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# Isolation and Characterization of Two Halophilic *Bacillus* (*B. licheniformis* and *Bacillus* sp) with Antifungal Activity

*E H Nabti**, *N Mokrane**, *M Ghoul, H Manyani, M Dary and M G. Megias**

Laboratory of Mastery of Renewable Energies, University of Bejaia, Targa Ouzemmour, 06000 Bejaia, Algeria

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**Abstract:** New bacterial strains of *Bacillus licheniformis* and sp were isolated from the rhizosphere of potato, in front of the sea (Bejaia, Algeria). Strains were identified by phenotypic analyses: Gram staining, mobility, catalase, oxidase, form of colony and cell morphology. Metabolic characters such carbon source, sugars, organic acids, aerobic/anaerobic respiratory system are revealed by API system 50CHB/E. The 16SrRNA gene was isolated and sequenced (1500 bp), and the phylogenic tree was established (bootstrap method). The first strain (RBA-E23) showed a very high similarity to Bacillus licheniformis (DQ082997.1) (98%) and *B. licheniformis* (AF372616.1), but the second (RBA-E32) is classified as the out-group. Strains are halotolerants 300 and 400 mM/l NaCl, respectively. They used glycine betaine and proline as synthetic osmoprotectants, and marine algal extracts of *Ulva lactuca* as natural osmoprotectant. The two strains produce: high amount of auxine-phytohormone (IAA: 78 and 101 mg/l); different enzymes such as: cellulase, chitinase, protease, lipase, amylase and urease; siderophores and solubilize inorganic phosphorus. The study of antifungal strains activity toward nine phyto pathogenic fungi *Verticillium dahlia, Fusarium oxysporum, Botryotinia fuckiliana, Phytophthora cinamomi, Phytophthora cactorum, Colletotrichum acutatum, Botrytis cinerea, Aspergillus Niger* and *Aspergillus flavus*, shows an interesting inhibition activity with variable percentage of Growth inhibition (PGI %) against phytopathogens. Both of bacteria displayed more benefic characters, while the enzymes can be involved in the organic matter decomposition; utilization of the phytohormone-IAA as simulator of vegetable growth. Siderophores and phosphate solubilization are interesting in the fertilization of soil. Finally, strains could have potential application against phytopathogenic fungi.

**Keywords:** *Bacillus*, saline stress, osmoprotection, antifungal activity

## 1 Introduction

The human nourishment with plants and animals is largely dependant on grounds, because, soil is not only the support for vegetable production, but it contains and accumulates common of nutritive elements crucial for life [4].

The soil is the main base for plants production. It provides vegetable growth by essential resources, such as, minerals, water and roots anchoring. To produce food within the framework of durable agriculture, it is indispensable to protect the soil against deterioration [4].

Each year, 20 million hectares of arable lands becomes unusable. Agricultural grounds are degraded and destructed by erosion; salinity and loss of organic matter. Tropical and Mediterranean arid and semi-arid regions are exclusively menaced by salinity. So, the excessive saltiness in soil inhibits the vegetable growth and development, consequently, products yield and quality will be decreased [5,10]. Additionally, pathogenic micro-organisms affecting plants health constitute a major threat for food production and the ecosystems stability. Farmers are gradually more depend on the chemical pesticides as a single method to protect harvests. However, the excessive employment of pesticides generates several negative effects, such as: phytopathogenic resistance and agricultural grounds polluting [10]. *Bacillus* belongs to the PGPR group (Plant growth promoting Rhizobacteria) and considered as stimulator of vegetable growth and bio-fertilizer. It is also involved in plants health. In addition, among PGPR, the genus *Bacillus* is known to have more characters promoting plant growth: ability to synthesize

* Corresponding author e-mail: elhnabti1977@yahoo.fr,nadjim_mokrane@hotmail.com,megiasg@us.es

phytohormones such as IAA (auxins); nitrogen fixation; antifungal activity by siderophores production; antibiotics, enzymes and phosphorus solubilization. It so, appears as a potential candidate to be a good bio-fertilizer through its ability to sporulate. Thus, it resists toward extreme conditions (water and salt stress).
This paper presents two osmotolerants *Bacillus* strains (RBA-E23 and RBA-E32), isolated from Algerian saline soil, with interesting characteristics: IAA; enzymes and siderophores production, utilization of marine alga (*Ulva lactuca*) as osmoprotectant. Both of strains showed an antifungal activity.

## 2 Material and Methods

### *2.1 Soil Sampling*

The work was conducted in the laboratory of Applied Microbiology, university of Bjaia-Algeria, located at 36.75° N, 5.08° E and 2 m above mean sea level. In order to isolate osmotolerant PGPR, soil samples were taken from the rhizosphere of potato. They are randomly chosen in greenhouse. The salinity, pH, water capacity, N, P and K of the sampled soil were 1.14%, 6.9, 28%, 69 μg/g, 3 μg/g, 6 μg/g, respectively.

### *2.2 Osmotolerant PGPR Screening*

Samples were prepared, after appropriate dilutions, then inoculated on the following agar medium (30 mM/l succinate, 10 mM/l $NH_4Cl$, 14 g $K_2HPO_4$, 2.7 g $KH_2PO_4$, 2.5 M NaCl, 5 ml 2% $MgSO_4$ solution, 4 mM/l $FeSO_4$ and 50 μl fungicide, pH 7.2). Plates were incubated for 7 days at 30°C. In parallel, to screen osmotolerant bacteria, the same medium was used with different NaCl-concentrations ranging from 1.72 to 1000 mM/l NaCl. After incubation at 30°C for 7 days, the number of bacteria in the soil sample was calculated by colony forming unit (UFC) in Petri dish. Only 10 colonies were gone in presence of 0.5 M NaCl, while no growth was observed up then 0.5 M NaCl. Screening was continued on the same agar medium (1.72-1000 mM/l NaCl) containing proline (Pro), glycine betaine (GB) and *Ulva lactuca* (UL) extracts with respective final concentrations of 1 mM/l, 1 mM/l and 10% [5,6]. Two bacterial strains (RBA-E23 and RBA-E32) only showed the best growth at 1 M NaCl in presence of (UL). The first one is grown in presence of (Pro) at 700 mM/l NaCl, and the other one is grown in 800 mM/l NaCl when (GB) is added. Both of strains were finally chosen to be identified.

### *2.3 Strains identification*

Identification based on Gram staining, mobility was performed. For other biochemical characters, the API system 50CHB/E was carried out (bio-Merieux, Marcy lEtoile, France). 16S rDNA-Based strain characterization was conducted after total DNA isolation. 16SrDNA partial sequences (1500 bp) were compared to databases using BLASTN 2.2.24. Sequences alignment and phylogenetic reconstruction were inferred.

### *2.4 PGPR characters*

Plant growth promoting characters were studied: IAA production, phosphorus solubilization and siderophores. Different benefic enzymes were searched: chitinase, cellulase, amylase, protease, urease and lipase [11].

### *2.5 Antifungal activity*

These strains are then tested for their antagonistic activity against various phytopathogenic fungi: *Verticillium dahliae (Vd), Fusarium oxysporum (Fo), Botryotinia fuckiliana (Bf), Phytophthora cinamomi (Pc), Phytophthora cactorum (Pca), Colletotrichum acutatum (Ca), Botrytis cinerea (Bc), Aspergillus Niger (An)* and *Aspergillus flavus (Af)*.

## 3 Results and Discussion

### *3.1 Strains identification*

Gram staining of the two strains (RBA-E23 and RBA-E32) revealed Gram positive cells. The first strain (RBA-E23) showed 74% of similarity with *B. amyloliquefciens* and 68% with *B. licheniformis*. While, the second (RBA-E32) has similarities of 62% and 60% with *B. amyloliquefciens* and *B. licheniformis*, respectively. The 16SrDNA partial sequence placed the isolate RBA-E23 within the *Bacillus licheniformis* cluster with 98% 16SrDNA of similarity to the strains *B. licheniformis* DQ082997.1 and *Bacillus licheniformis* MM/lL2501 (gene Bank). RBA-E32 is located in another cluster (out group), so, the strain can be classified as *Bacillus Sp*. with similarities of 99% to *Bacillus Sp*. R-(ABO21181.1) and *Bacillus Sp*. R-30632 (Fig 2).

### *3.2 Osmotelarence*

*Bacillus licheniformis* and *Sp* have the respective optimal growth of 400 mM/l and 300 mM/l NaCl, in absence of osmoprotectants (Figs 1a, 1b). In this case, these strains can be classified as slightly halophilic. Growth of both strains increased significantly in the presence of (GB), (PR) and (UL): values reached 800 mM/l (RBA-E23) and 500 mM/l (RBA-E32) in presence of (GB), 500 mM/l (RBA-E23) and 700 mM/l NaCl (RBA-E32) in addition

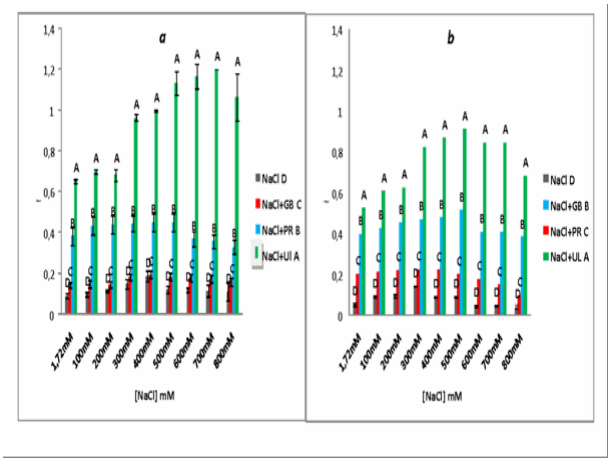


**Fig. 1:** Growth of *Bacillus licheniformis* and *sp* in saline medium with or without osmoprotectants.

The letters (A, B, C and D) denote the homogeneous groups obtained by the Fischer LSD test ($p \leq 0.05$). a: (RBA-E23) or *Bacillus licheniformis*; b : (RBA-E32) or *Bacillus Sp*

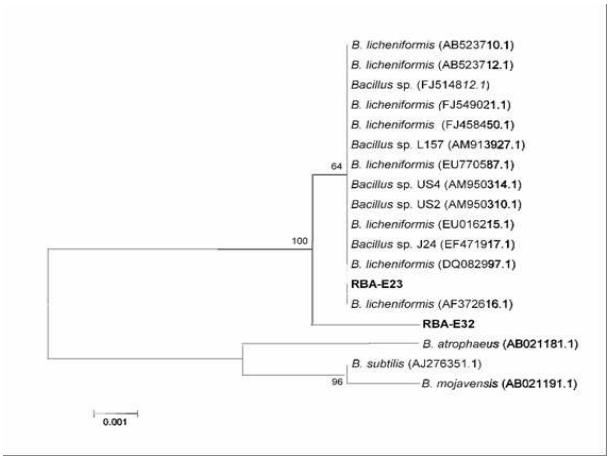


**Fig. 2:** The Phylogentic Tree of the both bacterial species (RBA-E32 and RBA-E23) using 16S RNA sequencing

of (PR) and 1000 mM/l NaCl for both of strains in presence of *U. lactuca* extracts. Thus, we highlight the best growth of the two strains when the marine algal extracts were added. Statistical analysis (ANOVA) using Fischers test (LSD) at interval of 95% ($p \leq 0.05$) supported our results (Fig 1). Bacteria usually use amine-containing compounds such as proline, glycine betaine as osmoprotectants. A large amount of GB is up taken by *Bacillus subtilis* from external environment in high saltiness, using three transport control systems [3]. Proline is an efficient osmoprotectant in various halophilic *Bacillus* and non-halophilic species as *Bacillus subtilis* [7]. *Halobacillus halophilus* accumulates proline in response to high salinity (> 2M NaCl). Proline may be uptake by *Bacillus subtilis* from the medium using a specific transport system *(Opu E)* [9]. Additionally, it is very clear that the presence of algal extracts enhanced and improved broadly the the two strains growth under salinity. Therefore, growth achieved the best value (> 1000 mM/l NaCl). A positive effect of (UL) as osmoprotectant has been already demonstrated [4]. The benefic characters of strains are summarized in Table 1.

## *3.3 PGPR characters*

Both of strains have an important characters for soil fertilization (cellulase, chitinase, protease, lipase, amylase and urease), phosphorus solubilization (Fig 3A), IAA-production 78 mg/l (RBA-E23) and 101 mg/l (RBA-E32). These strains could help plant growth by siderophores production (Fig 3B). As known, iron being an important micronutrient acts as a limiting factor in crop production even when plants do not show visual deficiency symptoms [8]. Too, with probable N2 fixation (positive growth on NFB-medium). Many strains of Bacillus with agricultural characters were isolated from soybean rhizosphere (*Glycine max L.*). These strains produced IAA (0.81 to 86.82 mg/l), thus, they enhanced seeds germination and growth of soybean. Our strains produce a very high IAA quantity (78 to 101 mg/l). The two *Bacillus* isolates solubilize tricalcium phosphate *in vitro*. This character is important for an eventual application in agriculture.*Pseudomonas Sp* and *Bacillus Sp* increased the availability of phosphorus in soil; as a result, they are used as biofertilizer in alkaline soils [1].

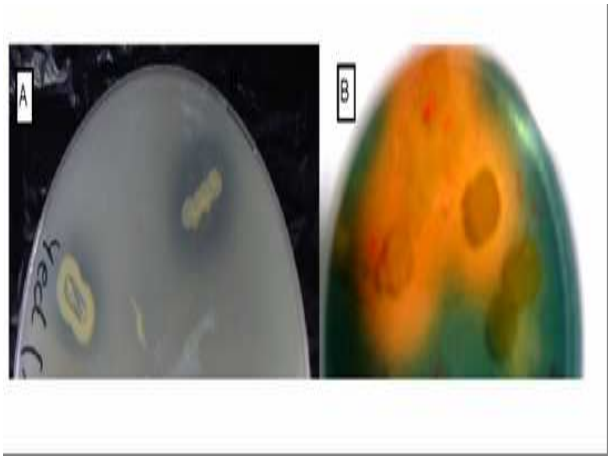


**Fig. 3:** phosphorus solubilization

**Table 1:** Different characters of *Bacillus licheniformis* and *Bacillus Sp*

| Characters | IAA (mg/L) | Cel | Chit) | Prot | Lip | Amy | Ure | Phos | Sid | NFB |
|---|---|---|---|---|---|---|---|---|---|---|
| *Bacillus licheniformis* | 78 | +++ | +++ | +++ | +++ | +++ | +++ | YED+ | ++ | ++ |
| *Bacillus Sp* | 101 | + | +++ | +++ | + | +++ | ++ | YED + | + | + |

+ corresponds to the positive activity; +++ means strong positive activity. Cel: cellulase, Chit:Chitinase, Prot: Protease, Lip: Lipase, Amy: Amylase, Ure: urease, Pho: Phosphatase, Sider: Siderophore.

### *3.4 Antifungal activity*

Bacillus strains are tested against nine phytopathogenic fungi how cause significant losses in agriculture and reduce crops and are often targeted in biological control. The two Bacillus strains show a good mycelial growth inhibition ( $>$ 60%) of the majority of targets (Fig 4). Several mechanisms have been proposed to explain the inhibition of phytopathogenic fungi by *Bacillus spp*, including antibiotic production, hydrolytic enzymes synthesis, competition for nutrients, or a combination of these mechanisms in synergy. Siderophores production is a major mechanism involved in bio-control by many PGPR groups, including *Bacillus sp*., which produces a large variety. Siderophores chelate iron in the soil; therefore, this nutritive element will be decreased in environment. In this case, fungal growth will be limited or removed. The two *Bacillus strains* have a variable antagonistic activity on plant pathogenic fungi with PGI% target variables (Fig 4).

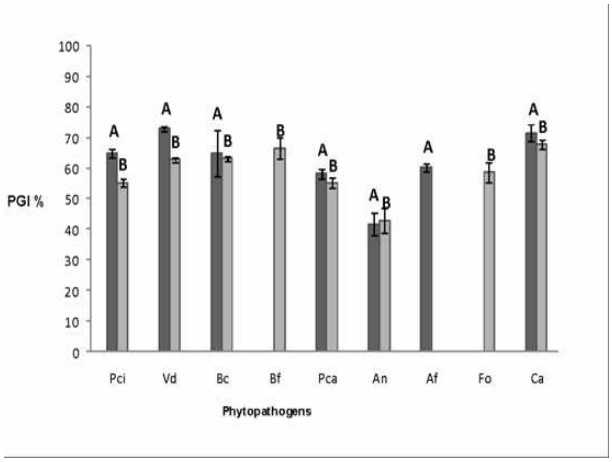


**Fig. 4:** Rate of mycelial growth inhibition (PGI%) plant pathogens in the presence of *Bacillus licheniformis* (A) and *Bacillus Sp* (B)

The letters (A and B) denote the homogeneous groups obtained by the Fischer LSD test (pgeq 0.05). *Vd: Verticillium dahliae; for: Fusarium oxysporum; Bf: Botryotinia fuckiliana; Pc: Phytophthora cinamomi; Pca: Phytophthora cactorum, Ca: Colletotrichum acutatum, and Bc: Botrytis cinerea, An: Aspergillus Niger, Af: Aspergillus flavus.*

In addition to siderophores, some microorganisms synthesize chitinases, $\beta$-1,3-glucanases or cellulases. These enzymes damage fungal cell wall components leading to cell lysis [2]. Some studies revealed a relationship between chitinase of *Bacillus Sp*, *Pseudomonas Sp*, *Streptomyces Sp* and their ability to inhibit *Fusarium oxysporum* and *Fusarium solani* mycelial growth [2]. It was shown that cellulase is also involved in the abolishment of *Phytophthora cactorum* growth by removing the oospores from the mycelium [1]. However, different mechanisms can be gathered in synergy, including antibiotics production and volatile compounds. Our study showed that both of *Bacillus* strains (RBA-E32 and RBA-E23) produce siderophores and different enzymes with antifungal activities (cellulase, chitinase, protease and lipase).

Thus, these strains can be considered as halotolerant PGPR, with the capability to utilize glycine betaine, proline and algal extracts as osmoprotectants. Their capacity to produce high IAA-quantity is sufficient to stimulate plants growth. Additionally, phosphorus solubilization; siderophores and enzymes production are benefic for soils fertilization. Thus, these strains could be applied as bio-control agents because of their antagonist activity against phytopathogens fungi.

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