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Discovery of fructans in Archaea

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ABSTRACT

Fructans are fructose-based oligo- and polysaccharides derived from sucrose that occur in a plethora of Eubacteria and plants. While fructan-producing (fructanogenic) Eubacteria are abundant in hypersaline environments, fructan production by Archaea has never been reported before. Exopolysaccharides accumulated by various Archaea from the Halobacteria class (belonging to the genera of *Halomicrobium, Haloferax* and *Natronococcus*) originating from different locations on Earth were structurally characterized as either levans or inulins with varying branching degrees (10%–16%). Thus, we show for the first time in the literature that fructans are produced in all three domains of life, including Archaea. This proof of concept will not only provide insight into Archaeal glycans and evolution but it may also open new frontiers for innovative strategies to overcome the ever-increasing threat of excessive salinization.

1. Introduction

Fructans are a diverse class of sucrose (Suc)-derived oligo- and polyfructosyl saccharides that naturally occur in a wide variety of Eubacteria and plants, hereafter referred to as fructanogenic Eubacteria and plants, and they are believed to act as multifunctional components due to their key roles in biofilms (Dogsa, Brloznik, Stopar, & Mandic-Mulec, 2013; Koczan, McGrath, Zhao, & Sundin, 2009), prebiotic (Adamberg et al., 2015; Lei et al., 2017) and immunomodulatory effects (Peshev & Van den Ende, 2014; Xu et al., 2016) and roles in plant stress responses (Tarkowski, Van de Poel, Höfte, & Van den Ende, 2019; Wei et al., 2017). There are mainly two structural forms of fructans in nature: levans (β-2,6 linkages between fructofuranosyl units on the polymer backbone with occasional branching at β-2,1 positions) and inulins (β-2,1 linked fructofuranosyl units on the backbone with possible branching points at β-2,6 positions). In Eubacteria, fructans are synthesized as extracellular oligo- and polysaccharides (EPSs) of levan and inulin types by the catalytic action of fructosyltransferase (FT) enzymes levansucrases (EC 2.4.1.10) and inulosucrases (EC 2.4.1.9), respectively. While levans are synthesized by a plethora of both Gram-negative and Gram-positive Eubacteria, so far microbial inulin synthesis has only been observed in some Gram-positive species (mostly lactic acid bacteria; Zannini, Waters, Coffey, & Arendt, 2016; Kralj et al., 2018). Contrary to Eubacteria, in plants, fructans are accumulated intracellularly. In addition to levan- and inulin-type fructans, highly branched neo-fructans (*e.g.* agavins in *Agave* species) and graminans (*e.g.* in cereals) occur in plants, which are produced by at least three different types of plant FTs. (Avila-de Dios, Gomez-Vargas, Damian-Santos, & Simpson, 2015; Verspreet et al., 2015). It should be noted that bacterial and plant fructan types greatly differ in terms of their degree of polymerization (DP), such that on average, bacterial fructan DPs are several orders higher as compared to plants (Toksoy Öner, Hernández, & Combie, 2016). Some fungi can accumulate fructans as well (Choukade & Kango, 2019; Zhang et al., 2017), but fructans are completely absent in animals.

Hypersaline environments are habitats with salinity values greater than that of oceans and seas, and are predominantly inhabited by halophilic Archaea, followed by Eubacteria, viruses and some Eukarya (Loukas, Kappas, & Abatzopoulos, 2018; Oren, 2008). Our recent findings revealed that hypersaline environments are rich in a wide variety of fructanogenic Eubacteria (Kırtel, Versluys, Van den Ende, & Toksoy Öner, 2018). However, the presence of fructans has never been reported in Archaea so far. Although both the CAZy database (http://www.cazy.org) and preliminary bioinformatic studies carried out in our

Abbreviations: EPSs, extracellular polysaccharides; FTs, fructosyltransferases; GH32, glycoside hydrolase 32 family; GH68, glycoside hydrolase 68 family; GH-J, glycoside hydrolase J clan; gHSQC, gradient Heteronuclear Single Quantum Coherence; HMBC, Heteronuclear Multiple Bond Correlation; HSQC, Heteronuclear Single Quantum Coherence; HPAEC-IPAD, High Performance Anion Exchange Chromatography with Integrated Pulsed Amperometric Detector; NOESY, Nuclear Overhauser Spectroscopy; Suc, sucrose; TOCSY, Total Correlated Spectroscopy

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previous study (Kırtel et al., 2018a) suggested the presence of putative Archaeal fructan biosynthetic genes, the production of fructans by Archaea has never been experimentally demonstrated.

Bacterial FTs belong to the GH68 family enzymes. These enzymes use Suc as their preferential fructosyl donor; hydrolyzing it and transferring the resulting fructosyl moiety to another Suc, with successive transfers resulting in an elongated chain of fructan while releasing glucose. In plants, however, synthesis of fructans is accomplished by a different set of FTs belonging to GH32 family, which together with the GH68 family form the GH-J clan of enzymes. GH32 family enzymes are also present in many Eubacteria and fungi, with the best-known ones being invertases/β-fructofuranosidases (hydrolyzing Suc, but also showing some FT activity at elevated Suc concentrations), levanases (hydrolyze levans) and inulinases (hydrolyze inulins). All GH-J clan enzymes carry a 5-bladed β propeller domain with a deep central cavity where substrate is bound. In addition to the 5-bladed β propeller domain, GH32 family enzymes also carry a β-sandwich domain composed of six β-strands (Lammens et al., 2009).

Archaea form one of the three domains of life on Earth along with Eubacteria and Eukarya (Woese, Kandler, & Wheelis, 1990), and they were originally associated exclusively with extremely saline or hot environments. However, recent studies suggest that they are much more prevalent than previously thought, with novel Archaeal species being constantly discovered in human body (Borrel et al., 2017; Gaci, Borrel, Tottey, O'Toole, & Brugère, 2014), soil (Orellana, Chee-Sanford, Sanford, Löffler, & Konstantinidis, 2018), the plant apoplast (Hardoim et al., 2015) and many other mesophilic environments. Their functions in those habitats are still largely undiscovered and present an exciting research area. Moreover, not long ago it was suggested in a comprehensive study that Archaea actually share a more recent last common ancestor with Eukarya than Eubacteria (Zaremba-Niedzwiedzka et al., 2017), making them even more intriguing organisms to study to better understand the evolution of life on Earth as a whole. A recent work revealed that the genetic structures of Halobacteria (also known as Haloarchaea) have been under significant influence of Eubacteria via gene transfers throughout their evolution, enhancing their carbohydrate metabolism drastically by creating chimeric protein families which also include GH32-like genes (Méheust et al., 2018). Those adaptations may have helped Halobacteria to adopt an aerobic lifestyle by allowing them to utilize a wide range of carbohydrates via aerobic respiration. Also, it is possible that other aerobiosis-related genes and GH-J clan genes were acquired from Eubacteria at the same time, which may have rendered Halobacteria more successful in the environment. What's intriguing is that the putative GH-J clan genes appear only in the halophilic class of Halobacteria, and not in any other class of Archaea. Preservation of GH-J-like genes only in the Halobacteria class of all Archaeal lineages raises provoking questions: are those pseudogenes that have no effect on the phenotype, or are they translated and expressed as active enzymes? If so, what are the functions of fructans in hypersaline environments? Are they simply parts of the biofilms produced by these microorganisms? Considering their excellent water absorption capabilities (Kang et al., 2009), do fructans help those microorganisms survive extreme desiccation periods typically seen in hypersaline environments? To date, these questions have never been investigated, and they present a completely novel aspect regarding life under extreme salinity as a whole.

Here, we sought for evidence that fructans occur in Halobacterial species. Three type strains harboring putative GH-J clan genes from three different continents (*Halomicrobium mukohataei* DSM 12286 from Argentina, *Haloferax prahovense* DSM 18310 from Romania, and *Natronococcus jeotgali* DSM 18795 from Korea) together with four *Halomicrobium* strains isolated from crude salt samples of a Turkish saltern (Tuz Lake) were investigated for their fructanogenic traits, and they were all shown to be able to synthesize different types of fructans. This is the first report in the literature that demonstrates the presence of fructans in Archaea.

2. Materials and methods

2.1. Origin of strains

Several Archaeal colonies were isolated from crude salt samples (Estuz Ltd., Izmir, Turkey) originating from Tuz Lake, Turkey (38°50′N 35°20′ E). The other strains were purchased from DSMZ GmbH (Germany), namely *Hmc. mukohataei* DSM 12286 isolated from Salinas Grandes, Argentina (Ihara, Watanabe, & Tamura, 1997; Oren, Elevi, Watanabe, Ihara, & Corcelli, 2002), *Hfx. prahovense* DSM 18310 isolated from Telega Lake, Romania (Enache, Itoh, Kamekura, Teodosiu, & Dumitru, 2007), and *Ncc. jeotgali* DSM 18795 isolated from fermented shrimp in Korea (Roh et al., 2007). These particular strains were selected because of the presence of putative GH68 family genes in their genomes (Kırtel et al., 2018a).

2.2. Taxonomic classification of Tuz Lake isolates

For their genetic identification, Tuz Lake strains were grown in DSMZ Medium 372 under conditions mentioned above. After a week of incubation, genomic DNA of the isolates were extracted using DNeasy® UltraClean® Microbial Kit from QIAGEN, following manufacturer's instructions. Extracted genomic DNA samples were sent to Macrogen Ltd. (Amsterdam, The Netherlands) for 16S ribosomal RNA gene sequencing. The universal-archaeal primer 21 F (5′ TCCGGTTGATCCYGCCGG 3′) and the reverse primer 1492R (5′ TACGGYTACCTTGTTACGACTT 3′) were used. Sequence chromatograms were refined with Chromas (v2.6.5) software.

2.3. Isolation, media and culture conditions

For the isolation of Halobacterial strains from Tuz Lake, crude salt samples were dissolved in a sterile medium at a final concentration of 250 g/L, with the following final composition (g/L): $MgSO_4.7H_2O$, 20; KCl, 2; trisodium citrate, 3; casamino acids, 7.5; yeast extract, 1; and $FeSO_4.7H_2O$, 0.0023. pH was adjusted to 7.6 under sterile conditions (Sehgal & Gibbons, 1960). 50 mL of media were incubated in 250 mL Erlenmeyer flasks at 37 °C and 180 rpm for 10 days. At the end of this incubation period, 100 µL of cell suspensions from the media were spread onto agar (15 g/L) plates with the same composition, supplied with 50 g/L Suc for the selection of fructanogenic strains. Plates were incubated at 37 °C for 2 weeks. Colonies exhibiting mucoid, convex, shiny phenotype were streaked onto fresh agar plates several times to obtain single colonies. Since no putative genes that take part in α-glucan synthesis (GH70 family enzymes) could be found in any Halobacteria according to NCBI database, this morphology was considered as a strong indicator of fructan accumulation in sucrose-based media. Four single colonies were chosen randomly and used in the following experiments.

Type strains *Hmc. mukohataei* and *Hfx. prahovense* were grown in DSMZ Medium 372 with the following composition (g/L): NaCl, 200; $MgSO_4.7H_2O$, 20; KCl, 2; trisodium citrate, 3; casamino acids, 5; yeast extract, 5; glutamic acid, 1; $FeCl_2.4H_2O$, 0.036; $MnCl_2.4H_2O$, 0.00036; agar, for solid media 20; and Suc, for fructan production, 50. pH was adjusted to 7.0 under aseptic conditions after sterilization. For *Ncc. jeotgali*, DSMZ Medium 954 with the following composition was used (g/L): NaCl, 200; $MgCl_2.6H_2O$, 20; TRIS, 12; KCl, 2; $CaCl_2.2H_2O$, 0.2; casamino acids, 5; yeast extract, 5; agar, for solid media, 20; Suc, for fructan production, 50. pH was adjusted to 7.4 under aseptic conditions after sterilization. In all cases, the media were sterilized at 121 °C for 15 min except for Suc, which was filter-sterilized through 0.2 µm syringe filters and added to media afterwards. All cultures were incubated at 37 °C and 180 rpm agitation rate throughout the experiments in this study. All strains were stored at −80 °C in a 20% (w/v) NaCl solution in 25% (v/v) glycerol.

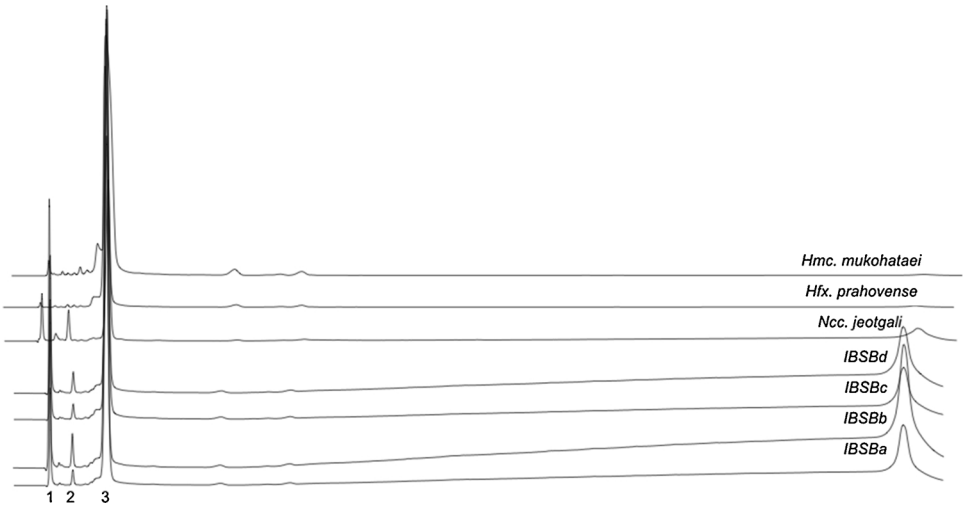

Fig. 1. Monomeric sugar compositions of Archaeal EPSs. HPAEC-IPAD chromatograms of acid-hydrolyzed Archaeal EPSs, showing their monomeric sugar compositions. Numbered peaks are: 1. rhamnose (internal standard), 2. glucose, 3. fructose. The peaks at the end of some chromatograms represent material retained on the column only after switching to 0.5 M Na-acetate as mobile phase.

2.4. Production and purification of fructans

For fructan production, all strains were grown in their respective media supplemented with 50 g/L Suc. Suc consumption throughout the incubation was recorded. Briefly, aliquots of 100 µl from each culture were taken at regular intervals and centrifuged at 10,000 × *g* for 5 min. Supernatants were analyzed for their Suc concentrations via HPAEC-IPAD as described in Section 2.5. When Suc depletion rate was no longer significant, cells were separated via centrifugation at 8000 x *g* for 20 min, and supernatants were dialyzed against several runs of distilled water at 4 °C through dialysis tubing with 13 kDa cut-off. After dialysis, fructan polymers were precipitated with 1:1 (v:v) ethanol and left at −20 °C overnight. Ethanol was removed via centrifugation at 8000 × *g* for 10 min, and polysaccharide pellets were redissolved in ultrapure water. To pellet proteins, samples were boiled for 30 min and centrifuged at 8000 × *g* for 20 min. Supernatants were passed through Dowex™ ion-exchange column (in Ac^- form) to remove residual proteins, followed by chloroform extraction for fractions with relatively higher total protein content. For chloroform extraction of proteins, samples were mixed with 1:1 (v:v) chloroform and vortexed vigorously, and then centrifuged at 10,000 × *g* for 5 min for phase separation. The upper phase, or the flow-through from Dowex™ column, was dried overnight at 30 °C in a Hetovac VR-I vacuum drier to obtain pure fructans.

2.5. Chemical characterization of fructans

Carbohydrate analyses of culture supernatants and purified EPSs were carried out via a Dionex ICS 5000 + HPAEC-IPAD (Thermo Scientific) system. Samples were diluted 100x in 20 µM rhamnose, which was used as the internal standard, then were run for 45 min. The flow rate was 0.25 ml/min, and the column used was Dionex CarboPac PA100 (2 × 50 mm). Starting with 100% mobile phase A (90 mM NaOH), a gradient was used for mobile phase B (90 mM NaOH + 0.5 M NaOAc), increasing from 0 to 35% in 26 min, followed by a cleaning step with 100% mobile phase B. Ag/AgCl (reference) and Au electrodes were utilized for amperometric detection, using a carbohydrate quadruple waveform. The reference solution used for the identification and quantification of the samples was a standard with 10 µM rhamnose (internal standard), D-glucose, D-fructose and Suc.

After their purification, EPSs obtained from Halobacterial cultures grown on Suc were analyzed for their monomeric sugar composition via acid hydrolysis in 60 mM HCl at 70 °C for 90 min, followed by carbohydrate analysis with HPAEC-IPAD. Inulin (Orafti® HP, plant origin, low DP) and *Halomonas* levan (levan produced via the recombinant levansucrase enzyme of *Halomonas smyrnensis* AAD6, high DP, Kırtel et al., 2018b) were used as control samples.

2.6. NMR analysis

To determine the linkage types of the purified EPSs, vacuum-dried pure fructan samples were dissolved in 0.5 ml D_2O (99.8%) prior to the measurement of Nuclear Magnetic Resonance (NMR) spectra that were recorded at Bruker Avance II 600 MHz at 293 K equipped with 5 mm TCI HCN Z gradient cryoprobe. In one-dimensional proton spectra HOD signal was suppressed by applying presaturation during relaxation delay for 1 s. Natural abundance gradient enhanced heteronuclear shift-correlated two-dimensional (2D) experiments (gHSQC) (Schleucher et al., 1994) were carried out using an F2 spectral width of 6000 Hz (1 H) and an F1 width of 5130 Hz (^{13}C); depending on concentration 4–220 time scans were accumulated with 4 K data points in F2 and 512 data points in F1 (256 in IBSBc sample due to low concentration), respectively. The structures of inulin (Orafti® HP) and *Halomonas* levan reference samples were confirmed by full assignment of ^{1}H and ^{13}C signals according to standard methods relying on TOCSY, NOESY, HSQC and HMBC spectra (Vanhaecke, Van den Ende, Van Laere, Herdewijn, & Lescrinier, 2006).

3. Results and discussion

3.1. Chemical characterization of archaeal EPSs

Monomeric sugar compositions of purified EPSs from Archaea were determined with HPAEC-IPAD after acid hydrolysis. The results show that all seven EPS samples from different Archaeal strains were majorly composed of fructose with only trace amounts of glucose (Fig. 1), a typical profile for fructans. This was also verified with almost identical HPAEC-IPAD profiles obtained from acid-hydrolyzed inulin and levan control samples (not shown). Minor glucose signals in HPAEC-IPAD profiles indicate either the single terminal glucose residue originating from the initial Suc molecule (fructosyl acceptor) on which the polymerization process begins, or the internal glucose residue found in neofructan structures.

3.2. NMR analysis

Due to stereoelectronic effects of the different glycosidic bonds that interconnect fructoses in levan and inulin, the chemical shifts of their ^{1}H and ^{13}C signals each have a characteristic distribution. Despite signal overlap, one-dimensional ^{1}H spectra of both fructans are significantly different (Fig. 2, left). To resolve ^{1}H NMR signals and obtain ^{13}C chemical shifts on natural abundance samples at low concentration,

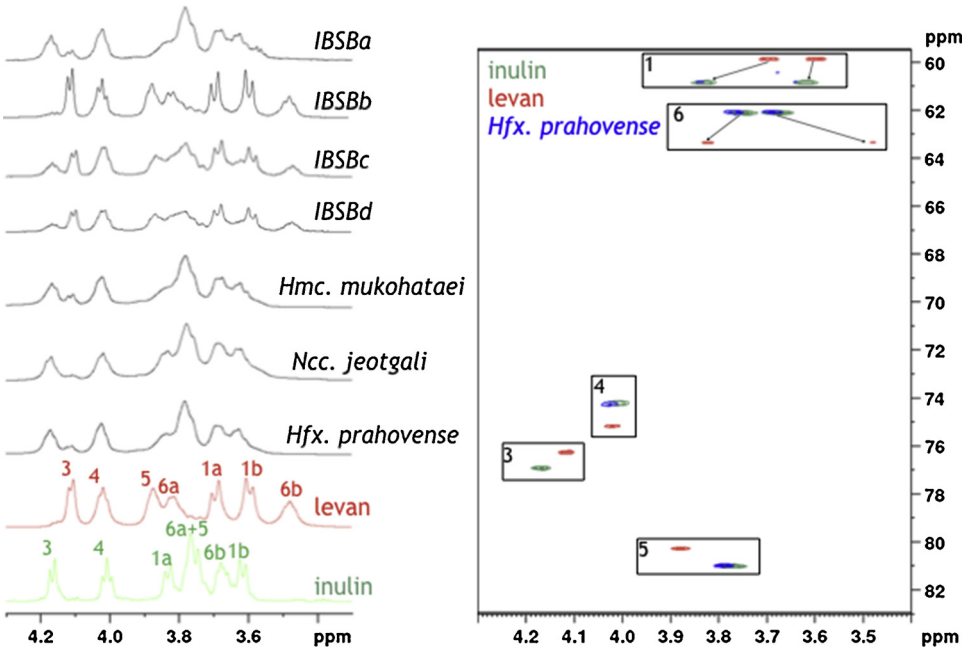

Fig. 2. NMR results of purified Archaeal fructans. Left: stacked plot of 1D ^{1}H spectra of reference samples and fructans isolated from a selection of Archaea, with strain and species names indicating their sources; **right:** overlay of main signals in the gHSQC of reference samples and the fructan isolated from *Hfx. prahovense* DSM 18310 (blue), demonstrating match with inulin. Arrows indicate the typical downfield shift of ^{13}C atoms that are involved in a glycosidic linkage. Numbers refer to assignment of signals. Each box in the right panel includes signals with the same assignment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

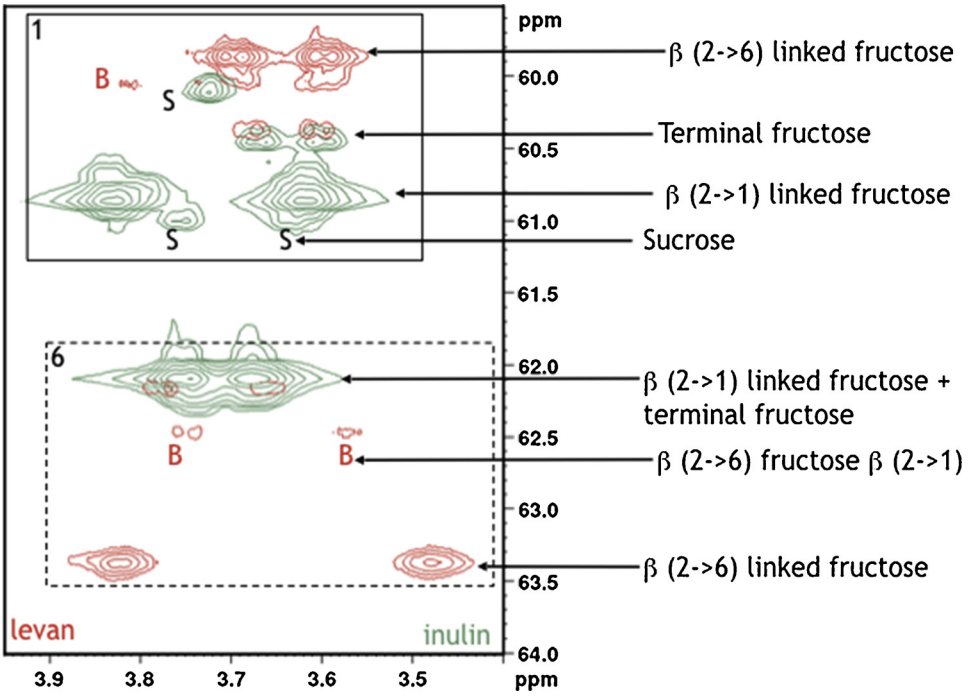

Fig. 3. Branching characteristics of levan and inulin. Section of gHSQC containing C1 and C6 signals at low threshold showing weaker signals of terminal fructose and fructose residues at a branchpoint (labeled with B). For inulin, also Suc signals are visible (labeled with S). Signals boxed in solid line: 1 − CH2; signals boxed in dashed line: 6 − CH2. Inulin reference sample was purchased from Orafti® (HP, plant origin, low DP), while levan was produced via recombinant levansucrase enzyme of *H. smyrnensis* AAD6 (high DP, Kırtel, Menéndez et al., 2018).

Table 1
Branching in fructans used in this study based on the cross peak volumes (V) of observed C6/H6 correlation in gHSQC.

Fructan	V (terminal)	V (β)	V (2-6)	Branching (%)
Halomonas levan[a]	26.04	23.45	183.11	10
strain IBSBb levan	21.98	20.10	123.40	12
	V (terminal) + V (2-1)			
Hmc. mukohataei inulin	329.36	61.99		16
Hfx. prahovense inulin	1003.90	170.90		15
Ncc. jeotgali inulin	180.60	19.10		10

[a] Synthesized via recombinant levansucrase enzyme of the bacterium *H. smyrnensis* AAD6 as reported in Kırtel et al. (2018b).

2D gHSQC spectra were recorded to obtain crosspeaks between hydrogens and the carbon to which they are directly bound. The characteristic downfield shift for carbons at linked positions to higher ppm is clearly visible in the overlayed gHSQC spectra of inulin and levan: in β-2,1 linked inulin C1 signals are downfield shifted compared with C1 in levan (+4.3 ppm) while in β-2,6 linked levan strong C6 signals in the main chain are downfield shifted compared with C6 in inulin (+3.6 ppm). Due to their different glycosidic bonds, also chemical shifts of other carbons and protons in the fructofuranosyl ring slightly differ in inulin versus levan, though this effect is less pronounced. The C2 signal is not observable in a gHSQC since it is not directly bound to a hydrogen. A small degree of branching was determined in the reference sample of levan, allowing to determine chemical shift signatures for terminal fructoses that are involved in a single glycosidic bond and

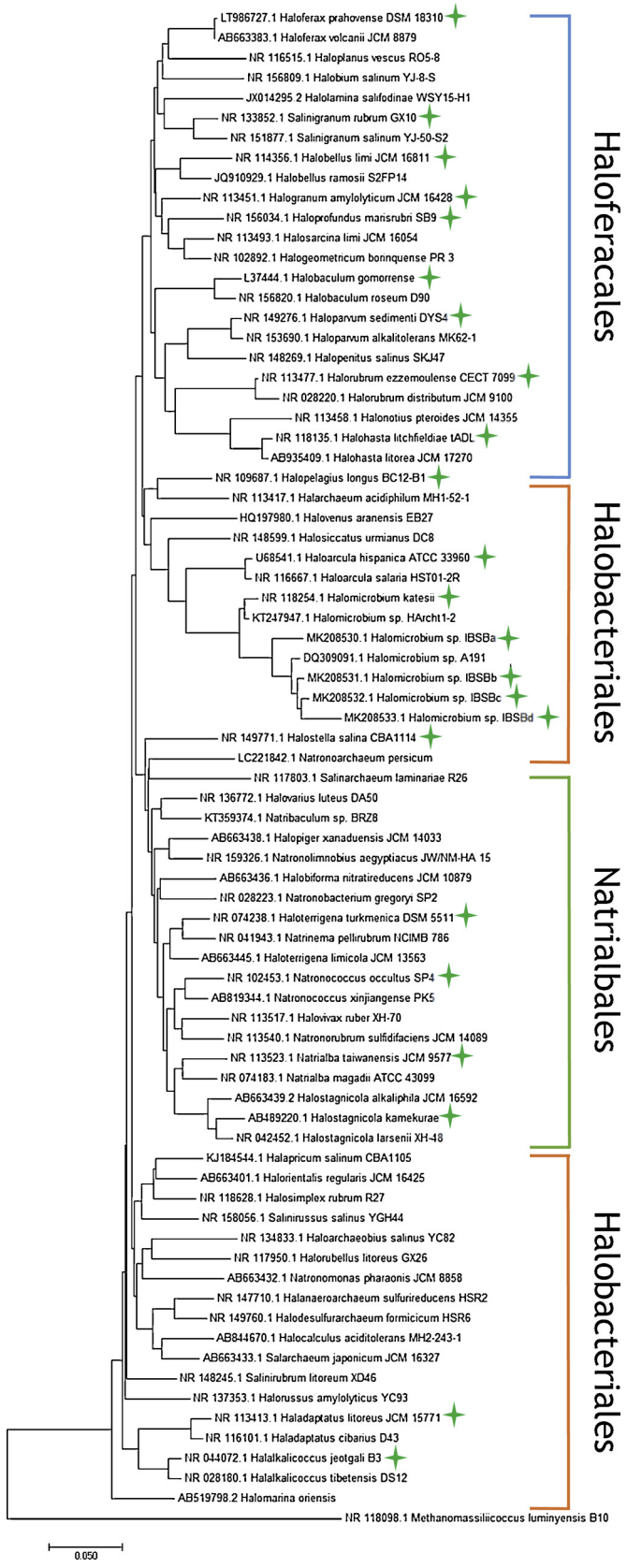

Fig. 4. Taxonomic distribution of fructanogenic Archaea. Distribution of some Archaea that harbor putative GH68 family genes within the Halobacteria class, each denoted with a star. The phylogenetic tree was based on 16S rRNA gene sequences of some Archaea from all three orders of the Halobacteria class, and built with MEGA7 (Kumar, Stecher, & Tamura, 2016) using neighbor-joining method. *Methanomassiliicoccus luminyensis* B10 was used as the outgroup.

fructoses at branch points with both β-2,1 and β-2,6 linkages.

The 1D proton spectra of fructans isolated from Archaea resemble results on inulin in samples from *Hmc. mukohataei* DSM 12286, *Hfx. prahovense* DSM 18310, *Ncc. jeotgali* DSM 18795 and strain IBSBa, while strain IBSBb matches the spectrum of levan. Fructans from strain IBSBc and strain IBSBd have a 1D ^{1}H spectrum with characteristics that could be attributed to levan as well as inulin, indicating the presence of both. gHSQC spectra of all Archaeal fructans can be found in Supplementary Material.

Usually, the degree of branching in polysaccharides is estimated based on the relative intensity of well separated anomeric proton signals (Gidley, 1985; Nilsson, Gorton, Bergquist, & Nilsson, 1996; Zhu, 2017). However, fructans do not have a proton in this position. Therefore, we used the relative intensity of C6/H6 crosspeaks in the gHSQC that are well dispersed depending on the linkages in which fructose units are involved (Fig. 3). Branching percentage calculations based on the cross peak volumes of observed C6/H6 correlation in gHSQC revealed that fructans from Halobacteria are branched with varying degrees (Table 1). Among the fructans of Tuz Lake isolates, only levan from strain IBSBb could be used for branching calculations, because the other three showed too weak signals at relative positions, due to low sample concentrations.

Branching characteristics of levans and inulins depend greatly on their sources. For instance, bacterial inulins are more branched than plant inulins (Shoaib et al., 2016). Levans from Eubacteria exhibit varying branching degrees, too: while levans of *Bacillus subtilis, Zymomonas mobilis* and *Erwinia herbicola* had branching degrees between 10–11% (Benigar et al., 2014), levans obtained from the cultures of *H. smyrnensis* AAD6 (Poli et al., 2009) and levansucrase of *Brenneria* sp. EniD312 (Xu et al., 2018) were highly linear. These results also indicate that the conditions under which the polymerization of levan is carried out affect its branching characteristics. *Halomonas* levan used in this study was produced via the recombinant levansucrase of *H. smyrnensis* AAD6 (Kırtel et al., 2018b), and contrary to microbially-produced *Halomonas* levan, this enzymatically-produced levan had a branching degree of 10% (Table 1). Except for *Ncc. jeotgali* inulin, fructans from Halobacteria used in this study had slightly higher branching degrees compared to Eubacterial ones, indicating that Halobacterial fructans may have distinctive and novel physicochemical properties.

3.3. Taxonomic classification of Tuz Lake isolates

The strains isolated from Tuz Lake were taxonomically classified according to their 16S rRNA sequences as members of the genus *Halomicrobium* and they were named as *Halomicrobium* sp. IBSBa, IBSBb, IBSBc and IBSBd with respective GenBank accession numbers of MK208530, MK208531, MK208532 and MK208533. When these sequences were aligned using ClustalW, identity percentages between them were as follows (%): IBSBa-IBSBb: 96, IBSBa-IBSBc: 97, IBSBa-IBSBd: 95, IBSBb-IBSBc: 99, IBSBb-IBSBd: 95, IBSBc-IBSBd: 96. NCBI BLAST analyses revealed that all Tuz Lake strains except for IBSBd showed a sequence identity of > 96% with *Hmc. mukohataei* DSM 12286, while for IBSBd the identity was 94%. Taxonomically closest strains to our isolates were either *Halomicrobium* sp. A191 (DQ309091.1), which was also isolated from Tuz Lake, or *Halomicrobium* sp. HArcht1-2 (KT247947.1). A phylogenetic tree was constructed using the 16S rRNA gene sequences from various Archaea, both predicted to be fructanogenic or not (Fig. 4). As seen in the tree, several *Halomicrobium* strains appear closest to Tuz Lake isolates. Thus, the full lineage of the Tuz Lake isolates was determined as follows: *Archaea; Euryarchaeota; Stenosarchaea* group*; Halobacteria; Halobacteriales; Haloarculaceae*; *Halomicrobium; Halomicrobium* sp. IBSB.

According to NCBI Taxonomy database, currently there are only three identified species in the *Halomicrobium* genus; namely *Halomicrobium mukohataei*, *Halomicrobium katesii* and *Halomicrobium zhouii*. Members of this genus stain Gram-negative, and show red to

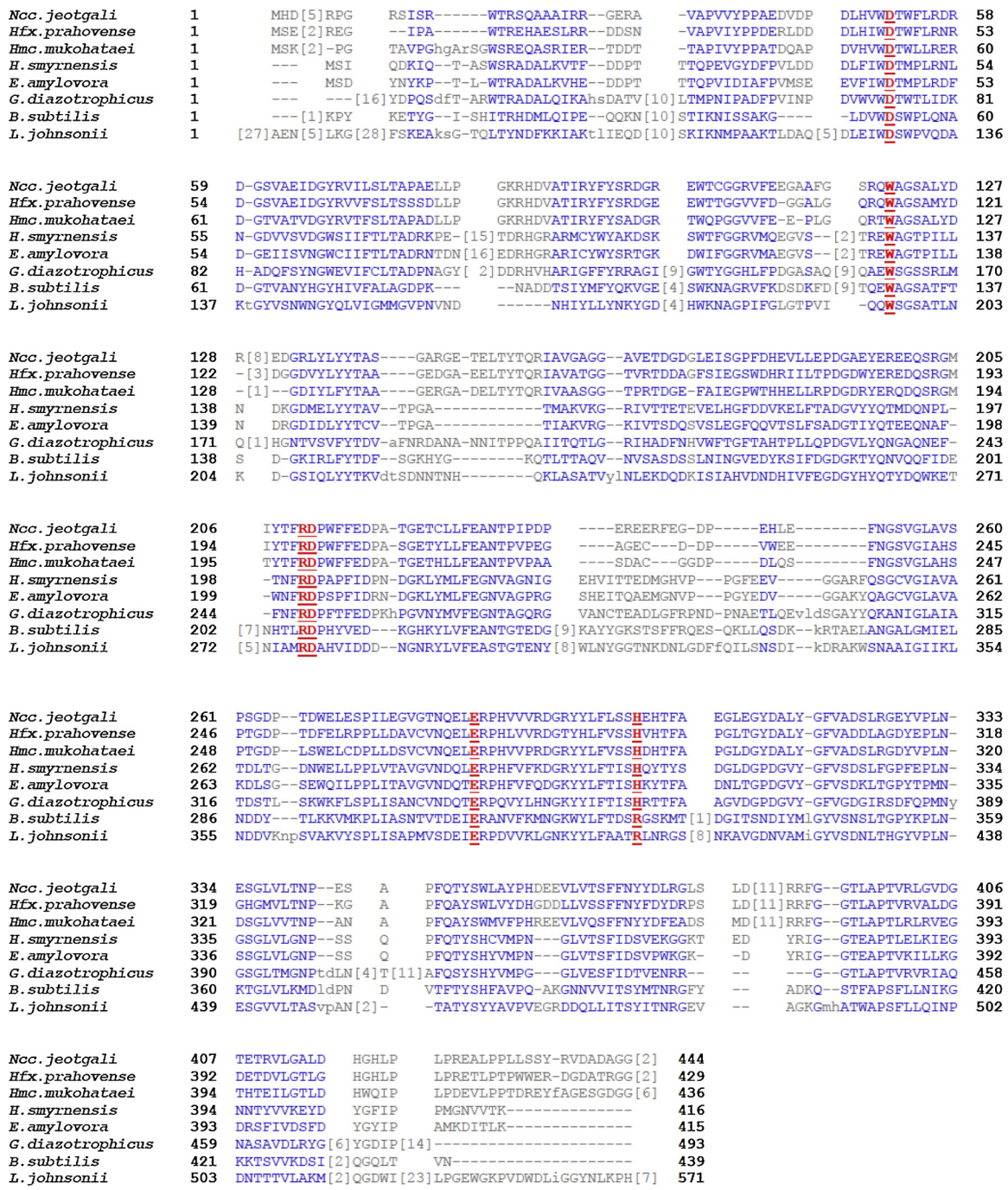

Fig. 5. Constraint-based multiple sequence alignment of several GH68 family enzymes. Alignment of Halobacterial GH68 family enzymes (with accession numbers: ELY66219.1 from *Ncc. jeotgali,* ELZ65257.1 from *Hfx. prahovense,* and ACV48051.1 from *Hmc. mukohataei)* with their selected Eubacterial homologues (levansucrase from *H. smyrnensis* AAD6, accession number: AGG11046.1; levansucrase from *E. amylovora,* PDB ID: 4D47; levansucrase from *Gluconacetobacter diazotrophicus,* PDB ID: 1W18; levansucrase from *B. subtilis,* PDB ID: 2VDT; inulosucrase from *L.johnsonii*, PDB ID: 2YFR). The alignment was carried out via NCBI COBALT. Conservation setting was set to 4. Bold red letters indicate strictly conserved amino acids that take part in catalysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

orange-red pigmentation. They are all extreme halophiles, requiring at least 1.4–3.4 M NaCl for growth, and have been isolated from various salt lakes, salterns and salt flats (Oren, 2015). Since *Halomicrobium* spp. typically carry multiple copies of divergent 16S rRNA genes (Cui, Zhou, Oren, & Liu, 2009), taxonomic identification on the species level requires extensive work.

As discussed in Kırtel et al. (2018a), putative GH-J clan genes are present in only extremely halophilic Archaea. Fructanogenic Archaea show a wide distribution within all three orders of the Halobacteria class (Fig. 4), dismissing the possibility that fructan synthesis is a rare, exceptional phenomenon in Halobacteria. As revealed in the phylogenetic tree, both fructanogenic and non-fructanogenic species are commonly encountered within the same genera. In the Halobacteriales order, 17 different species were predicted to harbor putative GH68 family genes, with the majority belonging to the *Haloarcula* genus; a close relative of the genus *Halomicrobium.* In the order of Haloferacales, fructanogenic species show a wider distribution with 32 different species, 12 of them belonging to the *Halorubrum* genus. As for the

Natrialbales order, only 9 species in the genera of *Halostagnicola, Haloterrigena, Natrialba* and *Natronococcus* carry putative GH68 family genes. Thus, approximately 21%, 31% and 16% of all known species of Halobacteriales, Haloferacales and Natrialbales orders, respectively, were predicted to be fructanogenic.

As discussed by Méheust et al. (2018), hundreds of chimeric protein families including GH-J clan enzymes in Halobacteria are most probably of Eubacterial origin, occuring as a result of gene transfer events throughout their evolution. This enabled Halobacteria to adapt to an aerobic lifestyle, unlike other classes of the archaeal domain of life, thus giving them the ability to utilize a wide variety of carbohydrates via aerobic respiration. Absence of any GH-J clan gene in methanogenic Archaea, which are the closest relatives and ancestors of Halobacteria, support the possibility that fructan metabolism appeared in Halobacteria in parallel with the emergence of their aerobic capabilities.

3.4. Structural investigation into halobacterial GH-J clan enzymes

All type strains used in this study, *Hmc. mukohataei, Hfx. prahovense* and *Ncc. jeotgali*, harbor one GH68 gene and one GH32 gene each. Multiple alignment and homology modelling analyses of these GH68 protein sequences via NCBICOBALT (Papadopoulos & Agarwala, 2007) and SWISS-MODEL (Biasini et al., 2014) were carried out. Multiple alignment of these four Halobacterial GH68 enzymes with some of their well-characterized Eubacterial homologues revealed that all critical amino acid residues that take part in catalysis are strictly conserved (Fig. 5). Numbered according to their homologues in *H. smyrnensis* AAD6 levansucrase, these residues are as follows: D47 (the nucleophile), W130 (plays a role in substrate binding), R201 (suggested to form a salt bridge with E286, Martínez-Fleites et al., 2005), D202 (the transition-state stabilizer), E286 (general acid/base catalyst) and H304 (crucial for the polymerization process). These residues were also evaluated for their positions and they were all observed to be superimposed towards the substrate located inside the deep central cavity of the 5-bladed β-propeller domain. Interestingly, although they all produce inulin, these Halobacterial GH68 enzymes show higher sequence similarity with known levansucrases (around 35%) compared to inulosucrases (around 25% for inulosucrase from *Lactobacillus johnsonii*, PDB ID: 2YFR), even with homologues of all the above-mentioned amino acid residues being also strictly conserved in inulosucrases. In fact, the mechanism that determines whether a fructan produced by the enzyme will carry either β-2,6 (levan-type) or β-2,1 (inulin-type) fructofuranosidic linkages on its backbone is still not understood and these Halobacterial enzymes may be the key to resolve this issue in the future.

4. Conclusions

The exact physiological functions of fructans in Halobacteria are yet unkown. Fructan and fructan metabolizing localization studies are required to shed light on this. The fact that our isolated strains can also grow on minimal media without Suc suggests that fructan formation is not strictly required for survival. Halobacteria are strictly heterotrophic and therefore Suc, the substrate for fructan synthesis, should have an exogenous origin. To shed light upon this subject, future studies should specifically focus on halophilic Suc-producers in hypersaline environments (Oren, 2015). For our four fructanogenic isolates, from Tuz lake, a rather “closed” ecosystem almost entirely fed by rain and ground water (Kashima, 2002), we expect that only one or a few autotrophic (Suc-producing) microorganism(s) are required to deliver Suc (Oren, 2014). The fructanogenic character appeared in Halobacterial members isolated from different places all over the world and the encoding GH-J genes seem to be widespread among Halobacteria, suggesting that fructans may provide benefits in hypersaline environments where Suc is available. We also detected a different branching degree on native Halobacterial inulins, and these distinctive and novel physicochemical properties are promising in terms of biotechnological applications. Future studies will involve obtaining the whole genome sequences of Tuz Lake isolates to get more solid information on Archaeal FTs. Overall, this work sets a milestone for carbohydrate/protein science in Archaea. Also, deeper insights into novel halophilic enzymes may provide strategies that help to overcome salinization, a serious threat to all life forms in the future. Now that it is demonstrated here that fructans are present in all three domains of life, in the future these polymers may prove to be key molecules to reveal yet-unknown dynamics shaping life in the presence of (salt and) Suc.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.carbpol.2019.05.064.

References

Adamberg, K., Tomson, K., Talve, T., Pudova, K., Puurand, M., Visnapuu, T., ... Adamberg, S. (2015). Levan enhances associated growth of *Bacteroides, Escherichia, Streptococcus and Faecalibacterium* in fecal microbiota. *PloS One, 10*, e0144042.

Avila-de Dios, E., Gomez-Vargas, A. D., Damian-Santos, M. L., & Simpson, J. (2015). New insights into plant glycoside hydrolase family 32 in Agave species. *Frontiers in Plant Science, 6*, 594.

Benigar, E., Dogsa, I., Stopar, D., Jamnik, A., Cigić, I. K., & Tomšič, M. (2014). Structure and dynamics of a polysaccharide matrix: aqueous solutions of bacterial levan. *Langmuir, 30*, 4172–4182.

Biasini, M., Bienert, S., Waterhouse, A., Arnold, K., Studer, G., Schmidt, T., ... Schwede, T. (2014). SWISS-MODEL: Modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Research, 42*, W252–W258.

Borrel, G., McCann, A., Deane, J., Neto, M. C., Lynch, D. B., Brugère, J. F., & O'Toole, P. W. (2017). Genomics and metagenomics of trimethylamine-utilizing Archaea in the human gut microbiome. *The ISME Journal, 11*, 2059–2074.

Choukade, R., & Kango, N. (2019). Characterization of a mycelial fructosyltransferase from *Aspergillus tamarii* NKRC 1229 for efficient synthesis of fructooligosaccharides. *Food Chemistry, 286*, 434–440.

Cui, H. L., Zhou, P. J., Oren, A., & Liu, S. J. (2009). Intraspecific polymorphism of 16S rRNA genes in two halophilic archaeal genera, *Haloarcula* and *Halomicrobium*. *Extremophiles, 13*, 31–37.

Dogsa, I., Brloznik, M., Stopar, D., & Mandic-Mulec, I. (2013). Exopolymer diversity and the role of levan in *Bacillus subtilis* biofilms. *PloS One, 8*, e62044.

Enache, M., Itoh, T., Kamekura, M., Teodosiu, G., & Dumitru, L. (2007). *Haloferax prahovense* sp. nov., an extremely halophilic archaeon isolated from a Romanian salt lake. *International Journal of Systematic and Evolutionary Microbiology, 57*, 393–397.

Gaci, N., Borrel, G., Tottey, W., O'Toole, P. W., & Brugère, J. F. (2014). Archaea and the human gut: New beginning of an old story. *World Journal of Gastroenterology: WJG, 20*, 16062.

Gidley, M. J. (1985). Quantification of the structural features of starch polysaccharides by NMR spectroscopy. *Carbohydrate Research, 139*, 85–93.

Hardoim, P. R., Van Overbeek, L. S., Berg, G., Pirttilä, A. M., Compant, S., Campisano, A., ... Sessitsch, A. (2015). The hidden world within plants: Ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiology and Molecular Biology Reviews, 79*, 293–320.

Ihara, K., Watanabe, S., & Tamura, T. (1997). *Haloarcula argentinensis* sp. nov. and *Haloarcula mukohataei* sp. nov., two new extremely halophilic archaea collected in Argentina. *International Journal of Systematic and Evolutionary Microbiology, 47*, 73–77.

Kang, S. A., Jang, K. H., Seo, J. W., Kim, K. H., Kim, Y. H., Rairakhwada, D., ... Rhee, S. K. (2009). *Levan: Applications and perspectives. Microbial production of biopolymers and polymer precursors: Applications and perspectives*145–161.

Kashima, K. (2002). Environmental and climatic changes during the last 20,000 years at Lake Tuz, central Turkey. *Catena, 48*, 3–20.

Kırtel, O., Versluys, M., Van den Ende, W., & Toksoy Öner, E. (2018). Fructans of the saline world. *Biotechnology Advances, 36*, 1524–1539.

Kırtel, O., Menéndez, C., Versluys, M., Van den Ende, W., Hernández, L., & Toksoy Öner, E. (2018). Levansucrase from *Halomonas smyrnensis* AAD6^T: First halophilic GH-J clan enzyme recombinantly expressed, purified, and characterized. *Applied Microbiology and Biotechnology, 102*, 9207–9220.

Koczan, J. M., McGrath, M. J., Zhao, Y., & Sundin, G. W. (2009). Contribution of *Erwinia amylovora* exopolysaccharides amylovoran and levan to biofilm formation: Implications in pathogenicity. *Phytopathology, 99*, 1237–1244.

Kralj, S., Leeflang, C., Sierra, E. I., Kempiński, B., Alkan, V., & Kolkman, M. (2018). Synthesis of fructooligosaccharides (FosA) and inulin (InuO) by GH68 fructosyltransferases from Bacillus agaradhaerens strain WDG185. *Carbohydrate Polymers, 179*, 350–359.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution, 33*, 1870–1874.

Lammens, W., Le Roy, K., Schroeven, L., Van Laere, A., Rabijns, A., & Van den Ende, W. (2009). Structural insights into glycoside hydrolase family 32 and 68 enzymes: Functional implications. *Journal of Experimental Botany, 60*, 727–740.

Lei, X. J., Kim, Y. M., Park, J. H., Baek, D. H., Nyachoti, C. M., & Kim, I. H. (2017). Effects of levan-type fructan on growth performance, nutrient digestibility, diarrhoea scores, faecal shedding of total lactic acid bacteria and coliform bacteria, and faecal gas emission in weaning pigs. *Journal of the Science of Food and Agriculture, 98*, 1539–1544.

Loukas, A., Kappas, I., & Abatzopoulos, T. J. (2018). HaloDom: A new database of halophiles across all life domains. *Journal of Biological Research-Thessaloniki, 25*, 2.

Martínez-Fleites, C., Ortíz-Lombardía, M., Pons, T., Tarbouriech, N., Taylor, E. J., Arrieta, J. G., ... Davies, G. J. (2005). Crystal structure of levansucrase from the Gram-negative bacterium *Gluconacetobacter diazotrophicus*. *The Biochemical Journal, 390*, 19–27.

Méheust, R., Watson, A. K., Lapointe, F. J., Papke, R. T., Lopez, P., & Bapteste, E. (2018). Hundreds of novel composite genes and chimeric genes with bacterial origins contributed to haloarchaeal evolution. *Genome Biology, 19*, 75.

Nilsson, G. S., Gorton, L., Bergquist, K. E., & Nilsson, U. (1996). Determination of the degree of branching in normal and amylopectin type potato starch with 1H-NMR spectroscopy: Improved resolution and two-dimensional spectroscopy. *Starch-Stärke, 48*, 352–357.

Orellana, L. H., Chee-Sanford, J. C., Sanford, R. A., Löffler, F. E., & Konstantinidis, K. T. (2018). Year-round shotgun metagenomes reveal stable microbial communities in agricultural soils and novel ammonia oxidizers responding to fertilization. *Applied and Environmental Microbiology, 84*, e01646–17.

Oren, A., Elevi, R., Watanabe, S., Ihara, K., & Corcelli, A. (2002). *Halomicrobium mukohataei* gen. nov., comb. nov., and emended description of *Halomicrobium mukohataei*. *International Journal of Systematic and Evolutionary Microbiology, 52*, 1831–1835.

Oren, A. (2008). Microbial life at high salt concentrations: Phylogenetic and metabolic diversity. *Saline Systems, 4*, 2.

Oren, A. (2014). The ecology of *Dunaliella* in high-salt environments. *Journal of Biological Research-Thessaloniki, 21*, 23.

Oren, A. (2015). Cyanobacteria in hypersaline environments: Biodiversity and physiological properties. *Biodiversity and Conservation, 24*, 781–798.

Papadopoulos, J. S., & Agarwala, R. (2007). COBALT: Constraint-based alignment tool for multiple protein sequences. *Bioinformatics, 23*, 1073–1079.

Peshev, D., & Van den Ende, W. (2014). Fructans: Prebiotics and immunomodulators. *Journal of Functional Foods, 8*, 348–357.

Poli, A., Kazak, H., Gürleyendağ, B., Tommonaro, G., Pieretti, G., Toksoy Öner, E., & Nicolaus, B. (2009). High level synthesis of levan by a novel *Halomonas* species growing on defined media. *Carbohydrate Polymers, 78*, 651–657.

Roh, S. W., Nam, Y. D., Chang, H. W., Sung, Y., Kim, K. H., Lee, H. J., ... Bae, J. W. (2007). *Natronococcus jeotgali* sp. nov., a halophilic archaeon isolated from shrimp jeotgal, a traditional fermented seafood from Korea. *International Journal of Systematic and Evolutionary Microbiology, 57*, 2129–2131.

Schleucher, J., Schwendinger, M., Sattler, M., Schmidt, P., Schedletzky, O., Glaser, S. J., ... Griesinger, C. (1994). A general enhancement scheme in heteronuclear multidimensional NMR employing pulsed field gradients. *Journal of Biomolecular NMR, 4*, 301–306.

Sehgal, S. N., & Gibbons, N. E. (1960). Effect of some metal ions on the growth of *Halobacterium cutirubrum*. *Canadian Journal of Microbiology, 6*, 165–169.

Shoaib, M., Shehzad, A., Omar, M., Rakha, A., Raza, H., Sharif, H. R., ... Niazi, S. (2016). Inulin: Properties, health benefits and food applications. *Carbohydrate Polymers, 147*, 444–454.

Tarkowski, Ł. P., Van de Poel, B., Höfte, M., & Van den Ende, W. (2019). Sweet immunity: Inulin boosts resistance of lettuce (*Lactuca sativa*) against grey mold (*Botrytis cinerea*) in an ethylene-dependent manner. *International Journal of Molecular Sciences, 20*, 1052.

Toksoy Öner, E. T., Hernández, L., & Combie, J. (2016). Review of levan polysaccharide: From a century of past experiences to future prospects. *Biotechnology Advances, 34*, 827–844.

Vanhaecke, M., Van den Ende, W., Van Laere, A., Herdewijn, P., & Lescrinier, E. (2006). Complete NMR characterization of lychnose from *Stellaria media* (L.) Vill. *Carbohydrate Research, 341*, 2744–2750.

Verspreet, J., Hansen, A. H., Dornez, E., Delcour, J. A., Van den Ende, W., Harrison, S. J., & Courtin, C. M. (2015). LC-MS analysis reveals the presence of graminan-and neotype fructans in wheat grains. *Journal of Cereal Science, 61*, 133–138.

Wei, H., Zhao, H., Su, T., Bausewein, A., Greiner, S., Harms, K., & Rausch, T. (2017). Chicory R2R3-MYB transcription factors CiMYB5 and CiMYB3 regulate fructan 1-exohydrolase expression in response to abiotic stress and hormonal cues. *Journal of Experimental Botany, 68*, 4323–4338.

Woese, C. R., Kandler, O., & Wheelis, M. L. (1990). Towards a natural system of organisms: Proposal for the domains Archaea, bacteria, and eucarya. *Proceedings of the National Academy of Sciences U. S. A. 87*, 4576–4579.

Xu, X., Gao, C., Liu, Z., Wu, J., Han, J., Yan, M., & Wu, Z. (2016). Characterization of the levan produced by *Paenibacillus bovis* sp. nov BD3526 and its immunological activity. *Carbohydrate Polymers, 144*, 178–186.

Xu, W., Liu, Q., Bai, Y., Yu, S., Zhang, T., Jiang, B., & Mu, W. (2018). Physicochemical properties of a high molecular weight levan from *Brenneria* sp. EniD312. *International Journal of Biological Macromolecules, 109*, 810–818.

Zannini, E., Waters, D. M., Coffey, A., & Arendt, E. K. (2016). Production, properties, and industrial food application of lactic acid bacteria-derived exopolysaccharides. *Applied Microbiology and Biotechnology, 100*, 1121–1135.

Zaremba-Niedzwiedzka, K., Caceres, E. F., Saw, J. H., Bäckström, D., Juzokaite, L., Vancaester, E., ... Stott, M. B. (2017). Asgard archaea illuminate the origin of eukaryotic cellular complexity. *Nature, 541*, 353–358.

Zhang, J., Liu, C., Xie, Y., Li, N., Ning, Z., Du, N., ... Zhong, Y. (2017). Enhancing fructooligosaccharides production by genetic improvement of the industrial fungus *Aspergillus niger* ATCC 20611. *Journal of Biotechnology, 249*, 25–33.

Zhu, F. (2017). NMR spectroscopy of starch systems. *Food Hydrocolloids, 63*, 611–624.