Original Paper

Phenotypic and genotypic insights into a halotolerant bacterium with antimicrobial potential from El Golea Lake

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Abstract

Introduction: The antimicrobial potential of a halotolerant bacterial strain, LMB3982, isolated from the hypersaline Lake El Golea in Algeria at 25% NaCl (w/v), was investigated. The study aimed to evaluate the antimicrobial activity of this strain and to characterize its phenotypic and genotypic properties, with a focus on its bioactive compounds and environmental adaptability.
Methods: The strain was subjected to primary and secondary screenings for antimicrobial activity against a panel of test microorganisms. The influence of growth medium and incubation time on the production of antimicrobial metabolites was assessed. Various organic solvents were used to extract antimicrobial compounds, which were then evaluated for their efficacy. Thin-layer chromatography (TLC) was performed on chloroformic extracts using three solvent systems, and chemical properties were analyzed using infrared (IR) spectroscopy. Additionally, a taxonomic study based on phenotypic and genotypic identification, including 16S rRNA gene sequencing, was conducted.
Results: Strain LMB3982 demonstrated significant antimicrobial activity against most test microorganisms, with maximum activity observed after 72 hours of culture on nutrient agar. Chloroform was identified as the most effective solvent for extracting bioactive compounds. TLC analysis revealed the presence of two types of molecules: amines and phenols. IR spectroscopy confirmed these findings, showing characteristic peaks corresponding to these compounds. Biochemical tests indicated that the strain could produce various enzymes, utilize multiple sugars, and grow under a wide range of conditions: NaCl concentrations of 0–25%, pH levels of 5–11, and temperatures of 15–37°C. Phylogenetic analysis of the 16S rRNA gene sequence positioned strain LMB3982 as a novel taxon within the *Bacillales* order, showing 96% similarity to *Bacillus pseudofirmus* and 93.3% to *Bacillus halodurans*
Conclusion:. These findings highlight the potential of strain LMB3982 for biotechnological applications, particularly in the production of novel antimicrobial compounds and enzymes adapted to extreme conditions.

Keywords: Halotolerance, antibacterial, phenotypic characterization, genotypic characterization, hypersalin environment.

Introduction

Microorganisms are ubiquitous in nature and can exhibit remarkable adaptability to extreme environments, defying biological norms and physical-chemical boundaries. Known as extremophiles, these organisms include halophilic and halotolerant species capable of thriving in high-salinity environments such as hypersaline lakes, solar salterns, and subsurface salt formations. A critical distinction exists between halophilic microorganisms, which require salt for growth, and halotolerant microorganisms, which can survive across a wide range of salinity levels without relying on salt for development (Menasria et al. 2019 ; Singh et al. 2019).
The ability to withstand high salinity endows halophilic and halotolerant microorganisms with unique traits, positioning them as promising candidates for various biotechnological applications (Nas et al. 2022). Simultaneously, the global surge in multidrug-resistant pathogens has created an urgent demand for novel antimicrobial compounds. In response, halophilic and halotolerant microorganisms have emerged as a promising

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area of exploration. These extremophiles represent a new frontier for discovering bioactive compounds, as they are known to produce highly stable secondary metabolites capable of withstanding extreme environmental conditions (Nas et al. 2022 ; Corral et al. 2020; Giani et al. 2019; Quadri et al. 2016).
In this study, we investigated the antimicrobial potential of a halotolerant bacterial strain, designated LMB3982, isolated from the hypersaline environment of El Golea Lake in the Algerian Sahara. The strain was characterized using phenotypic and genotypic approaches, and its antimicrobial activity was analyzed through chromatographic and spectroscopic techniques.

Materiel and methods

Primary Screening of Antimicrobial Activity

The antimicrobial activity of strain LMB3982 was assessed using the agar plug methodBalouiri et al. (2016), against five Gram positives bacteria (*Bacillus cereus* ATCC11778, *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* ATCC 19115, *Enterococcus feacalis* ATCC 25212, *Staphylococcus aureus* ATCC 25923), five Gram negatives bacteria (*E. coli* ATCC 25922, *Salmonella enteritidis* ATCC 2453, *Acinetobacter baumanii* ATCC 19606, *Klebsiella pneumoniae* ATCC 70603, *Pseudomonas aeruginosa* ATCC 27853) and one yeast (*Candida albicans* ATCC 10231).
The strain was grown on KN agar medium (composition : yeast extract 2 g, peptone 2 g, NaCl 2 g, NH_4Cl 1 g, KH_2PO_4 3 g, K_2HPO_4 3 g, $CaCl_2.2H_2O$ 0.1 g, $MgCl_2.6H_2O$ 0.5 g, KCl 0.1 g, agar 15 g, distilled water 1000 mL, pH 7). After incubation for 72 hours at 30°C, 6 mm agar plugs were excised and placed on Muller-Hinton and Sabouraud agar plates previously inoculated with test microorganisms, standardized to 0.5 McFarland. Plates were refrigerated for 2 hours to allow compound diffusion, then incubated for 24 hours at 37°C for bacterial strains and at 25°C for *Candida albicans*. The presence of inhibition zones indicated antimicrobial activity. All microbial pathogens used in this study were obtained from Laboratory of Applied Microbiology in Agri food, Biomedical and Environment (L.A.M.A.A.B.E) (Tlemcen- Algeria).

Antimicrobial activity of the supernatant

The strainLMB3982 was cultivated into KN broth medium for 72 hours at 30°C. After centrifugation at 10000 g for 20 minutes, the supernatant was tested for antimicrobial activity using filter paper disc method (100 µL / disc) against *B. cereus, E. feacalis, S. aureus, E. coli, S. enteritidis, K. pneumoniae, P. aeruginosa* and *C. albicans*(Khanna et al. 2011).

Optimization of antimicrobial compound production

Production of antimicrobial activity in various media

The production of antimicrobial activity was tested in various media: nutrient agar, KN agar, and Mossel agar. Agar plugs from cultures grown in these media were tested for inhibition against standard pathogens. The medium yielding the highest activity was selected for further experiments.

Effect of incubation period on antimicrobial activity

To determine the optimal incubation period, strain LMB3982 was grown in nutrient agar and tested for activity every 24 hours over a 72 hour incubation period. Agar plugs were assayed against the same microbial panel used in the primary screening.

Solvent selection for compound extraction

Five solvent of varying polarity were used (ether, chloroform, ethyl acetate, methanol and water) in order to choice the best solvent for extraction of bioactive compounds. The extraction was realized from solid medium by adopting the method of Kim et al. (2016). After 72 hour of culture at 30°C in nutrient agar, the medium was cut into cubes and agitated with each solvent for 2 hours. The organic extract obtained was filtered and then the vacuum was evaporated at 45°c using a rotary evaporator. The dry residue was then taken again in 5 ml of the same solvent used for extraction.
The best solvent for extraction was used for extraction from liquid medium (nutrient broth medium) according to the method of Chen et al. (2010). Fermentation supernatant was mixed with an equivalent volume of solvent.

Extracts were evaporated under reduced pressure at 45°C using a rotary evaporator, reconstituted, and tested for antimicrobial activity.

Antimicrobial activity of crude extracts

The antimicrobial activities of the different crude extracts was investigated using disc paper diffusion method (Balouiri et al. 2011)against *B. cereus, E. feacalis, S. aureus, E. coli, S. enteritidis, K. pneumoniae, P. aeruginosa* and *C. albicans*.
Sterile paper disks (Whatman N°3) with 6 mm diameter are loaded with 100 µl of organic extract and placed on the surface of Muller-Hinton agar and Sabouraud agar media which are seeded with test microbial pathogens (0.5 McFarland standards). Control disc were loaded with 100 µL of a pure organic solvent. The extract was allowed to diffuse into the agar at 4 °C for at least 2 h, and then incubated 24 hours at 37ºC for test bacterial and at 25°C for *Candida albicans*. At the end of the incubation period, the plates were observed for zones of inhibition around the disc.
The extract obtained from a liquid medium was also tested against two plant pathogen fungi which are *Aspergillus sp.* and *Cladosporium sp.* using thefood empoisoning technic **(Soliman et Badeaa 2002).**100 µL of the crud extract was added to 20 mL of Sabouraud agar and put in petri dishes. Plug agar seeded with 5 to 7 days old fungal culture was inoculated in the center of the Sabouraud agar and incubated at 25°C for 5 to 7 days. Sabouraud agar plate without the chloroformic extract inoculated with plug agar of 5 to 7 days old fungal culture was used as control.
The measure of diameter ofthe growth zone of the fungi on the Sabouraud agar added with chloroformic extract, was done by referring to the control dishes and calculated according to the formulation

$$T = \frac{Db - Da}{Db} \times 100$$

T : rate of inhibition of the mycelium growth (%).
Da : Diameter of the growth zone of mycelium at Sabouraud agar added with the chloroformic extract (test)
Db : Diameter of the growth zone of mycelium at Sabouraud agar without the chloroformic extract (control)

Preliminary characterization of the antimicrobial compounds

Thin-layer chromatography analysis

Thin-layer chromatography (TLC) was employed to analyze the bioactive compounds in chloroformic extracts using three solvent systems: chloroform-methanol (60:40), ethyl acetate-methanol (100:15), and chloroform-methanol-ammonia (8:1:1). The spots were visualized using chemical reagents (ninhydrin, FeCl3, and Molisch).

Spectral study of the antimicrobial compound by Infra-Red (IR)

To determine the functional groups of antimicrobial compound produced by the strain LMB3982, Infra-Red spectrum was recorded on Agilent Technologies Cary 600 FTIR model Spectrometer. A dry residue of the chlololormic extract was analyzed in the range of 700- 4000 cm^{-1}.

Phenotypic and genotypic characterization of strain LMB3982

The characterization of the strain LMB3982 was performed according to the minimal standards for describing new taxa within the aerobic, endospore-forming bacteria proposed by Logan et al. (2009).
The phenotypic characterization of the strain LMB3982 was done by studying microscopicall characters by means of a combination tests (Gram reaction, cell morphology and motility), using the API 20E system and other conventional microbiological methods: Mannitol-motility, respiratory type, catalase and oxidase, complemented with physiological tests.
The physiology of the strain was determined by evaluation her growth every 6 hours for 72 hours of incubation in different conditions of temperature, pH and salinity by measuring the increase in turbidity at 580 nm. Temperature and pH range for growth was determined following incubation of the strains at 4, 15, 25, 30, 37 and 45 °C and pH 2, 5, 7, 9, 11 and in KN Broth. Halotolerance was tested in KN broth (pH=7) supplemented with 0, 2, 5, 7.5, 10, 15, 17.5, 20, 25 and 30% (w/v) NaCl for 72 hours of incubation in a rotary shaker at 30°C .
Resistance to antibiotic was determined by using the standard disc diffusion technique (Bauer et al. 1966). The antibiotics used (mcg/disk): Rifampicin (30), Chloramphénicol (30), Pristinamycin (15), Céfotaxim (30),

Gentamycin (10), Oxacillin (5), Amoxicillin – clavulanic acid (20-10), Fosfomycin (10), Tobramycin (10), Kanamycin (30) and Pénicillin-Novobiocin (10-30).
The strain's phylogenetic identity was determined through 16S rRNA gene sequencing. DNA was amplified via polymerase chain reaction (PCR) and compared with sequences in GenBank using the neighbor-joining method as described in Nas et al (2022).

Results

Primary screening of antimicrobial activity

The primary screening of antimicrobial activity of the strain LMB3982 using plug agar method show that it exhibited antimicrobial activity against several test organisms. Significant inhibition zones were observed against Gram-positive bacteria, including *Bacillus cereus* (12 mm), *Bacillus subtilis* (9 mm), *Listeria monocytogenes* (10 mm), and *Staphylococcus aureus* (13 mm). Among Gram-negative bacteria, activity was detected against *Escherichia coli* (12 mm), *Klebsiella pneumoniae* (11 mm), and *Pseudomonas aeruginosa* (13 mm). No activity was observed against *Enterococcus faecalis*, *Salmonella enteritidis*, *Acinetobacter baumannii*, or the yeast *Candida albicans*.

Evaluation of culture supernatant activity

The antimicrobial activity of the culture supernatant was tested against the same microbial panel. No inhibition zones were observed, indicating that the bioactive compounds were likely not secreted into the medium or were present in insufficient concentrations.

Optimization of antimicrobial compound production

Production of antimicrobial activity in various media

Production of the antibacterial activity by LMB3982 strain was tested in various media, it was highest in nutrient agar, with inhibition zones reaching 13 mm against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Production in KN agar and Mossel agar was wake.

Effect of Incubation Period on Antimicrobial Activity

Maximum antimicrobial activity was observed after 72 hours of incubation in nutrient agar, coinciding with the sporulation phase of the strain. The inhibitory effect was most pronounced against *S. aureus* and *P. aeruginosa*.

Solvent selection for compound extraction

Chloroform was identified as the most effective solvent for extracting bioactive compounds, demonstrating stronger inhibition zones compared to other solvents. Extracts from liquid media exhibited higher antimicrobial activity than those from solid media. The chloroformic extract also inhibited the growth of plant pathogens *Aspergillus sp.* by 23.33% in a food poisoning assay.

Preliminary characterization of antimicrobial compounds

Thin-layer chromatography

Thin-layer chromatography (TLC) analysis revealed two bioactive molecules in the chloroformic extract. A peptide-like compound was detected with an Rf value of 0.93 (ninhydrin positive), and a phenolic compound was identified with an Rf value of 0.97 (staining with $FeCl_3$). The solvent system chloroform-methanol (60:40) provided the best separation and migration.

Infrared spectroscopy analysis

Infrared (IR) spectroscopy confirmed the presence of functional groups corresponding to peptides (peak at 1275 cm^{-1}) and phenols (peak at 1653 cm^{-1}), supporting the TLC results.

Phenotypic and genotypic characterization of strain LMB3982

The isolate was characterized as Gram positive, facultative aerobic, rod-shape no motile strain, able of forming endospore sub-terminally positioned. Colonies on standard agar growth medium (KN) are 2-3 mm in diameter, circular, convex with regular boards and smooth surface.
Growth was found to be optimal at 30°C with a range from 15 to 37°C, at pH 7 with a range from 5 to 11and at NaCl concentration of 0% with a range from 0 to 25% (w/v).
Biochemically, the strain demonstrated catalase and oxidase activity. Antibiotic susceptibility tests revealed resistance to cefotaxime and fosfomycin, intermediate sensitivity to several antibiotics, and susceptibility to penicillin/novobiocin and rifampin.
Phenotypic features determined using API 20E system are includedin Table 1.

Table 1: Biochemical characteristic of the isolate LMB3982, *Bacillus pseudofirmus* and *Bacillus halodurans*using API 20Esystem.

	LMB9832	***Bacillus pseudofirmus***	***Bacillus halodurans***
Api 20^E system			
ONPG	+	/	/
ADH	+	/	/
LDC	-	/	/
ODC	-	/	/
Citrate Simmons	-	/	/
H_2S	-	/	/
Urea	-	/	/
TDA	-	/	/
Indol	-	/	/
VP	+	/	/
Gelatin hydrolysis	+	+	+
GLU	-	+	+
MAN	+	/	/
INO	-	+*	+
SOR	-	-	-
RHA	-	-	+
SAC	+	/	/
MEL	-	-	+
AMY	+	+	/
ARA	+	+	/

/ : no determinant ; *** :** weakly positive

The molecular identification of the isolate was performedby sequencing 16S rRNA gene. Obtained sequenceswere compared with sequences of previously reported strains. The phylogenetic tree was cons tructed using closely related neighbors. Results showed that the isolate is neighboring to the *Bacilluspseudofirmus* with a similarity of 95% and *Bacillushalodurans* with a similarity of 93,3% (Figure 1). The results of this analysis suggested that strain is a new taxon in the order of *Bacillales* with a similarity of 96%.

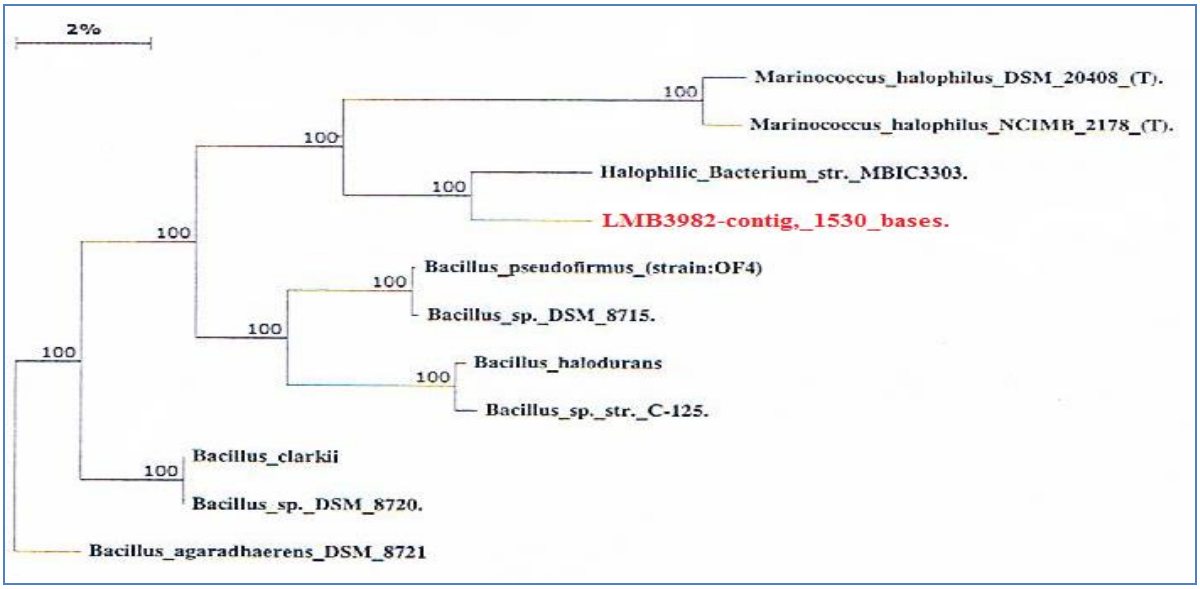

Figure 1: Phylogenetic tree obtained by neighbour-joining analysis based on 16S rRNA gene sequences showing the positions of strain LMB3982 within the *Bacillales* order.

Discussion

Saline and hypersaline ecosystems represent an important reservoir of diverse microbial communities, characterized by remarkable metabolic and physiological adaptations. Among the most extensively studied sites are the Great Salt Lake, the Dead Sea, the highly alkaline brines of Wadi Natrun in Egypt, Chott Djerid in Tunisia, and Lake Magadi in Kenya. In Algeria, such environments are widespread and have been reported to host unique, rare, and novel extremophilic microorganisms. These microorganisms have shown potential for producing various secondary metabolites with significant biotechnological applications. Notable examples of such ecosystems in Algeria include Sebkha Ezzemoul (Kharroub et al. 2006), El Golea Lake (Hacene et al. 2004), and the Sebkha of Kenadsa (Messaoudi et al. 2015).

This study focus on the highlight of the antimicrobial potential of an halotolerant strain LMB3982, isolated from El Golea Lake.

The phylogenetic study (16S rRNA gene sequence) reveal that the strain belong to the order Bacillales with a similarity of 96% what means that the strain LMB3982 is a new species in within this order (Devereux et al. 1990), also the strain LMB3982 is phylogenetically neighboring to the Bacillus pseudofirmus and Bacillus halodurans with a similarity of 95% and 93,3% respectively what means that the strain LMB3982 belong to the different genus and present a new taxon.

Primary screening of antimicrobial activities revealed broad-spectrum activity against both Gram-positive and Gram negative bacteria, particularly against notable pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Phenotypic and genotypic characterization show that the isolate is affilated to the *Bacillus* genus. *Bacillus species*can colonize different habitats even underextreme conditions, they have long been recognized for their potential to produce diverse array of secondary metabolites(Nas et al. 2022 ; Caulier et al. 2019 ; Sumi et al. 2015).

The absence of activity in the culture supernatant suggests that the antimicrobial compounds are primarily cell-associated, necessitating direct extraction from the biomass or the growth medium.

Nutrient agar proved to be the optimal medium for the production of antimicrobial compounds. The production of antimicrobial secondary metabolites is highly dependent on the specific conditions of the culture environment (Wu et al. 2023). Even small changes in the composition of the growth medium or adjustments to fermentation parameters can have a profound impact on the quantity and quality of the metabolites produced. Additionally, such modifications can alter the overall metabolic profile of the microorganisms involved.

The observed peak in activity after 72 hours of incubation aligns with previous studies, which have shown that sporulation triggers the synthesis of secondary metabolites, including antimicrobial agents. Many research examining the impact of incubation time on secondary metabolite production found that optimal antibacterial activity in *Bacillus* speciesrequires more than 48 hours. While antibiotic synthesis often begins during the

logarithmic growth phase, secondary metabolites are generally more abundant during the later stages of exponential growth and throughout the stationary phase (Hellany et al. 2024 ; Seyedsayamdost 2019).
Chloroform was identified as the best solvent for extracting bioactive compounds, outperforming other solvents in terms of extraction efficiency and inhibitory activity. Thin-layer chromatography and IR spectroscopy analyses confirmed the presence of peptide and phenolic compounds as the primary bioactive molecules. These compound classes are known for their antimicrobial properties, with peptides often targeting bacterial cell membranes and phenols disrupting microbial metabolic pathways.

Conclusion

This study demonstrates the antimicrobial potential of the halotolerant bacterium LMB3982, isolated from the hypersaline environment of El Golea Lake in the Algerian Sahara. The strain exhibited activity against a range of pathogenic bacteria, with optimal production achieved after 72 hours of incubation on nutrient agar. Chloroform extraction and subsequent characterization identified peptide and phenolic compounds as the primary bioactive molecules.
The phylogenetic analysis indicated that LMB3982 represents a novel taxon within the *Bacillales* order, highlighting the untapped biodiversity of hypersaline environments. These findings open avenues for further studies to fully elucidate the chemical structure and mechanism of action of antimicrobial compounds produced by this strain.
Future work will focus on scaling up compound production, identifying the complete structure of the bioactive molecules using advanced spectroscopic techniques (e.g. HPLC, mass spectrometry), and assessing their in vivo efficacy and toxicity. Additionally, the strain's salt tolerance present opportunities for broader applications in industrial and pharmaceutical biotechnology.

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Conflict of interest

The author states that he has no conficts of interest.

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