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Characterization of β-galactosidase and α-galactosidase activities from the halophilic bacterium *Gracilibacillus dipsosauri*

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Abstract

Purpose: *Gracilibacillus dipsosauri* strain DD1 is a salt-tolerant Gram-positive bacterium that can hydrolyze the synthetic substrates *o*-nitrophenyl-β-D-galactopyranoside (β-ONP-galactose) and *p*-nitrophenyl-α-D-galactopyranoside (α-PNP-galactose). The goals of this project were to characterize the enzymes responsible for these activities and to identify the genes encoding them.

Methods: *G. dipsosauri* strain DD1 was grown in tryptic soy broth containing various carbohydrates at 37 °C with aeration. Enzyme activities in cell extracts and whole cells were measured colorimetrically by hydrolysis of synthetic substrates containing nitrophenyl moieties. Two enzymes with β-galactosidase activity and one with α-galactosidase activity were partially purified by ammonium sulfate fractionation, ion-exchange chromatography, and gel-filtration chromatography from *G. dipsosauri*. Coomassie Blue-stained bands corresponding to each activity were excised from nondenaturing polyacrylamide gels and subjected to peptide sequencing after trypsin digestion and HPLC/MS analysis.

Result: Formation of β-galactosidase and α-galactosidase activities was repressed by D-glucose and not induced by lactose or D-melibiose. β-Galactosidase I had hydrolytic and transgalactosylation activity with lactose as the substrate but β-galactosidase II showed no activity towards lactose. The α-galactosidase had hydrolytic and transgalactosylation activity with D-melibiose but not with D-raffinose. β-Galactosidase I had a lower K_m with β-ONP-galactose as the substrate (0.693 mmol l^{-1}) than β-galactosidase II (1.662 mmol l^{-1}), was active at more alkaline pH, and was inhibited by the product D-galactose. β-Galactosidase II was active at more acidic pH, was partially inhibited by ammonium salts, and showed higher activity with α-PNP-arabinose as a substrate. The α-galactosidase had a low K_m with α-PNP-galactose as the substrate (0.338 mmol l^{-1}), a pH optimum of about 7, and was inhibited by chloride-containing salts. β-Galactosidase I activity was found to be due to the protein A0A317L6F0 (encoded by gene DLJ74_04930), β-galactosidase II activity to the protein A0A317KZG3 (encoded by gene DLJ74_12640), and the α-galactosidase activity to the protein A0A317KU47 (encoded by gene DLJ74_17745).

Conclusions: *G. dipsosauri* forms three intracellular enzymes with different physiological properties which are responsible for the hydrolysis of β-ONP-galactose and α-PNP-galactose. BLAST analysis indicated that similar

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β-galactosidases may be formed by *G. ureilyticus*, *G. orientalis*, and *G. kekensis* and similar α-galactosidases by these bacteria and *G. halophilus*.

Keywords: Enzyme, α-Galactosidase, β-Galactosidase, *Gracilibacillus*, Protein purification

Background

The genus *Gracilibacillus* includes a diverse group of halotolerant Gram-positive bacteria, which usually form thin motile rods with terminal spherical or ellipsoidal endospores (Editorial Board 2015). Many species were originally isolated for saline lakes or soils, including *G. halotolerans* (Wainø et al. 1999), *G. orientalis* (Carrasco et al. 2006), *G. boraciitolerans* (Ahmed et al. 2007), *G. quinghaiensis* (Chen et al. 2008a), *G. lacisalsi* (Jeon et al. 2008), *G. halophilus* (Chen et al. 2008b), *G. saliphilus* (Tang et al. 2009), *G. ureilyticus* (Huo et al. 2010), *G. kekensis* (Gao et al. 2012), *G. bigeumensis* (Kim et al. 2012), *G. marinus* (Huang et al. 2013), *G. xinjiangensis* (Yang et al. 2013), *G. aidingensis* (Guan et al. 2017), *G. eburneus* (Guan et al. 2018), and *G. salitolerans* (Gan et al. 2020). Others were isolated from animals (*G. dipsosauri* (Deutch 1994), *G. timonensis* (Diop et al. 2018), *G. phocaeensis* (Senghor et al. 2017)) or from food products (*G. thailandensis* (Chamroensaksri et al. 2010), *G. kimchii* (Oh et al. 2016), *G. massiliensis* (Diop et al. 2016), and *G. oryzae* (He et al. 2020)).

As part of their initial characterization and identification, most of these bacteria were subjected to a standard set of physiological tests which included hydrolysis of the synthetic substrate β-ONPG (β-ONP-galactose, *o*-nitrophenyl-β-D-galactopyranoside, 2-nitrophenyl-β-D-galactopyranoside). A positive result is indicative of the presence of an enzyme with β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) activity. In the same way, hydrolysis of α-PNPG (α-PNP-Galactose, *p*-nitrophenyl-α-D-galactopyranoside, 4-nitrophenyl-α-D-galactopyranoside) is an indication of an enzyme with α-galactosidase activity (α-galactoside galactohydrolase, EC 1.2.1.22). A partial summary of the species of *Gracilibacillus* with positive or negative results for these activities is included in the papers by Kim et al. (2012) and Diop et al. (2016). β-Galactosidases and α-galactosidases from other sources have been found to be industrially significant due to their ability to hydrolyze a wide range of carbohydrates or to form prebiotics and probiotics (Husain 2010; Saqib et al. 2017; Vera et al. 2020; Katrolia et al. 2014; Bhatia et al. 2020).

Gracilibacillus dipsosauri strain DD1 was originally isolated from the nasal cavity of a desert iguana and identified as *Bacillus dipsosauri* (Deutch 1994). It grew in KCl or NaCl concentrations up to 2.5 mol l^{-1} and lysed in the absence of added salt; its temperature optimum was 45 °C. The bacterium was later reclassified as *Gracilibacillus dipsosauri* after reorganization of several halotolerant genera (Lawson et al. 1996; Wainø et al. 1999). In its initial characterization, *G. dipsosauri* strain DD1 gave positive results for the hydrolysis of starch, triacylglycerides, esculin, and β-ONPG (Deutch 1994). Starch hydrolysis was subsequently shown to be due to an extracellular salt-tolerant α-amylase (Deutch 2002). The sequence of the *G. dipsosauri* genome was recently determined and the gene encoding this enzyme identified (Deutch and Yang 2020). The α-amylase activity in strain DD1 is associated with a protein (A0A317L430) that can also function as a cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19). The genome of *G. dipsosauri* strain DD1 contains multiple genes with the potential to encode either β-galactosidases or α-galactosidases. We have now characterized three of these hydrolytic activities in detail and identified the genes that encode them.

Results

Detection of glycosyl hydrolase activities from *G. dipsosauri* strain DD1

To determine if *G. dipsosauri* strain DD1 forms additional glycosyl hydrolases besides the extracellular α-amylase previously identified (Deutch 2002) and recently characterized as a cyclodextrin glycosyltransferase (Deutch and Yang 2020), exponential-phase bacteria grown in tryptic soy broth without dextrose containing 1.0 mol l^{-1} KCl were disrupted with a Bead Beater and a crude extract prepared by centrifugation. The extract was tested for hydrolytic activity using a series of synthetic substrates (Table 1). There was good activity with β-ONP-galactose (*o*-nitrophenyl-β-D-galactopyranoside, 2-nitrophenyl-β-D-galactopyranoside), β-PNP-galactose (*p*-nitrophenyl-β-D-galactopyranoside, 4-nitrophenyl-β-D-galactopyranoside), α-ONP-galactose (*o*-nitrophenyl-α-D-galactopyranoside, 2-nitrophenyl-α-D-galactopyranoside), and α-PNP-galactose (*p*-nitrophenyl-α-D-galactopyranoside, 4-nitrophenyl-α-D-galactopyranoside). There was lower activity with β-ONP-glucose (*o*-nitrophenyl-β-D-glucopyranoside, 2-nitrophenyl-β-D-glucopyranoside), α-PNP-glucose (*p*-nitrophenyl-α-D-glucopyranoside, 4-nitrophenyl-α-D-glucopyranoside), and α-PNP-arabinose (*p*-nitrophenyl-α-L-arabinopyranoside, 4-nitrophenyl-α-L-arabinopyranoside. There was no activity with α-PNP-Mannose (4-nitrophenyl-α-D-mannopyranoside), α-PNP-Xylose (4-nitrophenyl-α-D-xylopyranoside),

Table 1 Specific activities of glycosyl hydrolases from *G. dipsosauri* strain DD1

Substrate	Specific activity (μmoles min^{-1} (mg protein)$^{-1}$)	
	Crude extract	50 to 80% $NH_4(SO_4)_2$ fraction
β-ONP-galactose (*o*-nitrophenyl-β-D-galactopyranoside) (2-nitrophenyl-β-D-galactopyranoside)	1.11 ± 0.07	2.27 ± 0.03
β-PNP-galactose (*p*-nitrophenyl-β-D-galactopyranoside) (4-nitrophenyl-β-D-galactopyranoside)	0.71 ± 0.02	1.30 ± 0.04
α-ONP-galactose (*o*-nitrophenyl-α-D-galactopyranoside) (2-nitrophenyl-α-D-galactopyranoside)	2.85 ± 0.21	4.55 ± 0.10
α-PNP-galactose (*p*-nitrophenyl-α-D-galactopyranoside) (4-nitrophenyl-α-D-galactopyranoside)	1.54 ± 0.01	2.78 ± 0.04
β-PNP-glucose (*p*-nitrophenyl-β-D-glucopyranoside) (4-nitrophenyl-β-D-glucopyranoside)	0.026 ± 0	0.049 ± 0.008
α-ONP-glucose (*o*-nitrophenyl-α-D-glucopyranoside) (2-nitrophenyl-α-D-glucopyranoside)	0.015 ± 0.001	0.029 ± 0.001
α-PNP-arabinose (*p*-nitrophenyl-α-L-arabinopyranoside) (4-nitrophenyl-α-L-arabinopyranoside)	0.160 ± 0.003	0.285 ± 0.001

Specific activities are the means ± one standard deviation of the three replicate assays with each substrate from one of several independent experiments

α-PNP-Rhamnose (4-nitrophenyl-α-L-rhamnopyranoside, or α-PNP-Fucose (4-nitrophenyl-α-L-fucopyranoside). None of the activities found in the crude extract was present in significant amounts in the culture fluid from the exponential-phase cells. When the proteins in the crude extract were subjected to ammonium sulfate fractionation, 80 to 90% of each enzyme activity was found in the 50 to 80% ammonium sulfate fraction with a corresponding two-fold increase in specific activity (Table 1).

Detection of β-galactosidase and α-galactosidase activities in whole cells of *G. dipsosauri*

Because the β-galactosidase and α-galactosidase activities were found in the highest amounts in the crude extract from *G. dipsosauri* strain DD1 and in the 50 to 80% ammonium sulfate fraction, we focused on these enzymes. When bacteria were streaked on agar plates of tryptic soy broth containing 1.0 mol l^{-1} KCl or tryptic soy broth without dextrose containing 1.0 mol l^{-1} KCl which had been spread with 50 μl of 20 mg ml^{-1} solutions of 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside (X-β-galactose) or 5-bromo-4-chloro-3-indoxyl-α-D-galactopyranoside (X-α-galactose), they formed blue colonies after incubation at 37 °C for 24 to 48 h. The intensity of the color was greater on the plates of tryptic soy broth without dextrose. There was no blue color in the medium surrounding the colonies.

To quantify these results in whole cells of *G. dipsosauri*, strain DD1 was grown to exponential phase in tryptic soy broth without dextrose containing 1.0 mol l^{-1} KCl and harvested by centrifugation. The bacteria were washed with 0.85% (w/v) NaCl, resuspended in 0.85% (w/v) NaCl, and frozen at 20 °C. After thawing and treatment with 0.005% (v/v) Triton X-100 to increase their permeability to the substrates, the cells were tested for enzyme activities with β-ONP-galactose, β-PNP-galactose, α-ONP-galactose, and α-PNP-galactose as substrates. The results were consistent with those shown in Table 1 (Supplementary Fig. 1). The specific activity of α-galactosidase was about 2.5 times that of β-galactosidase. The activities seen with the *p*-nitrophenyl-galactoside (4-nitrophenyl-galactoside) substrates were about 60% of those seen with the *o*-nitrophenyl-galactoside (2-nitrophenyl-galactoside) substrates. Because α-ONP-galactose is no longer commercially available, most of the subsequent experiments were done with β-ONP-galactose and with α-PNP-galactose.

Effect of growth medium on the formation of β-galactosidase and α-galactosidase activities from *G. dipsosauri*

To determine if the formation of the β-galactosidase and α-galactosidase activities in *G. dipsosauri* was inducible by the substrates or repressible by D-glucose, the bacteria were grown in regular tryptic soy broth containing 1.0 mol l^{-1} KCl or in tryptic soy broth without dextrose containing 1.0 mol l^{-1} KCl supplemented with various sugars. The specific activities of β-galactosidase and α-galactosidase were determined in permeabilized whole cells using β-ONP-galactose and α-PNP-galactose as the substrates (Fig. 1). The activities of both enzymes in regular tryptic soy broth, which contains 0.25% (w/v) D-glucose, or in tryptic soy broth without dextrose supplemented with 0.5% (w/v) D-glucose were very low. The β-galactosidase specific activity was not increased by addition of 0.5% (w/v) D-galactose but the α-galactosidase specific activity was reduced. Addition of 0.5% (w/v) lactose (β-D-galactopyranosyl-(1,4)-D-glucopyranoside) did not induce the β-galactosidase activity but actually reduced both specific activities. Addition of 0.5% (w/v) D-melibiose (α-D-galactopyranosyl-(1,6)-D-glucopyranoside) or 0.5% (w/v) raffinose (α-D-galactopyranosyl-(1,6)-α-D-glucopyranosyl-(1,2)-β-D-fructofuranoside) did not affect the β-galactosidase

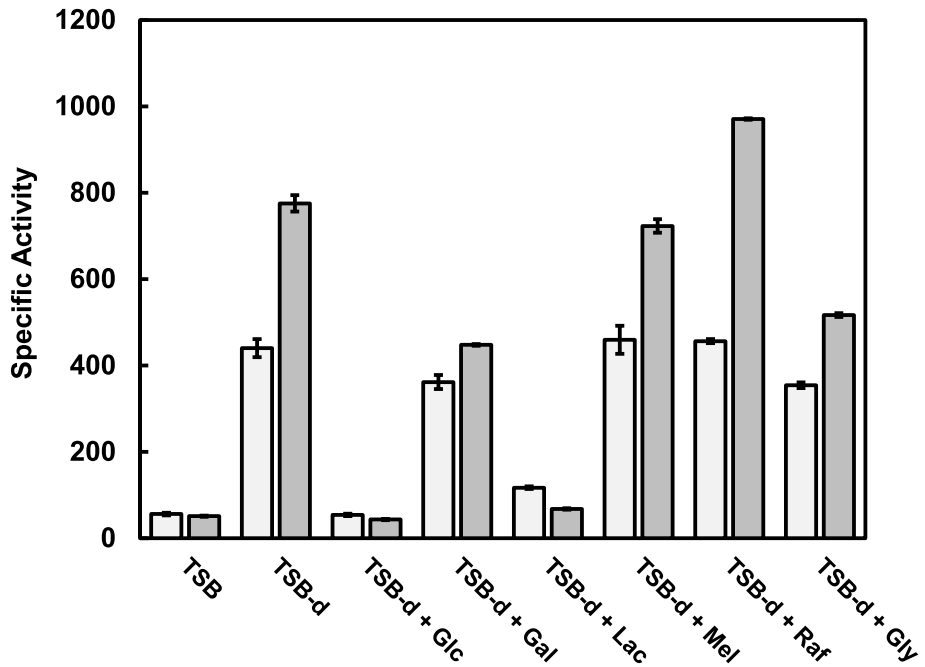

Fig. 1 Effect of growth medium on the formation of β-galactosidase and α-galactosidase activities in whole cells of *G. dipsosauri* strain DD1. Bacteria were grown to exponential phase in tryptic soy broth containing 1.0 mol l^{-1} KCl (TSB), tryptic soy broth without dextrose containing 1.0 mol l^{-1} KCl (TSB-d), tryptic soy broth without dextrose containing 1.0 mol l^{-1} KCl and 0.5% (w/v) D-glucose (TSB-d + Glc), tryptic soy broth without dextrose containing 1.0 mol l^{-1} KCl and 0.5% (w/v) D-galactose (TSB-d + Gal), tryptic soy broth without dextrose containing 1.0 mol l^{-1} KCl and 0.5% (w/v) lactose (TSB-d + Lac), tryptic soy broth without dextrose containing 1.0 mol l^{-1} KCl and 0.5% (w/v) D-melibiose (TSB-d + Mel), tryptic soy broth without dextrose containing 1.0 mol l^{-1} KCl and 0.5% (w/v) D-raffinose (TSB-d + Raf), or tryptic soy broth without dextrose containing 1.0 mol l^{-1} KCl and 0.5% (v/v) glycerol (TSB-d + Gly). Bacteria were harvested, washed with 0.85% NaCl, resuspended in 0.85% NaCl to give a suspension with 100 Klett Units, frozen, and thawed. Cells were treated with 0.005% Triton X-100 prior to enzyme assays with β-ONPG as the substrate for β-galactosidase activity (light bars) or with α-PNPG as the substrate for α-galactosidase activity (dark bars). Data show the means and standard deviations of assays from one of several experiments

activity. These sugars did not induce the α-galactosidase activity although the specific activity after growth with 0.5% raffinose was slightly higher than that with 0.5% D-melibiose. Addition of 0.5% glycerol (v/v) gave intermediate specific activities for both enzymes.

Partial purification of the β-galactosidase and α-galactosidase activities from *G. dipsosauri*

The β-galactosidase and α-galactosidase activities from *G. dipsosauri* were partially purified from the 50 to 80% ammonium sulfate fraction. After desalting this fraction on a column of Bio-Gel P-6 DG, the proteins were separated on a column of DEAE-Sepharose (Fig. 2 panel (a)). After stepwise elution with buffers containing 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mol l^{-1} KCl, there were two well-separated peaks of β-galactosidase activity which were designated β-galactosidase I and β-galactosidase II. There was one peak of α-galactosidase activity which overlapped with the second peak of β-galactosidase activity. Because the β-galactosidase II and α-galactosidase activities in the desalted 50 to 80% ammonium sulfate fraction could not be separated on the column of DEAE-Sepharose, the same fraction was applied to a column of MacroPrep-DEAE. Using this more hydrophobic resin, the β-galactosidase II activity eluted before the α-galactosidase activity after stepwise elution with buffers containing 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mol l^{-1} KCl (Fig. 2 panel (b)).

The fractions that were enriched in β-galactosidase I, β-galactosidase II, or α-galactosidase activity were then applied separately to a column of Sephadex G-200. The β-galactosidase I activity from either the DEAE-Sepharose column or the MacroPrep-DEAE column was eluted with Z buffer as a single peak of activity just after the void volume (Supplementary Fig. 2 panel (a)). The β-galactosidase II and α-galactosidase activities from the MacroPrep-DEAE column eluted from the Sephadex G-200 column as single peaks of activity with only minor contamination by the other enzyme (Supplementary Fig. 2 panel (b) and Supplementary Fig. 2 panel (c)).

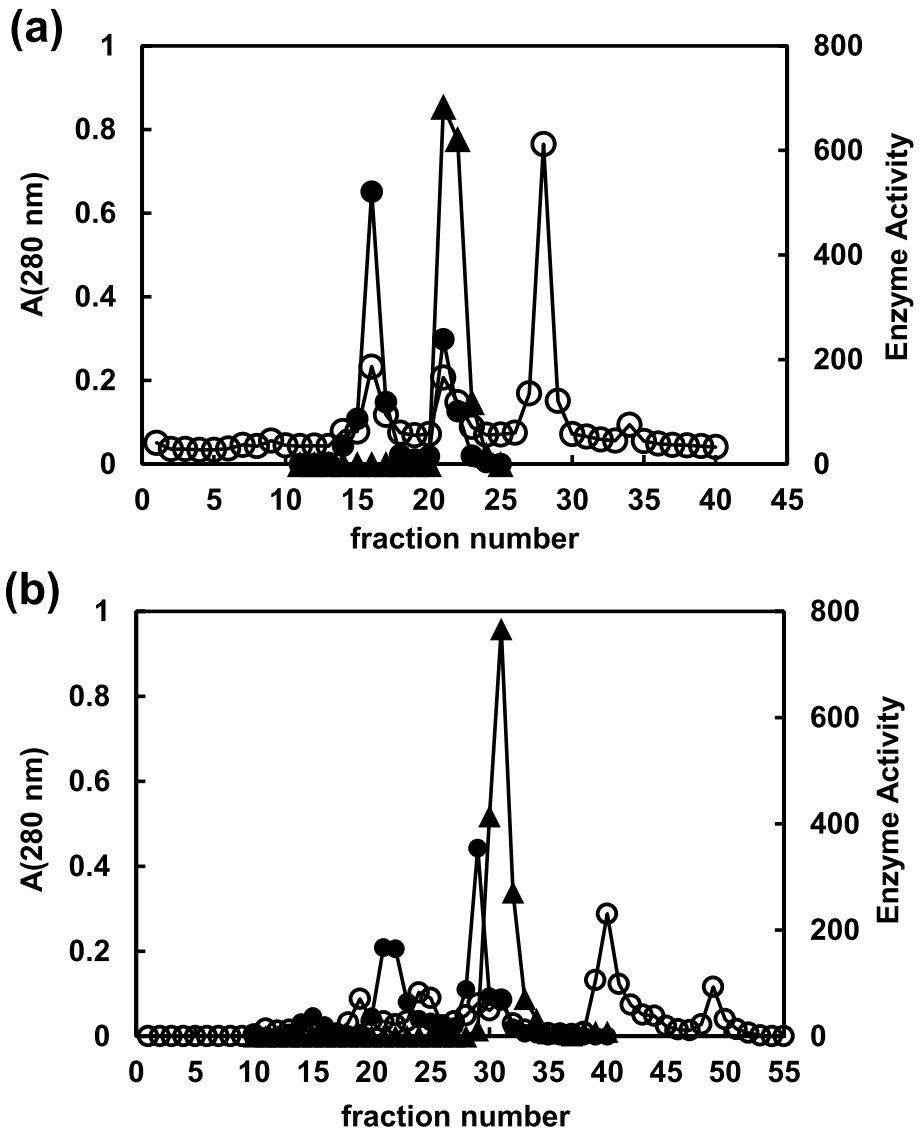

Fig. 2 Ion-exchange chromatography of proteins in the desalted P80 fraction from *G. dipsosauri* strain DD1. A portion of the desalted P80 fraction (2–3 ml) was loaded onto a column of DEAE-Sepharose (panel (**a**)) or MacroPrep-DEAE (panel (**b**)) and eluted by washing the resin with Z buffer or Z buffers containing 0.2, 0.4, 0.6, 0.8, or 1.0 mol l^{-1} KCl. Fractions (100 drops) were collected and tested for β-galactosidase activity with β-ONPG as the substrate (black circle), for α-galactosidase activity with α-PNPG as the substrate (black triangle), or for absorbance at 280 nm (white circle). Enzyme activities are shown in nmol min^{-1} ml^{-1} based on a single assay of each fraction.

To confirm that these enzyme activities were associated with three different proteins, the proteins in the desalted P80 fraction, the pools of activity from the MacroPrep-DEAE columns, and the Sephadex G-200 columns were separated by nondenaturing gel electrophoresis in polyacrylamide gels (Fig. 3). Exposure of the gels to X-β-galactose resulted in the staining of bands for β-galactosidase I and β-galactosidase II, with apparent native molecular masses of approximately 180 kDa and 120 kDa, respectively. Exposure of the gels to X-α-galactose resulted in staining of a different band for α-galactosidase with an intermediate apparent molecular mass of about 160 kDa. There was almost no detectable β-galactosidase activity in the MacroPrep-DEAE samples that were enriched in α-galactosidase (lanes 7 and 8) but some α-galactosidase in the MacroPrep-DEAE samples that were enriched in β-galactosidase (lanes 9 and 10). In some cases, slower-moving bands with β-galactosidase activity (lane 2) or α-galactosidase activity (lane 11) were visible in samples with high activity.

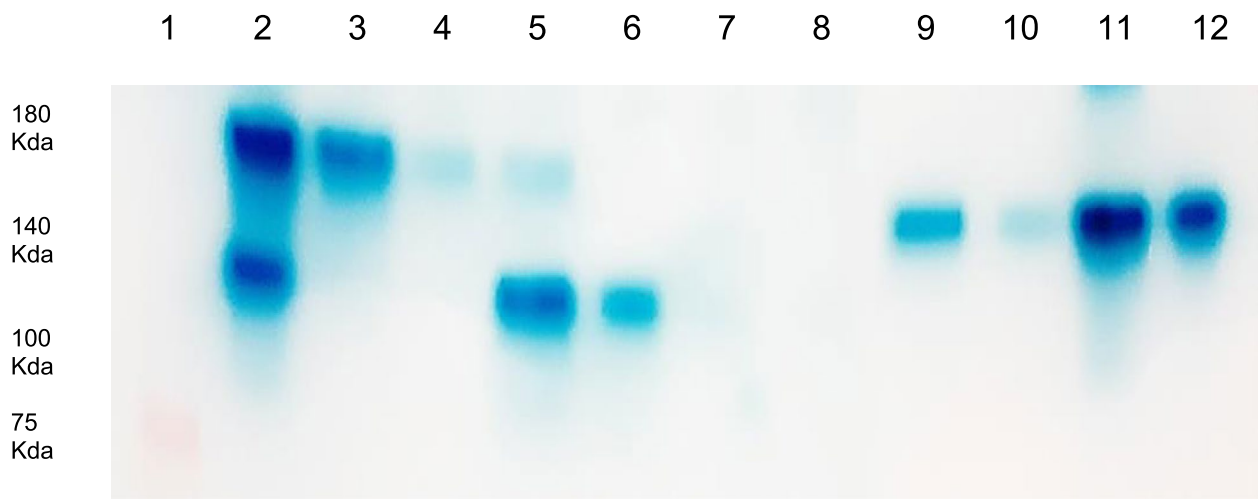

Fig. 3 Nondenaturing gel electrophoresis of *G. dipsosauri* strain DD1 samples after staining with X-β-Gal and X-α-Gal. Samples (10 µl) were combined with nondenaturing sample buffer (10 µl) and separated in a 12-well 4–15% Tris-glycine gel. Samples were as follows: (1) PM2500 molecular markers; (2) desalted P80 fraction; (3) protein pool from MacroPrep DEAE column containing β-galactosidase I activity; (4) fraction 22 from the Sephadex G-200 column using this pool; (5) protein pool from the MacroPrep DEAE column containing β-galactosidase II activity and some α-galactosidase activity; (6) fraction 23 from the Sephadex G-200 column using this pool; (7) protein pool from the MacroPrep-DEAE column containing α-galactosidase activity and some β-galactosidase activity; (8) fraction 21 from the Sephadex G-200 column using this pool; (9) protein pool from the MacroPrep DEAE column containing β-galactosidase II activity and some α-galactosidase activity; (10) fraction 23 from the Sephadex G-200 column using this pool; (11) protein pool from the MacroPrep-DEAE column containing α-galactosidase activity and some β-galactosidase activity; and (12) fraction 21 from the Sephadex G-200 column using this pool. After electrophoresis, the gel was divided into two parts: the part with lanes 1 to 8 was stained with X-β-Gal and the part with lanes 9 to 12 was stained X-α-Gal. The two parts were pushed back together prior to photography. Because of washing and processing, only the red-stained band at 75 kDa in the molecular marker mixtures was clearly visible. The positions of the other markers in lane 1 are indicated in the text box on the left

Table 2 Substrate specificities of Sephadex G-200-purified hydrolytic enzymes

Substrate	β-Galactosidase I	β-Galactosidase II	α-Galactosidase
β-ONP-Gal	100	100	5.6
α-ONP-Gal	2.7	3.1	164.6
β-PNP-Gal	41.6	183.8	3.5
α-PNP-Gal	0.7	2.7	100
β-PNP-Glc	1.	3.1	3.8
α-PNP-Glc	0.4	1.2	0.5
α-PNP-Ara	9.1	41.7	0.6

Activities are expressed as a percentage of the activity with the primary substrate for each enzyme. The activities of the control reaction were 50 nmol min^{-1} ml^{-1} for β-galactosidase I with β-ONPG as the substrate, 43 nmol min^{-1} ml^{-1} for β-galactosidase II with β-ONPG as the substrate, and 127 nmol min^{-1} ml^{-1} for α-galactosidase with α-PNPG as the substrate

Biochemical characterization of β-galactosidase and α-galactosidase activities from *G. dipsosauri*

Measurement of substrate specificity using the Sephadex G-200 purified fractions with various synthetic substrates indicated that β-galactosidase I had good activity with β-ONP-galactose and with β-PNP-galactose (Table 2). There was low activity with α-PNP-L-Arabinose but no activity with any of the glucose-containing substrates. The β-galactosidase II had good activity with β-ONP-galactose and with β-PNP-galactose, but there was more substantial activity with α-PNP-L-arabinose. The α-galactosidase had activity only with α-ONP-galactose and with α-PNP-galactose. The hydrolytic activities with the glucose-containing substrates seen in the crude extract and 50 to 80% ammonium sulfate fraction thus were most likely due to other proteins.

Many β-galactosidases and α-galactosidases have both hydrolytic and transgalactosylation activities. To determine if this applies to the enzymes from *G. dipsosauri*, the proteins from the Sephadex G-200 columns were combined with samples of lactose, D- melibiose, and raffinose. The products were separated by thin-layer chromatography (TLC, Fig. 4). Activity with lactose was low and only visible with a 10% (w/v) substrate solution. Purified β-galactosidase I had both hydrolytic activity, leading to the formation of D-galactose and D-glucose, and transgalactosylation activity, leading to the formation of a mixture of more slowly-moving galactosyl-oligosaccharides. It had no effect on melibiose or raffinose. By contrast, purified β-galactosidase II showed neither hydrolytic nor transgalactosylation activity with lactose; it had no effect on melibiose or raffinose. The purified

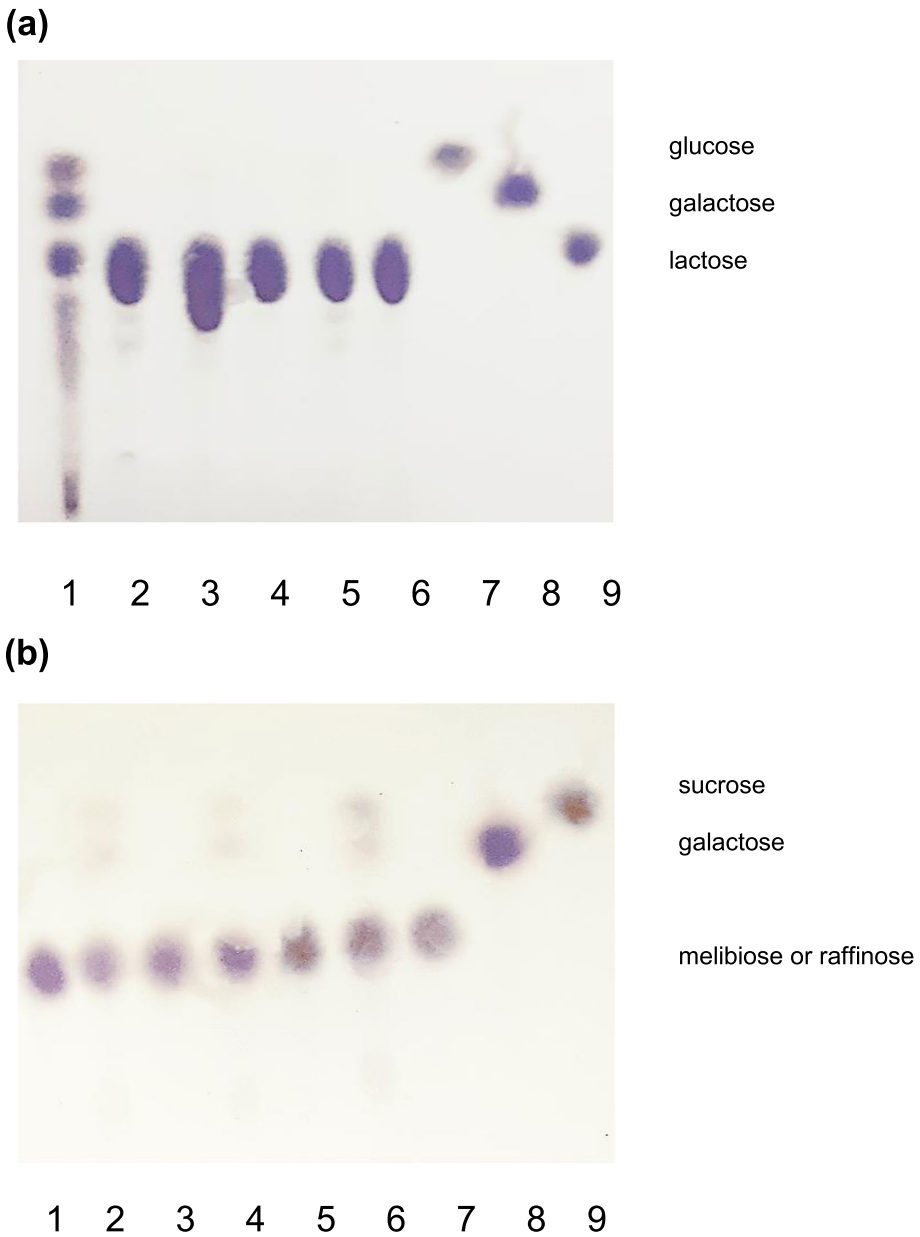

Fig. 4 Thin-layer chromatography of the products of β-galactosidase and α-galactosidase activities from *G. dipsosauri* strain DD1. Small samples (2 µl) were spotted on sheets of Merck Silica Gel F60 and developed in a solvent of *n*-butanol-*n*-propanol-ethanol-water (20:30:30:20). The spots were identified by spraying with an orcinol-sulfuric acid mixture and heating at 60 ° °C. Panel (**a**) shows the results with a 10% (w/v) lactose substrate solution. The samples were as follows: (1) lactose + P80 fraction; (2) lactose + desalted P80 fraction; (3) lactose + MacroPrep DEAE pool containing β-galactosidase I; (4) lactose + MacroPrep DEAE pool containing β-galactosidase II; (5) Sephadex G-200 fraction 16 from MacroPrep DEAE pool containing β-galactosidase I; (6) Sephadex G-200 fraction 21 from the MacroPrep DEAE pool containing β-galactosidase II; (7) 2% (w/v) D-glucose standard; (8) 2% (w/v) D-galactose standard; and (9) 2% (w/v) lactose standard. Panel (**b**) shows the results with 2% (w/v) D-melibiose or D-raffinose substrate solutions. The samples were as follow: (1) 2% (w/v) D-melibiose standard; (2) melibiose + Sephadex G-200 fraction 22 from the MacroPrep-DEAE pool enriched in α-galactosidase; (3) melibiose + Sephadex G-200 fraction 16 from MacroPrep DEAE pool containing β-galactosidase I; (4) melibiose + Sephadex G-200 fraction 21 from the MacroPrep DEAE pool containing α-galactosidase and β-galactosidase II; (5) 2% D-raffinose standard; (6) raffinose + Sephadex G-200 fraction 22 from the MacroPrep-DEAE pool containing α-galactosidase; (7) raffinose + Sephadex G-200 fraction 16 from the MacroPrep-DEAE pool containing β-galactosidase I; (8) 2% (w/v) D-galactose standard; and (9) 2% (w/v) sucrose standard. The D-glucose standard moved to the same position as sucrose but had a brownish color after staining rather than a purplish color

α-galactosidase had hydrolytic activity with 2% (w/v) D-melibiose and D-raffinose, leading to the formation of D-galactose and D-glucose or sucrose. It also had transgalactosylation activity with both sugars but no activity with lactose as the substrate.

Each enzyme activity from *G. dipsosauri* showed simple Michaelis-Menten kinetics (Fig. 5 panel (a)). The calculated K_m values were 0.693 mmol l^{-1} for β-galactosidase I with β-ONP-galactose as the substrate, 1.662 mmol l^{-1} for β-galactosidase II with β-ONP-galactose as the substrate, and 0.338 mmol l^{-1} for α-galactosidase with α-PNP-galactose as the substrate, respectively. The pH optimum curves for the three activities were different but centered around pH 7 (Fig. 5 panel (b)). β-Galactosidase I was more active at alkaline pH while β-galactosidase II was more active at acidic pH. The pH optimum curve for α-galactosidase was narrower but like that of β-galactosidase II. The salt-sensitivities of the three activities were also different (Fig. 5 panel (c)). β-Galactosidase I was partially inhibited by 1.0 mol l^{-1} concentrations of KCl, K_2SO_4, NH_4Cl, and $(NH_4)_2SO_4$. β-Galactosidase II was partially stimulated by $Na_2(SO_4)$ and $K_2(SO_4)$, but more strongly inhibited by NH_4Cl and $(NH_4)_2SO_4$. The α-galactosidase activity was partially inhibited by KCl and NaCl and completely inhibited by NH_4Cl. The three activities were affected in different ways by 10 mmol l^{-1} concentrations of the sugars that can be substrates and products (Fig. 5 panel (d)). β-Galactosidase I was stimulated about 20% by D-glucose while β-galactosidase II was inhibited about 80% by D-galactose and about 20% by D-melibiose. The α-galactosidase activity was inhibited about 20% by lactose and D-melibiose but was not affected by D-raffinose. Similar experiments to test the thermal stability of enzymes indicated that all three proteins were almost completely inactivated after 15 min at 55 °C (data not shown).

Identification of β-galactosidase and α-galactosidase proteins from *G. dipsosauri*

The genome of *G. dipsosauri* strain DD1 has been sequenced (Deutch and Yang 2020) and a search of the UniProtKB database indicates the potential to form nine β-galactosidases (A0A317L1K1, A0A317L6F0, A0A317KXR4, A0A317KZG3, A0A317L660, A0A317L0K1, A0A317KZF9, A0A317KX90, A0A317L417) and four α-galactosidases/α-glucosidases (A0A317KU47, A0A317KWL7, A0A317KVX0, A0A317KUT0). To determine which of these proteins was found in the extracts of *G. dipsosauri* strain DD1 and characterized in these experiments, the proteins in the Sephadex G-200 fractions derived from the MacroPrep-DEAE purification were concentrated and subjected to electrophoresis in both nondenaturing polyacrylamide gels (Fig. 6 panel (a)) and denaturing SDS-polyacrylamide gels (Fig. 6 panel (b)). There were multiple bands in each sample after staining with Coomassie Blue.

The gel segments in the nondenaturing gels corresponding to each activity as seen after staining with to X-β-galactose or X-α-galactose were excised and subjected to in-gel trypsin digestion and HPLC/mass spectrometry analysis at MSBioworks (Ann Arbor, MI, USA). Assembly of the peptides from the gel slice containing β-galactosidase I activity indicated unambiguously that it corresponds to protein A0A317L6F0 in the UniProtKB database (1034 amino acids, molecular mass of 119,214, encoded by gene DLJ74_04930, also designated PWU69329). The gel slice containing β-galactosidase II activity contained a mixture of peptides associated with several different proteins. However, those related to beta-galactosidase clearly indicated that it corresponds to protein A0A317KZG3 (684 amino acids, molecular mass of 78,122, encoded by gene DLJ74_12640, also designated PWU67948). Assembly of the peptides from the gel slice containing α-galactosidase activity indicated that it unambiguously corresponds to protein A0A317KU47 (742 amino acids, molecular mass of 85,657, encoded by gene DLJ74_17745, also designated PWU67062). Coomassie-Blue stained bands of the expected subunit sizes were apparent in the denaturing SDS-PAGE gel, although some unrelated proteins were still present as well.

(See figure on next page.)

Fig. 5 Biochemical characteristics of β-galactosidase I (white circle), β-galactosidase II (white triangle) and α-galactosidase (white square) from *G. dipsosauri* strain DD1. Partially-purified proteins from the DEAE-Sepharose column were tested in triplicate using β-ONPG as the substrate for β-galactosidase and α-PNPG as the substrate for α-galactosidase activity, respectively. Data points show the mean ± one standard deviation of each assay. Panel (**a**) shows Lineweaver-Burke plots of a kinetic analysis in which the concentration of the substrate was varied. Linear regression lines were fitted to each set of data points using Excel and both the equation and R^2 value for each line are shown. Panel (**b**) shows the effects of pH on each enzyme activity using Z buffers adjusted to the pH indicated. The control activities (pH 7.0) were 143 nmol min^{-1} ml^{-1} for β-galactosidase I, 196 nmol min^{-1} ml^{-1} for β-galactosidase II, and 677 nmol min^{-1} ml^{-1} for α-galactosidasePanel (**c**) shows the effects of added salts (1.0 mol l^{-1}) on the β-galactosidase I (open bars), β-galactosidase II (light bars), and α-galactosidase (dark bars) activities. The control activities were 132 nmol min^{-1} ml^{-1} for β-galactosidase I, 163 nmol min^{-1} ml^{-1} for β-galactosidase II, and 685 nmol min^{-1} ml^{-1} for α-galactosidase. Panel (**d**) shows the effects of added sugars (10 mmol l^{-1}) on the β-galactosidase I (open bars), β-galactosidase II (light bars), and α-galactosidase (dark bars) activities. The control activities were 229 nmol min^{-1} ml^{-1} for β-galactosidase I, 191 nmol min^{-1} ml^{-1} for β-galactosidase II, and 692 nmol min^{-1} ml^{-1} for α-galactosidase

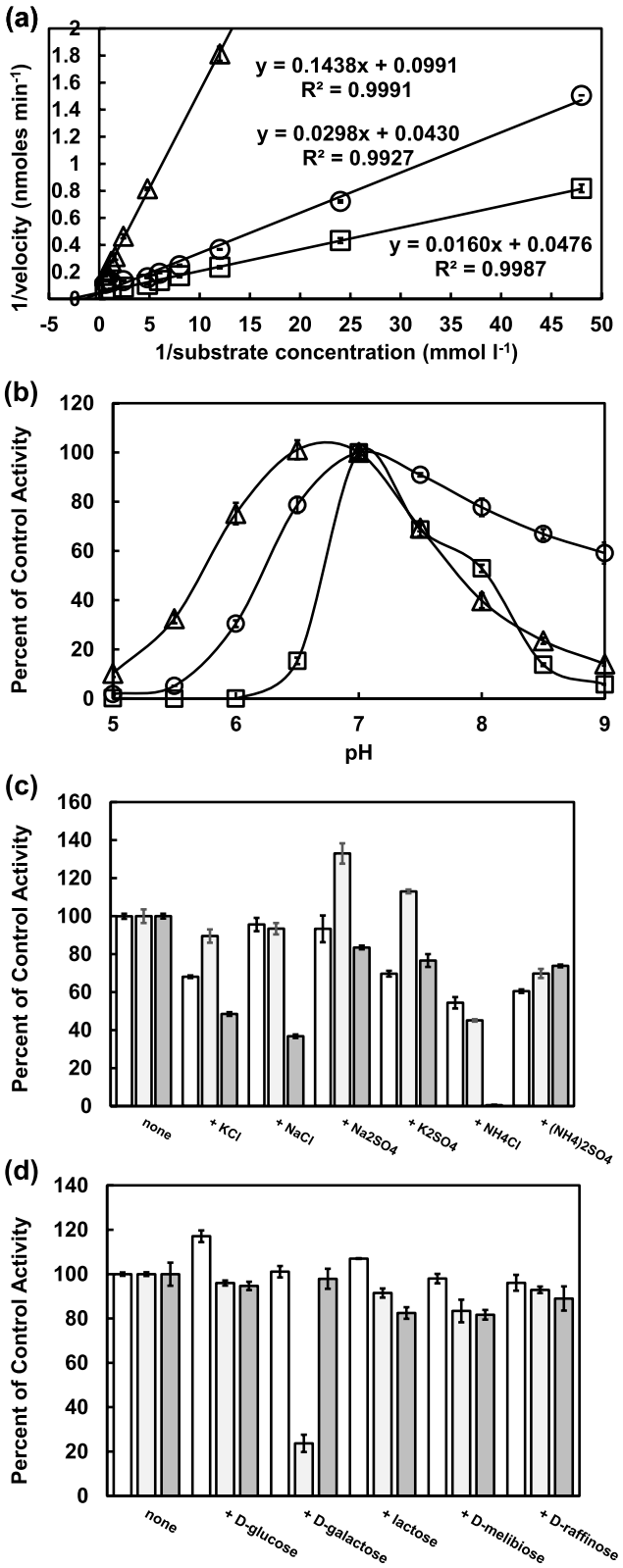

Fig. 5 (See legend on previous page.)

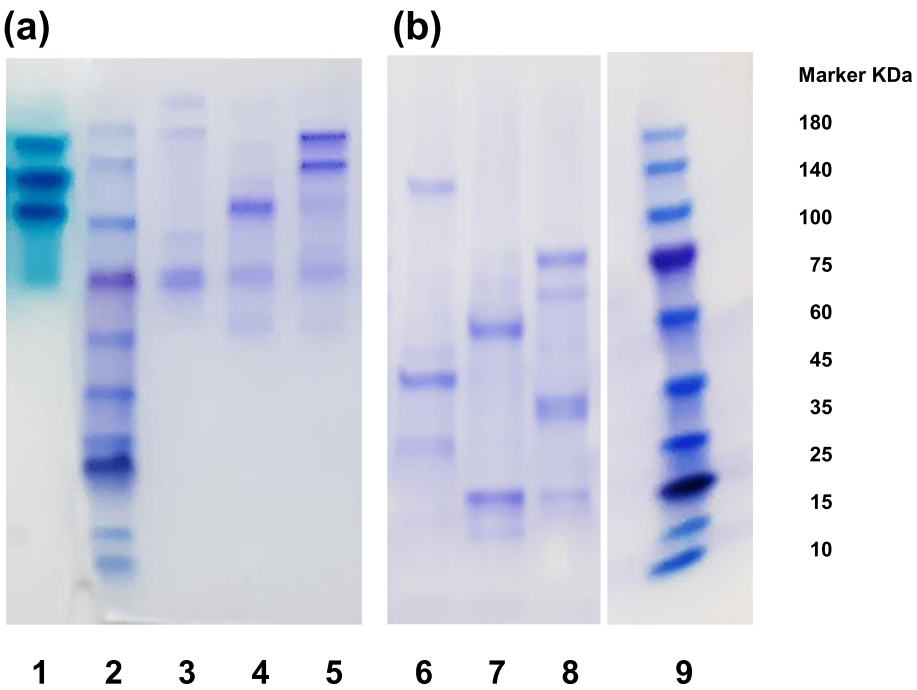

Fig. 6 Gel electrophoresis of proteins from the purification of β-galactosidase and α-galactosidase activities from *G. dipsosauri* strain DD1. Panel (**a**) shows a nondenaturing 4–15% polyacrylamide gel. The samples were as follows: (1) a mixture of proteins from the Sephadex-G-200 columns stained for β-galactosidase activity with X-β-Gal and for α-galactosidase with X-α-Gal; (2) PM2500 molecular markers—the sizes of these proteins are indicated in the text box to the right of panel (**b**); (3) concentrated MacroPrep-DEAE pool containing β-galactosidase I; (4) concentrated MacroPrep-DEAE pool containing β-galactosidase II; (5) concentrated MacroPrep-DEAE pool containing α-galactosidase. Panel (**b**) shows the corresponding 4-15% denaturing (SDS-PAGE) gel. The samples were as follows: (6) concentrated MacroPrep-DEAE pool containing β-galactosidase I; (7) concentrated MacroPrep-DEAE pool containing β-galactosidase II; (8) concentrated MacroPrep-DEAE pool containing α-galactosidase; and 9) PM2500 molecular markers. The two gels were run separately and the proteins in lanes 2 to 9 stained with Coomassie Blue. The images of the relevant parts of the two gels were aligned so that the molecular markers matched as much as possible

Table 3 Summary of BLAST analysis of *G. dipsosauri* protein sequences with those of other *Gracilibacillus* species

***G. dipsosauri* sequence**	**Bacterium**	**Sequence**	**Percent identity**
β-Galactosidase I A0A317L6F0	*G. ureilyticus*	A0A1H9PUC9	70.6
	G. orientalis	A0A1I4ICB3	67.6
	G. kekensis	A0A1M7N3A6	67.2
β-Galactosidase II A0A317KZG3	*G. ureilyticus*	A0A1H9T22	84.3
	G. orientalis	A0A1I4NW10	82.7
	G. kekensis	A0A1M7KQE9	82.6
α-Galactosidase A0A317KU47	*G. kekensis*	A0A1M7KL20	80.2
	G. ureilyticus	A0A1H9SYD0	79.9
	G. orientalis	A0A1I4NSX4	78.6
	G. halophilus	N4WNP7	78.5

To determine if there are similar proteins in other species of *Gracilibacillus*, a BLAST analysis was done using the UniProtKB database and the existing genomic sequences (Table 3) There are homologs to the β-galactosidase I activity of *G. dipsosauri* strain DD1 (A0A317L6F0) in *G. ureilyticus*, *G. orientalis*, and *G. kekensis*. There are homologs to the β-galactosidase II activity of *G. dipsosauri* strain DD1 (A0A317KZG3) in *G. ureilyticus*, *G. orientalis*, and *G. kekensis*. There are homologs to the α-galactosidase activity in *G. dipsosauri* strain DD1 (A0A317KU47) in these same bacteria as well as in *G. halophilus*.

Discussion

The hydrolysis of synthetic substrates containing nitrophenyl moieties is commonly used in the characterization of bacteria. These experiments indicated that positive tests for the hydrolysis of β-ONP-galactose (*o*-nitrophenyl-β-D-galactopyranoside, 2-nitrophenyl-β-D-galactopyranoside) and α-PNP-galactose (*p*-nitrophenyl-α-D-galactopyranoside, 4-nitrophenyl-α-D-galactopyranoside) by *G. dipsosauri* strain DD1 were due to the presence of two different β-galactosidases and an α-galactosidase. The three activities could be separated by ion-exchange chromatography on

DEAE-Sepharose or MacroPrep-DEAE columns and further purified by gel-filtration chromatography on a Sephadex G-200 column. β-Galactosidase I activity was found to be due to the protein A0A317L6F0 and β-galactosidase II activity to the protein A0A317KZG3. Both proteins appeared to occur in nondenaturing gels as dimers with molecular masses about twice those predicted for each protein from its amino acid sequence. Formation of both proteins was repressed by the presence of D-glucose in the growth medium but was not induced by lactose. β-Galactosidase I had both hydrolytic and transgalactosylation activity with lactose as the substrate, but β-galactosidase II showed no activity towards lactose. β-Galactosidase I had lower K_m with β-ONP-galactose as the substrate than β-galactosidase II, was active at more alkaline pH, and inhibited by the product D-galactose. β-Galactosidase II was active at more acidic pH and showed higher activity with α-PNP-Arabinose as an alternative substrate. The hydrolysis of α-arabinosides by β-galactosidases has been observed by others (Li et al. 2001). The α-galactosidase activity was found to be due to the protein A0A317KU47, which also appeared to occur as a dimer in nondenaturing gels. Formation of this enzyme was repressed by D-glucose but somewhat induced by D-raffinose. The enzyme showed both hydrolytic and transgalactosylation activity with D-melibiose and D-raffinose. It had a low K_m with α-PNP-galactose as the substrate, a pH optimum of about 7, and was inhibited by various chloride-containing salts.

There is a very extensive literature on β-galactosidases and α-galactosidases, and the PubMed database contains thousands of papers about them. The UniProtKB database (www.expasy.org) has 385 reviewed and 114,896 unreviewed β-galactosidase protein sequences and 151 reviewed and 68,938 unreviewed α-galactosidase protein sequences. These proteins vary in length and fall into different families of glycosyl hydrolases (Cantarel et al. 2009). The β-galactosidase I from *G. dipsosauri* is predicted to belong to the glycosyl hydrolase 2 family while the β-galactosidase II to the glycosyl hydrolase 42 family; the α-galactosidase is predicted to belong to glycosyl hydrolase family 36. The basic mechanism of hydrolysis of the glycosidic bond is believed to involved acid/base catalysis (Matthews 2005). To determine if the proteins from *G. dipsosauri* share this mechanism, the amino acid sequences of the three proteins described in this paper were aligned with other known proteins. The best studied example of a β-galactosidase is the LacZ protein of *Escherichia coli* (glycosyl hydrolase family 2, Juers et al. 2012). The key residues that are thought to participate in catalysis of LacZ include Glu537 and Glu461, but other residues such as Asp201, Glu416, His357, His391, His418, His540, Phe601, Trp568, Trp999, Tyr100, and Tyr503 are used to bind hydroxyl groups, water, or monovalent or divalent cations. The sequence of the β-galactosidase I from *G. dipsosauri* was aligned with the LacZ protein of *E. coli* and the sequence from the unrelated but industrially important Gram-positive bacterium *Lactobacillus delbrueckii* (glycosyl hydrolase family 2). The results are shown in Supplementary Fig. 3, part A. The three sequences showed 21.8% identity, and in particular, all of the key residues thought to contribute to binding and catalysis in the *E. coli* LacZ enzyme were conserved. By contrast, the β-galactosidase II from *G. dipsosauri* showed only 11.2% identity to the *E. coli* LacZ protein and most of the key residues needed for catalysis in *E. coli* were not apparent. The proteins are very different in size (1024 amino acids vs. 684 amino acids) and so the β-galactosidase II from *G. dipsosauri* was aligned with the YesZ sequence from *Bacillus subtilis* (glycosyl hydrolase family 42) which has 663 amino acids (Supplementary Fig. 3, part B). In this case, there was 23.1% identity, which included residues Glu145 and Glu295 that have been shown to be key to acid/base catalysis in this enzyme (Shaikh et al. 2007). The α-galactosidase from *G. dipsosauri* showed little sequence homology to the MelA α-galactosidase protein from *E. coli* but 43.3% identity to the MelA protein from *Lactobacillus acidophilus* (glycosyl hydrolase family 36). When these sequences were aligned, there was 43.3% identity and all of the key catalytic residues in the *Lactobacillus* protein (Fredslund et al. 2011) also occur in the α-galactosidase from *G. dipsosauri* (Supplementary Fig. 3, part C). These included Asp552, Asp371, Asp482, Arg447, Cys530, Glu608, Gly533, Lys480, Trp418, and Trp549. Thus, while *G. dipsosauri* is highly salt-tolerant and the proteins described here exhibit some unusual properties, their basic mechanism of action seems to be the same as that found in other members of the same glycosyl hydrolase families in which they occur.

The enzymes from *G. dipsosauri* described here were found entirely inside the bacteria, so it was not too surprising that they exhibited less tolerance to extreme conditions than the previously described α-amylase from *G. dipsosauri* strain DD1 (Deutch 2002). β-Galactosidase I was partially inhibited by 1.0 mol l^{-1} concentrations of some salts. β-Galactosidase II was partially stimulated by $Na_2(SO_4)$ and $K_2(SO_4)$, but more strongly inhibited by NH_4Cl and $(NH_4)_2SO_4$. The α-galactosidase activity was partially inhibited by KCl and NaCl and completely inhibited by NH_4Cl. None of the enzymes functioned well at extreme pH or temperatures. Accordingly, their potential use for industrial applications may be more limited than the corresponding extracellular enzymes from other sources (Patil et al. 2010; Chauhan et al. 2015; Thongaram et al. 2017; Martarello et al. 2019).

The nucleotide sequence of the genome of *G. dipsosauri* strain DD1 has been determined (Deutch and Yang 2020) and contains predicted sequences that may encode seven other β-galactosidases and three other α-galactosidases. It is not clear why there are so many genes for glycosyl hydrolases in this bacterium. A system for producing genetic mutants of *Gracilibacillus* is not currently available and it is possible that other proteins might be expressed if the genes for the enzymes identified here were altered or eliminated. *G. dipsosauri* strain DD1 cannot yet be grown in a chemically defined medium and it is also possible that other enzymes are expressed under different growth conditions. The genomes of about 10 other *Gracilibacillus* species have been determined (Deutch and Yang 2020). A BLAST analysis of the protein sequences from *G. dipsosauri* indicated that similar β-galactosidase proteins may occur in *G. ureilyticus*, *G. orientalis*, *G. kekensis*. Related α-galactosidases may also occur in these three species as well as in *G. halophilus*. For these and other bacteria that show β-ONP-galactose or α-PNP-galactose hydrolysis, it will be important to determine which proteins with β-galactosidase or α-galactosidase activity are expressed and may be useful for biotechnology applications.

Conclusions

Hydrolysis of β-ONP-galactose or α-PNP-galactose is commonly used to differentiate among new species of *Gracilibacillus* and often employed in the characterization of other bacteria. The results shown here indicate that the proteins with β-galactosidase or α-galactosidase activity which carry out these reactions in *G. dipsosauri* differ in their biochemical properties but are not inducible by their corresponding substrates. Because they are intracellular enzymes, their potential for industrial applications may be more limited than the corresponding extracellular enzymes. The observation that there are multiple genes for these enzymes within the genome of *G. dipsosauri* suggests that further biochemical and evolutionary studies of these bacteria would be useful.

Methods

Bacteria and growth conditions

G. dipsosauri strain DD1 was stored as frozen glycerol-treated cultures at − 20 °C and maintained on plates of tryptic soy broth (Becton Dickinson and Company) containing 1.0 mol l^{-1} KCl and 1.5% (w/v) agar. The bacteria were routinely grown at 37 °C with aeration in tryptic soy broth without dextrose (Becton Dickinson and Company) containing 1.0 mol l^{-1} KCl in a shaker-incubator at 250 rpm.

Enzyme assays

Synthetic substrates were obtained from Sigma-Aldrich or from Gold Biotechnology. β-Galactosidase and α-galactosidase activities were measured in reaction mixtures containing 5 to 50 µl of extract or enzyme fraction in Z buffer containing per liter: 16.1 g $Na_2HPO_4.7H_2O$, 5.5 g $NaH_2PO_4.H_20$, 0.75 g KCl, 0.246 MgSO4.$7H_2O$, 16.1 mg dithiothreitol (Miller 1972) to give a total volume of 1000 µl. After equilibration to 37 °C, reactions were started by the addition of 200 µl of a 10 mmol l^{-1} substrate solution and stopped when a pale-yellow color had developed by addition of 500 µl of 1.0 mol l^{-1} sodium carbonate. The absorbance of the reactions was measured in a Shimadzu U-160 spectrophotometer at 420 nm (for *o*-nitrophenyl-containing substrates) or 405 nm (for *p*-nitrophenyl-containing substrates). The amount of product was quantitated using a *o*-nitrophenol or *p*-nitrophenol standard curve in Z buffer and sodium carbonate. Enzyme assays were normally done in triplicate and varied by < 10%. The results are reported as the mean ± one standard deviation.

Whole cell assays

To measure the β-galactosidase or α-galactosidase activities in whole cells, *G. dipsosauri* strain DD1 was grown to exponential phase at 37 °C with aeration in tryptic soy broth without dextrose containing 1.0 mol l^{-1} KCl. The medium was modified by the addition of different carbohydrates as specified in each experiment. The bacteria were harvested by centrifugation at 10,000 × *g* in a Bio-Lion XC-H165 centrifuge, washed once with 0.85% (w/v) NaCl, resuspended in 0.85% (w/v) NaCl, and frozen at − 20 °C. After thawing, the cells were treated with 0.005% (v/v) Triton X-100 to increase their permeability to the substrate prior to the enzyme assays. All whole cell experiments were done at least twice.

Preparation of cell extracts

G. dipsosauri strain DD1 was grown to exponential phase at 37 °C with aeration in 1000 ml of tryptic soy broth without dextrose containing 1.0 mol l^{-1} KCl (Deutch 1994; Deutch and Yang 2020). The bacteria were harvested by centrifugation at 10,000 × *g* in a Bio-Lion XC-H165 centrifuge for 10 min, washed once with 0.85% (w/v) NaCl, resuspended in 0.85% (w/v) NaCl, and frozen at − 20 °C. The cell pellet was thawed, resuspended in Z buffer, and the bacteria were disrupted by agitation with 0.1-mm glass beads in a Bead Beater (Biospec Products) for 6 one-minute cycles (Deutch 2011; Deutch 2019). Unbroken bacteria and beads removed by centrifugation in a Bio-Lion XC-H165 centrifuge first at 2000 × *g* for 5 min and then at 5000 × *g* for 15 min. Solid enzyme-grade ammonium sulfate was slowly added to the crude extract

to give 50% saturation (Burgess 2009) and the suspension centrifuged in a Bio-Lion XC-H165 centrifuge at 10,000 × *g* for 15 min. The supernatant was saved and more ammonium sulfate added to give an 80% saturated solution (Burgess 2009). The suspension was centrifuged in a Bio-Lion XC-H165 centrifuge at 10,000 × *g* for 15 min. The ammonium sulfate precipitate that contained both enzyme activities was resuspended in a small volume of Z buffer and saved as the P80 fraction at 4 °C. All experiments with cell extracts were done at least three times.

Partial purification of enzyme activities

To purify the proteins with β-galactosidase and α-galactosidase activity, portions of the P80 fraction were applied to a 2.5 × 20-cm column of Bio-Gel P-6 desalting gel (Bio-Rad Laboratories) and 100-drop fractions collected during elution with Z buffer. The fractions were tested for both enzyme activities and the active ones pooled as the desalted P80 fraction. To further purify the enzymes of interest, 3- to 4-ml portions of the desalted P80 fraction were applied to a 1.5 × 12 cm column of DEAE-Sepharose (GE Healthcare) or a 1.5 × 24-cm column of MacroPrep-DEAE (Bio-Rad Laboratories) equilibrated with Z buffer. Unbound proteins were eluted as series of 100-drop fractions with Z buffer. Bound proteins then were eluted as additional 100-drop fractions with 20 ml or 30 ml portions of Z buffer containing 0.2, 0.4, 0.6, 0.8, or 1.0 mol l^{-1} KCl. Fractions with each activity pooled and saved. The active pools then were subjected to gel-filtration chromatography of a 1.5 × 45-cm column of Sephadex G-200 (Pharmacia). Proteins were eluted with Z buffer as a series of 50-drop fractions and those with β-galactosidase and α-galactosidase activity saved for further analysis. Enzyme purification experiments were done at least three times.

Thin layer chromatography analysis of sugar hydrolysis

Fractions (10 μl) from the ammonium sulfate fractionation, an ion-exchange column, or a gel filtration column were combined 100 μl of a 10% (w/v) or 2% (w/v) sugar solution and incubated at 37 °C for 24 or 48 h. Samples (2 μl) of the mixtures along with untreated standard sugar solutions were spotted on Merck Silica Gel F60 thin layer chromatography sheets (Splechtna et al. 2006). The chromatograms were developed in a solvent of *n*-butanol-*n*-propanol-ethanol-water (20:30:30:20). The spots were identified by spraying with an orcinol-sulfuric acid mixture (180 mg, 5 ml water, 75 ml sulfuric acid) and heating at 60 °C. TLC analysis was done three times.

Gel electrophoresis

Proteins in cell extracts or partially purified fractions were analyzed by polyacrylamide gel electrophoresis (Hames 1998). Dilute samples from the Sephadex G-200 columns were first concentrated about 25-fold using Nanosep 10K Omega (Pall Corporation) centrifugal filter devices. For nondenaturing (native) gel electrophoresis, samples (10 μl) were combined with 10 μl of a 2× sample buffer containing 62.5 mmol l^{-1} Tris-HCl buffer, pH 6.8, 25% (v/v) glycerol, and 0.01% (w/v) bromphenol blue. For denaturing gel electrophoresis, samples (10 μl) were combined with 10 μl of a similar 2× sample buffer that contained 2% (w/v) SDS and 5% (v/v) 2-mercaptothanol. The native gel samples were not heated but the denatured gel samples were heated at 100 °C for 5 min. The proteins were separated on 4-15% SMOBIO Q-PAGE (QP-4510) precast gels in Tris-glycine electrophoresis buffer (3.0 g l^{-1} glycine, 14.4 g l^{-1} Tris base, pH 9) without or with 0.1% (w/v) SDS. A mixture of prestained proteins (SMOBIO PM2500) was used as molecular mass markers. For nondenaturing gel electrophoresis, the protein bands containing β-galactosidase or α-galactosidase activity were identified by washing the gel with Z buffer and overlaying it with a 20 mg ml^{-1} solution of X-β-Gal (5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside) or X-α-Gal (5-bromo-4-chloro-3-indoxyl-α-D-galactopyranoside) in dimethylsulfoxide. For both nondenaturing and denaturing gel electrophoresis, the other proteins in the gels were stained with Coomassie Blue in acetic acid and methanol (Ausubel et al. 1995). Gels were photographed with a Samsung A20 cell phone camera and the images pasted into Word documents.

Protein identification

Coomassie Blue-stained bands corresponding to the β-galactosidase and α-galactosidase activities in nondenaturing polyacrylamide gels were excised and sent to MSBioworks (Ann Arbor, MI) for peptide analysis. Briefly, the samples were subjected to in-gel trypsin digestion followed by nano-LC-MS/MS analysis with a Waters M-Class HPLC system interfaced with a ThermoFisher Lumos. Peptide data were analyzed using Scaffold proteome software and the proteins identified using the UniProtKB database.

Protein assays

Protein concentrations were determined by the bicinchoninic acid method (Smith et al. 1985) using reagents from Gene and Cell Technologies and bovine serum albumin as the standard.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13213-021-01657-1.

Additional file 1: Supplementary Fig. 1. Specific activities [nmol min^{-1} (mg protein)$^{-1}$] of β-galactosidase and α-galactosidase in whole cells of *G. dipsosauri* strain DD1 after growth to exponential phase in tryptic soy broth without dextrose containing 1.0 mol l^{-1} KCl. Bacteria were harvested, washed with 0.85% NaCl, resuspended in 0.85% NaCl to give a suspension with 100 Klett Units, frozen, and thawed. Cells were treated with 0.005% Triton X-100 prior to enzyme assays with β-ONPG (*o*-nitrophenyl-β-D-galactopyranoside, 2-nitrophenyl-β-D-galactopyranoside), β-PNPG (*p*-nitrophenyl-β-D-galactopyranoside, 4-nitrophenyl-β-D-galactopyranoside), α-ONPG (*o*-nitrophenyl-α-D-galactopyranoside, 2-nitrophenyl-α-D-galactopyranoside), and α-PNPG (*p*-nitrophenyl-α-D-galactopyranoside, 4-nitrophenyl-α-D-galactopyranoside) as substrates. Data show the means and standard deviations of assays from one of several experiments. **Supplementary Fig. 2.** Gel filtration chromatography of *G. dipsosauri* strain DD1 β-galactosidase and α-galactosidase activities from the ion-exchange columns. 2-3 ml of the pooled proteins from the MacroPrep-DEAE column with β-galactosidase I activity [panel (a)], β-galactosidase II activity [panel (b)], and α-galactosidase activity [panel (c)] were applied to a column of Sephadex G-200 and eluted with Z buffer. Fractions (50 drop) were tested for β-galactosidase activity with β-ONPG as the substrate (●) or for α-galactosidase activity with α-PNPG as the substrate (▲). Enzyme activities are shown in nmol min^{-1} ml^{-1} based on a single assay of each fraction. **Supplementary Fig. 3.** Alignment of the amino acid sequences of the purified enzymes from *G. dipsosauri* with similar sequences from other bacteria in the same glycosyl hydrolase families. Part A shows the alignment of β-galactosidase I from *G. dipsosauri* (A0A317L6F0) with the LacZ sequences from *Escherichia coli* (P00722) and *Lactobacillus delbrueckii* (A0A061CJR5). Part B shows the alignment of β-galactosidase II from *G. dipsosauri* (A0A317KZG3) with the YesZ sequence from *Bacillus subtilis* (O31529). Part C shows the alignment of α-galactosidase from *G. dipsosauri* (A0A317KU47) with the MelA protein from *Lactobacillus acidophilus* (G1UB44). In each case, a * below the sequences indicates identical residues and : or . indicate functionally similar residues. The residues thought to be essential for substrate binding and catalysis are highlighted in yellow and identified above the sequences.

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Authors' contributions
Charles E. Deutch did all the microbiological and biochemical experiments included in this paper and wrote the manuscript. Amy M. Farden and Emily S. DiCesare did the initial studies of the β-galactosidase activity in strain DD1 as undergraduate students at Elmira College and their work was presented as a poster at the 102nd General Meeting of the American Society for Microbiology, Salt Lake City, Utah, May 2002. The authors read and approved the final manuscript.

Availability of data and materials
Original data found in lab notebooks can be obtained from Dr. Charles E. Deutch. There are no large data sets.

Declarations

Ethics approval and consent to participate
Not applicable: no animals or human subjects were used in this project.

Consent for publication
All of the authors consent to the publication of this manuscript in Annals of Microbiology.

Competing interests
The authors declare no competing interests in publishing this manuscript.

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