A family 3 glycosyl hydrolase of _Dickeya dadantii_ 3937 is involved in the cleavage of aromatic glucosides

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_Dickeya dadantii_ is a phytopathogenic bacterium secreting a large array of plant-cell-wall-degrading enzymes that participate in the infection and maceration of the host plant tissue. Sequencing of the _D. dadantii_ 3937 genome predicted several genes encoding potential glucosidases. One of these genes, _bgxA_, encodes a protein classified in family 3 of glycosyl hydrolases. Inactivation of _bgxA_ and the use of a gene fusion revealed that this gene is not essential for _D. dadantii_ pathogenicity but that it is expressed during plant infection. The _bgxA_ expression is induced in the presence of glycosidic or non-glycosidic aromatic compounds, notably ferulic acid, cinnamic acid, vanillic acid and salicin. The _BgxA_ enzyme has a principal β-D-glucopyranosidase activity and a secondary β-D-xylopyranosidase activity (ratio 70:1). This enzyme activity is inhibited by different aromatic glucosides or phenolic compounds, in particular salicin, arbutin, ferulic acid and vanillic acid. Together, the induction effects and the enzyme inhibition suggest that _BgxA_ is mostly involved in the cleavage of aromatic β-glucosides. There is evidence of functional redundancy in the _D. dadantii_ β-glucoside assimilation pathway. In contrast to other β-glucoside assimilation systems, involving cytoplasmic phospho-β-glucosidases, the cleavage of aromatic glucosides in the periplasmic space by _BgxA_ may avoid the release of a toxic phenolic aglycone into the cytoplasm while still allowing for catabolism of the glucose moiety.

INTRODUCTION

β-Glucosidases (β-D-glucopyranoside glucohydrolases, E.C.3.2.1.21) are a heterogeneous group of enzymes, which hydrolyse glycosidic bonds to release non-reducing terminal glucosyl residues from glucosides and oligosaccharides. These enzymes catalyse the hydrolysis of cellulose and various β-glucosides, the aglycone of which can be an aromatic compound (Ketudat Cairns & Esen, 2010). β-Glucosidases are universally found in all domains of living organisms, archaea, eubacteria and eukaryotes. Most microbial β-glucosidases have been investigated in the soil and plant microflora and they have been considered as the ultimate enzymic step in the biological conversion of cellulose into glucose. However, other β-glucosidases preferentially hydrolyse aryl β-glucosides, such as arbutin and salicin (Faure et al., 1999). β-Glucosidases have been the focus of several studies because of their roles in a variety of biological processes, namely, the conversion of cellulose to glucose, the release of aromatic compounds from flavourless glucosidic precursors, the detoxification of cyanogenic glucosides, and the synthesis of useful β-glucosides (Bhatia et al., 2002; Ismail & Hayes, 2005; Ketudut-Cairns & Esen, 2010). The classic enzyme classification (EC) system groups together glucoside hydrolases according to their substrate specificity, for example, the β-glucosidases all have the designation EC3.2.1.21. An alternative classification system has been developed for glycoside hydrolases, based on amino acid sequence and structural similarity (Henrissat & Davies, 1997; Cantarel et al., 2009). The β-glucosidases currently identified are in the glycoside hydrolase (GH) families GH1, GH3, GH5, GH9 and GH30 (Ketudat-Cairns & Esen, 2010).

Pectinolytic enterobacteria are phytopathogens that infect a wide range of host plants, affecting many crops of economic importance such as vegetables and ornamentals (Toth et al., 2003; Charkowski et al., 2012). These pathogens belong to two genera, _Pectobacterium_ (formerly _Erwinia carotovora_) and _Dickeya_ (formerly _Erwinia chrysanthemi_), recently divided into multiple species. The genus _Dickeya_ includes _Dickeya chrysanthemi_, _D. paradiensa_, _D. dadantii_, _D. dianthicola_, _D. dieffenbachiae_ and _D. zeae_ (Hauben et al., 1998; Samson et al., 2005). These pathogens secrete a large array of plant-cell-wall-degrading enzymes. The classification and the use of a gene fusion revealed that this gene is not essential for _D. dadantii_ pathogenicity but that it is expressed during plant infection. The _bgxA_ expression is induced in the presence of glycosidic or non-glycosidic aromatic compounds, notably ferulic acid, cinnamic acid, vanillic acid and salicin. The _BgxA_ enzyme has a principal β-D-glucopyranosidase activity and a secondary β-D-xylopyranosidase activity (ratio 70:1). This enzyme activity is inhibited by different aromatic glucosides or phenolic compounds, in particular salicin, arbutin, ferulic acid and vanillic acid. Together, the induction effects and the enzyme inhibition suggest that _BgxA_ is mostly involved in the cleavage of aromatic β-glucosides. There is evidence of functional redundancy in the _D. dadantii_ β-glucoside assimilation pathway. In contrast to other β-glucoside assimilation systems, involving cytoplasmic phospho-β-glucosidases, the cleavage of aromatic glucosides in the periplasmic space by _BgxA_ may avoid the release of a toxic phenolic aglycone into the cytoplasm while still allowing for catabolism of the glucose moiety.

**Abbreviations**: pNPG, _p_ -nitropheryl-β-D-glucopyranoside; pNPX, _p_ -nitropheryl-β-D-xylopyranoside.
enzymes that participate in the infection and maceration of the plant tissue (Hugouvieux-Cotte-Pattat et al., 1996). In the genus Dickeya, most studies have been performed on the D. dadantii strain 3937 isolated from African violet (Saintpaulia ionantha). This strain has been chosen as a model by the Dickeya international community for complete genome sequencing (Glaser et al., 2011).

Plants represent an important reservoir of β-glucosidases such as cellulose and aryl β-glucosidases in which a glucose residue is linked to an aromatic compound, as in salicin and arbutin. Like several bacteria of the soil microflora or of the mammalian digestive system, D. dadantii can grow on aryl β-glucosides as the sole carbon source (el Hassouni et al., 1990). During analysis of the xylolytic activities of strains that selectively attack grasses, an enzyme with both β-glucosidase and β-xylosidase activity was described in the corn strain D1 of the genus Dickeya (Er. chrysanthemi, new species classification unknown). This protein, BgxA, was classified in the GH3 family, one of the largest glycoside hydrolase families, which includes members with distinct substrate specificities. In this work, Vroemen et al. (1995) observed the β-glucosidase/β-xylosidase activity in all corn strains but not in strains collected from dicot plants. The D. dadantii strain 3937 was isolated from the dicot plant S. ionantha. Several genes encoding potential glycosidases were predicted from its genome sequence (Glaser et al., 2011); one of these genes is homologous to bgxA of strain D1. In the present work, we further analysed bgxA expression and BgxA specificity to understand the biological role of this enzyme in the model dicot strain D. dadantii 3937.

**METHODS**

**Bacterial strains and growth conditions.** The bacterial strains of D. dadantii and Escherichia coli and the plasmids used in this study are listed in Table 1. The phage Phi-EC2 was used to transfer the uidA–Km fusions in the regulator mutants by generalized transduction (Resibois et al., 1984). Bacteria were grown in LB or in M63 medium (Miller, 1992) and, when required, the media were solidified with 15 g agar l\(^{-1}\). D. dadantii cells were normally incubated at 30 °C and E. coli cells at 37 °C. Carbon sources were usually added at 2 g \(l^{-1}\). When required, antibiotics were added at the following concentrations: kanamycin (Km), 20 μg ml\(^{-1}\); ampicillin (Ap), 50 μg ml\(^{-1}\); and chloramphenicol (Cm), 20 μg ml\(^{-1}\). Plant extract was prepared by autoclaving 10 g of chrysanthemum leaves in 100 ml M63 (10% extract). The extract was diluted 10-fold in the growth medium.

**Recombinant DNA techniques.** Preparation of plasmid DNA, restriction digestions, ligations, DNA electrophoresis and transformations were carried out as described by Sambrook & Russell (2001). Sequence data were obtained from the genome sequencing project of D. dadantii strain 3937 (Glaser et al., 2011). PCR primers (23- and 27-mers) were designed to amplify 2 kb of D. dadantii 3937 chromosomal DNA overlapping bgxA (Table 1). Restriction sites were added to each primer to help in determining the DNA orientation in the vector (BamHI at the 5’ end and XbaI at the 3’ end). The PCR products were purified (QIAquick PCR purification kit, Qiagen) and ligated to the pGEM-T vector (Promega). A genetic fusion was constructed by insertion of the uidA–Km cassette (Bardonnet & Blanco, 1992) into the HpaI site of bgxA. The orientation of the uidA–Km cassette was determined by restriction analysis and a plasmid in which uidA and bgxA had the same transcriptional direction was retained. This plasmid was introduced into D. dadantii cells by electroporation. The insertion was integrated into the D. dadantii chromosome by marker-exchange recombination, after successive cultures in low phosphate medium in the presence of kanamycin (Roeder & Collmer, 1985). The correct recombination of the insertion was verified by PCR.

**In situ detection of β-glucosidase.** The β-glucosidase activity was detected in situ using the chromogenic substrate 5-bromo-6-chloro-3-indolyl-β-D-glucopyranoside (X-Gluc; BioChemika). After 24 h of growth on the appropriate medium, each colony was covered with 50 μl 1 mM X-Gluc solution. The plates were incubated at 30 °C and observed every 10 min until the appearance of a purple colour.

**Assay of β-glucosidase and β-xylosidase activities.** To measure the glycosidase activity, the formation of p-nitrophenol from the cleavage of a synthetic substrate was monitored at 405 nm. The β-glucosidase and β-xylosidase activities were measured by following the degradation of p-nitrophenyl-β-D-glucopyranoside (pNPG) and p-nitrophenyl-β-D-xylopyranoside (pNPX), respectively, for 5 min at 37 °C. The standard assay mixture consisted of 50 mM phosphate buffer at pH 7.0, 1 mM substrate and the extract in a total volume of 1 ml. The β-glucosidase and β-xylosidase specific activities are expressed as nanomoles of product liberated per minute per milligram of bacterial dry weight (nmol min\(^{-1}\) mg\(^{-1}\)).

Different assay conditions were tested on the BgxA activity. All assays were performed in triplicate. The optimal pH was determined using phosphate buffer pH 6.0–8.0 and Tris/HCl buffer pH 7.0–8.0. Kinetic experiments were performed using pNPG at concentrations ranging from 0.01 to 1 mM. The kinetic parameters \(K_\text{m}\) and \(V_\text{max}\) were determined from the Lineweaver–Burk plot of the Michaelis–Menten equation.

The effect of various cations was determined by incubating the enzyme in 0.1 mM of the chloride salt of Ba\(^{2+}\), Ca\(^{2+}\), Co\(^{2+}\), Cu\(^{2+}\), Fe\(^{3+}\), Mg\(^{2+}\), Mn\(^{2+}\), Ni\(^{2+}\) or Zn\(^{2+}\). EDTA and DTT were added at a final concentration of 10 mM, and NaCl at a concentration of 50 mM.

Potential inhibitors were used at the final concentrations of 10, 20, 40 and 80 mM. The inhibition tests were performed after addition of the following compounds to the reaction mixture: glucose, xylose, cellobiose, gentiobiose, arbutin, salicin, galactose, arabinose, fucose, lactose galacturonate, vanillic acid, phenyl β-D-glucopyranoside or ferulic acid. After incubation for 5 min at 37 °C, the substrate (pNPG or pNPX, 1 mM) was added to start the reaction. The residual activity was expressed as a percentage of enzyme activity in the standard assay mixture. The inhibition constants were determined from the corresponding Lineweaver–Burk plot. The \(K_i\) values are the means of three independent experiments.

**Assay of pectate lyase and β-gluconoridase activities.** Pectate lyase activity was determined by monitoring spectrophotometrically the formation of unsaturated products from polygalacturonate at 235 nm (Tardy et al., 1997). Specific activity is expressed as micromoles of unsaturated products liberated per minute per milligram of bacterial dry weight (μmol min\(^{-1}\) mg\(^{-1}\)).

To estimate the expression of gene fusions, β-glucuronidase (GUS) activity was measured by following the degradation of p-nitrophenyl-β-D-glucuronide (3.2 mM) into p-nitrophenol at 405 nm. The specific activity is expressed as nanomoles of product liberated per minute per milligram of bacterial dry weight (nmol min\(^{-1}\) mg\(^{-1}\)).
Table 1. Bacterial strains, plasmids and primers used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid/primer</th>
<th>Description/genotype</th>
<th>Reference/origin</th>
</tr>
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<tbody>
<tr>
<td><strong>Strain</strong></td>
<td></td>
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<tr>
<td><em>Dickeya dadantii</em></td>
<td></td>
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<tr>
<td>3937</td>
<td>Wild-type</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>A4415</td>
<td><em>pelD::uidA–Km</em></td>
<td>Hugouvieux-Cotte-Pattat <em>et al.</em> (1992)</td>
</tr>
<tr>
<td>A4655</td>
<td><em>bgxA::uidA–Km</em></td>
<td>This work</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM522</td>
<td><em>F′ proAB lacZΔlacZYAΔ(λac–proAB) thi hsd-5</em></td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td><em>E. coli B, F− dcm ompT hsdS gal d(ΔDE3), T7 polymerase gene under the lacUV5 promoter</em></td>
<td>Studier &amp; Moffatt (1986)</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEM-T</td>
<td>Vector for direct cloning of PCR products, Ap+</td>
<td>Promega</td>
</tr>
<tr>
<td>p3177</td>
<td>pGEM-T bgxA+</td>
<td>This work</td>
</tr>
<tr>
<td>p13349</td>
<td>pT7-5 bgxA+</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Primer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bgxAAG</td>
<td>GCGGATTCTGCAACTTGAAGTACGG</td>
<td>This work</td>
</tr>
<tr>
<td>bgxAAD</td>
<td>GCTCTAGAAGGTCAGTGGG</td>
<td>This work</td>
</tr>
</tbody>
</table>

*Restriction sites are underlined.

**Overproduction of the protein BgxA.** The T7 promoter–T7 RNA polymerase system (Tabor & Richardson, 1985) was used to overproduce BgxA. The 2 kb *BamHII*-XbaI fragment overlapping the *bgxA* gene was inserted into the pT7-5 vector. The nucleotide sequence of the resulting plasmid p13349 was verified. The plasmid was introduced into the *E. coli* strain BL21(DE3), which contains a chromosomal copy of the T7 RNA polymerase gene under the control of the lacUV5 promoter (Studier & Moffatt, 1986). After transformation, the β-glucosidase activity was checked in the presence of IPTG, using the chromogenic substrate X-Gluc. The BL21(DE3)/p13349 cells were grown at 28 °C in LB medium with ampicillin (100 μg ml⁻¹). When the OD of the culture at 600 nm reached 0.4 to 0.6, the synthesis of T7 RNA polymerase was induced by the addition of IPTG at a final concentration of 0.2 mM, and cells were grown for an additional 4 h.

**Cellular fractionation.** Different cellular fractions were obtained from the *E. coli* BL21(DE3)/p13349 induced cells. The bacterial cells were recovered by centrifugation (Eppendorf centrifuge 5415D) for 2 min at 8000 r.p.m. The pellet was suspended in 0.7 ml 80 mM Tris/HCl buffer pH 8.0 and 0.1% Triton X-100, and the cells were broken by sonication. Centrifugation for 2 min at 10 000 r.p.m. led to the recovery of the supernatant containing the soluble proteins. The pellet, corresponding to the insoluble proteins and cell debris, was suspended in 0.7 ml 80 mM Tris/HCl buffer, pH 8. The periplasmic fraction was recovered by osmotic shock (Copeland *et al.*, 1982).

**Analytical procedures.** The protein concentration was determined in each fraction by the Bradford method, using a commercial protein assay kit (Bio-Rad) and BSA as the standard. SDS-PAGE was performed on slab gels (4% stacking gel and 12% separating gel) using the Mini-Protean II system (Bio-Rad). The proteins were stained with Coomassie blue G-250.

**Pathogenicity tests.** Chicory leaves were inoculated as previously described (Hugouvieux-Cotte-Pattat & Charaoui-Boxekerzaa, 2009). Prior to infection, leaves were slightly wounded in their centre to define the inoculation site. Twenty leaves were infected for each strain using 10⁶ bacteria per inoculation site. After incubation in a dew chamber for 24 h at 30 °C, the length of rotted tissue was measured to estimate the disease severity. After infection, the macerated plant tissue was recovered and used to perform bacterial cell enumerations by dilution plating and β-glucuronidase assays (Van Gijsenberg *et al.*, 2008). The specific activity was calculated as nanomoles of product formed per 10⁶ bacteria. The wild-type strain, used as a negative control, showed no detectable β-glucuronidase activity. A fusion in the *pelD* gene was used as a positive reference in these experiments (Hugouvieux-Cotte-Pattat *et al.*, 1992).

**RESULTS**

The *bgxA* gene encodes the major β-glucosidase activity of *D. dadantii*

The genome of strain 3937 (Glasner *et al.*, 2011) was analysed for the presence of genes encoding potential glycosidases. One of these genes (ID 20230) encodes a protein of the GH3 family that is homologous to the *bgxA* product of the *Dickeya* strain D1 (92% identity) (Vroemen *et al.*, 1995). It is also conserved in the pectinolytic enterobacteria *D. chrysanthemi* Ech1591 and *D. zeae* Ech586 (92% identity) (Vroemen *et al.*, 1995). It is also conserved in some other enterobacteria: *Rahnella aquatilis* (68%), *Citrobacter* sp. (60%), and *Brenneria* sp. (57%). Homologues are also encoded by distant genera, such as *Caulobacter* sp. (60%), *Agrobacterium vitis* (54%) and *Azospirilum irakense* (51%). These bacteria either are found in aquatic environments or are plant-associated. The sequence identity (100%) observed between the *bgxA* products of strain D1 and of Ech586 could suggest that strain D1, isolated from corn, belongs to the species *D. zeae.*
The bgxA gene of the *D. dadantii* strain 3937 is situated in a locus containing no other potential glycosidase or plant-cell-wall-degrading enzymes. It is preceded and followed by proteins of unknown function (separated by 147 nt and 247 nt, respectively). Thus, considering its genetic organization, bgxA most probably constitutes an independent transcriptional unit.

We used a chromogenic substrate to detect β-glucosidase activity directly on bacterial colonies (Fig. 1a). The purple colour of colonies of the wild-type strain 3937 confirmed that this strain has β-glucosidase activity. In contrast, colonies of the bgxA mutant A4655 remained colourless, like those of the negative control *E. coli* strain NM522. This suggested that bgxA encodes a major β-glucosidase of *D. dadantii* 3937. However, the bgxA mutant was not affected for growth on β-glucosides such as cellobiose, arbutin or salicin when these were used as the sole carbon source, indicating a redundancy of β-glucoside assimilation pathways in *D. dadantii*. When bgxA was cloned in *E. coli* (Fig. 1b), colonies of the recombinant strain BL21(DE3)/pI3349 stained purple in the presence of IPTG, confirming that the *D. dadantii* bgxA gene encodes a protein with β-glucosidase activity.

### Expression of the bgxA gene under different conditions

In order to analyse its regulation, a transcriptional fusion in bgxA was constructed by insertion of a *uidA*-Km cassette. Different polysaccharides and carbon sources were added to the culture medium to detect any potential inducer of bgxA transcription. The expression of the bgxA fusion was not modified in the presence of cellulose, polygalacturionate, xylan, galactose, galacturonate or xylose (data not shown). It decreased by about fourfold in the presence of glucose (Table 2). While cellobiose and arbutin had no effect, the bgxA expression increased in the presence of some glycosides, by about fourfold with salicin and twofold with phenol β-D-glucopyranoside. Phenolic compounds also induced the bgxA fusion, by a factor of about fivefold for ferulic acid, *trans*-cinnamic acid and vanillic acid (Table 2).

The expression of the bgxA fusion was also followed during bacterial growth, showing maximal expression after 20 to 24 h of growth, in the early stationary phase (data not shown). When assayed in the same cultures, pectate lyase activity was maximal at between 16 and 24 h of growth, at the end of the exponential growth phase. The bgxA expression was analysed at three temperatures that are favourable for *D. dadantii* growth (25, 30 and 37 °C) (Fig. 2a). Expression was optimal at 30 °C and it was slightly reduced at higher or lower temperatures. Change in the medium osmolarity demonstrated that bgxA is better expressed under conditions of high osmolarity (Fig. 2b). In each case, the variations due to a modification of the environmental conditions were less pronounced than the inducing effects observed following the addition of phenolic compounds (Table 2).

### Virulence of the mutant and bgxA expression during infection

Chicory leaves were infected with the bgxA mutant to analyse the effect of the mutation on virulence. No significant difference was observed in the degree of maceration caused by the bgxA mutant and the parental strain 3937 (Fig. 3a). The expression of the bgxA transcriptional fusion was assayed in the macerated tissues to determine whether or not it is expressed during infection. A fusion in the plant-inducible pectate lyase gene *pelD* (Hugouvieux-Cotte-Pattat et al., 1992) was used as the control. The level of bgxA expression in the macerated tissue was nearly 30% of the *pelD* expression (Fig. 3b). These results indicate that, although bgxA is not essential for the development of the soft rot disease, it is expressed during plant infection.

### Biochemical characterization of the *D. dadantii* BgxA activity

To characterize the BgxA protein, its overproduction was achieved from the cloned gene using the T7 RNA polymerase system in *E. coli* BL21(DE3). Different cellular fractions were analysed by SDS-PAGE (Fig. 1c). Two additional bands were observed after IPTG induction, with apparent sizes of 71 and 69 kDa, respectively. The 71 kDa
Table 2. Expression of the bgxA transcriptional fusion

<table>
<thead>
<tr>
<th>Carbon source, potential inducer*</th>
<th>β-Glucuronidase specific activity†</th>
</tr>
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<tbody>
<tr>
<td>Glycerol</td>
<td>53</td>
</tr>
<tr>
<td>Glucose</td>
<td>16</td>
</tr>
<tr>
<td>Glycerol + plant extract</td>
<td>81</td>
</tr>
<tr>
<td>Glycerol + cellobiose</td>
<td>58</td>
</tr>
<tr>
<td>Glycerol + arbutin</td>
<td>57</td>
</tr>
<tr>
<td>Glycerol + salicyclic acid</td>
<td>193</td>
</tr>
<tr>
<td>Glycerol + phenyl β-D-glucopyranoside</td>
<td>96</td>
</tr>
<tr>
<td>Glycerol + ferulic acid</td>
<td>277</td>
</tr>
<tr>
<td>Glycerol + trans-cinnamic acid</td>
<td>307</td>
</tr>
<tr>
<td>Glycerol + vanillic acid</td>
<td>260</td>
</tr>
</tbody>
</table>

*Bacteria were grown to early stationary phase in M63 medium containing glycerol or glucose (2 g l⁻¹) as the carbon source. The potential inducers were added at the following concentrations: plant extract 1% (v/v); cellobiose, arbutin, salicyclic and phenyl β-D-glucopyranoside 1 g l⁻¹; ferulic acid, trans-cinnamic acid and vanillic acid 0.25 g l⁻¹.

†The values given (in nmol min⁻¹ mg⁻¹) are the mean of at least three independent experiments, with standard deviations corresponding to less than 20%.

This confirms that the BgxA signal sequence is cleaved during its exportation into the E. coli periplasm.

The periplasmic fraction of BL21/pI3349, containing the overproduced BgxA protein, is able to cleave pNPG, a β-glucosidase substrate. Since enzymes of the GH3 family frequently combine different activities (Vroemen et al., 1995; Faure, 2002; Shipkowski & Brenchley, 2005; Dodd et al., 2010) we tested potential α-L-arabinosidase, β-D-galactosidase, or β-D-xylosidase activities using different PNP-glycosides as substrates. A low level of β-xylosidase activity was observed when pN PX was used as the substrate. Comparison of its capacity to cleave pNPX and pN PX suggested that the β-D-glucosidase activity of BgxA is 70-fold higher than its β-D-xylosidase activity.

The effect of pH on the BgxA activity was analysed using 50 mM phosphate buffer at different pH (Fig. 4a). The BgxA β-glucosidase activity increased up to pH 7.8, where it reached its optimal value. This value was also the optimal pH for BgxA β-xylosidase activity (data not shown). To determine the BgxA kinetic parameters, the initial velocities were measured using 0.01–1 mM pNPG. The apparent $K_m$ and $V_{max}$ values for the hydrolysis of pNPG by BgxA were 0.20 mM and 2.7 μmol min⁻¹ mg⁻¹, respectively.

The effect of metal ions on the β-glucosidase activity was also tested. In comparison with the control, β-glucosidase activity was slightly enhanced in the presence of the divalent cations Co²⁺, Mg²⁺ and Mn²⁺, whereas Fe²⁺, Ni²⁺ and Zn²⁺ had an inhibitory effect (Fig. 4b). The inhibition reached 53% and 78% with Fe²⁺ and Zn²⁺, respectively. NaCl, EDTA and DTT also had a partial inhibitory effect on the β-glucosidase activity.

Inhibitors of the BgxA activity

After preliminary observations indicating BgxA inhibition by some glucosides, we analysed the effect of different
mono- and disaccharides on the β-glucosidase activity (Fig. 5). In the presence of xylose, cellobiose and gentiobiose, BgxA activity was not significantly affected, even at a high sugar concentration (80 mM). In contrast, a marked inhibition was observed in the presence of aromatic glycosides such as arbutin, salacin and phenyl-glucoside (Fig. 5). Glucose exerts an intermediate degree of inhibition, more clearly visible at a high concentration (Fig. 5). Strong inhibition was also observed in the presence of non-glycosidic phenolic compounds, such as ferulic acid or vanillic acid. When the β-xylosidase activity of BgxA was assayed, similar inhibitory effects were observed in the presence of these compounds (data not shown). These data suggest that the BgxA enzyme has an affinity for glycosidic or non-glycosidic phenolic compounds.

In order to characterize the inhibitory effect, the kinetic parameters of BgxA were determined in the presence of arbutin or salacin. In the presence of an aromatic glucoside, the apparent $V_{\text{max}}$ values did not change while the apparent $K_m$ values clearly increased. This is characteristic of competitive inhibition, indicating that arbutin and salacin exert a competitive inhibitory effect on the pNPG hydrolysis. The $K_i$ values for arbutin and salacin were 0.31 mM and 0.33 mM, respectively. These $K_i$ values are close to the $K_m$ for pNPG (0.20 mM), indicating a high affinity of the BgxA enzyme for these aromatic glycosides.

**DISCUSSION**

The *D. dadantii* 3937 genome includes 46 genes encoding potential glycoside hydrolases (http://www.cazy.org/) and, among these, some are found mostly in the plant-associated bacteria. This is the case for BgxA, a GH3 enzyme which is found in all the sequenced strains of the genus *Dickeya* and is also conserved in certain *Enterobacteriaceae*, *P. carotovorum*, *R. aquatilis*, *Citrobacter* sp. and *Brenneria* sp., as well as in other plant-associated bacteria, such as *Ag. vitis* and *Az. trakense*. An orthologous enzyme has been previously described in the *Dickeya* strain D1, isolated from corn (Vroemen *et al.*, 1995). However, this study gave no information on the bgxA expression and the enzyme specificity remained unclear.

The main information concerning the potential role of BgxA in *D. dadantii* 3937 was derived from an analysis of the gene expression. The bgxA transcription was not affected by the addition of various polysaccharides, oligosaccharides or sugars, except for glucose, which caused a downregulation. However, it was induced in the presence of aromatic glucosides, such as salacin, and even more so in the presence of phenolic compounds, such as ferulic acid (Table 2). Interestingly, Antunez-Lamas *et al.* (2009) also detected an induction of the *D. dadantii* bgxA gene in the presence of a plant hormone, jasmonate. This induction pattern indicated that BgxA may play a role in the cleavage of plant aromatic β-glucosides rather than in the assimilation of oligosaccharides.

The BgxA activity is not essential for the maceration of chicory leaves caused by *D. dadantii* but, nevertheless, the bgxA gene is expressed during infection (Fig. 3). Similarly, BgxA activity did not appear to be necessary for the virulence of strain D1 on corn leaves (Vroemen *et al.*, 1995). As was proposed for strain D1, the BgxA protein of *D. dadantii* 3937 appears to be a periplasmic enzyme with a 29 aa signal sequence cleaved in *E. coli* (Fig. 1c). Both enzymes show major activity on β-glucosides and secondary activity on β-xylosides. The ratios of these two activities, β-glucosidase: β-xylosidase, as determined on pNP-glycosides, is 70 for the BgxA enzyme of strain 3937 (this study) and 40 for the BgxA of strain D1 (Vroemen *et al.*, 1995).
The GH3 family is one of the most abundant families in the carbohydrate-active enzymes and it includes members that possess diverse enzymic activities, either with unique substrate specificity or as bifunctional enzymes (http://www.cazy.org/). The characteristics of BgxA are similar to those of other bacterial GH3 enzymes. The association of β-glucosidase and β-xylosidase activities, in varying ratios, is commonly found (Faure et al., 1999; Vroemen et al., 1995; Breves et al., 1997; Watt et al., 1998). Several GH3 enzymes are localized within the periplasmic space, such as BglX in E. coli (Yang et al., 1996) and SalA or SalB in Az. irakense (Faure et al., 1999). The affinity of D. dadantii BgxA for pNPG is comparable to that determined for other bacterial GH3 enzymes. The characteristics of BgxA are similar to substrate specificity or as bifunctional enzymes (http://www.cazy.org/). The crystal structure of a GH3 enzyme, the barley exohydrolase, provided information on the catalytic mechanism and on substrate binding (Varghese et al., 1999). A pair of residues, Asp and Glu, act as nucleophile and acid/base in the hydrolytic reaction (Fig. 6). In addition to these two critical residues, GH3 enzymes possess a core set of highly conserved residues surrounding the −1 subsite that interact with the terminal non-reducing sugar of the substrate (glucose for glucosidases). The GH3 enzymes are organized in two domains, A and B, corresponding to the N-terminal and C-terminal part of the protein, respectively (Faure et al., 1999; Varghese et al., 1999). The active site of the enzyme is a pocket situated at the interface of the two domains (Varghese et al., 1999). Domain A contains the catalytic nucleophile residue and most of the residues of the −1 subsite which, by directly interacting with the sugar pyranose ring, contribute to the substrate specificity. Domain B contains the catalytic acid/base residue. Like the SalB enzyme of Az. irakense (Faure et al., 1999), BgxA has a large domain A, about 400 residues, but a short domain B of about 230 residues. The residues that are conserved in BgxA and its orthologues either participate in catalysis (the nucleophile Asp364, with the BgxA numbering) or in hydrogen bonding interactions with the terminal non-reducing sugar of the substrate (Asp193, Arg231, Lys271, His272, Tyr322, Met319, Trp365) (Fig. 6). Xylose is a pentose sugar whose pyranose form does not possess the CH2OH substituent of C5 that is present on the hexose sugar glucose. The −1 subsite of BgxA has the potential to interact more with glucose than with xylose. For example, the residue Asp193 is predicted to interact with C6OH and the residue Met319 with C6H (Varghese et al., 1999). These observations could explain the higher affinity of BgxA for β-glucosides than for β-xylosides. BgxA activity was shown to be slightly enhanced by Mg2+ but inhibited by Zn2+, Fe2+ or the presence of EDTA (Fig. 4b). These cation effects could be related to the presence of several charged residues and the importance of ionic interactions in catalysis and substrate binding. The reduction of BgxA activity in the presence of DTT (Fig. 4b) suggests that this enzyme contains disulfide bond(s). Since there are only two cysteine residues in the BgxA sequence, a potential disulfide bond could link the two residues Cys371 and Cys375 (Fig. 6).

A new property of BgxA was revealed from observation of the marked inhibition by aromatic glucosides, such as arbutin and salicin, and by phenolic compounds, such as ferulic acid. The Ki values measured for arbutin and salicin, 0.31 mM and 0.33 mM, were close to the Km value for pNPG, 0.20 mM. Together, the induction effect and the enzymic inhibition suggest that the physiological role of BgxA is linked to the cleavage of aromatic glycosides.

Growth of the bgxA mutant was not affected when arbutin or salicin was used as the sole carbon source. However, a potential role of bgxA in the assimilation of these
β-glucosides could be masked by the redundancy of functionally equivalent pathways. Indeed, a previous analysis of β-glucoside assimilation in D. dadantii 3937 demonstrated the role of the *arbGFB* locus in growth with arbutin or salicin, and also predicted the presence of at least one other non-identified system, called *cbr*, that allows the assimilation of cellubiose, arbutin and salicin (el Hassouni et al., 1990). The *arbGFB* operon encodes a classical β-glucoside degradation pathway, including a phosphorylation-coupled uptake and the cytoplasmic cleavage of the phosphorylated substrate by a phospho-β-glucosidase of the GH1 family (el Hassouni et al., 1990). Analysis of the *D. dadantii* 3937 genome indicated that two other regions, in addition to the *arbGFB* cluster, encode proteins that could be involved in β-glucoside assimilation. A large region, probably corresponding to the *cbr* system, encodes three proteins of the GH1 family (ID18465, 18466, 1846), together with potential transporters and regulators. The second region encodes only one protein of the GH1 family (ID16574). Thus, *D. dadantii* has redundant β-glucoside assimilation pathways including cytoplasmic enzymes. The role of a β-glucosidase of the GH3 family, such as BgaA, could be linked to growth on arbutin and salicin when the *arb* and *cbr* systems are not expressed, and also to the hydrolysis of aromatic β-glucosidase outside the cytoplasm. The BgaA protein is exported to the periplasmic space and it was not found in the *D. dadantii* extracellular fraction. The cellular location of BgaA may avoid the release in the cytoplasm of the aromatic part of the substrate, which could have toxic effects (Hassan & Hugouvieux-Cotte-Pattat, 2011). Assimilation of the glucose moiety of the substrate remains possible via the classical glucose uptake system. Thus, the BgaA subcellular localization could be an important element in its specialization as regards aromatic glycosides originating from the host or the environment.

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