

RESEARCH ARTICLE

Characterization of the GH13 and GH57 glycogen branching enzymes from *Petrotoga mobilis* SJ95 and potential role in glycogen biosynthesis

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OPEN ACCESS

Citation: Zhang X, Leemhuis H, van der Maarel MJEC (2019) Characterization of the GH13 and GH57 glycogen branching enzymes from *Petrotoga mobilis* SJ95 and potential role in glycogen biosynthesis. PLoS ONE 14(7): e0219844. <https://doi.org/10.1371/journal.pone.0219844>

Editor: Israel Silman, Weizmann Institute of Science, ISRAEL

Received: April 15, 2019

Accepted: July 3, 2019

Published: July 15, 2019

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: The authors received no specific funding for this work.

Competing interests: The authors have declared that no competing interests exist.

Abstract

Glycogen is a highly branched α -glucan polymer widely used as energy and carbon reserve by many microorganisms. The branches are introduced by glycogen branching enzymes (EC 2.4.1.18), that are classified into glycoside hydrolase families 13 (GH13) and 57 (GH57). Most microorganisms have typically only a single glycogen branching enzyme (*gbe*) gene. Only a few microorganisms carry both GH13 and GH57 *gbe* genes, such as *Petrotoga mobilis* and *Mycobacterium tuberculosis*. Here we report the basic characteristics of the GH13 and GH57 GBE of *P. mobilis*, both heterologously expressed in *E. coli*. The GH13 GBE has a considerably higher branching activity towards the linear α -glucan amylose, and produces a highly branched α -glucan with a high molecular weight which is very similar to glycogen. The GH57 GBE, on the contrary, makes a much smaller branched α -glucan. While the GH13 GBE acts as a classical glycogen branching enzyme involved in glycogen synthesis, the role of GH57 GBE remains unclear.

Introduction

The phylum *Thermotogae* is currently composed of 50 species spread across 13 genera. All its members have a characteristic outer membrane that lies loosely around the cells. The genera are grouped into 5 families [1, 2]: (i) *Fervidobacteriaceae*, comprising the genera *Fervidobacterium* [1] and *Thermosipho* [3]; (ii) *Kosmotogaceae*, comprising the genera *Kosmotoga* [4] and *Oceanotoga*; (iii) *Mesoaciditogaceae*, comprising the genera *Mesoaciditoga* [5, 6] and *Athalassotoga*; (iv) *Petrotogaceae*, comprising the genera *Petrotoga* [7], *Defluviitoga* [8], *Geotoga* [7], *Marinitoga* [9] and *Oceanotoga* [10]; and (v) *Thermotogaceae*, comprising the genera *Thermotoga* and *Pseudothermotoga* [2]. These thermophilic bacteria live in hot, anaerobic environments, such as hot springs, the deep-sea floor, or oil reservoirs. Thirty-six genome sequences of members of the *Thermotogae* phylum have been reported in the last few years covering all five families. All the genomes contain the key enzymes for glycogen synthesis, namely ADP-

glucose pyrophosphorylase (GlgC, EC 2.7.7.27), glycogen synthase (GlgA, EC 2.4.1.11), and glycogen branching enzyme (GlgB, EC 2.4.1.18), together catalyzing the conversion of glucose-1-phosphate into glycogen [11].

Glycogen is an intracellular α -glucan reserve polymer of many microorganisms and eukaryotes [11]. It is composed of anhydroglucose residues with approximately 90% α -1,4-glycosidic linkages and branched by approximately 10% α -1,6-glycosidic linkages. In many bacteria, glycogen is the major reserve polymer accumulated during exponential growth [11]. Besides an energy reserve, glycogen can have other functions in bacteria: as a protectant of proteins and membranes [12], as a transcriptional regulator [13], as a structural constituent in the cell wall [14], and as a regulatory molecule in glucose metabolism [15]. Glycogen branching enzyme (GBE), being one of the key enzymes in glycogen synthesis, cleaves an α -1,4-glycosidic linkage in a growing α -1,6 glucan chain and subsequently attaches the cleaved-off fragment onto the C6 hydroxyl of the anhydroglucose moiety within an α -1,4 glucan chain [11, 16] *i.e.* the branch. All GBEs known so far are found in the glycoside hydrolase families 13 (GH13) and 57 (GH57) [17, 18].

Whereas most bacteria only have either a GH13 or a GH57 GBE, some bacteria have two GBE encoding genes, one GH13 and one GH57 [19]. An example of this is *Mycobacterium tuberculosis*, the causative agent of tuberculosis, a disease killing more than one million people each year, which has a GH13 and a GH57 GBE [20, 21]. The genomes of different *M. tuberculosis* strains have been sequenced and almost all these strains contain both a GH13 and a GH57 *gbe* indicating that both enzymes are required (21). Deletion of the GH13 *gbe* (Rv1326c) of *M. tuberculosis* did not give viable mutants, indicating an essential role for the GH13 GBE [22]. The putative GH57 GBE (Rv3031) has only been predicted from protein sequence homology [20], and has so far not been characterized; it has been proposed that this protein plays a role in lipopolysaccharide synthesis [23, 24].

In the genomes of the members of the *Petrotoga*aceae, except *Geotoga petraea*, *Petrotoga mexicana*, and *Petrotoga sibirica*, two *gbe* genes are present, one encoding a GH13 GBE and one a GH57 GBE. Several GH13 and GH57 GBEs have been characterized in detail [20, 21, 25–28]. However, characterization of the GH13 and GH57 GBEs from a single species has not been reported and thus it is not clear if both GBEs play a role in glycogen synthesis or only one of the two, as is suggested for *M. tuberculosis*. In this work, the genes encoding the putative GH13 and GH57 GBE from *P. mobilis* were overexpressed and both enzymes were shown to be functional GBEs *in vitro*. PmGBE13 (GH13) shows 130 folds higher activity than PmGBE57 (GH57) with long chain amylose V as substrate. These two GBEs also differ in the degree of branching of the final branched α -glucans; the PmGBE57 produces an 8.5% branched product while the PmGBE13 makes a 12.4% branched product with more short chains.

Materials and methods

Materials

Amylose V was provided by Avebe (Veendam, Netherlands). Lithium bromide was obtained from Acros Organics. Isoamylase (EC 3.2.1.68, specific activity 260 U/mg), pullulanase M1 (EC 3.2.1.41, specific activity 34 U/mg) and β -amylase (EC 3.2.1.2, specific activity 10,000 U/mL) were obtained from Megazyme (Wicklow, Ireland). DHBS was obtained by debranching highly branched starch (HBS, 8% α -1,6-linkages) with isoamylase and pullulanase; HBS was obtained by modifying gelatinized potato starch with the Branchzyme, an enzyme preparation produced by Novozymes (Bagsvaerd, Denmark) containing the glycogen branching enzyme from *Rhodothermus obamensis*. The oligosaccharide kit was purchased from Sigma-Aldrich (Zwijndrecht, Netherlands).

Sequence collection

The sequences of GH13 GBEs from *Thermotoga* bacteria were collected based on the preliminary analysis of family GH13_9, from the CAZy database [29]. The sequences of GH57 GBEs were collected based on a BLAST search using the complete sequences of the specified GBEs from *Thermus thermophilus* (UniProt accession no. Q5SH28) and *Thermococcus kodakarensis* KOD1 (UniProt accession no. Q5JDJ7) with the genome sequences of *Thermotoga* bacteria. All the selected potential sequences had to possess the GH57 characteristic signatures, such as five conserved sequence regions (CSRs), both catalytic residues and a $(\beta/\alpha)_7$ barrel domain [18].

Expression and purification of PmGBE13 and PmGBE57

Codon optimized genes (*glgB13* and *glgB57*) encoding the GH13 (PmGBE13) and GH57 (PmGBE57) GBE of *P. mobilis* SJ95 were synthesized by GenScript, and cloned into the *NdeI*-*Bam*HI sites of the pET28a vector (Novagen), with a 6×His-tag fused at the C-terminal. Sequence details are provided in the supplemental information. *glgB13* and *glgB57* were over-expressed in *Escherichia coli* BL21(DE3) cultivated in Luria-Bertani (LB) medium (10 g/L of tryptone, 5 g/L yeast extract, and 10 g/L NaCl) supplemented with 50 µg/mL kanamycin at 16°C for 20 h and 150 rpm. Cells were harvested by centrifugation (5,000×g, 10 min, 4°C). Cells were washed twice with 10 mM sodium phosphate buffer pH 7.0 and lysed by high-pressure homogenizer (Emulsiflex-B15; Avestin). The cell free extract was collected by centrifugation (20,000×g, 20 min, 4°C). PmGBE13 and PmGBE57 were purified in two steps. Firstly, the cell free extracts were heated at 65°C for 20 min, followed by removal of the denatured proteins by centrifugation (20,000×g, 20 min, 4°C). Subsequently, the His-tagged proteins were purified using HisPur™ Ni-NTA Resin (ThermoFisher Scientific) according to the manufacturer's protocol. Protein concentration was quantified using the Quick Start Bradford Protein Assay kit (Bio-Rad Laboratories). Purity and molecular mass of the proteins were checked by SDS-PAGE.

Enzyme activity assays

Activity of PmGBE13 and PmGBE57 was measured with amylose V as substrate. Reaction progress was followed by iodine staining, which is based on monitoring the decrease of the absorbance of the glucan-iodine complex [30]. Amylose V was dissolved in 1 M sodium hydroxide, and then neutralized to pH 7.0 with 1 M HCl. The reaction mixture consisted of 0.125% (w/v) amylose V in 50 mM sodium phosphate buffer (pH 7.0). Reactions were performed at 50°C and started by the addition of enzyme, 3 µg/mL PmGBE1 or 60 µg/mL PmGBE2. Reaction progress was followed in time by taking 10 µL aliquots and adding them to 150 µL iodine reagent (aqueous solution of 0.0127% I₂ (w/v) and 0.035% KI (w/v)). The absorption at 660 nm was measured. One unit of activity is defined as the decrease in absorbance of 1.0 per min at 660 nm.

The total activity, being the sum of hydrolytic and branching activity, was determined by measuring the reducing ends of the product after debranching by isoamylase and pullulanase. The reducing ends were measured by the BCA method [31]. Prior to debranching the products, enzymes were inactivated by incubating the samples at 100°C for 10 min. One unit of total activity is defined as 1 µmol total reducing ends synthesized per minute.

Branching activity, representing the newly synthesized α -1,6-glycosidic linkages, was determined by measuring the increase in reducing ends before and after debranching of the product by isoamylase and pullulanase. One unit of branching activity is defined as 1 µmol of α -

1,6-linkage synthesized per minute. The data used to calculate different activities were from 0 to 30 min of incubation.

The influence of temperature and pH on the activity of PmGBE13 and PmGBE57 was tested in 50 mM sodium phosphate buffer at pH 7.0 or 50 °C, respectively. The temperature ranged from 40 to 80 °C and the pH from 6.0 to 9.0. Amylose V at a concentration of 0.125% (w/v) was used as substrate and the activity was quantified using the iodine staining assay.

High performance anion exchange chromatography

Oligosaccharide analysis was carried out by High Performance Anion Exchange Chromatography (HPAEC) on a Dionex ICS-3000 system (Thermo Scientific, USA) equipped with a 4×250 mm CarboPac PA-1 column. A pulsed amperometric detector with a gold electrode and an Ag/AgCl pH reference electrode were used. The system was run with a gradient of 30–600 mM NaAc in 100 mM NaOH run at 1 mL/min. Chromatograms were analyzed using Chromeleon 6.8 chromatography data system software (Thermo Scientific). A mixture of glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose (0.1 mg/mL of each component) was used as reference for qualitative determination of elution time of each component. The decay rate of detector signal from DP 2 to DP 7 calculated from reference sample is 4.44:2.76:2.02:1.45:1.36:1.00, which is used to correct the DP 2 to DP 7 of all samples and other components are without correction.

¹H-NMR spectroscopy

¹H-NMR spectra were recorded at a probe temperature of 323 K on a Varian Inova 500 spectrometer (NMR Center, University of Groningen). All samples produced by PmGBE1 or/and PmGBE2 were dialyzed by dialysis tubing with cutoff size of 100 to 500 Da. Subsequently, all samples were freeze dry. Before analysis, samples were exchanged twice in D₂O (99.9 atom% D, Sigma-Aldrich Chemical) with intermediate lyophilization, and then dissolved in 0.6 mL D₂O. Spectra were processed using MestReNova 5.3 software (Mestrelabs Research SL, Santiago de Compostella, Spain), using Whittaker Smoother baseline correction and zero filling to 32 k complex points. Chemical shifts (δ) are expressed in ppm by reference to internal acetone (δ 2.225 for ¹H). Carbohydrate structures were determined using the previously developed ¹H-NMR structural-reporter-group concept of α -D-glucans [32]. The α -1,6 signal is presented at δ 4.98, originating from H1 in 1,4- α -glucose-1,6 and α -1,4 signal is at δ 5.36 from the H1 in 1,4- α -glucose-1,4 and 1-4,6- α -glucose-1,4 residues.

Molecular weight distribution analysis

Molecular weight distributions were measured by Gel Permeation Chromatograph (GPC). DMSO-LiBr (0.05 M) was prepared by stirring 3 h at room temperature. Samples were dissolved at a concentration of 2 mg/mL in DMSO-LiBr at 80 °C with shaking for 3h and filtered through a 0.45 μ m Millex PTFE membrane (Millipore Corporation, Billerica, USA). The Size Exclusion Chromatography (SEC) system setup (Agilent Technologies 1260 Infinity) from PSS (Mainz, Germany) consisted of an isocratic pump, auto sampler without temperature regulation, an online degasser, an inline 0.2 μ m filter, a refractive index detector (G1362A 1260 RID Agilent Technologies), viscometer (ETA-2010 PSS, Mainz), and MALLS (SLD 7000 PSS, Mainz). WinGPC Unity software (PSS, Mainz) was used for data processing. The samples were injected with a flow rate of 0.5 mL min⁻¹ into a PFG guard-column and three PFG SEC columns 100, 300 and 4000, which were also purchased from PSS. The columns were held at 80 °C, the visco-detector at 60 °C (Visco) and the RI detector at 45 °C (RI). A universal calibration curve was generated using a standard pullulan kit (PSS, Mainz, Germany) with molecular

weights from 342 to 805,000 Da, in order to determine the hydrodynamic volume from the elution volume. The specific RI increment value dn/dc was measured by PSS and is 0.072.

Chain length analysis

The structure of a branched α -glucan can be described by the average length of the linear chains in total (ACL) and the average length of the linear chains between two branch points, i.e. the internal chain length (ICL). The ACL was calculated from peak area of HPAEC profiles. Based on the chain length distribution three fractions were classified as DP 2–4, DP 5–10 and DP above 10. The percentage of each fraction was calculated from the peak area. In order to reduce the influence of attenuate signal, the attenuate ratio was tested by standard sample containing the same mass concentration fractions glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose.

Based on the relations of one chain with others the chains of α -glucan are classified into three types [33, 34]: the A chain is linked only through its reducing terminus to carbon 6 of a glucose unit of another chain; the B chain is linked at its reducing end to another B or to a C chain while at the same time it carries one or more A and/or B chains as branches; the C chain is the chain with the only free reducing end in the molecule. The internal chain length (ICL) is defined as the average number of glucose units between two branching points in B chains. The ICL is determined by first trimming the exterior chains using the exo-acting enzyme β -amylase. A-chains are shortened to 2 or 3 anhydroglucose units and, for B-chains to 1 or 2 anhydroglucoses [35]. Briefly, branched α -glucan products (2 mg) were treated with 10 units β -amylase at 40°C in 5 mM sodium citrate buffer pH 6.5 for 24 h. β -amylase was inactivated by boiling for 5 min. Subsequently the pH was lowered to 5.0 with citric acid, followed by overnight isoamylase and pullulanase debranching at 40°C. The debranched samples were analyzed by HPAEC. The chain length distribution was then compared to the distribution of the samples without β -amylase treatment, as described. The percentage of A-chains was calculated as the ratio between two folds peak area of maltotriose (A-chains were hydrolyzed to maltose and maltotriose by β -amylase) and total peak area. The AICL was calculated as follow:

$$\text{AICL} = \frac{\text{BAM} - 1.5}{100/(100 - \text{A}\%)} - 1$$

BAM: the average chain length of β -amylase treated α -glucans was calculated from peak area of $\text{DP} \geq 4$ in HPAEC spectra. A%: the percentage of A-chains in α -glucans.

Results and discussion

Distribution of genes encoding glycogen branching enzymes in *Thermotoga* species

GBEs are key enzymes in the synthesis of glycogen, a reserve polymer of many microorganisms, invertebrates, and animals [36]. In this study the distribution of GBEs in 42 *Thermotoga* species, of which the whole genome sequence has been published, is reported. All these 42 *Thermotoga* species have one or two glycogen branching enzyme genes, a glycogen synthase gene, and a glucose-1-phosphate adenylyltransferase gene, indicating that they all have the capacity to synthesize glycogen (Table 1). All Thermotogaceae, Fervidobacteriaceae, Kosmotogaceae, and Mesoacidotogaceae have a single GH57 *glgB*. Within the Petrotogaceae, *Geotoga petraea* and *Petrotoga sibirica* possess a single GH13 *gbe* while *Petrotoga hypogea* has two GH57 *gbes* (Table 1). *Defluviitoga tunisiensis*, *Marinitoga* sp., *M. hydrogenotolerans*, *M. peizophilis*, *Petrotoga mobilis*, *Petrotoga halophile*, and *P. miotherma* are remarkable as they possess

Table 1. Occurrence of the key enzymes in glycogen synthesis in members of *Thermotogaceae*. GBE: glycogen branching enzyme; GSE: glycogen synthase; AGPase: ADP-glucose pyrophosphorylase.

Families	Name	GH13 GBE	GH57 GBE	GSE	AGPase	
Thermotogaceae	<i>Thermotoga caldifontis</i> AZM44c09		WP_041077987.1	WP_041077756.1	WP_041075563.1	
	<i>Thermotoga maritima</i> MSB8		WP_004081707.1	NP_228703.1	NP_228053.1	
	<i>Thermotoga naphthophila</i> RKU-10		WP_012896461.1	WP_011942738.1	WP_012896196.1	
	<i>Thermotoga neapolitana</i> DSM 4359		WP_038067483.1	ACM23857.1	ACM22623.1	
	<i>Thermotoga petrophila</i> RKU-1		WP_011943829.1	WP_011942738.1	WP_012310694.1	
	<i>Thermotoga profunda</i> AZM34c06		WP_041082268.1	WP_041082835.1	WP_041083424.1	
	<i>Thermotoga sp.</i> 2812B		WP_008195099.1	WP_004080686.1	WP_004082940.1	
	<i>Pseudothermotoga elfii</i> DSM 9442		WP_012003808.1	WP_012003430.1	WP_028843492.1	
	<i>Pseudothermotoga hypogea</i> DSM 11164		WP_031503953.1 WP_081836282.1	WP_031504818.1	WP_031504508.1	
	<i>Pseudothermotoga lettingae</i> TMO		WP_012003808.1	WP_012003430.1	WP_012002332.1	
	<i>Pseudothermotoga thermarum</i> DSM 5069		WP_013933073.1	WP_013933165.1	WP_013932574.1	
	Fervidobacteriaceae	<i>Fervidobacterium islandicum</i> DSM 17883		WP_033191873.1	WP_033191209.1	WP_033191528.1
		<i>Fervidobacterium gondwanense</i> DSM 13020		WP_072759036.1	WP_072760135.1	WP_072757324.1
<i>Fervidobacterium nodosum</i> Rt17-B1			WP_011994035.1	WP_011993493.1	WP_011994654.1	
<i>Fervidobacterium pennivorans</i> DSM 9078			WP_041262849.1	WP_014451068.1	WP_014450695.1	
<i>Fervidobacterium thailandensis</i>			WP_069293104.1	WP_069293649.1	WP_069292357.1	
<i>Thermosipho affectus</i>			WP_075665539.1	WP_077197850.1	WP_075665454.1	
<i>Thermosipho africanus</i> H17ap60334			WP_012579629.1	WP_012579478.1	WP_012579605.1	
<i>Thermosipho africanus</i> Ob7			WP_114702187.1	WP_114702310.1	WP_114702215.1	
<i>Thermosipho africanus</i> TCF52B			WP_012579629.1	WP_012579478.1	WP_012579604.1	
<i>Thermosipho atlanticus</i> DSM 15807			WP_073073190.1	WP_073072419.1	WP_073072127.1	
<i>Thermosipho globiformans</i>			WP_126992764.1	WP_126992938.1	WP_126993134.1	
<i>Thermosipho melanesiensis</i> BI429			WP_012056570.1	WP_012056316.1	WP_012056477.1	
<i>Thermosipho sp.</i> 1063			WP_008195099.1	WP_075665323.1	WP_075665454.1	
Petrotogaceae		<i>Defluviitoga tunisiensis</i> DSM 23805	WP_045087168.1	WP_045087488.1	WP_045087536.1	WP_045087221.1
	<i>Geotoga petraea</i> ATCC 51226	WP_091403612.1		WP_091405813.1	WP_091402621.1	
	<i>Marinitoga hydrogenitolerans</i> DSM 16785	WP_072864394.1	WP_072862856.1	WP_072863441.1	WP_072864199.1	
	<i>Marinitoga piezophila</i> KA3	WP_014296184.1	WP_014297024.1	WP_014296945.1	WP_014296021.1	
	<i>Marinitoga sp.</i> 1155	WP_047265238.1	WP_047265848.1	WP_075780662.1	WP_075780313.1	
	<i>Petrotoga mobilis</i> SJ95	WP_012209120.1	WP_012208426.1	WP_012208925.1	WP_012208734.1	
	<i>Petrotoga halophila</i> DSM 16923	WP_103898766.1	WP_103898294.1	POZ92479.1	WP_012208734.1	
	<i>Petrotoga mexicana</i> DSM 14811		WP_103077822.1	PNR98227.1	PNR98748.1	
	<i>Petrotoga miotherma</i> DSM 10691	WP_103079200.1	WP_103078532.1	PNS02142.1	PNS02470.1	
	<i>Petrotoga olearia</i> DSM 13574	WP_103066081.1	WP_103066988.1	PNS02142.1	PNS02470.1	
	<i>Petrotoga sibirica</i> DSM 13575	WP_103876993.1		WP_103876724.1	WP_103876533.1	
Kosmotogaceae	<i>Kosmotoga arenicorallina</i> S304		WP_068347510.1	WP_068347125.1	WP_068346683.1	
	<i>Kosmotoga olearia</i> TBF 19.5.1		WP_012744890.1	WP_015869246.1	WP_015868560.1	
	<i>Kosmotoga pacifica</i>		WP_047754759.1	WP_047755003.1	WP_047754440.1	
	<i>Kosmotoga sp.</i> DU53		WP_012744890.1	WP_015869246.1	WP_015868270.1	
	<i>Mesotoga infera</i>		KUK67992.1	CCU85914.1	CCU83670.1	
	<i>Mesotoga prima</i> MesG1.Ag.4.2		WP_006486989.1	WP_014730450.1	WP_006486752.1	
Mesoaciditogaceae	<i>Mesoaciditoga lauensis</i> DSM 25116		WP_036226301.1	WP_036221777.1	WP_036226373.1	

<https://doi.org/10.1371/journal.pone.0219844.t001>

both a GH13 and a GH57 *gbe*. The presence of a GH13 and putative GH57 *gbe* in one and the same species raises the question whether the corresponding GBEs are functional and if they

both play a role in glycogen biosynthesis. To shed some light on this question, the GH13 and GH57 GBE genes and corresponding enzymes of *P. mobilis* were studied in more detail.

The gene *pmgbe13* (encoding the GH13 GBE of *P. mobilis*) has a complete ORF with a start codon at position 1,405,056 and stop codon at position 1,407,239 in the genome sequence. A clear promoter was predicted in the upstream region (2,000 bp) of the *pmgbe13* gene with a clear -10 (TTTTATAAT) and -35 (TTTAAA) consensus sequence (<http://www.softberry.com/>). *pmgbe57* (encoding the GH57 GBE of *P. mobilis*) also has a complete ORF from position 639,260 to position 640,876 in the genome sequence. The promoter was predicted in the upstream region (2,000 bp) with a -10 (CTCTACTAT) and -35 (TTTAAT) consensus sequence. Several transcription factor-binding sites were predicted in the upstream sequences for both genes. These results taken together indicate that both genes are functional and can be translated and regulated *in-vivo*. As expected for GBEs no signal sequences were identified, suggesting that these two GBEs are not excreted and are active intracellularly. As the *P. mobilis* genome sequence also contains a glycogen synthase and a glycogen debranching enzyme, it is concluded that *P. mobilis* contains all the key enzymes for glycogen synthesis making it likely that it synthesizes glycogen. So far no studies on the presence and structure of the glycogen from any of the *Petrotogaceae* have been reported.

Biochemical properties of PmGBE13 and PmGBE57

To investigate if the putative PmGBE13 and putative PmGBE57 have glycogen branching activity, the corresponding genes were over expressed in *E. coli* BL21(DE3). The obtained enzymes were purified to homogeneity, as judged by SDS-page (Fig 1). Both purified proteins convert amylose V at 60 °C and pH 7.0, as revealed by a decrease in iodine staining, thus demonstrating that both putative GBEs are functional α -glucan modifying enzymes. Subsequent ¹H-NMR analysis demonstrated that both enzymes convert amylose V in branched α -glucans with a degree of branching of 12.4% for the PmGBE13 and 8.5% for the PmGBE57 (Fig 2). The branching degree of the branched α -glucan products of PmGBE57 is in the range of those found for other GBEs [37–39]. The branching degree of the PmGBE13 product is one of the highest values reported so far, being in the same range as the branched α -glucan product (13.5%) made by the *Geobacillus thermoglucosidans* GBE with amylose as substrate [40]. The PmGBE13 enzyme is an interesting enzyme to further explore as it is not only thermostable, which is an advantage in starch processing as this is done at temperatures above 60 °C [41], but also the high degree of branching of the products could contribute to a slower digestion in the small intestine, possibly making this branched α -glucan a slow digestible starch [37, 42, 43].

The influence of temperature and pH on the activity of PmGBE1 and PmGBE2 was investigated. Both enzymes showed maximum activity at 50 °C and pH 7.0, and lost activity at temperatures of 70 °C and higher (Fig 3). Of the two enzymes, the activity of PmGBE13 is most sensitive to changes in temperature and pH. The maximum activity at neutral pH also supports the earlier conclusion that both enzymes are very likely to be active in the cytosol.

GBEs create branches via a transglycosylation reaction, in which a new α -1,4-gluco-oligosaccharide chain is used as an acceptor. A side reaction of GBEs is hydrolysis, in which water is used as an acceptor, resulting in the formation of short α -1,4-gluco chains. The branching and hydrolysis reaction of both GBEs with amylose V as substrate was followed in time (Fig 4). The use of amylose V as substrate has the advantage that it is virtually free from α -1,6-linkages, so any α -1,6-bond present in the product is the result of the branching activity of the GBE. PmGBE13 rapidly branches amylose V into a branched α -glucan within the first 50 min of the reaction (Fig 4A). Differently, the PmGBE57 is clearly slower in introducing α -1,6-linkages

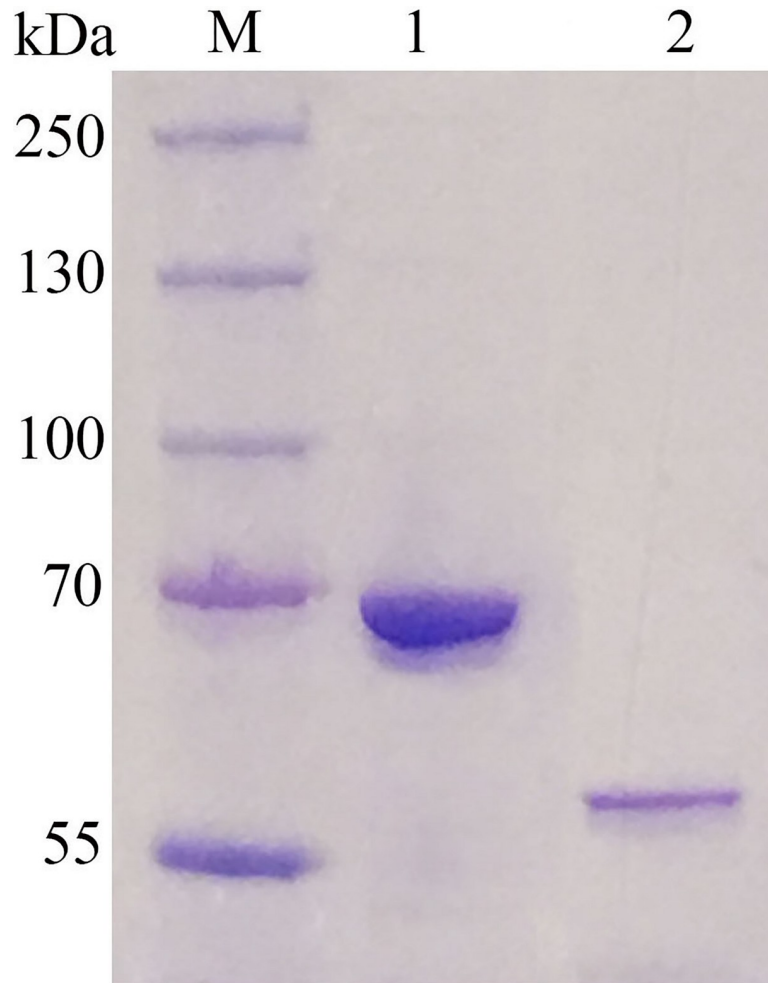


Fig 1. SDS-PAGE of purified PmGBE13 (lane 1) and PmGBE57 (lane 2). M: protein standard.

<https://doi.org/10.1371/journal.pone.0219844.g001>

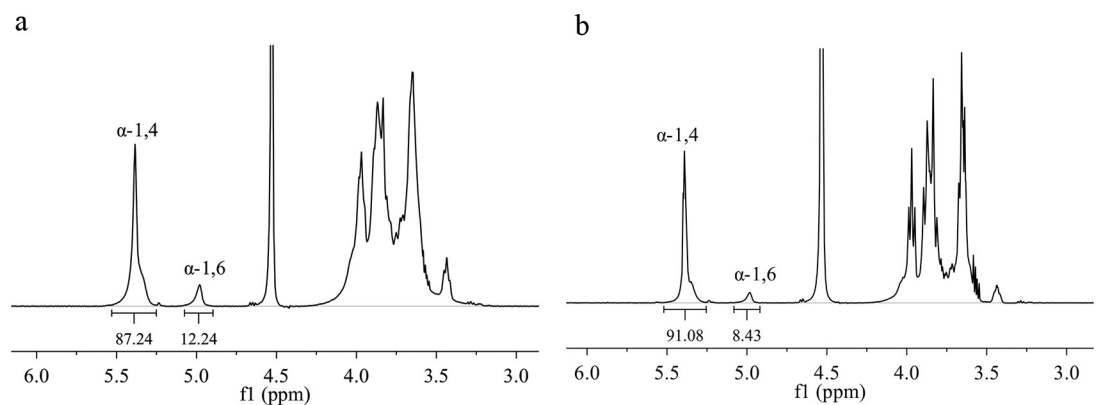


Fig 2. $^1\text{H-NMR}$ spectrum of the branched α -glucans derived from amylose V by the action of PmGBE13 (a) and PmGBE57 (b). Reactions were performed in phosphate buffer pH 7.0 at 50°C for 24 h. The spectra were recorded in D_2O at 323 K. The signal originated by the residual water in the sample (HOD peak at 4.24 ppm) was cut off from the spectrum.

<https://doi.org/10.1371/journal.pone.0219844.g002>

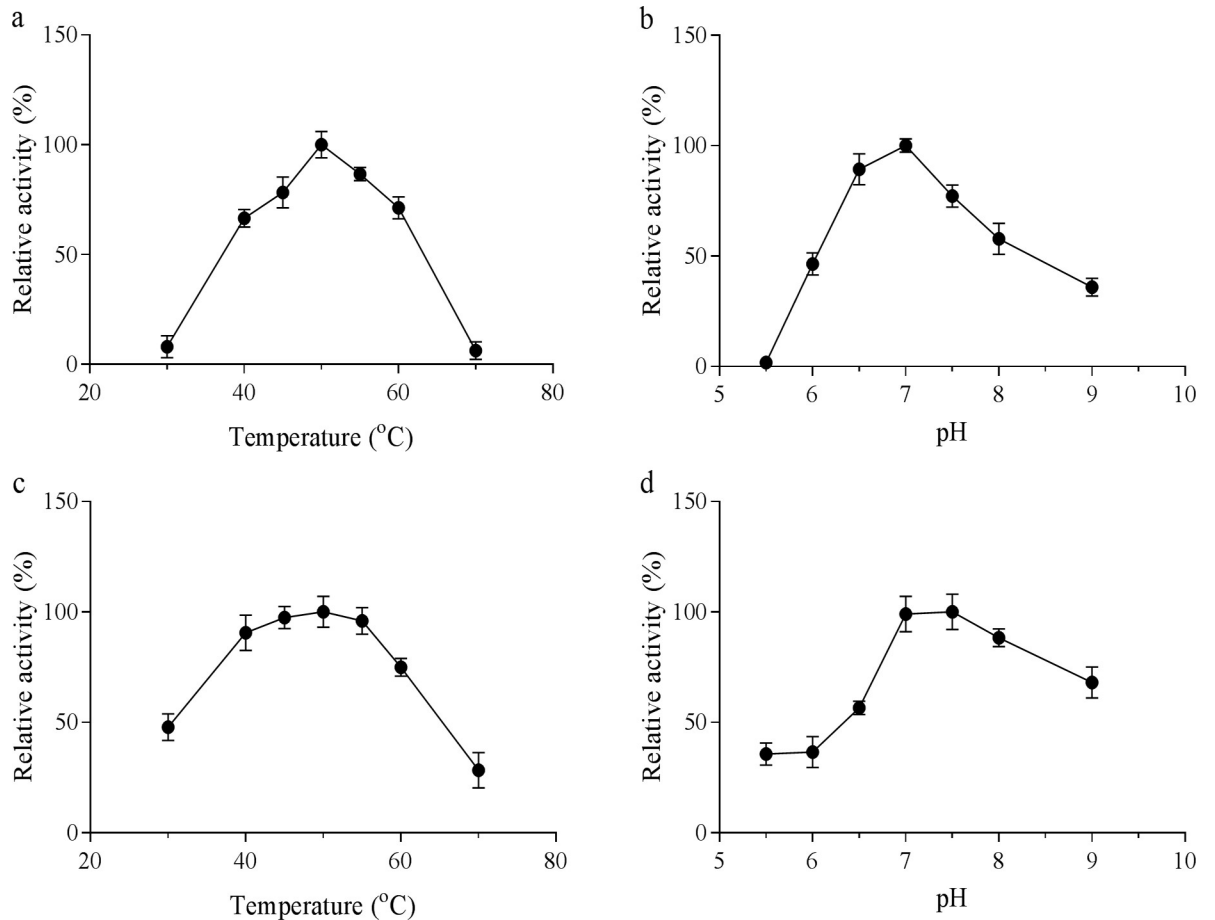


Fig 3. Temperature and pH activity profiles of PmGBE13 (a, b) and PmGBE57 (c, d).

<https://doi.org/10.1371/journal.pone.0219844.g003>

(Fig 4B) when given amylose. The branching activity of PmGBE13 and PmGBE57 is 6 U/mg protein and 0.04 U/mg protein, respectively. The branching activity of PmGBE13 is similar to

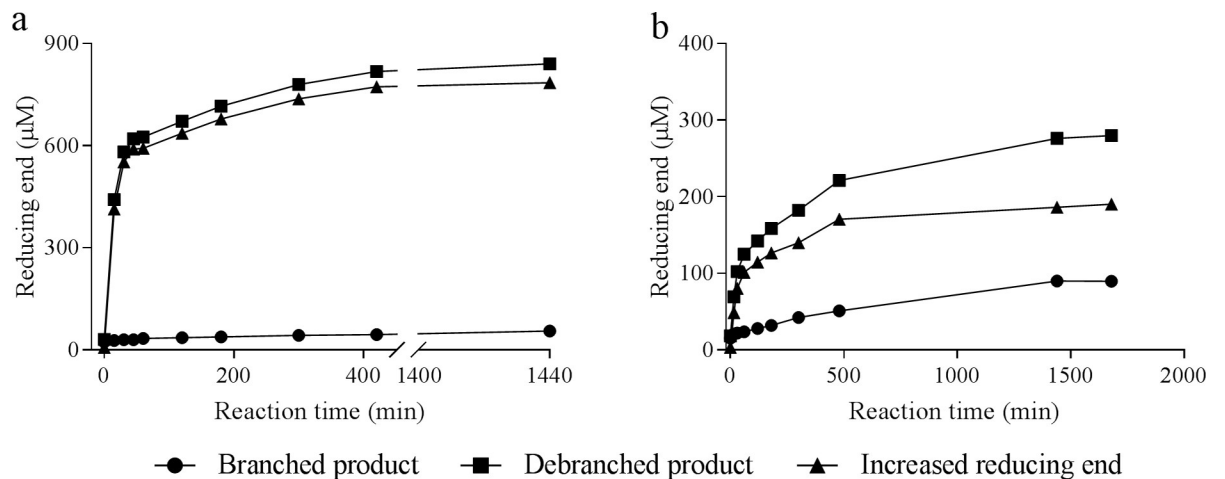


Fig 4. Increase in reducing end following the branching and hydrolytic activity in time.

<https://doi.org/10.1371/journal.pone.0219844.g004>

that reported for the GH13 GBE from *Deinococcus geothermalis* [44], and relatively high compared to the previously reported GBEs from *E. coli*, *Aquifex aeolicus*, *Geobacillus stearothermophilus* and *Anaerobranca gottschalkii* [45–48]. The PmGBE57 showed relatively lower activity than GH57 GBEs from *T. thermophilus*, *Pyrococcus horikoshii* [26, 28]. The hydrolytic activity of PmGBE13 and PmGBE57 is 0.006 U/mg and 0.003 U/mg, being comparable to the hydrolytic activity of the GBE of *D. geothermalis* [44], and *T. thermophilus* [28], resp.

Structural properties of the branched α -glucans

PmGBE13 made branched α -glucans with an average molecular mass of 1.7×10^6 Da, which is 8.5 times larger than that of amylose V, having an average molecular mass of 2×10^5 Da (Fig 5). The PmGBE13 product shows a relatively high molecular mass compared to GBE modified waxy corn starch [37], while it is lower than the glycogen from *Sphaerotilus natans*, *Arthrobacter viscosus* and oyster [49]. On the other hand, PmGBE57 made a branched α -glucan that was considerably smaller than amylose V; the average molecular mass was 1.4×10^4 Da (approx. 85 glucose units), which is much smaller than glycogen produced by bacteria, and even 10 times smaller than the glycogen-like carbohydrate polymers extracted from the cell wall of *M. tuberculosis* [50, 51].

To further elucidate the structure of the branched α -glucans, the chain length distribution, average chain length (ACL), and average internal chain length (AICL) were determined (Table 2). The PmGBE13 product has side chains ranging from 2 to 15 residues, showing a bell-shaped distribution (Fig 6A); the ACL is 8 and the AICL is 2.6. The PmGBE57 product has, in contrast, considerable shorter side chains of 3 to 5 residues (Fig 6B). The ACL of PmGBE57 is 7 while the AICL is DP 2.4. Although the ACL and AICL of the PmGBE13 and PmGBE57 products did not differ significantly, a clear difference was found for the percentage of A-chains in the PmGBE13 and PmGBE57 products; the PmGBE13 product contained 28% A-chains while the PmGBE57 product contained more than 44% A-chains (Table 2). All these results taken together show that PmGBE13 makes a completely different branched α -glucan than PmGBE57, hinting at the involvement of the two enzymes in different biosynthetic

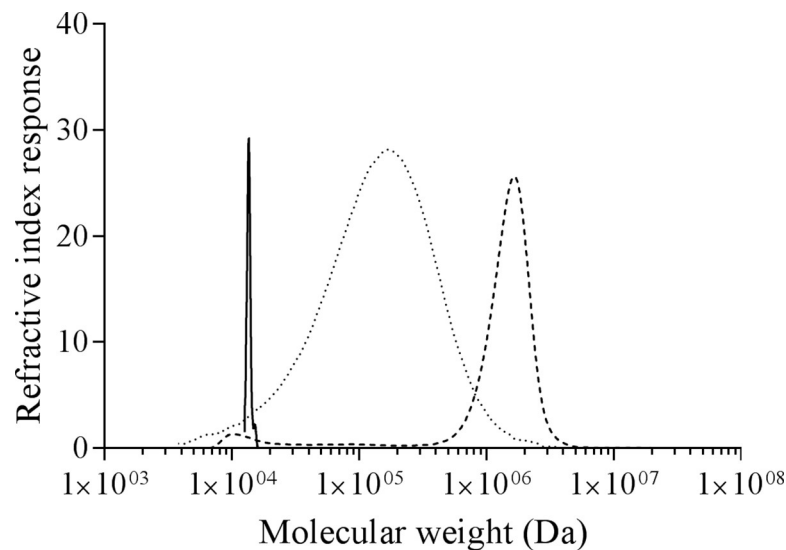


Fig 5. Molecular size distribution of amylose V (.....) and the branched α -glucan products made by PmGBE13 (---) and PmGBE57 (-).

<https://doi.org/10.1371/journal.pone.0219844.g005>

Table 2. Chain length distribution, average chain length (ACL), average internal chain length (AICL), and A-chain content of PmGBE13 and PmGBE57 branched α -glucans from amylose.

	PmGBE13	PmGBE57
Σ DP 2–4 (%)	19	28
Σ DP 5–10 (%)	75	59
Σ DP > 10 (%)	6	13
ACL (DP)	8	7
AICL (DP)	2.6	2.4
A-chain (%)	28	44

<https://doi.org/10.1371/journal.pone.0219844.t002>

pathways or the presence of two structurally different intracellular branched α -glucans in *P. mobilis*.

Minimal donor substrate length

GBEs require a minimum length of substrate before a branching or hydrolysis reaction can start [45]. As such, the minimum chain length of the donor substrate is an important parameter of GBEs. To assess this specificity, PmGBE13 and PmGBE57 were incubated with a mixture of linear chains in the DP range of 2 to 30 (DHBS). Subsequent analysis of the debranched products showed that PmGBE13 had converted all linear oligosaccharides with a DP of 13 and more (Fig 7). PmGBE57 requires slightly longer linear oligosaccharides, as it had converted all linear oligosaccharides of DP 17 and longer (Fig 7). The PmGBE13 branched α -glucan product is rich in side chains with 5 and 6 residues while the PmGBE57 branched glucan product is composed of longer chains with a maximum at 9 to 10 residues (Fig 7), this being in line with the results found with amylose V as substrate (Fig 3). Thus, the minimum substrate length for PmGBE13 is 13 residues, this being very close to what was found for the GH13 GBEs of *Rhodothermus obamensis* and *E. coli* that use donor substrates of minimally 12 residues [27, 45]. For GH57 GBEs, no minimum substrate lengths have been published so far.

Role of GH13 and GH57 GBEs

The human pathogen *M. tuberculosis* has two *glgB* genes, one encoding a GH13 GBE (Rv1326c) and another encoding a GH57 GBE (Rv3031). A knock-out of the *glgB* gene

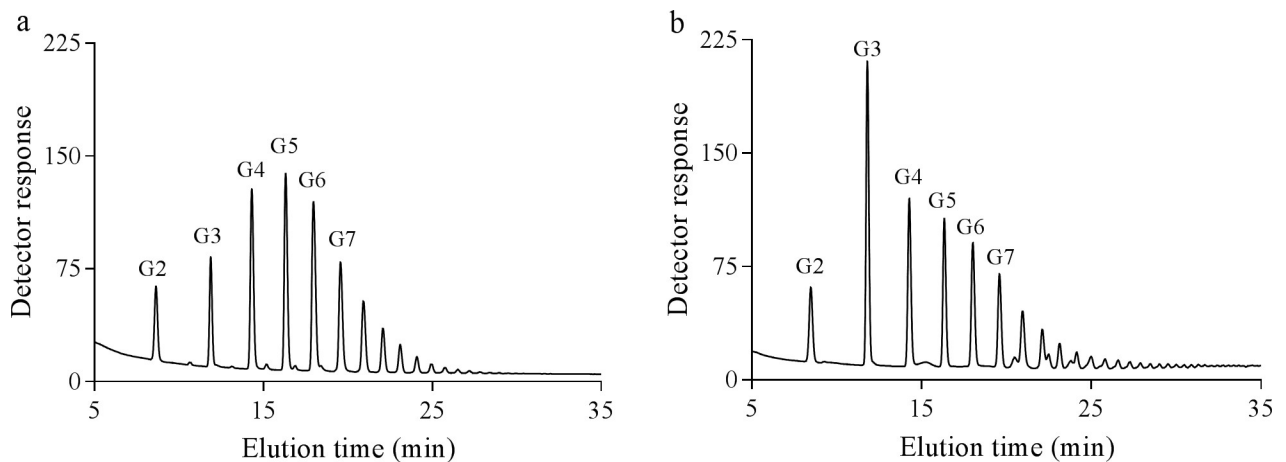


Fig 6. The chain length distribution of the branched α -glucans derived from amylose V incubated with PmGBE13 (a) and PmGBE57 (b) in phosphate buffer pH 7.0 at 50°C for 24 h.

<https://doi.org/10.1371/journal.pone.0219844.g006>

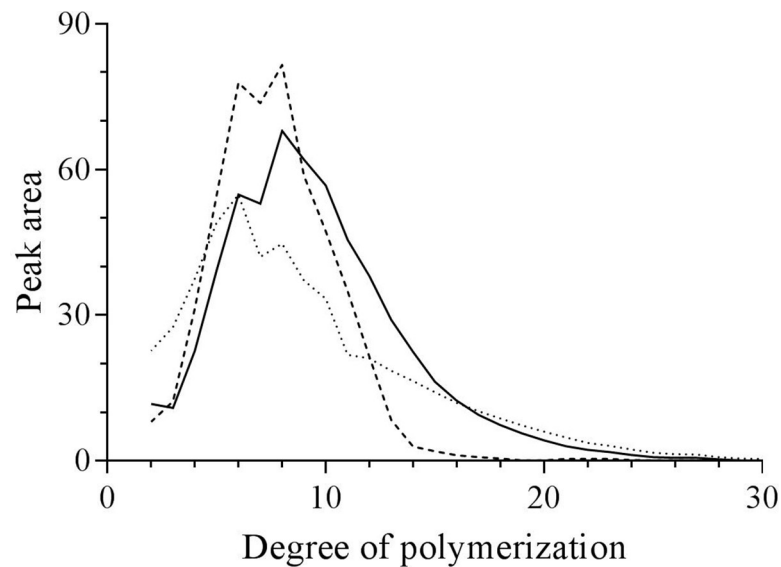


Fig 7. The chain length distribution of the products derived when debranched HBS (....) was incubated with PmGBE13 (---) or PmGBE57 (-) at 50°C for 24 h.

<https://doi.org/10.1371/journal.pone.0219844.g007>

Rv1326c in *M. tuberculosis* H37Rv did not give any viable mutants. The knock-out mutation could be compensated by a plasmid carrying either the *gbe* gene from *M. tuberculosis* or *E. coli*, demonstrating that the *gbe* gene Rv1326c and its corresponding enzyme are essential for growth [22]. It remains unclear what the role of Rv3031 in *M. tuberculosis* is. Apparently Rv3031 does not take over the role of Rv1326c in glycogen biosynthesis, either because the gene was not expressed in the Rv1326c knock-out mutant or because the GH57 GBE enzyme does not act on a growing linear α -glucan chain as the GH13 GBE does [21]. The Rv3031 gene has been linked to the synthesis of the capsular glucans typical for *M. tuberculosis*, although without experimental evidence [52, 53]. The results reported in this paper show that the GH13 and the GH57 GBE of *P. mobilis* differ considerably with respect to activity towards amylose and the structure of the branched α -glucan produced. The high molecular mass of the branched α -glucan product points at a role for the PmGBE1 in glycogen biosynthesis as glycogen is a large molecule of 10^6 to 10^7 Da [49], this being in line with the role Rv1326c plays in glycogen production in *M. tuberculosis* [22]. The role of PmGBE2 as is the role of the GH57 GBE in *M. tuberculosis* remains unclear and calls for further investigation.

Conclusions

The majority of the genome sequences of *Petrotogaceae* harbor two *glgB* genes, encoding a GH13 and a GH57 GBE. Both genes have all features to encode the corresponding proteins and overexpression in *E. coli* resulted in active GBEs. The lack of a clearly recognizable signal sequence and the activity at neutral pH point at an intracellular localization of both enzymes. The PmGH13 seems a common GBE with a high activity and synthesizing highly branched and relative high MW α -glucans of 10^6 – 10^7 Da. The PmGBE57, in contrast has a very low branching activity with amylose as substrate and forms branched α -glucans of considerably lower MW (10^4 Da) with a lower degree of branching making it very unlikely that this enzyme plays a role in glycogen biosynthesis. Further studies including detailed analysis of the structure of *Petrotoga* glycogen structure, gene expression, and gene knock-outs should shed some light on the role of the GH13 and GH57 GBE during the biosynthesis of glycogen and/or other α -glucans in *P. mobilis* and other *Petrotogaceae*.

Supporting information

S1 Appendix. Gene sequence of *glgB13*.

(DOCX)

S2 Appendix. Gene sequence of *glgB57*.

(DOCX)

Acknowledgments

We thank prof. dr. Lubbert Dijkhuizen (Microbial Physiology, University of Groningen) for kindly allowing us to use the HPAEC-PAD equipment.

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Formal analysis: Xuewen Zhang.

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Methodology: Xuewen Zhang.

Software: Xuewen Zhang.

Supervision: Hans Leemhuis, Marc J. E. C. van der Maarel.

Writing – original draft: Xuewen Zhang.

Writing – review & editing: Xuewen Zhang, Hans Leemhuis, Marc J. E. C. van der Maarel.

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