

Insights into Genomic Features and Potential Biotechnological Applications of *Bacillus halotolerans* Strain HGR5

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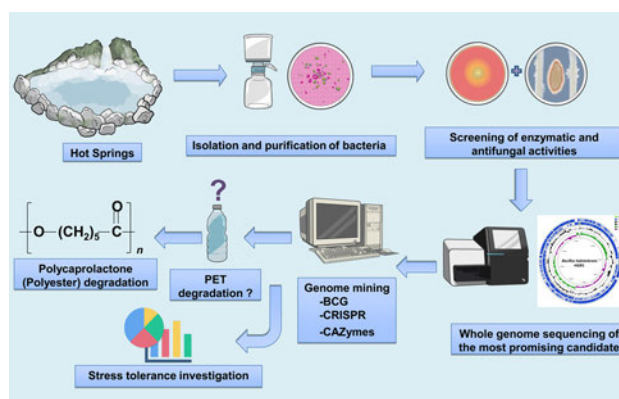
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Abstract

Algeria is one of the wealthiest countries in terms of hydrothermal sources, with more than two hundred hot springs. However, diverse and little-described microbial communities colonize these habitats, making them an intriguing research subject. This work reports the isolation of bacteria from two hot springs water samples in north-eastern Algeria, evaluating their enzymatic activities and effect on plant pathogens. Out of the obtained 72 bacterial isolates and based on the 16S rRNA gene sequence analysis, the strain HGR5 belonging to *Bacillus halotolerans* had the most interesting activity profile. Interestingly, HGR5 was substantially active against *Fusarium graminearum*, *Phytophthora infestans*, and *Alternaria alternata*. Furthermore, this strain presented a high ability to degrade casein, Tween 80, starch, chitin, cellulose, and xylan. The genome sequence of HGR5 allowed taxonomic validation and screening of specific genetic traits, determining its antagonistic and enzymatic activities. Genome mining revealed that strain HGR5 encloses several secondary metabolite biosynthetic gene clusters (SM-BGCs) involved in metabolite production with antimicrobial properties. Thus, antimicrobial metabolites included bacillaene, fengycin, laterocidine, bacilysin, subtilosin, bacillibactin, surfactin, myxovirescin, dumulmycin, and elansolid A1. HGR5 strain genome was also mined for



CAZymes associated with antifungal activity. Finally, the HGR5 strain exhibited the capacity to degrade polycaprolactone (PCL), a model substrate for polyester biodegradation. Overall, these results suggest that this strain may be a promising novel biocontrol agent with interesting plastic-degradation capability, opening the possibilities of its use in various biotechnological applications.

Key words: *Bacillus*, genome mining, hydrolytic enzymes, secondary metabolites

Introduction

Thermophilic bacteria have recently received particular attention for their ability to produce thermostable enzymes (i.e., proteases, lipases, amylases, cellulases, xylanases, and DNA polymerases), which can also be active under extreme conditions (Sharma et al. 2019; Oztas Gulmus and Gormez 2020). Therefore, these

enzymes can be used in high-temperature industrial fermenters, which accelerates the biochemical reactions, reduces contamination rates, and facilitates product recovery (Kambourova 2018; Wohlgemuth et al. 2018; Jin et al. 2019). In addition, thermophilic bacteria have several other biotechnological applications, including the production of bioactive compounds (e.g., exopolysaccharides, antibiotics, and biosurfactants) (Nicolaus

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et al. 2010; Mahajan and Balachandran 2017; Schultz et al. 2022). Moreover, they can be used for the removal of heavy metals by bioremediation as well as for the biodegradation of plastic polymers and hydrocarbons (Ilyas et al. 2014; Rajkumari et al. 2019; Ru et al. 2020).

Thermophilic microorganisms grow in various marine and terrestrial ecosystems such as hydrothermal vents, deep waters, compost, and hot springs; most of them come from the latter habitat (Oztas Gulmus and Gormez 2020). Several studies have investigated the microbial diversity in different hot springs in Algeria, the USA, Iceland, New Zealand, India, Japan, China, Turkey, and Russia by culture-dependent or independent methods (Verma et al. 2018). Hence, they provide insights into the composition of microbial communities colonizing these habitats, which leads to the discovery of new taxa and the understanding of the functional role of microbes in such ecosystems (Li and Ma 2019). These studies suggest that the dominant phyla and taxa depend on temperature, water geochemistry, and pH (Cihan et al. 2012). Algeria has more than 240 hot springs across the country. However, research on the isolation and characterization of microorganisms from these sources remains very limited. Therefore, it would be interesting to investigate these new ecological niches and study their diversity (Saibi 2009; Benammar et al. 2020). Benammar et al. (2020) examined the biodiversity of eight hot springs in Algeria using cultivation methods. They discovered that Firmicutes was the most abundant phylum (95%); however, strains belonging to *Deinococcus-Thermus* (5%), and Actinobacteria (2%) were also present. It was also reported that *Bacillus* and related genera are ubiquitous in all the studied hot springs and represent 64 % of all the isolates (Benammar et al. 2020). Moreover, according to a report on the biodiversity of two hot springs located in Guelma (northeastern Algeria), the three most abundant families are *Planococcaceae*, *Bacillaceae*, and *Paenibacillaceae* (Gomri et al. 2018).

The *Bacillus* genus includes various Gram-positive, rod-shaped, and endospore-forming species with different physiological characteristics (thermophilic, halotolerant, acidophilic, or alkali tolerant strains). They can be isolated from various habitats, including soil, compost, lakes, marine sediments, and animal intestine (Zeigler and Perkins 2008; Fira et al. 2018). Since the 1980s, the application of molecular techniques (i.e., PCR) and, more recently, the NGS (Next-generation sequencing) methods has expanded the biodiversity of *Bacillus*, leading to the discovery of more than seventy species (Baltaci et al. 2020). This diversity explains their use in many fields of biotechnology, such as the production of antibiotics, enzymes, and vitamins (Zalma and El-Sharoud 2021). *Bacillus* sp. also has beneficial effects on plant growth and protection against patho-

gens (Ongena and Jacques 2008; Fira et al. 2018; Saxena et al. 2020). Several formulations of biopesticides and fertilizers produced by some species (i.e., *Bacillus amyloliquefaciens* and *Bacillus velezensis*), have been developed and commercialized (Rabbee et al. 2019).

Recently, it was found that some microbes can degrade plastic polymers through the action of particular enzymes (Yoshida et al. 2016; Danso et al. 2019; Wei et al. 2020). Thus, many hydrolases from various bacteria and fungi have been identified and investigated for their ability to degrade aliphatic and aromatic polyesters, including polyethylene terephthalate (PET). Microorganisms producing PET hydrolases (PETases) are attracting much interest, especially for their potential application in treating PET waste, one of the most commonly produced plastics in the world (Carr et al. 2020; Kawai 2021).

Thus, the aim of this study was to isolate bacteria from two hot springs located in eastern Algeria and analyze their hydrolytic enzyme and antifungal activities. Among the seventy-two isolated bacteria, the HGR5 strain belonging to *Bacillus* sp. presented the most interesting properties. Therefore, we conducted a whole genome sequencing of this strain for a finer taxonomic affiliation, indicating that HGR5 belongs to *B. halotolerans* species. We also performed *in silico* analysis of genes related to their potential antimicrobial and hydrolytic activities, with the identification of different biosynthetic gene clusters (BGCs) of several secondary metabolites. Furthermore, we evaluated its resistance to various abiotic stimuli, including high temperatures, salt, extreme pH, and its capacity to break down polycaprolactone.

Experimental

Materials and Methods

Hydrothermal springs sampling and physico-chemical measurements. Six water samples were collected aseptically from two hydrothermal springs in Hammam Ouled Tebben and Hammam Guergour (GPS: 35°47'40.32"N, 5°07'30.18"E; 36°19'17.19"N, 5°03'19.36"E, respectively) (Wilaya of Sétif) for the isolation of thermotolerant bacteria. Water temperature was measured on-site with an electronic thermometer (Electronic temperature controller; Ahlborn Mess- und Regelungstechnik GmbH, Germany), and pH measurements (pH 211 Microprocessor pH Meter; HANNA Instruments Deutschland GmbH, Germany) were performed in the laboratory.

Bacterial isolation. Dilutions from 10^{-1} to 10^{-7} were prepared from each sample with physiological water and used for bacterial isolation on three different

media: GYM, R2A, and King's B, by streaking 100 μ l of each dilution on the Petri dishes. The plates were incubated at 30°C for 96 hours. The colony-forming units (CFU) per plate were measured for all dilutions in the three different media. The bacterial colonies were selected based on their macroscopic characteristics (i.e., color, shape), and successive streaks on TSA medium obtained pure cultures. Finally, pure colonies were suspended in a TSB medium containing 20% glycerol and stored at -20°C.

Preparation of bacterial inoculum. The isolates ($n=72$) were cultivated at 30°C on TSA medium for 48 h. The bacterial colonies were harvested and suspended in saline solution, and the concentration was adjusted to 10^8 CFU/ml, corresponding to an optical density (OD_{600}) of 0.1.

Screening of enzymatic activities. All isolates were screened in triplicate for lipase, protease, amylase, chitinase, cellulase, and xylanase activity on agar media containing specific substrates for each enzyme. 2 μ l of bacterial suspension was spread over the surface of the agar. The dishes were incubated at 30°C for 48 hours. The enzymatic index was measured for all these activities.

Lipolytic activity. Lipolytic activity was screened using Tween 80 as substrate. The degradation was performed in media containing 1.0% peptone, 0.5% NaCl, 0.01% $CaCl_2 \cdot H_2O$, 1.5% agar, and 1.0% substrate (Tween 80) (Ramnath et al. 2017). After incubation, precipitations around colonies show Tween 80 degradation (Ramnath et al. 2017).

Proteolytic activity. The detection of proteolytic activity was screened on media supplemented with 3% casein, a zone of hydrolysis around the colonies indicates caseinolytic activity (Zhang et al. 2015).

Starch, chitin, cellulose and xylan degradation. Starch degradation was measured on agar supplemented with 1% starch. After two days of incubation, the starch degradation was detected by flooding the dishes with Lugol iodine solution; clear zones around colonies indicate positive activity (Benammar et al. 2020). Colloidal chitin agar was used to screen chitinolytic activity, according to Gasmi et al. (2019). Positive isolates were identified based on the presence of hydrolysis zones. Whereas assays for cellulase and xylanase were carried out on agar mediums enriched with CMC and xylan, respectively (Liu et al. 2011; Liang et al. 2014). The plates were flooded in 1% Congo red solution for 15 minutes after 48 hours of incubation, then washed with 1 M NaCl solution to visualize hydrolysis clearance zones (Shanthi and Roymon 2018).

Antifungal activity. Nineteen of the isolates were examined through a confrontation test on agar for activity against the three phytopathogenic fungus *Fusarium graminearum* (FG), *Phytophthora infestans*

(PI), and *Alternaria alternata* (ALT). Co-cultivation was performed in PDA medium by placing an 8 mm mycelial agar disc in the center of the dishes. Then, two bacterial spots were inoculated 2.5 cm away from the fungi, toward the periphery. Controls were prepared by growing only the fungus under the same conditions. Dishes were incubated at 28°C and monitored after 5 and 14 days (Alenezi et al. 2016). The inhibition rate (IR), which is used to assess antifungal activity, was calculated using the following formula:

$$IR (\%) = \frac{(Dc - Dt)}{Dc} \times 100\%$$

where:

Dc – control fungus diameter (average of 3 replicates),
Dt – treated fungus diameter (average of 3 replicates).

Molecular identification of strains HGG15, HGR5, and HGK11. The extraction of the genomic DNA was performed using a Wizard® Genomic DNA Purification Kit (Promega, USA) following the manufacturer's instructions. The two primers, 27F (5'AGAGTTTGATCCTGGCTCAG3') and 1492R (5'GGTTACCTTGTTACGACTT-3') were used for the amplification of the 16S rRNA genes using HOT FIREPol® DNA polymerase (Solis BioDyne, Estonia). The PCR amplification mixture was composed of a 1 $\times$ concentration of reaction buffer, 2 mM magnesium $MgCl_2$, 200 μ M of each dNTP, 0.3 μ M of both the forward and reverse primers, 1 unit (U) of HOT FIREPol® DNA polymerase enzyme, and 2 μ l of isolated template DNA. The total reaction volume was adjusted to 20 μ l using nuclease-free water. Cycling conditions were: initial activation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 30 sec, primers annealing at 58°C for 30 sec, and extension at 72°C for 1 min, then the final extension at 72°C for 10 min. Sequencing was performed by Eurofins Genomics (France), and based on the fluorescence-adapted Sanger method. Fragments were separated by capillary electrophoresis on an Applied Biosystems™ 3730xl Genetic Analyzer (Thermo Fisher Scientific, Inc., USA), and the resulting gene sequences were analyzed using the 16S Based-ID tool of EzBioCloud (<https://www.ezbiocloud.net>; Yoon et al. 2017). 16S rDNA-based phylogenetic tree was constructed using a neighbor-joining method in MEGA X (Kumar et al. 2018) using the 16S gene sequences from the closest *Bacillus* species.

Genome sequencing, assembly, and annotation. The genomic DNA of *B. halotolerans* HGR5 was obtained, as mentioned above. The quantity and purity of the DNA were checked using the NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific, Inc., USA). The sequencing library was prepared using the Illumina® TruSeq® DNA PCR-Free kit (Illumina, Inc., USA) according to the manufacturer's

recommendations. Whole genome sequencing was performed on the NovaSeq™ 6000 (Illumina, Inc., USA) platform using the S4 reagent kit v1.5 with a 2 × 151-bp paired-end read protocol.

Default parameters were used for all bioinformatic tools unless otherwise noted. TrimGalore v0.6.2 (Krueger 2015) was used for read trimming, and read quality was checked with FastQC v0.11.9 (Andrews 2010). Raw paired-end reads were *de novo* assembled with Unicycler v0.5.0 (Wick et al. 2017), and assembly metrics were calculated using QUAST v0.5.2 (Mikheenko et al. 2018). Whole genome annotation was carried out using the Bakta pipeline v1.8.1 (Schwengers et al. 2021). ResFinder (Bortolaia et al. 2020) and VFDB (Liu et al. 2019) were used to screen the genome sequence for antibiotic resistance and virulence genes, respectively, in addition to Pathfinder v1.1 (Cosentino et al. 2013) used to assess the pathogenicity of the strain.

The whole-genome shotgun project of *B. halotolerans* HGR5 has been deposited at GenBank under the accession number JASXRX000000000. The associated BioSample and BioProject accessions are SAMN35725142 and PRJNA983391, respectively.

Phylogenetic analysis. Nucleotide sequences for the four housekeeping genes 16S rDNA, *cdsA*, *gyrA*, and *gyrB* were either extracted from the assembled genome of HGR5 isolate or retrieved from GenBank for the sixty-seven *Bacillus* type strains. For each gene, nucleotide sequences were aligned using muscle (default parameters) (Edgar 2004a; 2004b), and a maximum likelihood tree was constructed using mega X (Kumar et al. 2018) with Hasegawa-Kishino-Yano model, Gamma distribution with invariant sites G + I, and bootstrapping (n = 1,000) parameters.

Functional genome analysis. Functional sequence annotation to establish clusters of orthologous groups (COG) categories was achieved using EggNOG-mapper (Cantalapiedra et al. 2021), where gene sequences were assigned gene ontology (GO) terms. Then, the distribution analysis of GO terms was performed and displayed using TB tools (Chen et al. 2020).

The biosynthetic gene clusters (BGCs) involved in the synthesis of secondary metabolites (SMs) were identified using the online tools antiSMASH v7.0.0 (Blin et al. 2023) and BAGEL4 (van Heel et al. 2018). Also, the ClusterBlast and KnownClusterBlast modules were employed for the comparative analysis of the obtained gene clusters as described by (Tsalgatidou et al. 2022). The detection of CRISPR-Cas systems was carried out online by the CRISPRCasFinder (Couvin et al. 2018).

Annotation of genes involved in antifungal activities was achieved using the carbohydrate-active enzymes (CAZymes) (<http://www.cazy.org>; Drula et al. 2022) database through the dbCAN3 annotation tool Run-dbcAn v4.0.0 (<https://github.com/linnabrown/>

run_dbcan; Zhang et al. 2018). Then, CAZymes were grouped based on the substrates and the corresponding Merops families (Rawlings et al. 2016).

General esterase and polyesterase activity assays.

The biodegradation of coconut oil, tributyrin, and polycaprolactone diol (PLCD; $M_n \sim 2,000$) by *B. halotolerans* HGR5 was performed on LB agar according to the protocol developed by (Molitor et al. 2020). A 50% (v/v) substrate emulsion was prepared in sterile distilled water containing 0.5% gum arabic. A volume of 30 ml of this emulsion was then added to 1 liter of LB agar and mixed using a homogenizer (POLYTRON® PT 1200 E; Kinematica AG, Switzerland), then poured into Petri dishes. Clear zones around the colonies reveal positive activities against tributyrin and PCLD (Molitor et al. 2020). At the same time, coconut oil degradation was detected under UV radiation at 350 nm by observing fluorescent halos around positive colonies (Ramnath et al. 2017).

Tolerance to heat, salt, and extreme pH stress. To investigate the thermotolerance of strain HGR5, tubes containing 5 ml of TSB broth were inoculated with 50 µl of the bacterial suspension and then incubated with shaking at 160 rpm at 30, 35, 40, 45, 50, 55 and 60°C respectively. After 48 h, the growth was recorded by measuring the optical density at 600 nm (Baltaci et al. 2020). For halotolerance, 50 µl of the bacterial suspension was inoculated into 5 ml of TSB broth with NaCl concentrations of 0; 2.5; 5; 7.5; 10; 12.5; 15%. The tubes were then incubated at 35°C (growth optimal temperature) under shaking at 160 rpm for 96 hours. Every 24 h, a volume of 300 µl was collected, and the OD_{600} was measured using a microplate reader (VEG 500;

ESSE 3, Italy). Finally, the optimal pH was also determined by inoculating 50 µl of bacterial suspension into 5 ml of TSB broth with a pH range adjusted from 5 to 11 (Baltaci et al. 2020). The optical density of the bacteria was recorded at 24, 48, 72, and 96 h using a microplate reader (Wu et al. 2021). All experiments described above were performed with three repetitions.

Results and Discussion

Measurements of water parameters. The water temperature and pH of Hammam Ouled Tebben and Hammam Guergour were at 52°C; 44.5°C and 6; 6.9, respectively.

Bacterial isolation. Three media (i.e., R2A, King B, and GYM) were employed for bacterial isolation, and enumeration was performed by membrane filtration of 100 ml of water from each source. The highest densities were observed in the R2A medium, around 39 CFU/100 ml for Hammam Ouled Tebben and 49 CFU/100 ml for Hammam Guergour. However,

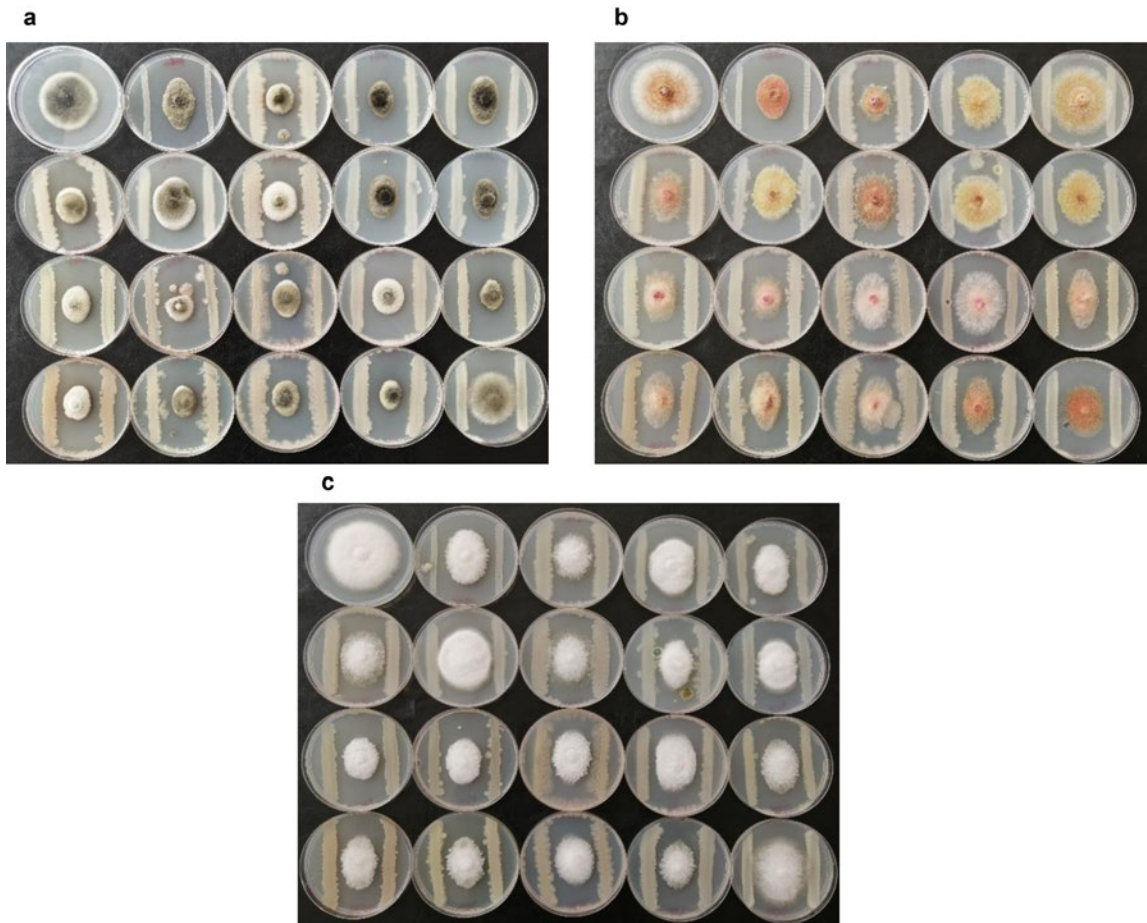


Fig. 1. Antagonism effect of the nineteen selected strains based on their enzymatic activities. Control on the top left followed by each bacterial strain to the bottom right of each panel: HGG11, OTG3', OTG9', OTG6, OTR1, OTR2, OTK3, OTK1, OTK4, OTK8, OTK9, HGG7, HGG9, HGG15, HGG16, HGR5, HGK5, HGK11, HGK1, against a) *Alternaria alternata*; b) *Fusarium gramine arum*; c) *Phytophthora infestans*.

the other two media also showed significant densities, with the GYM medium exhibiting 33 CFU/100 ml for the two samples. For the King's B medium, an average density of 35 CFU/100 ml and 30 CFU/100 ml was obtained for Hammam Ouled Tebben and Hammam Guergour, respectively. Thus, 72 isolates were obtained, 35 from Hammam Ouled Tebben and 37 from Hammam Guergour.

In vitro enzymatic and antifungal activities. Screening of enzymatic activities revealed that 68% (n = 49/72) of the isolates produced protease, whereas 66,6% (n = 48/72) exhibited lipolytic activity against Tween 80. On the other hand, for glycosyl hydrolases, 62.5%, 73.6%, 50%, and 5.55% of the isolates were able to degrade starch, chitin, cellulose, and xylan, respectively (Table SI). The enzymatic profiles of bacteria and actinomycetes isolated from Algerian hot springs have been described in several studies (Gomri et al. 2018; Benamar et al. 2020; Medjemadj et al. 2020; Bouacem et al. 2022). These bacteria displayed a variety of typical extracellular enzymatic activities, suggesting their potential applications in various biotechnological applications.

Nineteen of the isolates with the best enzymatic profiles (Fig. 1, highlighted in bold in Table SI) were selected for the antifungal activity against *F. graminearum*, *P. infestans*, and *A. alternata*. Sixteen of them showed significant antifungal activity in the confrontation test (Table SII). Our selection criteria, which included a < 50% inhibition rate on ALT, PI, and FG, enabled us to select three strains with the highest antifungal activities, which are HGG15, HGK11, and HGR5. Their inhibition rates were 60, 64, and 60% for ALT, 53.13, 79.69, and 53.13% for PI, 56.90, 51,72, and 58.62% for FG, respectively.

Numerous studies have highlighted the promising antifungal activity of *B. halotolerans*. For instance, *B. halotolerans* MS50-18A had an antagonistic efficacy towards *Phytophthora capsici*, *Fusarium solani*, *Rhizoctonia solani*, and *Fusarium oxysporum*, which was higher than 60% (Sagredo-Beltrán et al. 2018). Also, four strains (named BFOA1 to BFOA4) belonging to this species have been isolated from the roots of plants grown in semi-arid areas of North Africa (Algeria and Tunisia). They had a considerable antagonistic potential

toward *F. oxysporum* f. sp. *albedinis*, and 16 other phytopathogenic fungi of the genus *Fusarium* (Slama et al. 2019). They also exhibited antifungal activity against *A. alternata*, *Rhizoctonia bataticola*, *P. infestans*, and *Botrytis cinerea*. In a different investigation, *B. halotolerans* strain KLBC XJ-5 was employed as a biocontrol agent against *B. cinerea* mold on harvested strawberries. It was able to maintain the nutrient quality of the fruit while also controlling mycelial development (Wang et al. 2021).

On the other hand, the *B. halotolerans* KKD1 strain showed a potent antagonistic effect against *F. graminearum*, with an average size of the zone of inhibition above 11 mm. Additionally, the lipopeptide within the extracts of this strain presented a suppressive effect on this phytopathogen (Wu et al. 2021). The mining of its genome revealed that this antifungal activity resulted from the production of several bioactive molecules. The production of fengycin/plipastatin, surfactin, bacillibactin, and polyketides was mostly responsible for the activity attained. Therefore, the expression of surfactin and fengycin lipopeptides, which have been recognized as potent antifungals of KKD1, was confirmed by HPLC and MALDI-TOF-MS (Wu et al. 2021). In addition, the *B. halotolerans* KDD1 strain displayed positive protease and amylase activities, which aligns with our findings (Wu et al. 2022).

The two *B. halotolerans* strains Hil4 and Cal.l.30, isolated from medicinal plants, demonstrated antifungal activity against *B. cinerea*. They successfully controlled gray mold on harvested grapes and cherry tomatoes. Moreover, the supernatants of these strains also showed high activity against this plant pathogen. On the other hand, UHPLC-HRMS analysis of the extracts showed that *B. halotolerans* Hil4 and Cal.l.30 synthesized and secreted various secondary metabolites with antibacterial activity, such as fengycin, surfactin, mojavensin A, and others (Thomludi et al. 2021; Tsalgatidou et al. 2022). Similarly, the culture supernatant of *B. halotolerans* strain QTH8 exhibited antifungal activity by significantly reducing the conidial germination of *Fusarium pseudograminearum*. Fengycin, iturin, and surfactin were identified by MALDI-TOF-MS in the QTH8 extract. Therefore, PCR was used to confirm the presence of the required genes to produce these compounds (Li et al. 2022).

Molecular identification of HGG15, HGR5, and HGK11. The generated 16S DNA genes sequences (i.e., MN420494.2, MN420495.1, MN420495.1 GenBank accession numbers for HGR5, HGG15, and HGK11 strains, respectively) were submitted to the EzBioCloud database through the 16S-Based ID tool. Unfortunately, the obtained sequences did not cover the whole 16S rDNA gene sequence (i.e., completeness of about 80%), making partial taxonomic identification with a valida-

tion at least of the genus. Thus, we obtained a 98.01% and 97.46% similarity sequence with *B. halotolerans* ATCC® 25096™ for HGG15 and HGK11 strains, respectively. Nevertheless, the highest percentage of identity for HGR5 was 94.57%, which is below the 97% threshold needed to suggest a potential species affiliation. Thus, identifying this strain required further molecular analyses, such as whole genome sequencing.

Genomic analyses of strain HGR5. Genome features. Whole genome sequencing of *B. halotolerans* HGR5 yielded 28,626,338 paired-end reads with an average length of 150 bp. The assembled draft genome was 4,209,419 bp within 21 contigs, with the largest one of 2,224,912 bp size, a GC content of 43.52%, and 1,011× coverage. Bakta annotation indicated 88.6% coding density with 4,120 coding sequences (CDS), 62 tRNA genes, 30 and 63 ncRNA and ncRNA regions, respectively, and three rRNA genes (Fig. 2).

Taxonomic classification. Several phylogenetic and phylogenomic analyses were conducted to validate the taxonomic position of the biotechnologically interesting isolate HGR5. First, the 16S rDNA-based phylogenetic tree showed that HGR5 is more closely related to the *B. halotolerans* species (Fig. 3). Similar results were obtained for the analysis of *cdaA*, *gyrA*, and *gyrB* gene markers (Fig. S1, S2 and S3). Then, to confirm this classification, the whole-genome sequence of HGR5 was further compared to the sixty-seven *Bacillus* species by alignment-based ANI and digital DDH, indicating 99.06% and 92.5% scores by comparison to *B. halotolerans* ATCC® 25096™ type-strain, respectively. According to the recommended species cut-off, defined as being 96%, HGR5 was found to be a representative of *B. halotolerans*, which is consistent with the results obtained for single gene phylogenetic analysis.

CRISPR analysis. The *B. halotolerans* HGR5 genome contains two CRISPR elements of 49 bp and 24 bp size located in the genomic regions 213244–213373 and 417946–418052, respectively. While *B. halotolerans* KDD1 has been reported to contain six CRISPR-Cas systems involved in antiphage activity (Wu et al. 2021), the presence of such genetic signatures may contribute to similar antiphage activity in HGR5.

Genome functional characterization. A total of 3,821 (92.74%) genes were identified and assigned clusters of orthologous groups (COG) category using eggNOG-mapper (Fig. 4). The majority of annotated genes were found associated with “Translation, ribosomal structure and biogenesis”, indicating the importance of protein synthesis in this microorganism. Other prominent categories include “Transcription”, “Replication, recombination and repair”, and “Signal transduction mechanisms” categories, highlighting key processes involved in genetic information management and cellular communication. Additionally, numerous

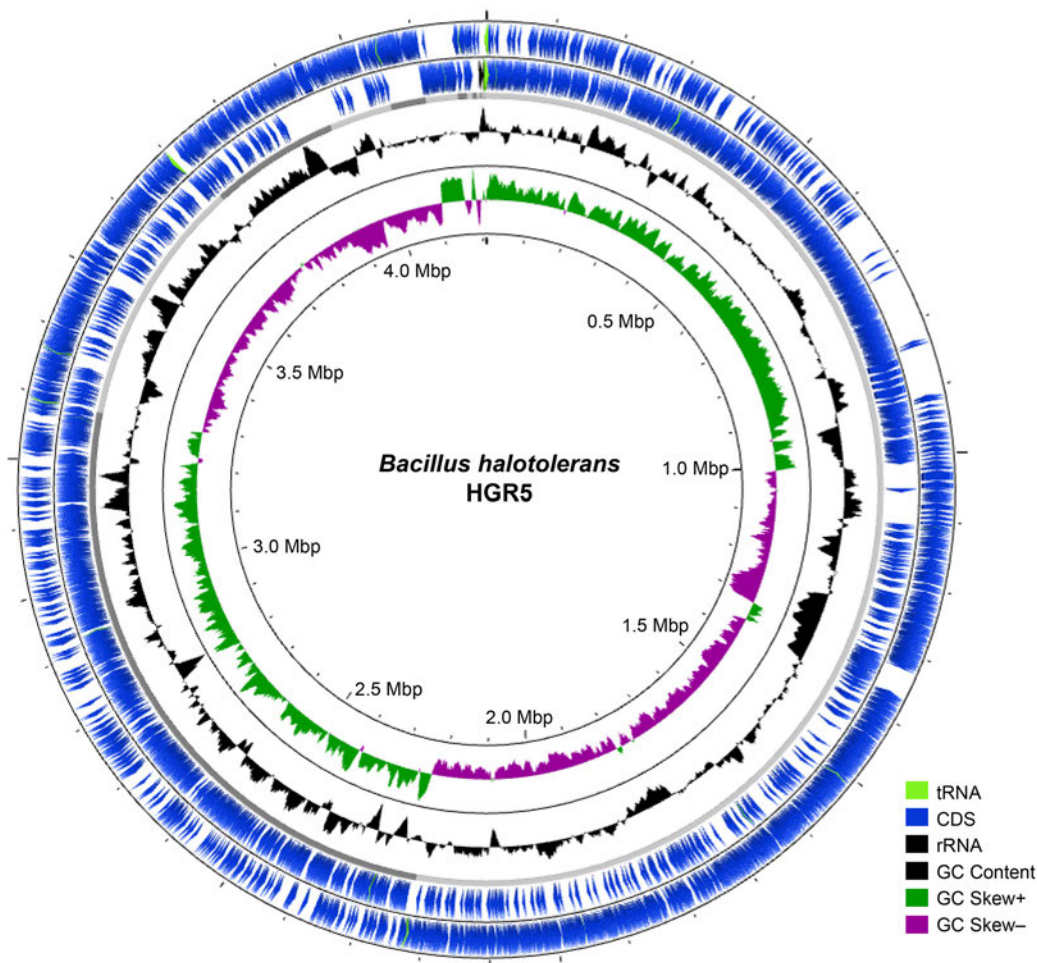


Fig. 2. Whole-genome map of *Bacillus halotolerans* HGR5 generated by CGview. The genome map consists of six different circles from the inner to the outer circle: 1) measuring scale, 2) GC skew, 3) G + C content, 4) contig positions, 5) forward CDS, and 6) reverse CDS.

genes were found involved in “Cell wall/membrane/envelope biogenesis”, “Energy production and conversion”, “Carbohydrate transport and metabolism”, and “Amino acid transport and metabolism”, suggesting a versatile metabolic capability of HGR5. Interestingly, a substantial number of genes were classified as having “General function prediction only” implying that their specific functions are not yet well-defined. Furthermore, a significant proportion of genes fell under the category of “Function unknown,” highlighting the need for further investigation to uncover their roles and potential functions.

Genomic insights into the biocontrol potential of HGR5. Genome mining for secondary metabolites. Identifying novel genes encoding for secondary metabolites through genome mining has recently emerged as an alternative to more traditional approaches. The clusters encoding these molecules in microorganisms generally have the same composition, enabling the development of tools to search and identify BGCs of secondary metabolites in genomes and metagenomes

(Weber et al. 2015). Therefore, an approach based on the antiSMASH and ClusterBlast algorithms platforms was used to identify these clusters within the genome of the HGR5 strain. Thus, fourteen BGCs encoding for secondary metabolites were found. They are distributed over 10 biosynthetic regions, covering a significant proportion (13.5 %) of its genome (Table I, Fig. S4). These regions include clusters encoding for known metabolites but also those that code for substances that are not listed in published databases. A similar proportion was found in the genome of *B. halotolerans* Cal.1.30 (Tsalgaidou 2022), while other reports indicated a proportion of around 9% identified in the other *B. halotolerans* strains (Wang et al. 2021; Wu et al. 2021).

The first four BGCs successively code for bacilysin, subtilosin A, bacillibactin, and bacillaene. These regions show 100% similarity to known BGCs in the Minimum Information about a Biosynthetic Gene Cluster (MIBiG) database (Terlouw et al. 2023). Furthermore, ClusterBlast analysis revealed that the genetic organization of these regions was 100% similar to other

Table I
BGCs encoding secondary metabolites discovered by the AntiSMASH server in the *Bacillus halotolerans* HGR5 genome along with the closest biosynthetic gene clusters detected by ClusterKnownBlast and listed in the MIBiG database.

Cluster	Predicted size (bp)	BGC type	Compound	MIBiG accession	Similarity	Closest strain
1	41,419	other	bacilycin	BGC0001184	<i>Bacillus velezensis</i> FZB42	100%
2	21,613	sactipeptide	subtilosin A	BGC0000602	<i>Bacillus subtilis</i> subsp. <i>spizizenii</i> ATCC® 6633™	100%
3	47,140	NRPS	bacillibactin	BGC0000309	<i>Bacillus subtilis</i> subsp. <i>subtilis</i> str. 168	100%
4	106,121	NRPS, transAT-PKS	bacillaene	BGC0001089	<i>Bacillus velezensis</i> FZB42	100%
5	79,855	NRPS	fengycin	BGC0001095	<i>Bacillus velezensis</i> FZB42	80%
		NRPS, transAT-PKS	mycosubtilin	BGC0001103	<i>Bacillus subtilis</i> subsp. <i>spizizenii</i> ATCC® 6633™	100%
6	41,098	T3PKS	–	–	–	–
7	21,899	terpene	–	–	–	–
8	65,396	NRPS	surfactin	BGC0000433	<i>Bacillus velezensis</i> FZB42	86%
9	20,807	terpene	–	–	–	–
10	51,225	PKS	myxovirescin A1	BGC0001025	<i>Myxococcus xanthus</i> DK 1622	13%
			macrolactin H	BGC0000181	<i>Bacillus velezensis</i> FZB42	40%
11	32,261	PKS	macrobrevin	BGC0001470	<i>Brevibacillus</i> sp. Leaf182	26%
			phormidolide	BGC0001350	<i>Leptolyngbya</i> sp. ISBN3-Nov-94-8	21%
12	13,175	NRPS	fengycin	BGC0001095	<i>Bacillus velezensis</i> FZB42	20%
			plipastatin	BGC0000407	<i>Bacillus subtilis</i> subsp. <i>subtilis</i>	38%
13	10,326	NRPS	plipastatin	BGC0000407	<i>Bacillus subtilis</i> subsp. <i>subtilis</i>	23%
			fengycin	BGC0001095	<i>Bacillus velezensis</i> FZB42	20%
14	7,314	PKS	bryostatin	BGC0000174	<i>Candidatus Endobugula sertula</i>	100%

B. halotolerans strains. At the same time, the surfactin is encoded by an NRPS (non-ribosomal peptide synthesis) cluster that exhibits 86% similarity to the *B. velezensis* FZB42 surfactin-encoding cluster.

The fifth BGC contains two biosynthetic gene clusters encoding fengycin and mojavencin, respectively, like those of *B. velezensis* FZB42 and *B. subtilis* subsp. *spizizenii* ATCC® 6633™. However, the fengycin-encoding cluster was found to be incomplete, showing 80 and 73% similarity to those of *B. velezensis* FZB42 and *B. halotolerans* FJAT-2398, respectively. While the mojavensin cluster showed 100% gene homology with mycosubtilin, it also exhibits 60% and 44% gene homologies with bacillomycin D and iturin, respectively. Nevertheless, the presence of the C module indicates that the amino acid sequence of this BGC encodes mojavensin synthase (Dunlap et al. 2019). The presence of fengycin/mojavensin BGC in *B. halotolerans* strain Hil4 was also reported by Thomloui et al. (2021), though they noticed that this BGC is not present in all strains of this species. The cluster encoding mojavensin was acquired by a horizontal transfer, according to an analysis of the clusters coding secondary metabolites of these strains (Thomloui et al. 2021). This conclusion is backed up by comparing all the BGCs coding SM in *B. halotolerans* strains. All strains that had the mojavensin cluster were found phylogenetically close to each

other. This comparison also showed that the clusters encoding for surfactin, fengycin, bacilysin, subtilosin, bacillaene, and bacillibactin are present in all strains of *B. halotolerans* (Tsalgatidou et al. 2022). Wu et al. (2021) also found that the BGCs coding for secondary metabolites in *B. halotolerans* are species-specific and independent of environmental factors.

Moreover, the HGR5 strain also has two NRPS-like BGCs, which, according to the KnownClusterBlast analysis, contain two clusters that have partial similarities to the clusters encoding plipastatin and fengycin from *Bacillus subtilis* subsp. FZB42 and *B. velezensis*, respectively (Table I). Interestingly, the missing region of the 5th BGC fengycin cluster is present in the 12th BGC with an identical genomic organization to *B. velezensis* FZB42. Moreover, the HGR5 strain possesses a BGC encoding a type III polyketide synthase and two terpene BGCs that are not matched in the database. On the other hand, according to ClusterBlast analysis, these regions are also present in other strains of this species. Thus, several recent studies have reported the presence of these BGCs coding regions in the genome of *B. halotolerans* strains (Thomloui et al. 2021; Wu et al. 2021; Tsalgatidou et al. 2022).

AntiSMASH analysis revealed that HGR5 contains three BGCs encoding polyketide synthases, with relatively low similarity to known BGCs in the MIBiG

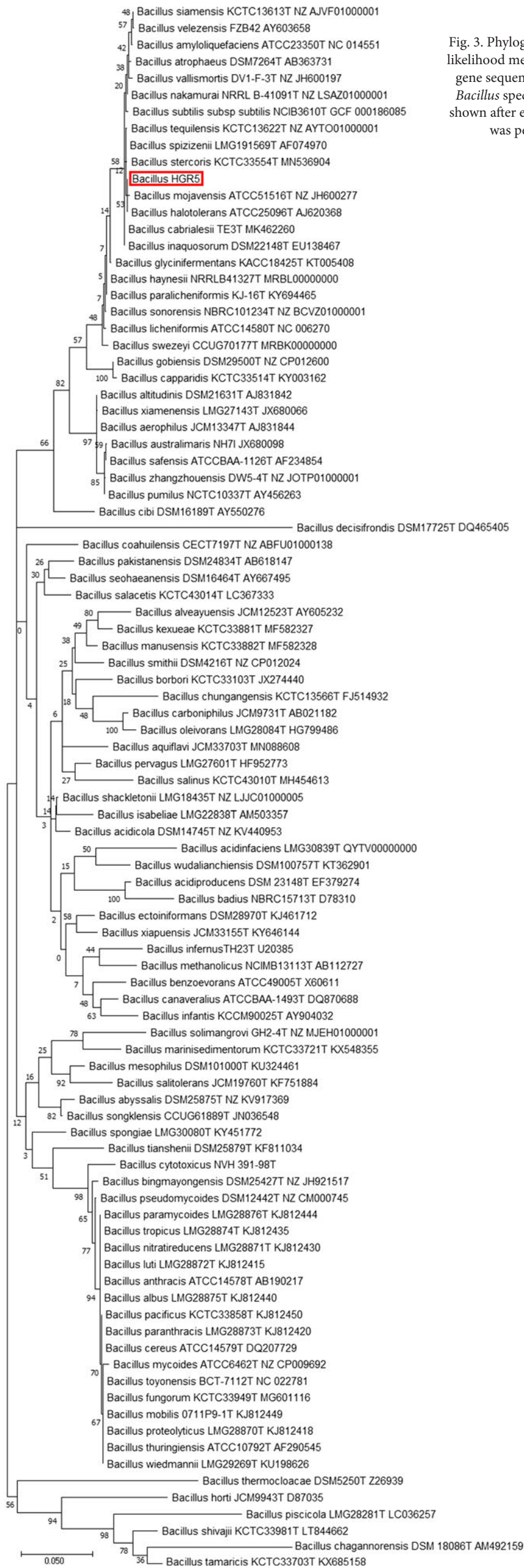


Fig. 3. Phylogenetic analysis by maximum likelihood method based on the 16S rRNA gene sequences of HGR5 and its related *Bacillus* species. Accession numbers are shown after each strain ID. Bootstrapping was performed 1,000 times.

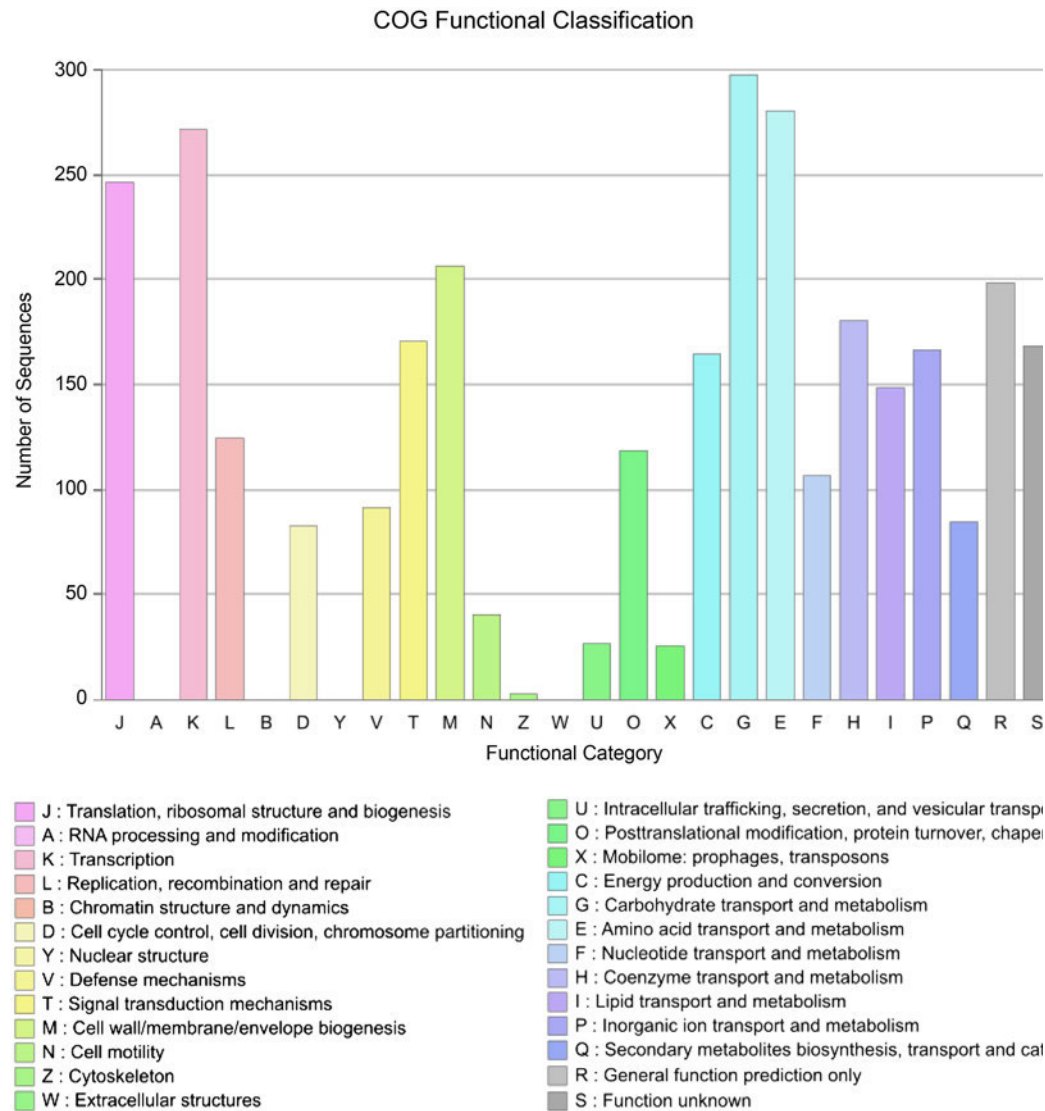


Fig. 4. GO classifications of the genome sequence of *Bacillus halotolerans* HGR5. Bar chart represents the number of genes which were assigned a known GO function by the eggNOG-mapper tool.

database (Table I). ClusterBlast analysis of these regions also showed low similarity to gene clusters present in bacteria. The nucleotide sequences of these three BGCs were used as Blastn queries, and the results showed that they shared 99% similarity and 100% query coverage with regions found in *B. halotolerans* S-5 and *B. mojavensis* PS1. Furthermore, some gene clusters that composed these BGCs were significantly similar to those found in *B. halotolerans*, *B. mojavensis*, *B. velezensis*, and other *Bacillus* species. Based on the results of all these analyses, these three BGCs are thought to encode unidentified secondary metabolites.

Genome mining for CAZyme genes. This analysis revealed the presence of 155 CAZymes within the HGR5 genome sequence, encoding enzymes that target several components of the fungal cell wall i.e., chitin, chitosan, starch, cellulose, xylan, fructan, and pectin. Six classes were identified, including carbohydrate esterases (CE,

n = 14), glycoside hydrolases (GH, *n* = 55), and polysaccharide lyases (PL, *n* = 6) that are known as plant cell wall degrading enzymes. The three other classes with indirect roles in degrading carbohydrates are auxiliary activity (AA, *n* = 3), carbohydrate-binding module (CBM, *n* = 38), and glycosyl-transferases (GT, *n* = 38). 28.38% of these CAZyme proteins harbor amino-terminal signal peptides in their sequences, indicating their secreted enzymes status via protein export through the cytoplasmic membrane.

Degradation of coconut oil, tributyrin, and polycaprolactone. Solid-state activity assays have recently been developed to screen microbes that rapidly degrade specific polymers. Clear or colored zones that form around bacterial colonies indicate the production of catalytically active enzymes on the polymer incorporated in the medium. Polycaprolactone (PCL) and PET nanoparticles have been used several methods to

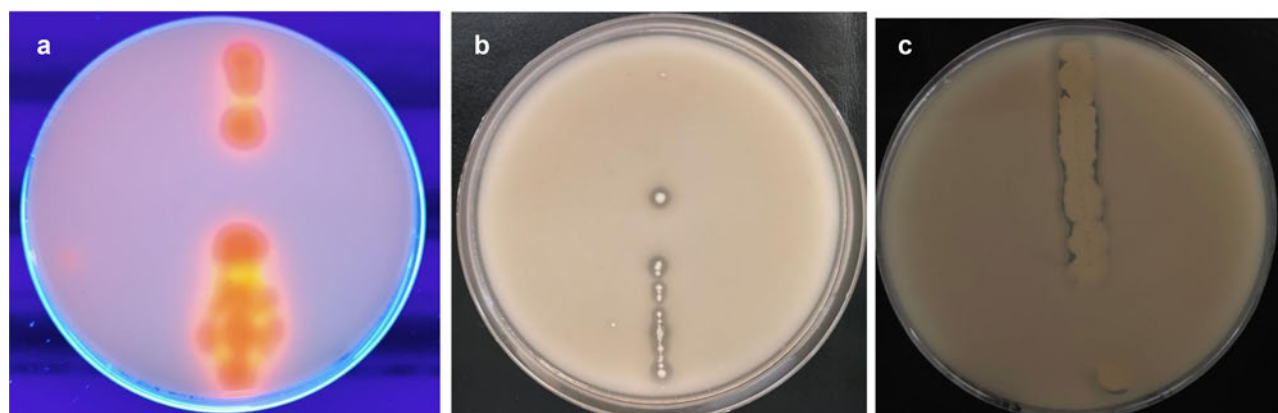


Fig. 5. Esterases activities of *Bacillus halotolerans* HGR5.

a) lipolytic activity on rhodamine B/coconut oil agar, b) lipolytic activity on tributyrin agar, c) biodegradation of PLCD.

identify bacteria with polyesterase activity (Jarrett et al. 1984; Nishida and Tokiwa 1993; Wei et al. 2014). Nevertheless, the production of these nanoparticles is based on the use of toxic or dangerous solvents. Hence, a new protocol was developed using polycaprolactone diol, a water-emulsifiable polyester (Molitor et al. 2020).

The microbial degradation of PCL, which is used as a model substrate for the hydrolysis of polyesters, indicates possible action against the most complex polyesters, including polyethylene terephthalate PET (Danso et al. 2018). Since polyesterases are lipolytic enzymes, they can be screened via substrates such as tributyrin and coconut oil, which are non-specific esterase assays (Molitor et al. 2020). These assays showed that HGR5 was able to degrade tributyrin and coconut oil. In addition, polyesterase activity screening showed that this strain was capable of degrading polycaprolactone diol by secreting one or several lipolytic enzymes (Fig. 5). Carr et al. (2022) examined 51 bacterial strains for their activity against PCLD; only five strains showed positive activity. Otherwise, some studies have reported lipolytic enzyme production from strains of this species. Hence, a 38 kDa lipase from *B. halotolerans* VSH 09 has been purified and characterized, and its optimal activity was observed at pH 7.0 and a temperature of 35°C (Mahnashi et al. 2022). Another report found that the *B. halotolerans* RCPS2 strain produces lipolytic enzymes (Chouhan et al. 2020).

Environmental stress tolerance of *B. halotolerans* HGR5. According to the results, HGR5 can grow in the pH range of 5 to 11, tolerate temperatures up to 55°C, and survive salinities up to 12.5%. It exhibits an optimum temperature of 35°C and an optimum pH of 7. It grows best in a media devoid of salts. Its growth is affected by high temperatures, extreme pH, and high NaCl concentrations (Fig. 6). Our findings are consistent with those of (Wu et al. 2021), who reported that *B. halotolerans* strain KDD1 tolerates salinity and alkalinity better than the two model strains *B. velezensis*

FZB42 and *B. subtilis* 168. This strain develops well at pH 10 and 13% salinity. They claim that KKD1 contains a variety of genes and gene families that enable it to survive various biotic and abiotic challenges. The expression of multiple genes, notably sodium transporter genes, proline biosynthesis genes, glycine betaine genes, and trehalose genes, relates to its ability to endure high salt concentrations. Hence, they observed a correlation between enhanced expression of these genes and tolerance to salt stress (Wu et al. 2021). The genome of strain HGR5 was explored, and it was found that all the gene families reported in strain KKD1 were present as well. This might explain the halotolerance of the strain.

Meanwhile, three strains of the genus *Bacillus*, isolated from a hot spring in India, could grow at temperatures of 40, 50, and 60°C, indicating that they can withstand unfavorable conditions (Verma et al. 2018). In contrast, bacteria isolated from hot springs in Algeria could grow at 55°C, with only 5.11 percent of these isolates could grow at 80°C. These isolates exhibited optimal growth at neutral pH and could survive a concentration of 15% NaCl (Benammar et al. 2020). In addition, in a different collection of bacteria isolated from two hydrothermal springs in Algeria, the isolates demonstrated optimal development at temperatures between 50 and 60°C, at a pH between 6.5 and 7.5, and they tolerated up to 10% salt (Gomri et al. 2018).

Conclusions

Based on these results, the hot springs of Hammam Ouled Tebben and Hammam Guergour in Algeria harbor diverse bacterial communities with interesting biotechnological applications, highlighting the importance of exploring the microbial diversity of such ecosystems and their potential for biotechnological applications. Their harsh conditions (i.e., water temperature and pH) may select specific bacteria and genotypes with

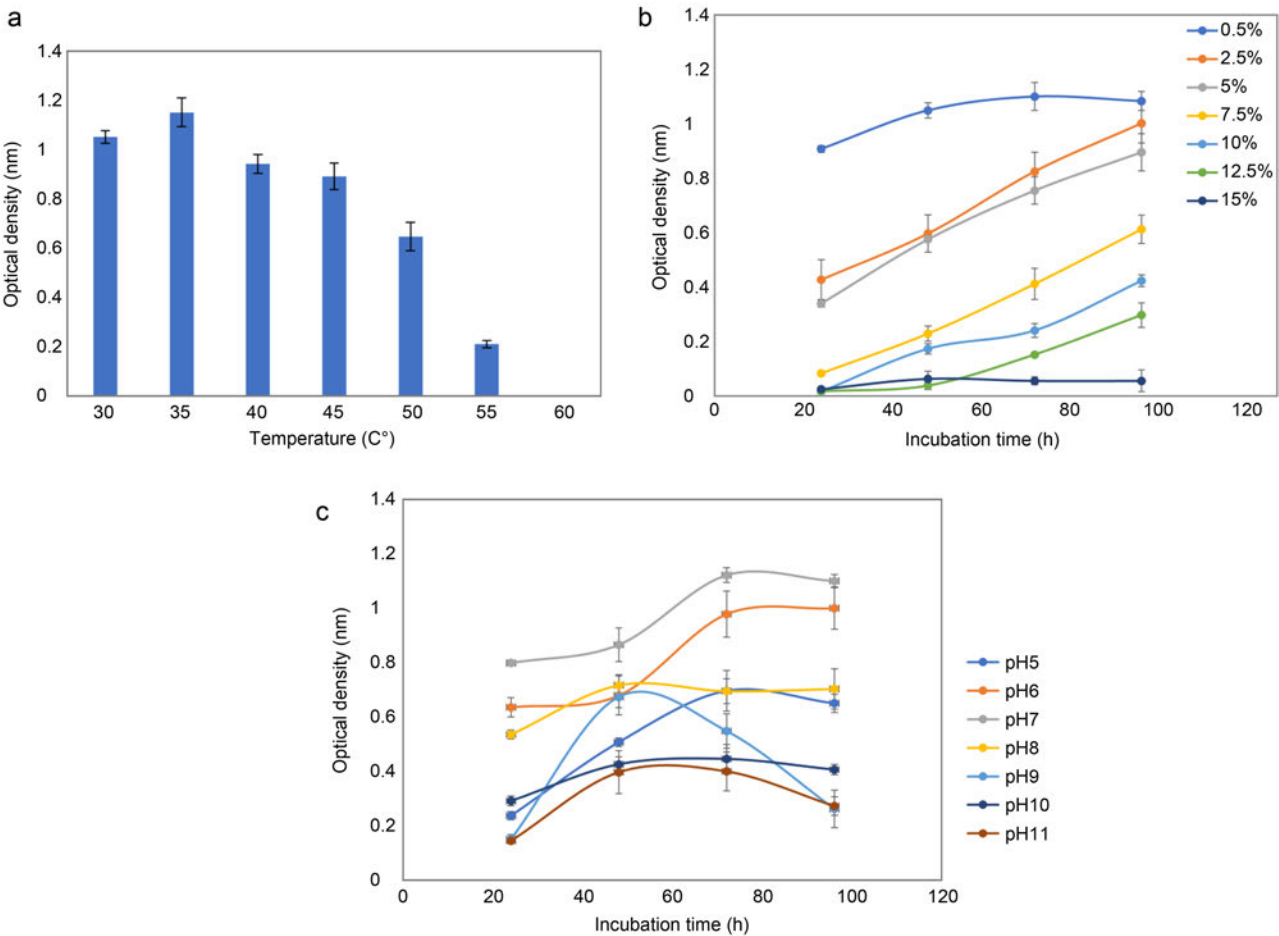


Fig. 6. Growth curve of *Bacillus halotolerans* HGR5 under various conditions of stress.
a) growth curve under various temperatures, b) growth curve under different pH, c) growth curve under increasing concentrations of NaCl.
The error bars show the average standard deviation of each treatment, which was replicated three times.

such interesting properties. *B. halotolerans* was a predominant species among the collected isolates, which has been previously reported to produce various bioactive compounds. The isolated strains exhibited various enzymatic activities, including protease, lipase, and glycosyl hydrolase, with differential levels, where *B. halotolerans* HGR5 presented the most significant yields. In addition, valuable insights into the functional landscape of the genome sequence of this isolate offer a foundation for studying the biology, physiology, and potential biotechnological applications of this halotolerant bacterium. Finally, the findings of this study can be helpful for the development of novel biotechnological products and processes. However, future studies will be conducted to explore the potential of the isolated strains and their bioactive compounds, with focusing on *B. halotolerans* HGR5.

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Conflict of interest
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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