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Identification and characterization of *Bacillus altitudinis* strain KA15 newly isolated from the highest summit of the Djurdjura Mountains in Kabylia, Algeria

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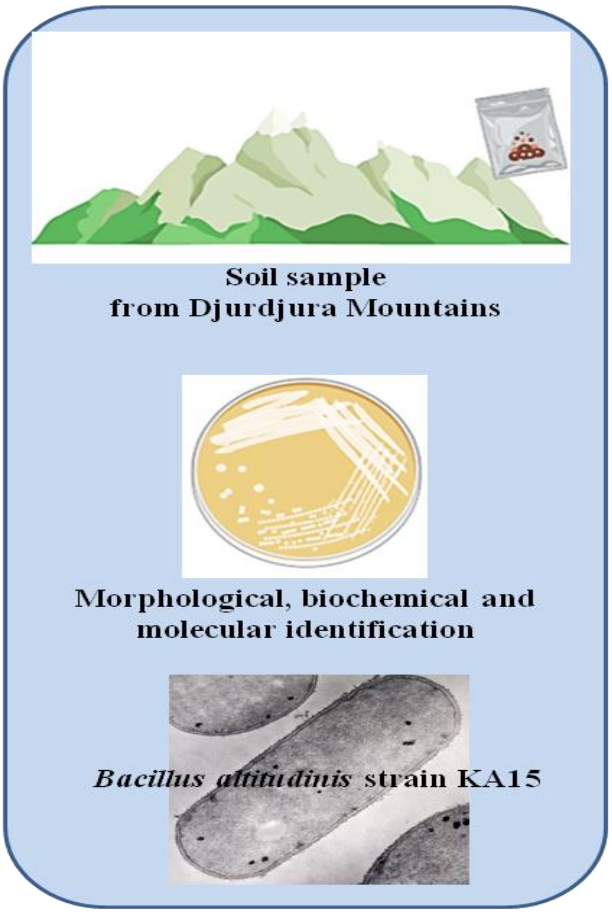
Graphical Abstract



Abstract

A novel bacterial strain was isolated from the highest summit of the Djurdjura Mountains in Kabylia (Algeria) at altitudes of about 23 km. For a long time, scientists have investigated in familiar world to identify novel microbial biocatalysts. However, the mountain soil has been shown as an almost entire reserve of novel enzymes with interesting properties for industrial and environmental applications. *Bacillus* sp. as a genus of aerobic and facultative anaerobic bacteria is widespread in nature. Many species of this genus produce different enzymes used in biodegradation, textiles, bakery, food industry, stationery, biopharmaceutical industries and in many other domains. Thus, the strain KA15 was isolated from Tikjda, in the Djurdjura Mountains, Algeria. The identification of this newly isolated bacterium was carried out using morphological, physiological, and biochemical characteristics. In addition, the *16S* rDNA gene was also amplified and sequenced. All the data obtained with regards to the physiological and biochemical properties of the isolate, confirmed that the KA15 strain belonged to the *Bacillus* genus. The growth temperature was 8–45 °C with an optimum Temperature at 25 °C, and pH range was 5–8 with an optimum pH around 5. Moreover, the nucleotide sequence and blast analyses confirmed that the KA15 strain (GenBank accession no.: MK874318) was closely related to those of the *Bacillus* strains. All the results obtained strongly suggested that this new isolate should be assigned as *Bacillus altitudinis* strain KA15.

1. Introduction

Studies on the qualitative and quantitative distribution of microorganisms in the upper troposphere-stratosphere (10–85 km altitude) in various parts of the Earth are important as they should help (*i*) in determining the role of the various atmospheric strata in the transport of microorganisms from one part of the globe to another and (*ii*) to test the theory that microorganisms might exist in space (Hoyle & Wickramasinghe, 1999) and form a part of the hundreds of tons of material that enters the atmosphere each day from space (Love & Brownlee, 1993). It is now well recognized that microorganisms can survive the harsh conditions of the upper atmosphere and the rigours of outer space. Theoretical studies have indicated that it is also possible for microorganisms of an appropriate size to escape into outer space and thus be transported from one planet to another. There have been, however, very few published studies on the quantity and nature of microorganisms in the upper atmosphere (Shivaji et al., 2006). These studies have used either a meteorological rocket or a specially designed direct-flow sampler sent up on a balloon. Both bacteria and fungi have been found at altitudes of up to 85 km. In another paper, Harris et al. (2001) detected bacteria from stratospheric air samples collected at 41 km using scanning electron microscopy and epifluorescence techniques. Using the same samples, Wainwright et al., (2003) described the presence of two bacterial species (*Bacillus simplex* and *Staphylococcus pasteurii*) and a fungus (*Engyotontium album*). In this short paper, a polyphasic taxonomic approach was used to characterize new bacterial strain from the highest summit of the Djurdjura Mountains in Kabylia (Algeria) at altitudes of about 23 km (Asmani et al., 2020).

For a long time, scientists have investigated in familiar world to identify novel microbial biocatalysts. However, the mountain soil has been shown as an almost entire reserve of novel enzymes with interesting properties for industrial and environmental applications.

Bacillus species are aerobic or facultatively anaerobic, sporulating, rod-shaped, and Gram-positive bacteria (Vos et al., 2011). Many species of this genus possesse a wide range of physiologic properties that allow them to live in different natural environment such as desert sands, hot springs, Arctic soils, fresh waters and marine sediments. This genus includes thermophilic, psychrophilic, alkaliphilic, acidophilic, halotolerant bacteria, which are able to withstand to temperatures, pH values and salt concentrations at which few organisms could survive (Turnbull, 1996). *Bacillus* species are used in many medical, pharmaceutical, agricultural, and industrial applications due to their ability to produce enzymes, antibiotics, and other metabolites (Meena et al., 2018; Pereyra et al., 2018; Hamiche et al., 2019). Moreover, *Bacillus altitudinis* produce enzymes that harbor a huge commercial potential thanks to their biotechnological applications (Adhyaru et al., 2017; Thite et al., 2020).

2. Materials and Methods

2. 1. Substrates and reagents

All of the other chemicals and reagents used were of analytical grade or the best grade commercially available, unless otherwise stated.

2.2. Methods

2.2.1. Isolation and cultivation of chitinase-producing microorganisms

Samples were collected from soil of Lalla Khedidja (Tamgut Aalayen) in Tikjda (GPS coordinates: Latitude 36°27′0″ N, Longitude 4°13′60″ E), the highest summit of the Djurdjura Mountains in Kabylia (2308 m), Algeria, using 1 L sterile thermal glass bottles. Samples were stored at 23±2°C. Enrichment cultures and isolation were performed as described by the authors (Yahiaoui et al., 2019). Different colonies were selected and restreaked several times to obtain pure cultures which were stored in nutrient agar until used.

2.2.2. Identification of microorganism

Analytical profiling index (API) strip tests and *16S* rDNA gene sequencing (ribotyping) were carried out for the identification of the genus to which the strain KA15 belonged. The API 50 CH strips (bioMerieux, SA, Marcy-l'Etoile, France) were used to investigate the physiological and biochemical characteristics of strain KA15 in accordance with the instructions of the manufacturer.

Phenotypic characteristics, including motility, cell morphology, Gram staining, catalase, and oxidase production, among others, were investigated. The fermentation of substrate belonging to carbohydrates and derivatives were determined using API 50 CH as recommended by the manufacturer. The colony morphologies were using cultures grown aerobically on nutrient agar. Cell morphology, motility, spore formation and presence of flagella were examined microscopically on fresh liquid cultures during exponential-phase after 18–24 h of incubation at 25 °C. Optimum temperature range for growth was determined by incubating the strain at temperatures ranging between 5.0 and 50 °C (in 5 °C increments) under aerobic conditions. The pH range for growth was examined in a range of pH values from 3.0 to 9.0. The effect of NaCl on bacterial growth was evaluated in presence of 1 to 5 % (w/v) NaCl. All the physiological tests were determined in nutrient agar medium.

Polymerase chain reaction (PCR) amplification of the *16S* rDNA gene was carried out with two universal primers, one forward and the other reverse, designed from the conserved zones within the rRNA operon of *E. coli* (Gurtler and Stanisich 1996). The forward primer (27F) was 5'-AGAGTTTGATCCTGGCTCAG-3' extended from base position 8 to 27; the reverse primer (1525R) was 5'-AAGGAGGTGATCCAAGCC-3' extended from base position 1,541 to 1,525. The genomic DNA of the KA15 strain was purified using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) and then used as a template for PCR amplification. After denaturation at 95 °C for 3 min, DNA samples were subjected to 35 cycles of amplification with denaturation at 94 °C for 30 s, annealing at 65 °C for 45 s, and extension at 72 °C for 90 s, followed by a final elongation step at 72 °C for 5 min. The PCR product (~1.5 kb) was then cloned in the pGEM-T Easy vector (Promega, Madison, WI, USA), leading to the pKA15-16S plasmid (This study). The *E. coli* DH5α was used as a host strain. All recombinant clones of *E. coli* were grown in LB media with the addition of ampicillin (100 µg/mL), isopropyl-thio-β-D-galactopyranoside (IPTG) (0.67 mM), and X-gal (360 µg/mL) for screening. DNA electrophoresis, DNA purification, restriction, ligation, and transformation were all performed according to the method previously described by Sambrook et al., (1989).

2.2.3. DNA sequencing and phylogenetic analysis

The nucleotide sequences of the cloned *16S* rDNA gene were determined on both strands by the automated DNA sequencer ABI PRISM® 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The obtained sequences were compared with sequences available in the public sequence databases and with the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/). Phylogenetic and molecular evolutionary genetic analyses were performed using the Molecular Evolutionary Genetics Analysis (MEGA) software v. 4.1. Distances and clustering were calculated using the neighbor-joining (NJ) method.

3. Results and discussion

3.1. Phenotypic identification of isolated bacteria

According to phenotypic results, the strain KA15 appears rod-shaped cells, aerobic growth, white colony color, motile, endospore-forming, Gram-positive, and positive for catalase and oxidase (Table 1). In addition, biochemical profile obtained with API 50 CH gallery test, exhibited that this isolate metabolize maltose, lactose, D-trehalose, D-fucose, D-arabinose, D-tagatose, starch, and rhamnose besides other simple sugars (Table 1). These results confirmed that the KA15 strain belonged to the *Bacillus* genus.

As shown in Table 1, the growth occurred within temperature range of 8.0–45 °C and pH range of 5.0–8.0, respectively. The KA15 strain tolerates up to 2 % NaCl.

Table 1
Phenotypic, physiologic, and biochemical characterization of the strain KA15.

Characteristic	Strain KA15
Colony morphology	Circular
Pigmentation	White
Cell shape	Rod
Cell arrangement	Single
Motility	+
Gram stain	Gram+
Temperature range (°C)	8.0–45
pH range	5.0–8.0
Salt concentration (%)	2
Carbon source utilization:	
Aesculine	+
Salicine	+
Cellobiose	+
Maltose	+
Lactose	+
Saccharose	-
Trehalose	-
Gentiobiose	+
Melibiose	+
Raffinose	+
Melezitose	-
Starch	+

Characteristic	Strain KA15
Carbon source utilization	
Glycogen	+
Inuline	+
D-Turanose	-
D-Tagatose	+
D-Lyxose	-
D-Arabitol	+
L-Arabitol	-
Xylitol	-
Gluconate	+
2-Ceto gluconate	+
5-Ceto gluconate	+
Glycerol	+
Erythritol	-
D-Arabinose	+
L-Arabinose	-
D-Ribose	-
L-Xylose	-
D-Xylose	+
Adonitol	-
β-Methyl xyloside	-
Galactose	+
Glucose	+
Fructose	+
Mannose	+
L-Sorbose	+
L-Rhamnose	+
Dulcitol	-
myo-Inositol	+
D-Sorbitol	+
D-Mannitol	-
L-Methyl-D-mannoside	+
D-Methyl-D-glucoside	+
N-Acetyl glucosamine	+
Amygdaline	+
Arbutine	+

3.2. Molecular phylogenetic analysis of strain KA15

The molecular identification of the strain KA15 was carried out. A single DNA fragment of about 1525 bp of the *16S* rDNA gene was amplified from genomic DNA of the isolate, cloned into the pGEM-T Easy vector and sequenced on both strands. Results of GenBank BLAST search analysis of the 16S *rDNA* gene sequence reveal high sequence homology with several cultured *Bacillus* strains, reaching a maximal of 98.62% sequence identity. The nearest *Bacillus* strains identified by the BLAST analysis were the *Bacillus alititudinis* 41KF2b^T (GenBank accession no.: AJ831842), *Bacillus pumilus* SAFN-032^T (GenBank accession no.: AB098578), *Bacillus stratosphericus* 41KF2a^T (GenBank accession no.: AJ831841), and *Bacillus aerophilus* 28K^T (GenBank accession no.: AJ831844). In addition, phylogenetic trees were constructed (Fig. 1) by aligning above sequences using MEGA software package version 4.1. The findings confirmed that the KA15 strain (GenBank accession no.: **MK874318**) was closely related to those of the *Bacillus* strains.

All the results obtained strongly suggested that this isolate should be assigned as *Bacillus altitudinis* strain KA15.

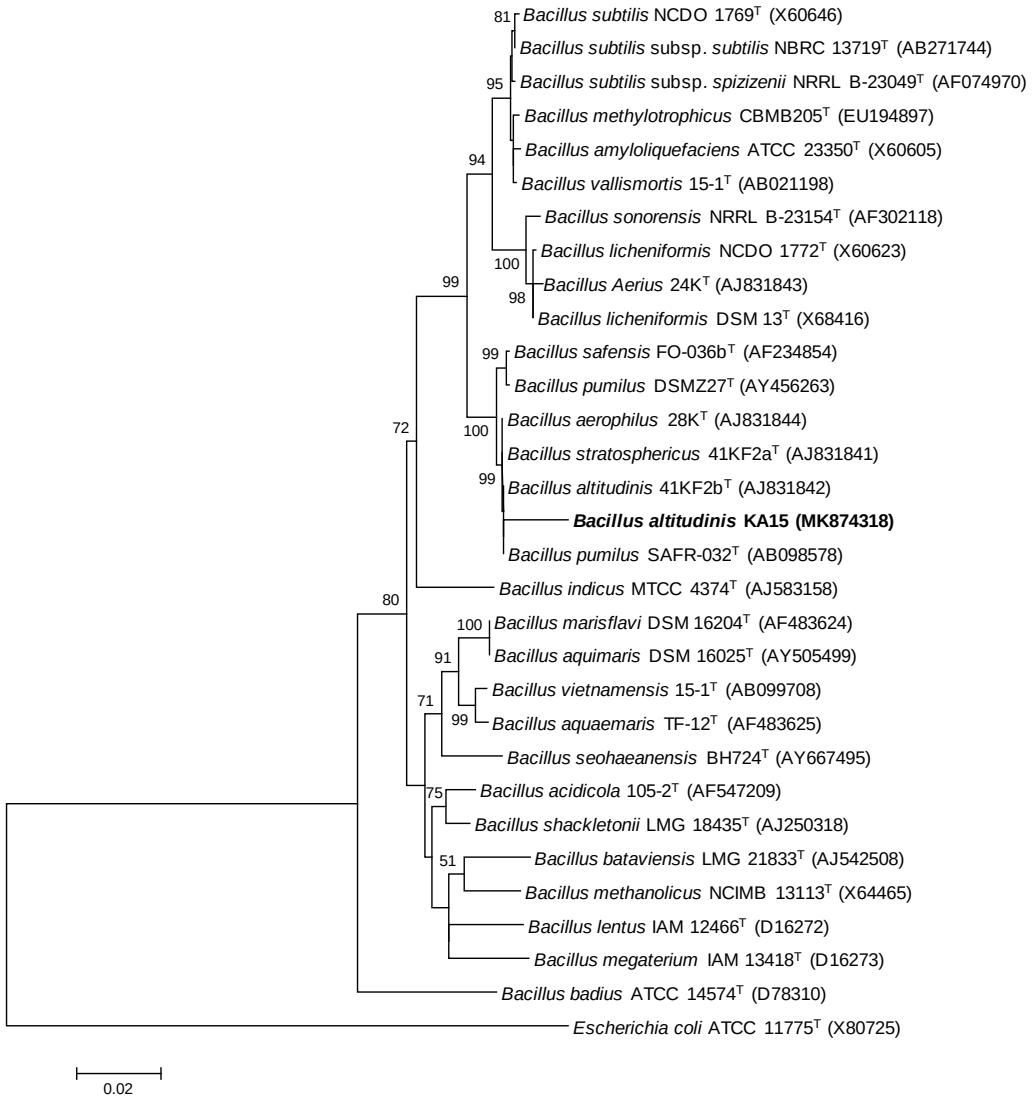


Fig. 1. Phylogenetic tree based on *16S* rDNA gene sequences showing the position of strain KA15 within the genus *Bacillus*. The sequence of *E. coli* ATCC 11775^T (GenBank accession no.: X80725) was used as an out-group. Bootstrap values (expressed as percentages of 1000 replications) > 50 % are given at nodes. Bar, 2 substitutions per 100 nucleotides.

4. Conclusions

The mountain soils contain a significant variety of microorganisms. On the basis of the phenotypic, biochemical, and phylogenetic evidence described above, the new strain KA15, isolated from the Djurdjura Mountains in Kabylia (Algeria), belongs to the bacterial genus *Bacillus* and it is closely related to *Bacillus altitudinis*.

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