et al. 1995; Verdoucqet al. 2004). The wide substrate specificity of Aaβ-gly, showing activity on aryl-β-D-gluco, -galacto, -xylo and fucosides and, to a limited extent, on mannosides and 4NP-β-L-arabinoside, is typical of GH1 enzymes. Interestingly, the Lineweaver–Burk plot of Aaβ-gly on 2NP-β-D-Glc substrate showed peculiar biphasic behaviour, showing a lower k_{cat}/K_M at higher substrate concentrations (Table 2). Similar results were obtained with the GH1 β-glycosidases from *Agrobacterium* sp. and from the hyperthermophilic archaeon *Pyrococcus furiosus* and have been explained suggesting that transglycosylations

Table 2 Steady-state kinetic constants of Aaβ-gly on different substrates[a]

Substrates	k_{cat} (per second)	K_M (mM)	k_{cat}/K_M ($s^{-1}mM^{-1}$)
4NP-β-D-Gal	2,877.40 ± 84.09	5.82 ± 0.47	494.40
2NP-β-D-Gal	8,045.50 ± 197.60	5.50 ± 0.42	1,462.82
4NP-β-D-Glc	803.66 ± 23.10	0.27 ± 0.03	2,976.52
2NP-β-D-Glc[b]	2,375.00 ± NS[d]	0.06 ± NS	39,583.34
2NP-β-D-Glc[c]	3,454.50 ± NS	1.18 ± NS	2,927.54
Lactose	2,388.30 ± 76.21	73.45 ± 8.68	32.52
Cellobiose	1,276.44 ± 50.04	6.89 ± 1.01	185.26

[a]Assays were performed in 50 mM sodium acetate buffer, pH 5.5, at 65°C
[b]Kinetic constants were measured at substrate concentrations between 0.1 and 2.5 mM
[c]Kinetic constants were measured at substrate concentrations between 2.5 and 37 mM
[d]The standard errors are smaller than 0.003, therefore, they were not shown (NS)

occurred at high substrate concentrations (Kempton and Withers 1992; Pouwels et al. 2000).

Multiple sequence alignments allowed us to identify the residues Glu166 and Glu354 of Aaβ-gly as the acid/base and nucleophile of the reaction, respectively. These amino acids correspond to the glutamic acid residues in the highly conserved motives NEP and ENG, respectively (Fig. 2). The pK_a values calculated from the bell-shaped curve reported in Fig. 5 are fully consistent with the function of these two residues, suggesting that the pK_a of about 4.0 and 6.8 correspond to the nucleophile Glu354 and the acid/base Glu166, respectively.

Interestingly, the sequencing of the DNA fragment cloned from the *A. acidocaldarius* genomic library revealed in the region upstream of the *glyB* gene the presence of an ORF encoding for a putative ATPase subunit of a ABC transport system. Though this ORF was not complete, the analysis of the amino acid sequence allowed us to conclude that *abc1* encodes for a protein putatively involved in the ATP hydrolysis associated with the uptake of the solute. In fact, this ORF showed the highest similarity with the ATPase components of the ABC transport systems from *Vibrio vulnificus* (AAO8187 and NP936192, 48% identity), *Bacillus licheniformis* (YP078425, 44%), *Streptomyces avermitilis* (BAC70476, 43%), and *Agrobacterium tumefaciens* (NP533934, 41%). ABC transporters are integral membrane proteins that couple substrate translocation across the cytoplasmic membrane to ATP hydrolysis (for reviews see Dassa and Schneider 2001; Jones and George 2004). These systems transport molecules such as ions, sugars, amino acids, vitamins, peptides, polysaccharides, hormones, lipids and xenobiotics. Among gram-positive bacteria, ABC systems are oligoprotein assemblies with a membrane-anchored substrate-binding protein exposed to the outside of the cell, two membrane-integral proteins forming the transport channel, and an intracellular ATPase (Ehrmann et. al. 1998). The arrangement in clusters or operons of the genes encoding for GH and ABC transporters of sugar substrates has been observed in *A. acidocaldarius* (Hulsmann et al. 2000) and it is not uncommon in gram-positive bacteria, however, the ATPase is usually not encoded in the same operon (Scheffel et al. 2004; Bertram et al. 2004). In addition, cello-oligosaccharide-specific ABC systems have been identified by computational and transcriptional analysis in the gram-positive bacterium *Streptomyces coelicolor* (Bertram et al. 2004).

Aaβ-gly is able to hydrolyse short length cello-oligosaccharides (Table 1); in *A. acidocaldarius* ATCC27009 a β-1,4-glucanase, named CelA, belonging to GH9, and a endoglucanase (CelB), which is a member of GH51, have been identified and characterized (Eckert and Schneider 2003; Eckert et al. 2002). The former is cytoplasmatic and active on lichenan, CMC, and 4-nitrophenyl-cello-oligosaccharides (Eckert et al. 2002) whilst CelB is cell associated and optimally active at acidic pH on CMC and oat spelt xylan (Eckert and Schneider 2003). Moreover, interestingly, at least other two polypeptides of unknown origin presenting xylanase activity are associated to the *A. acidocaldarius* cells grown on xylan (Eckert and Schneider 2003). It has been pointed out that CelB, and eventually other enzymatic activities, may hydrolyse glucans on the cell surface producing oligosaccharides that are imported in the cytosol becoming substrates of CelA (Eckert and Schneider 2003). In the study presented here, we showed that the intracellular β-glucosidase activity in *A. acidocaldarius* hydrolysed efficiently cello-oligosaccharides of up to five glucose units, suggesting that these compounds could be among the natural substrates of the enzyme. Therefore, Aaβ-gly could be the third, still missing member in this organism of the enzymatic cascade for the degradation of β-glucans to monosaccharides. In addition, we found a putative ATPase of a bacterial ABC transporter whose gene is co-transcribed in vivo with the β-glucosidase gene, supporting the hypothesis that this gene cluster is one of the components of a specific cello-oligosaccharide transporter.

The genus *Alicyclobacillus* has been described as noncellulolytic (Bergquist et al. 1999); therefore, the identification of enzymes involved in the degradation of glucans is a remarkable result. The genome of *A. acidocaldarius* has not been sequenced so far, therefore, to identify in this organism the other enzymatic components involved in the degradation of cellulose and hemicellulose is not an easy task. Further functional studies like the one reported here are required to shed more light on the ability of this microorganism to utilize plant cell wall material.

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