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Salt-tolerant extracellular enzymes from the genus *Gracilibacillus*: A review

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Abstract
The genus *Gracilibacillus* includes over 20 species of halophilic bacteria which have been isolated from salterns, saline lakes or soils, plants, or animals and can grow in media containing 0.5% to 20% NaCl. The bacteria are Gram-positive motile thin rods or filaments that form terminal endospores. They release a variety of extracellular enzymes that are active in the presence of high salt concentrations but often also tolerate high temperatures, extreme pH, solvents, detergents, or metal ions. This review summarizes the characterization of five well-studied examples of these enzymes: an α-amylase/cyclodextrin glycosyl transferase from *G. dipsosauri* strain DD1: a xylanase from *Gracilibacillus* sp. strain TSCPVG, a protease from *G. boraciitolerans* strain LO15, an azo-dye reductase from *Gracilibacillus* sp. GTY, and an alginate lyase from *Gracilibacillus* strain A7. These studies illustrate how these proteins can be studied and suggest ways by which additional salt-tolerant extracellular enzymes from this group can be investigated.

Keywords: Alginate lyase, α-amylase, cyclodextrin glycosyl transferase, *Gracilibacillus*, protease, salt-tolerant enzyme, xylanase

Introduction
Microorganisms that have adapted to grow in habitats characterized by high salt concentrations, very low or high temperatures, extremely acidic or alkaline pH, high pressures, or potentially damaging radiation have been termed extremophiles [1-5]. They illustrate the range of environments within which life as we understand it is possible and can serve as examples of how external conditions affect the physical and chemical properties of biomolecules. Extremophiles have been investigated as models for life outside of the planet Earth [6-8], as potential contributors to bioremediation on the Earth [9-11], and as sources of proteins for biotechnology [12-17]. Among the enzymes that have been isolated and purified from extremophilic bacteria and archaea are amylases, cellulases, chitinases, DNA polymerases, lipases, and proteases. Some of these proteins are found in the cytoplasm but others are released into the environment as extracellular enzymes or exoenzymes [18-20]. The availability of modern techniques for DNA sequencing and genome analysis has made it possible to identify genes for additional enzymes from bacteria that cannot yet be cultured in the laboratory [21-22]. Expression of these genes in commonly-used hosts such as *Escherichia coli* can allow the proteins to be synthesized in usable amounts and analyzed. [23]

Extracellular enzymes or exoenzymes from halophilic microorganisms are of particular interest in terms of their potential applications [24-29]. Although nonhalophilic microbes grow best in media containing 0 to 2% salt (about 0.2 M) and may not be able tolerate higher concentrations, those that are slightly halophilic are more halotolerant can grow in 2% to 5% salt (about 0.2 to 0.5 M) [30]. Microorganisms that are moderately halophilic grow best in 5% to 20% salt (about 0.5 to 2.5 M) while extreme halophiles grow best in 20% to 30% salt (about 2.5 to 5.2 M). The exact salt composition may vary in natural habitats but NaCl is most often used in the laboratory. Because exoenzymes formed by moderately and extremely halophilic microbes are released directly into the growth medium, they are usually active under the more extreme physical conditions in which these microorganisms are grown. Moreover, these proteins can be readily isolated from laboratory cultures without having to disrupt the microbes. Following centrifugation to remove the microorganisms, the proteins can be precipitated with solvents such as ethanol or acetone or with salts such as ammonium sulfate and purified.

Because many fewer proteins are present in the initial extract than in a whole cell extract, the steps required for complete purification are often much simpler. While extracellular enzymes from halophilic microorganisms can usually function in high salt concentrations, many can also tolerate high temperatures or extreme pH and can maintain activity in the presence of solvents, detergents, or metal ions. Among the recently characterized examples of salt-tolerant extracellular enzymes with good potential applications are an acid-alkali tolerant and surfactant stable α-amylase from *Bacillus siamensis* F2 isolated in India [31], a thermostable serine protease from *Melghiribacillus thermohalophilus* Nari2A isolated in Algeria [32], a cold active solvent-tolerant lipase from *Bacillus licheniformis* KM12 isolated in Iran [33], and two thermostable xylanases from *Bacillus* sp. Asc6BA isolated in Chile [34].

The Genus *Gracilibacillus*

The genus *Gracilibacillus* was established in 1999 by the merger of several existing genera and species of Gram-positive bacteria [35]. It now includes over 20 recognized species which have been isolated from environmental sites, plants, and animals. The general properties of the genus and the characteristics of some of the best studied species were summarized in Volume 3 (Firmicutes) of the second edition of *Bergey's Manual of Systematic Bacteriology* [36]. This entry was updated in the online version of *Bergey's Manual of Systematics of Archaea and Bacteria* [37]. The genus has more recently been classified in the Genome Taxonomy Database into the family Amphibacillaceae [38-39]. Bacteria in the genus *Gracilibacillus* typically have DNAs with a total length of about 4.0 to 4.5 Mbp, a G+C content of 36 to 39 mole%, and can potentially encode 3000 to 4500 protein products [40]. The bacteria are generally described as motile Gram-positive thin rods or filaments with the potential to form terminal spherical, oval, or ellipsoidal endospores in swollen sporangia. They are moderately salt-tolerant and can usually grow in media containing 0 to 20% NaCl. The cell wall peptidoglycan contains *meso*-diaminopimelic acid and the chains are directly cross-linked. The cell membranes contain $C_{15:0}$, $C_{16:0}$, and $C_{17:0}$ fatty acids with the most prominent polar lipids being phosphotidylglycerol and diphosphotidylglycerol. The bacteria are chemoheterotrophs that sometimes can produce acid from glucose but most gain energy primarily by aerobic respiration using electron transport chains containing menaquinone MK-7. They can usually hydrolyze starch and esculin but are negative for arginine dihydrolase, lysine and ornithine decarboxylase, and indole production.

Table 1 summarizes some of the key properties of the 25 species *Gracilibacillus* species currently given in the List of Prokaryotic Names with Standing in Nomenclature (LPSN) [41].

Phylogenetic analyses based on 16S rRNA sequences which include most of these *Gracilibacillus* species have been done by several investigators and the figures in their papers show their relationships [42-44]. In the table, the species are listed alphabetically and each listing gives the primary reference, the source of the microorganism, an indication of whether the genome has been sequenced, the shape and position of the endospore, and whether the bacteria can reduce nitrate to nitrite as an indication of anaerobic respiration. The table also shows the ability of the bacteria to degrade individual compounds as determined by the standard laboratory tests. Degradation of most of these compounds depends on the formation and release of a hydrolytic exoenzyme [45]. For example, a positive result in the starch hydrolysis test indicates the presence of an extracellular α-amylase activity, a positive result in the casein or gelatin hydrolysis test indicates the presence of a protease, a positive result in the Tween hydrolysis test indicates the presence of a lipase or esterase, and a positive result in the urea hydrolysis test indicates the presence of a urease.

Table 1: Current list of *Gracilbacillus* species and their properties

Species Name	Primary Reference	Original Source	Genome Sequenced	Endospores	Nitrate Reduction	Starch Hydrolysis	Gelatin Hydrolysis	Casein Hydrolysis	Tween Hydrolysis	Urea Hydrolysis
G. aidingensis	Guan *et al*. 2017 [46]	Saline soil China	No	Spherical	-	+	-	-	w	+
G. alcalifphilus	Hirota *et al*. 2014 [47]	Fermented *Polygonum* liquor Japan	Yes	Spherical Terminal	+	+	+	-	+	+
G. bigeumensis	Kim *et al*. 2012 [48]	Solar saltern soil South Korea	No	Spherical Terminal	-	-	w	-	-	-
G. boraciitolerans-1	Ahmed *et al*. 2007 [49]	Boron-enriched soil Turkey	Yes	Spherical Terminal or subterminal	-	NR	-	NR	NR	-
G. caseinilyticus	Subramanian *et al*. 2023 [51]	Saltern soil South Korea	Yes	Spherical Terminal	-	-	+	+	-	+
G. dipsosauri-2	Deutch 1994 [52]	Desert iguana United States	Yes:	Spherical Terminal	+	+	w	-	NR	-
G. eburneus	Guan *et al*. 2018 [54]	Salt Lake sediment China	No	Spherical Terminal	+	+	-	NR	-	+
G. halophilus	Chen *et al*. 2008 [55]	Saline soil China	Yes	Ellipsoidal Terminal	+	+	+	-	+	-
G. halotolerans	Wainø *et al*. 1999 [35]	Salt Lake United States	Yes	Ellipsoidal terminal	+	+	+	-	+	+
G. kekensis	Gao *et al*. 2012 [56]	Salt Lake sediment China	Yes	Ellipoidal terminal	-	+	-	+	+	-
G. kimchii	Oh *et al*. 2021 [57]	Fermented vegetables Japan	No	Ellipsoidal Terminal	-	+	+	NR	NR	-
G. lacisalsi	Jeon *et al*. 2008 [58]	Salt Lake Sediment China	Yes	Spherical Terminal	+	-	NR	-	-	-
G. marinus	Huang *et al*. 2024 [59]	Sea water China	No	Spherical Terminal	+	+	-	-	-	-
G. massiliensis	Diop *et al*. 2017	Table Salt France	Yes	None	-	NR	NR	NR	NR	+

	[60]									
G. orientalis	Carrasco *et al.* 2006 [61]	Salt Lake water China	Yes	Spherical Terminal	-	+	+	-	-	-
G. oryzae	He *et al.* 2020 [62]	Rice seeds China	Yes	Ellipsoidal	+	+	+	NR	NR	-
G. phocaeensis-3	Senghor *et al.* 2017 [42]	Human Stool Senegal	Yes	+	-	NR	NR	NR	NY	+
G. quinghaiensis	Chen *et al.* 2008 [64]	Salt Lake sediment China	No	Ellipsoidal Terminal	+	-	-	-	-	+
G. salinarum	Subramanian *et al.* 2023 [51]	Saltern soil South Korea	Yes	Spherical Terminal	+	-	-	-	NR	+
G. saliphilus	Tang *et al.* 2009 [65]	Lake soil China	Yes	Spherical Terminal	+	+	+	-	-	+
G. salitolerans	Gan *et al.* 2020 [66]	Saline soil China	Yes	Spherical Terminal	+	+	-	-	-	-
G. suaedae	Huang *et al.* 2021 [67]	Suaeda salsa root China	Yes	+	-	-	-	NR	-	+
G. thailandensis	Chamroensaksri *et al.* 2010 [68]	Fermented fish Thailand	Yes	Oval Terminal	+	-	+	-	-	-
G. timonenesis-4	Senghor *et al.* 2017 [69]	Human stool Senegal	Yes	+	-	NR	NR	NR	NR	NR
G. ureilyticus	Huo *et al.* 2010 [70]	Saline Soil China	Yes	+	+	+	+	-	-	+
G. xinjiangensis	Yang *et al.* 2013 [71]	Soil China	No	Ellipsoidal Terminal	-	-	+	NR	-	-

Indicates a positive result, a (w) indicates a weak result, a (-) indicates a negative result, and NR indicates a result was not reported.

Notes:

- *Gracilibacillus boraciitolerans* was isolated and described by Ahmed *et al.* (2007) [49] and a draft genome sequence later reported by Ahmed *et al.* (2014) [50].
- *Gracilibacillus dipsosauri* was isolated and originally described as a *Bacillus* sp. by Deutch (1994) [52]. It was renamed *Bacilus dipsosauri* based on the same phenotypic characteristics by Lawson *et al.* (1996) [53] and remaned *G. dipsosauri* by Waimø *et al.* (1999) [35]. The genome was described by Deutch and Yang (2020) [40].
- *Gracilibacillus phocaeensis* was described by Senghor *et al.* (2017) [42] and again by Ngom *et al.* (2020) [63] with the same characteristics but added genomic data.
- *Gracilibacillus timonensis* was described by Senghor *et al.* (2017) [69] and again by Diop *et al.* (2018) [44] with same characteristics but added genomic data.

Well-characterized salt-tolerant extracellular enzymes from *Gracilibacillus*

Although the initial description of most new isolates of *Gracilibacillus* indicates that they have the capacity to form extracellular enzymes, only a few of these enzymes have been characterized in detail both biochemically and genetically. This section focuses on the properties of the best-characterized salt-tolerant extracellular enzymes and their potential applications.

***A*-Amylase/Cyclodextrin Glycosyl Transferase from *G. dipsosauri* Strain DD1**

Gracilibacillus dipsosauri strain DD1 was originally isolated from the nasal cavity of a desert iguana (*Dipsosaurus dorsalis*) collected near Las Vegas, Nevada in the United States [52]. These animals have salt glands that allow them to excrete a concentrated brine of KCl when they are osmotically stressed, and it was thought that this might be a novel source of salt-tolerant bacteria. Of 40 isolates recovered from 10 different animals on agar plates containing increasing concentrations of KCl at 37 °C, 29 were Gram-positive cocci and 11 were Gram-positive rods. Strain DD1 was one of several strains that could grow on R2A or tryptic soy broth (TSB) agar plates containing 20% (2.68 M) KCl. When grown at 37 °C in liquid tryptic soy broth containing 1.0 M KCl, the bacteria formed thin motile rods. The bacteria could grow well at salt concentrations as high as 2.5 M KCl or NaCl, but the growth rate gradually decreased as the salt concentration increased and they became more filamentous. In medium with no added salt or KCl concentrations less than 0.5 M, the cells lysed at the end of exponential growth. On tryptic soy broth agar plates containing 1.0 M KCl, the strain DD1 formed refractile spherical endospores within swollen sporangia at the end of the rods or within the longer filaments after 3 days at 37 °C. The temperature optimum for growth in tryptic soy broth containing 1.0 KCl was 45 °C. Growth could occur in the same medium at pHs from 6.5 to 10.0 but was optimal at pH 7.5.

Initial characterization of the bacteria using standard microbiological tests indicated that strain DD1 was catalase- and oxidase-positive. It could reduce nitrate to nitrite and could grow anaerobically on agar plates containing TSB supplemented with 1.0 M KCl and nitrate concentrations as high as 1.0 M. There was no indication of fermentation in API fermentation strips or in bromcresol purple fermentation broths, but there was weak acid formation but no gas formation in phenol red fermentation broths containing 0.5% glucose, sucrose, mannitol, or dulcitol. Strain DD1 produced enzymes hydrolyzing starch, triacylglycerides, esculin, *o*-nitrophenylgalactoside, and *p*-nitrophenylgalactoside. It showed weak gelatin hydrolysis but did not break down casein, phospholipids, red blood cells, or urea. It did not exhibit arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, or tryptophan deaminase activity. It did not utilize citrate, form indole or hydrogen sulfide, and gave negative results in the MR-VP tests. The bacteria were resistant to ampicillin, bacitracin, and streptomycin, but they were sensitive to chloramphenicol, kanamycin, and triple sulfa.

On the basis of these characteristics, strain DD1 was identified as a member of the genus *Bacillus*. However, because it differed from other known halophilic examples,

strain DD1 was thought to represent a new species. Analysis of its 16S rRNA sequence and comparison of that sequence to those of other bacteria led to its designation as *Bacillus dipsosauri* [53]. It was found to be distantly related to most other *Bacillus* species but to show some phylogenetic association with *B. pantothenicus*, *Sporosarcina halophila*, and *Marinococcus albus*. Strain DD1 was later transferred into the new genus *Gracilibacillus* as *G. dipsosauri* along with the new species *Gracilibacillus halotolerans* [35]. However, *G. dipsosauri* differs from *G. halotolerans* in that it is capable of anaerobic growth but does not produce H_2S or break down urea.

G. dipsosauri strain DD1 gave a positive result for the hydrolysis of starch as part of its initial characterization. On TSB agar plates containing 0.5% soluble starch and 0 to 2.5 M KCl, there were clear zones of hydrolysis around bacterial streaks after 4 days of incubation at 37 °C and staining with Gram's iodine solution [72]. The width of the zone of clearing decreased with increasing salt concentrations. This was consistent with the formation of an *α*-amylase (EC 3.2.1.1) able to catalyze the endohydrolysis of (1→4)-α-D-glycosidic linkages in polysaccharides containing three or more (1→4)-α-linked D-glucose units. Attempts to measure amylase activity in liquid culture medium after removal of the bacteria by centrifugation with standard assays based on the binding of iodine to starch [73], the hydrolysis of dye-labelled polymeric substrates [74], or the formation of reducing sugars by reaction with the 3, 5-dinitrosalicylic acid reagent [75] gave inconsistent results. However, the activity could be assayed quantitatively using the synthetic substrate 2-chloro-4-nitrophenyl-α-D-maltotrioside (CNPG3) [76]. The formation of 2-chloro-4-nitrophenol could be measured spectrophotometrically at 405 nm and was linear with time and the amount of culture fluid. Synthesis of the enzyme by strain DD1 during growth in TSB containing 1.0 M KCl was induced by starch but repressed by D-glucose. It was highest after growth to stationary phase in TSB medium without glucose containing 1.0 M KCl and 0.5% soluble starch.

The proteins in the culture medium from a stationary phase culture were concentrated by precipitation with cold ethanol and resuspended in 50 mM MES buffer, pH 6.1. Nondenaturing gel electrophoresis indicated that there was a single band of amylase activity. In the assay with CNPG3 as the substrate, the activity increased with the KCl concentration from 0 to 2.0 M, had a pH optimum of 6.5, and increased with temperature up to 60ºC before it was rapidly lost. Enzyme activity was maintained in the presence of 1.0 M concentrations of various salts but stimulated about 4-fold by 1.0 Na_2SO_4. The enzyme was purified from about 500 ml of culture fluid by a combination of ethanol precipitation, passage through a BioGel P6-DG desalting column, ion-exchange chromatography on a column of DEAE-cellulose, and gel filtration chromatography of a column of BioGel-100. The purified material gave a single band with an apparent molecular mass of about 80 kDa after SDS-PAGE and staining with Coomassie Blue. It again gave a single band after activity staining of a nondenaturing gel but the apparent molecular mass was only about 30 kDa.

The purified enzyme showed simple Michaelis-Menten kinetics with an apparent K_m for CNPG3 of 12.5 mM. The enzyme did not hydrolyze *p*-nitrophenyl-*α*-D-glucopyranoside, *p*-nitrophenyl-*α*-D-galactopyranoside, or *p*-nitrophenyl-*α*-D-mannopyranoside. The activity was not inhibited by free D-glucose but was inhibited by glucose-containing oligosaccharides of increasing length. It was inhibited by a number of divalent cations including Cd^{2+} and Zn^{2+}. The range of starch substrates was determined by line streaks on TSB without glucose agar plates containing 1.0 M KCl and 1% starch samples. After 4 days at 37 °C and staining with Gram's iodine, wide zones of hydrolysis were seen with potato amylopectin, unmodified wheat starch, heat-hydrolyzed wheat starch, rice starch, and corn starch. There were narrower zones of hydrolysis around the streaks of potato starch and potato amylose.

Although these results were consistent with the formation and release of a salt-tolerant α-amylase by *G. dipsosauri* strain DD1, there were some issues that required clarification. Why could the activity be quantitatively measured with the synthetic substrate CNPG3 but not with the other standard assays for α-amylase? Why did the purified enzyme exhibit a denatured molecular mass of about 80 kDa but a native mass of about 30 kDa? Why was the protein able to maintain its activity at very high salt concentrations? Repeated attempts to clone the gene for the enzyme from genomic strain DD1 DNA into a variety of plasmids were unsuccessful. To resolve these issues, the whole genome of *G. dipsosauri* was sequenced, assembled, and analyzed [40]. The genome was found to be 4.19 Mb in size with an overall G+C content of 36.9%. It appeared to contain 4052 genes, of which 3941 could potentially code for proteins. It was similar in size and composition to *G. ureilyticus*. Analysis of the *G. dipsosauri* genome revealed the presence of a genetic locus (DLJ74_06065) which appeared to code for an α-amylase composed of 691 amino acids (PWU69658.1 in the NCBI Protein database and A0A317L430 in the UniProt KB database). The protein appeared to be a member of the glycosyl hydrolase 13 family and to have a molecular mass of 77,396. BLAST analysis revealed significant matches to the α-amylase from *G. orientalis* (70% amino acid identity) and to a cyclodextrin glycosyl transferase (CGT) from *Bacillus cereus* (66% identity).

The BLAST analysis showed sequence similarities to a number of other enzymes with CGT activity (EC 2.4.1.19, 1,4-alpha-D-glucan 4-alpha-D-(1,4-alpha-D-glucano)-transferase (Cyclizing)). These enzymes catalyze the breakdown of starches to form cyclic molecules with 6 (α-cyclodextrins), 7 (β-cyclodextrins), and 8 (γ-cyclodextrins) glycosyl units. Because the cyclodextrin products are internally linked, they behave as nonreducing sugars and do not react with reagents such as 3, 5-dinitrosalicylic acid used in the standard α-amylase assays. To determine if the enzyme from *G. dipsosauri* strain DD1 has CGT activity, the protein was again purified from culture fluid after growth of the bacteria to stationary phase at 37 °C in tryptic soy broth without glucose containing 1.0 M KCl and 0.5% soluble starch. The culture fluid gave a positive test for the presence of β-cyclodextrins using phenolphthalein as the colorimetric reagent [77-78] as well as a positive test for α-amylase activity with the CNPG3 substrate and with *p*-nitrophenyl-D-maltoheptoside (PNGP7) as another synthetic substrate. When the proteins in the culture fluid were precipitated with ethanol and concentrated, both CGT and α-amylase activities increased to the same extent. When the proteins were further purified on a BioGel-P6 desalting column and further separated on a DEAE-Sepharose column, both activities eluted together in the same fractions.

Although these results showed good formation of β-cyclodextrins, there was no formation of α-cyclodextrins or γ-cyclodextrins as indicated with reactions with methyl orange or bromcresol green. Unlike the α-amylase activity with the CNPG3 substrate which was maintained in the presence of 1.0 M concentrations of many salts and stimulated by even higher concentration of Na_2SO_4, the CGT activity was inhibited by added salts including Na_2SO_4. The CGT activity could be detected after growth in the presence of a variety of starch substrates including rice starch, potato starch, wheat starch, and corn starch. The formation of β-cyclodextrins by the enzyme from strain DD1 was much higher with amylopectin than with amylose as the substrate.

Modeling of the three-dimensional structure of the α-amylase/CGT protein from *G. dipsosauri* indicated that it has a very compact form with 59.9% α-helices and 43.6% β-strands. The predicted isoelectric point (pI) of the protein is 4.39 and 13.7% (95/691) of the amino acids in its sequence are aspartate or glutamate. Under non-denaturing conditions in the standard Laemmli buffer system, the protein would be negatively-charged and expected to move towards the positive electrode. In combination with the compact three-dimensional structure, this could explain its unusual native mobility and apparent small molecular mass compared to its actual size.

Previous studies have also shown that proteins with a high salt tolerance often have a high ratio of acidic to basic residues and a small number of hydrophobic residues. In contrast to the high content of aspartate and glutamate in the protein from strain DD1, only 5.6% (39/691) of the residues are arginine or lysine and only 16.6% (115/691) are phenylalanine, leucine, or isoleucine. The protein from *G. dipsosauri* only contains one cysteine residue so disulfide bridges play no role in the stabilization of the protein's structure. Although the α-amylase/CGT protein from *G. dipsosauri* is now well-characterized and the gene has been identified, that gene has not yet been cloned into an expression vector or the protein produced in large amounts. Any applications of this enzyme will still require further research.

Xylanase from *Gracilibacillus* sp. strain TSCPVG

Gracilibacillus sp. strain TSCPVG was isolated from soil samples collected in Sambhar Salt Lake in Rajasthan, India [79]. It was selected for its ability to degrade xylan, a complex plant hemicellulose consisting of a polymeric backbone of D-xylose residues linked 1, 4-β with attached acetyl, arabinose, glucuronic acid, or methyl glucuronic acid groups [80]. Complete hydrolysis of xylan normally requires endo-1, 4-β-D-xylanases, β-D-xylosidases, α-L-arabinofuranosidases, α-D-glucuronidases, and acetylxylanesterases. The organism was grown at 30 °C in a rich medium at pH 7.5 containing 3.5% NaCl, 0.75% Birchwood xylan, 0.2% tryptone, 0.2% yeast extract, and a complex mixture of salts and trace elements. The bacterium was an aerobic Gram-positive motile short rod that formed terminal endospores. It was positive for catalase and amylase but negative for oxidase, urease, nitrate reduction, and H_2S production. Analysis of its 16S rRNA sequence showed it was 94% homologous to *G. halophilus*.

Xylanase activity could be measured by combining 500 µl of a 1% birchwood xylan solution in 100 mM sodium phosphate buffer pH 7.5 containing 3.5% NaCl, 400 µl of buffer, and 100 µl of a diluted culture supernatant. After incubation at 60 °C for 10 minutes, the amount of reducing sugar formed was detected with the 3, 5-dinitrosalicylic acid reagent and quantified by comparison to a D-xylose standard curve [81]. Strain TSCPVG grew in the presence of 1 to 30% NaCl but there was no growth in the absence of NaCl. Growth and xylanase formation were maximal at 3.5% NaCl, and both total cellular protein and xylanase activity decreased as the salt concentration was increased further. The bacteria also grew well at pHs from 6.5 to 10 but was poor at pH 6.0. Total cellular protein and xylanase activity were maximal at pH 7.5 and decreased gradually at pHs up to 10. The temperature optimum for growth and xylanase formation was at 30 °C, and both total protein and total enzyme activity decreased rapidly at 25 °C and 20 °C or at 35 °C and 40 °C. The formation and release of the extracellular xylanase began at the end of exponential phase and continued during stationary phase. Addition of xylan or D-xylose induced xylanase formation, but addition of L-arabinose, D-ribose, or D-glucose repressed it. The activity was not induced by starch, dextrin, or pectin. Maximum growth and xylanase formation occurred with the addition of 1.0% yeast extract and 0.8% tryptone to the basal medium containing 0.75% xylan and 3.5% NaCl.

The xylanase activity in the culture supernatant was active over a wide range of NaCl concentrations from 0 to 30%. It was also active at pHs from 5.0 to 10.5. The enzyme showed maximal activity at 60 °C. The culture fluid was also found to contain a β-xylosidase activity. The xylanase activity in the culture supernatant could be precipitated with acetone. When the proteins were resuspended and analyzed by nondenaturing polyacrylamide gel electrophoresis, there were multiple bands in both the Coomassie Blue stained gel and in the activity gel. There was a broad prominent band at about 55 kDa, but additional bands at about 22 kDa and 18 kDa. It was not clear with these were completely different proteins or modified forms of the same protein. The products of the xylanase reaction were separated by HPLC and found to include xylose, xylobiose, xylotriose, and arabinose. This indicated that the enzyme is an endoxylanase.

Poosarla and Chandra [82] later described complete purification of one specific xylanase from *Gracilibacillus* sp. strain TSCPVG. The bacteria were grown for 60 hours in 50 ml of the basal medium used before containing 3.5% NaCl, 0.75% Birchwood xylan, 1.0% yeast extract, 0.8% tryptone, and 0.02% of proline, thiamine, and Tween 40. The bacteria were removed and the proteins in the supernatant precipitated with two volumes of cold acetone. The pellet was dissolved in 10 ml of 100 mM phosphate buffer pH 7.5 containing 3.5% NaCl. When this sample was analyzed by native PAGE, there were more than 10 bands of xylanase activity. Two ml of the resuspended material was applied to a phenyl-Sepharose column equilibrated with 100 mM phosphate buffer pH 7.5 and the proteins eluted with a linear gradient of ammonium sulfate from 1.5 to 0 M. There were two major peaks of activity: fractions 23-25 and fractions 42-49. Analysis of the first peak indicated the presence of multiple low molecular weight xylanases and was not studied further. Analysis of the second peak indicated the presence of high, medium, and low molecular weight xylanases. The most active fractions were concentrated and applied to a column of Sephadex G-75 and a peak of material was obtained containing medium and low

molecular weight xylanases. This sample was then subjected to preparative polyacrylamide gel electrophoresis and the major band recovered by electroelution. This sample contained a single protein in a nondenaturing gel with a mass of >40 kDa, indicating a 12-fold purification from the initial culture supernatant with a 22% yield in activity. Analysis of this protein by SDS-PAGE indicated it had a molecular mass of 42 kDa and an isoelectric point of 6.1.

The properties of the purified enzyme were similar to those reported for the activity in the initial supernatant. It retained high activity in the presence of 0 to 30% NaCl, had a broad pH range with an optimum of pH 7.5, and exhibited maximal activity at 60 °C. The enzyme hydrolyzed Birchwood and oat spelt xylans but no activity was detected with *p*-nitrophenyl-β-D-xylopyranoside, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-acetate, carboxmethyl cellulose, avicel, filter paper, soluble starch, or casein under standard conditions. The enzyme showed simple Michaelis-Menten kinetics with Birchwood xylan as the substrate and a K_m of 21 mg/ml. The major products of hydrolysis as indicated by HPLC were xylooligosaccharides, xylobiose, and xylose. Comparison of the properties of this enzyme with other xylanases indicated it is probably a member of the glycoside hydrolase family 10. However, the gene for this enzyme has not yet been identified or the protein expressed in another host. A search of the UniProtKB database does indicate that *Gracilibacillus halophilus* strain YIM-C55.5 has been reported to contain a gene for an endo-1,4-β-xylanase. The encoded protein (N4WKF3_BACI) is somewhat larger (497 amino acids) and appears to have a transmembrane segment that would anchor it to the plasma membrane.

Protease from *Gracilibacillus boraciitolerans* strain LO15

G. boraciitolerans strain LO15 was one of six bacteria isolated from the sediment at Lake Oubeira in Algeria which formed a clear zone of hydrolysis around the colonies on skim milk agar plates [83]. This was indicative of the formation of an extracellular protease capable of degrading casein. Strain LO15 was Gram-positive and formed thin motile rods with spores. It was catalase- and oxidase-positive but was negative for gelatin hydrolysis, nitrate reduction and urease activity. It was identified as *G. boraciitolerans* on the basis of its 16S rRNA sequence and similar to *G. boraciitolerans* strains FM14 and T-16X.

Optimal growth and protease formation occurred growth in a basal salts medium at pH 7.4 supplemented with 2% casein, 1% yeast extract 0.5% tryptone after 40 hr at 28ºC. Protease activity was measured quantitatively by the formation of free tyrosine amino acids from casein using the Folin-Ciocalteu reagent as previously described for a similar enzyme from *Anoxybacillus kamchatkensis* M1V [20]. The enzyme was precipitated from 500 ml of culture medium with 45% to 75% ammonium sulfate. After resuspension in 100 mM MOPS buffer pH containing 2 mM $CaCl_2$, the preparation was dialyzed, heated at 70 °C for 30 min, and fractionated on a column of Sephacryl S-200 HR. The purified enzyme had a specific activity of 85,000 units/mg protein with a yield of 51%. When analyzed by polyacrylamide gel electrophoresis, it showed a single band of protein and activity with an apparent molecular mass of about 30 kDa. MALDI-TOF analysis and mass spectrometry of the protein gave a corrected mass of 30, 285. N-terminal sequencing of the purified protein showed 88% sequence identity to a protease from *Virgibacillus natechei* strain FarD and sequence similarities of serine alkaline proteases from other species in the family *Bacillaciae*.

The effects of varying conditions on the activity and stability of the purified protease from *G. boraciitolerans* strain LO15 were determined using the casein hydrolysis assay. The enzyme gave a broad pH optimum curve with a maximum activity at pH 10. The enzyme was stable at pH 7 for 12 to 16 hours, but the stability decreased as the pH was raised to 8, 9, 10 or 11. The activity of the enzyme increased as the temperature was raised above 45ºC and was maximal at 65ºC. The enzyme showed higher activity in the presence of 2 mM $CaCl_2$ than in the absence of $CaCl_2$. Stability decreased as the temperature was increased. The protease activity was partially inhibited by 100 mg/ml of PEG 8000, PEG 6000, PEG 1500, and PEG 1000 but remained high in the presence of 100 mg/ml sorbitol. Activity gradually decreased in the presence of increasing concentrations of NaCl or KCl, but it was still greater than 50% at 5.0 osmolyte concentration. The activity of the enzyme was completely inhibited by 5 mM phenylmethylsulfonyl fluoride and 2 mM diisopropylfluorophosphate, showing that it is true serine protease. It was not inhibited by EDTA or EGTA, indicating no metal ions were required for catalysis. The enzyme showed good activity with azo-casein, albumin, albumin azure, gelatin, and keratin as substrates but had no activity towards collagen. The enzyme could hydrolyze a number of synthetic substrates including *N*-succinyl-phenyl-*p*-nitroanilide and *N*-succinyl-phenyl-alanyl-alanyl-phenyl-*p*-nitroanilide as measured by the release of *p*-nitroanilide at 410 nm. It only exhibited esterase activity towards *N*-acetyl-L-tyrosine ethyl ester and *N*-benzoyl-L-tyrosine ethyl ester. *The* K_m for casein was 0.255 mM and the K_m for *N*-succinyl-phenyl-alanyl-alanyl-phenyl-*p*-nitroanilide was 0.451 mM. The enzyme had higher protease activity in the presence of various organic solvents and detergents than other serine alkaline proteases.

The gene for the serine alkaline protease from *G. boraciitolerans* strain LO15 was amplified from genomic DNA using primers based on the sequences found in the genes from *Virgibacillus natechei* strain FarD and *Virgibacillus massilensis* DSM 28587 which code for similar proteins. The resulting PCR product called the *sapGB* gene was sequenced and analyzed and then cloned into *Escherichia coli* expression vectors. The complete nucleotide sequence revealed an open reading frame of 1158 bp, encoding a pre-pro-protein of 386 amino acids. Analysis of this sequence indicated that removal of the signal peptide and the pro-sequence would give a mature protease containing 276 amino acids. Expression of the recombinant protein in *E. coli* showed that it had the same physical and kinetic properties as the original enzyme. Inspection of the amino acid sequence revealed that it contained the catalytic triad of residues (D32, H62, S219) typical of serine proteases. There were no cysteine residues but the presence of 18 aspartate and 20 asparagine residues totaling 13.7 mole% suggested these might contribute to its unusual stability.

One of the potential uses of this enzyme is as an addition to laundry products to facilitate the removal of stains. To test this idea, pieces of clean cotton cloth were soaked with cow's blood or chicken egg and dried. The samples were then washed with tap water alone, in a solution containing a commercial detergent (ISIS), in a solution containing ISIS and a commercial enzyme called Esperase 8.0 L, or in a solution containing ISIS and the *G. boraciitolerans* strain

LO15 protease. The addition of the commercial enzyme or the *G. boraciitolerans* protease noticably enhanced stain removal.

Other Potentially Useful Salt-Tolerant Extracellular Enzymes from *Gracilibacillus*

In addition to the three enzymes described above, several other salt-tolerant extracellular enzymes have been identified from other *Gracilibacillus* species but studied in less detail.

Azo-dye reductase from *Gracilibacillus* sp. GTY

Azo-dyes include a wide range of natural and synthetic colored compounds containing a diazo group (-N=N-) linking various aromatic groups [84]. They are commonly used in various processes in the paper, clothing, cosmetic, and pharmaceutical industries, and tons of them are released each year into the wastewater resulting from these processes. Because of the stability of the -N=N- bond and the potential toxicity of these compounds, there has been a large international effort directed at their removal. Among the methods that have been used are chemical extraction, adsorption, precipitation, membrane filtration, and photolysis. Many microorganisms have been identified which can help decompose these compounds either by reduction or oxidation. This may occur under aerobic or anaerobic conditions by single or mixed cultures by bacteria that are halotolerant [85-86]. Azoreductases are enzymes that mediate bond reduction using electrons from NADH or an electron transport chain.

Gracilibacillus sp. GTY was isolated in the Dalian coastal area of Northern China as part of a mixed bacterial culture and purified as a single colony on Luria-Bertani (LB) medium containing high salt concentrations [87]. It was initially identified as an aerobic Gram-negative rod that was oxidase- and catalase-positive but negative for nitrate reduction. Subsequent analysis of its 16S rRNA sequence indicated that it clustered with other *Gracilibacillus* species and was most closely related to *G. dipsosauri* and *G. halotolerans*. The bacteria grew well in LB medium containing 2% to 25% NaCl. When the azo-dye acid red B was incorporated into the medium at a concentration of 100 mg/ml, there was a progressive decrease in the absorbance at 515 nm over a period of 96 hr. The rate of decolorization was most rapid in medium containing 10% to 15% NaCl. Decolorization was less efficient at 2% NaCl and at 25% NaCl.

Washed resting cells of *Gracilibacillus* sp. GTY could also decolorize a 100 mg/ml solution of acid red B dye in a 0.1 M phosphate buffer, pH 8.0 containing 15% NaCl at 30 °C. When the decolorized suspension was centrifuged at 22,000 x *g* to remove the bacteria, the resulting supernatant fraction could still reduce a solution of 50 mg/ml dye in the presence of NADH. When bacteria from an overnight 2000 ml LB culture containing 15% NaCl were harvested by centrifugation, washed, frozen, and then disrupted by ultrasonic treatment, a cell-free extract was obtained that could also reduce a 100 mg/ml solution of the dye in a 20 mM phosphate buffer containing NADH. There results indicated that *Gracilibacillus* sp. GTY is able to form a salt-tolerant NADH-dependent azo dye reductase and that at least some of the enzyme can be released as an extracellular protein. However, no additional biochemical or genetic studies of this exoenzyme have been reported.

Alginate Lyase from *Gracilibacillus* strain A7

Alginates are the primary polysaccharides found in brown algae or seaweeds [88]. They are linear polymers containing D-mannuronic acid (M residues) and L-guluronic acid (G residues) linked by 1, 4 glycosidic bonds. The sugars may occur as blocks of M residues, blocks of G residues, or as alternating heterodimers of MG and GM residues. Alignates have a relatively large molecular mass of 32 kDa to 200 kDa and form viscous aqueous solutions. They are used in the food industry as a thickening agent and in the pharmaceutical field as a scaffolding material. Alginates can also accumulate in the ocean as a result of eutrophication or the release of industrial waste. Because the accumulation of seaweed wastes can severely damage ocean ecosystems, there is a great deal of interest in their degradation. This can be accomplished using enzymes called alginate lyases or alginases. These enzymes cleave the glycosidic bonds between the sugars and have been isolated from a large number of different microorganisms [89-90].

Tang *et al*. described the isolation of *Gracilibacillus* strain A7 from wakame (*Undaria pinnatifada*) compost [91]. The Gram-positive bacteria were long thin rods that formed circular white colonies on alginate agar medium after 5 days at 30 ºC. When grown in a liquid medium containing 0.05% polypeptone, 0.03% yeast extract, 0.5% alginate 0.2% ammonium sulfate and 0.05% $MgSO_4$ in a phosphate buffer at pH 8.5, the bacteria released reducing and unsaturated sugars over a period of 72 to 96 hr. Analysis of the 16S rRNA of strain A7 indicated that it was 99% similar to *G. halotolerans*. The most rapid growth of this bacterium occurred at pH 8.5-9.5 in the presence of 0.5 M to 2.0 M NaCl at 30ºC in the presence of 0.2% to 0.5% polypeptone.

To characterize the enzyme involved in alginate degradation, *Gracilibacillus* strain A7 was grown for 96 hr in one liter of a liquid medium at pH 8.5 containing 5 g polypeptone, 1 g yeast extract, 5 g sodium alginate and 22.92 g NaCl [92]. The bacteria were removed by centrifugation and the proteins in the supernatant precipitated by adding ammonium sulfate to give an 80% saturated solution. The concentrated proteins were dissolved in a 0.02 M phosphate-citrate buffer at pH 7.0 and tested for enzyme activity. The enzyme solution (1 ml) was combined with 1 ml of 0.5% sodium alginate in a phosphate buffer at pH 7, incubated at specific temperatures for 30 min, and the concentration of reducing sugars determined with the 3,5-dinitrosalicylic acid reagent [93]. The optimal temperature for activity was found to be 40 °C and the optimal pH was 8.0. The amount of enzyme activity increased as the alginate concentration was raised from 0.1% to 0.3% but did not change at higher concentrations. Activity was stimulated by Mg^{2+} and Na^{+} ions but inhibited by Cu^{2+} and Zn^{2+} ions. During the first 60 minutes of the reaction, the concentration of reducing sugars increased rapidly but then began to slow down. The concentration of unsaturated sugars as measured by the change in absorbance at 235 nm lagged and increased more slowly over a period of 300 min. It was not clear if this was due to another enzyme in this relatively crude preparation. The enzyme from *Gracilibacillus* strain A7 could gradually reduce the degree of polymerization of the alginate over a period of 300 min as measured by dividing the total sugar concentration by the reducing sugar concentration. Although these studies were consistent with the formation of a salt-tolerant extracellular alginate lyase

by strain A7, no other experiments with this enzyme have been reported.

Identification of Other *Gracilibacillus* species Forming Salt-Tolerant Extracellular Enzymes

In addition to the bacteria described above, a number of other *Gracilibacillus* species that form extracellular enzymes have been identified. As part of a project screening for salt-tolerant microorganisms from Howz Sultan Lake in Iran, brine, solar salt, saline soil, and saline mud samples were collected at different locations and depths and cultured in a saline nutrient broth containing 5% yeast extract, various salts, and 10% or 20% NaCl [94]. After 3 to 7 days at 34 °C or 38 °C, individual bacteria were recovered on solid medium containing 10% NaCl for moderately halophilic microbes or 20% NaCl for extremely halophilic microbes. There were 231 moderately halophilic organisms and 49 extremely halophilic ones, of which 172 were Gram-positive rods, 56 were Gram-negative rods, and 52 were Gram-positive cocci. The individual organisms were tested for extracellular amylase, lipase, protease, DNase, xylanase, pullulanase, pectinase, cellulase (carboxymethyl cellulase or CMCase), and inulinase activities by looking for zones of clearing around colonies on agar test plates. Analysis of the 16S rRNA sequences of the positive strains indicated they were members of the following genera: *Salicola, Halovibrio, Halomonas, Bacillus, Oceanobacillus, Thalasobacillus, Virgibacillus, Gracilibacillus, Halobacillus, Piscibacillus,* and *Salinococcus.* Many of the *Gracilibacillus* isolates were positive for amylase, lipase, protease, xylanase, cellulase, and inulinase activities but none showed DNase, pullulanase, or pectinase activities.

One of these individual salt-tolerant *Gracilibacillus* strains designated 1-9 h was later characterized in more detail [95]. It was found to form thin Gram-positive rods with ovoid terminal endospores. The bacteria were motile with peritrichous flagella and capable of growth both aerobically and anaerobically. It was positive of catalase, nitrate reduction, formation of hydrogen sulfide, and hydrolysis of starch, Tween 80, and gelatin. It was negative for oxidase and hydrolysis of casein, xylan, and urea. Analysis of the 16S rRNA sequence indicated it was most closely related to *G. saliphilus* and *G. lacisalsi.* The optimum pH for growth in a medium containing 10% NaCl, 1% yeast extract, 0.05% proteose peptone, 0.01% glucose, and other salts was 7.5. The optimum salt concentration for growth was 15-21% but occurred in the presence of 9 to 32% NaCl. The optimum temperature for growth was 40-45ºC but could occur at temperatures as high as 65 °C. The organism was said to be hyperhalophilic and moderately thermophilic. However, the biochemical properties of its extracellular enzymes were not studied further.

More recently, 66 halophilic bacteria were isolated from saline soil at the former Lake Texoco in Mexico [96]. The bacteria were cultured on Marine agar plates or in liquid Marine broth. The isolates were identified as members of the genera *Kocuria, Micrococcus, Nesterenkonia, Halomonas, Halobacillus, Tenuibacillus, Salinococcus, Kurthia, Gracilibacillus, Virgibacillus,* and *Bacillus* based on their 16S rRNA sequences. Isolate HJ019 was most similar to *G. saliphilus* and *G. boraciitolerans* and renamed *Gracilibacillus* sp. M-18. The formation of salt-tolerant extracellular enzymes was determined on agar plates containing 0.5 M NaCl, 0.01% yeast extract, small amounts of sodium acetate and sodium pyruvate, and various potential polymeric substrates including Tween 80, skim milk, and soluble starch. Isolate HJ019 showed positive results for lipase/esterase, pullulanase, amylase, inulinase, xylanase, cellulase (CMCase), chitinase, and pectinase activity but not for protease and DNase activity. While some of the enzymes from this collection of bacteria were further characterized, the ones from isolate HJ019 were not.

Halophilic and halotolerant bacteria were also recovered from soil samples in the “Los Negritos” zone of Michoacán, Mexico [97]. Some were isolated after three cycles of enrichment following growth in medium containing 10% or 22% NaCl. Others were isolated after direct plating of diluted samples on agar medium containing 10% or 22% NaCl. Individual colonies were selected and purified as pure cultures. A total of 62 isolates were obtained, 51 after growth in the presence of 10% NaCl and 11 after growth in the presence of 20% NaCl. Of these isolates, 33 were Gram-positive rods, 11 were Gram-negative rods, and 18 were Gram-positive cocci. In addition, 74.2% were halotolerant while 25.8% required at leasr 2.5% NaCl for growth. The individual bacteria were identified based on their 16S rRNA sequences as members of the genera *Oceanobacillus, Bacillus, Gracilibacillus, Halobacillus, Marinococcus, Planococcus, Prieslia, Salibacterium, Salimicrobium, Salinicoccus, Terribacillus,* and *Virgibacillus*. *Gracilibacillus* sp. LNSP5103-2 was most closely similar to *G. halotolerans* NN^T. This strain was positive for cellulase activity but not for any of the other hydrolases that were tested.

Discussion

These studies indicate that bacteria in the genus *Gracilibacillus* represent a good but still underutilized source of salt-tolerant extracellular enzymes. Various species have been found to form enzymes degrading polysaccharides, proteins, and lipids in the presence of NaCl concentrations as high as 3.0 M. Some of these proteins can also tolerate moderately high temperatures and acid or alkaline pH. None of the enzymes characterized so far has been used in a commercial industrial or biotechnological application, although their potential is apparent in some cases. One of the advantages of using *Gracilibacillus* species for this purpose is that none of them has been found to be pathogenic to humans, plants, or animals. They are all listed as biosafety level 1 (BSL1) microorganisms and so are particularly suitable for use in projects with students.

Investigators who want to study other salt-tolerant extracellular enzymes from this group might utilize one of two general strategies. The first would be to isolate new microbes with properties related to the problem of interest. *Gracilibacillus* species have been recovered from a wide range of environmental saline habitats as well as from plant and animal sources. It is relatively easy to use enrichment cultures to find bacteria utilizing particular substrates under specific conditions. Most of these bacteria can be grown in the laboratory in relatively simple media under aerobic conditions. As indicated by the projects described above, there are convenient agar plate or spectrophotometric assays for many of these enzymes.

The second would be to directly isolate genes from *Gracilibacillus* species that have already been identified which code for proteins of interest. Many of the type strains of *Gracilibacillus* can be obtained in the United States from the American Type Culture Collection.

https://www.atcc.org/search#q=gracilibacillus&sort=relevancy&numberOfResults=24 or from the corresponding German collection

https://www.dsmz.de/collection/catalogue/microorganisms/catalogue or Japanese collection https://www.jcm.riken.jp/cgi-bin/jcm/jcm_db?AN=gracilibacillus&BN=. More than 29 complete *Gracilibacillus* genomic DNA sequences are now known and can be found on the NCBI Data Sets site (https://www.ncbi.nlm.nih.gov/datasets/genome/?taxon=74385). Based on these sequences, it has been possible to predict the structures of many different amylases, proteases, lipases, and cellulases. These can be found on the UniProKB site https://www.uniprot.org/uniprotkb?query=Gracilibacillus. One could isolate the corresponding gene from genomic DNA using the polymerase chain reaction, clone the sequence, and then express the protein in a suitable host. This might allow the full potential of *Gracilibacillus* to be realized.

Contributor Role

All of this manuscript was written by Charles E. Deutch.

Conflicts of Interest

The author declares there are no conflicts of interest.

Conclusion

Extremophiles, particularly those in the genus Gracilibacillus, demonstrate remarkable adaptability to harsh environmental conditions, including high salinity. These microorganisms produce a diverse array of extracellular enzymes, such as amylases, proteases, and xylanases that retain functionality under extreme conditions. The ability to produce these enzymes in significant quantities, coupled with advances in genomic analysis, highlights the potential for Gracilibacillus species to contribute to biotechnological applications. Further research into these organisms could lead to the development of industrial processes that harness their unique enzymatic properties, offering sustainable solutions to various environmental and industrial challenges.

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