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Halobacillus blutaparonensis sp. nov., a Moderately Halophilic Bacterium Isolated from *Blutaparon portulacoides* Roots in Brazil

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Abstract A moderately halophilic, Gram-positive, spore-forming bacterium was isolated from the roots of *Blutaparon portulacoides*, a plant found in sandy soil parallel to the beach line in Restinga de Jurubatiba, Rio de Janeiro, Brazil. The strain, designated M9^T, was motile and strictly aerobic with rod-shaped cells. It grew in the absence of NaCl and up to 20% NaCl, and was able to hydrolyze casein and starch. Strain M9^T had a cell-wall peptidoglycan based on L-Orn-D-Asp, the predominant menaquinone present was menaquinone-7 (MK-7), diaminopimelic acid was not found, and anteiso-$C_{15:0}$ and iso-$C_{15:0}$ were the major fatty acids. A phylogenetic analysis based on 16S rRNA gene sequences showed that strain M9^T belonged to the genus *Halobacillus* and exhibited 16S rRNA gene similarity levels of 97.8–99.4% with the type strains of the other nine *Halobacillus* species. The DNA-DNA relatedness of strain M9^T with *H. trueperi*, the closest relative as regards 16S rRNA gene similarity, and *H. locisalis* was 21% and 18%, respectively. Therefore, on the basis of phenotypic, genotypic, and phylogenetic data, strain M9^T (=ATCC BAA-1217^T, =CIP 108771^T, =KCTC 3980^T) should be placed in the genus *Halobacillus* as a member of a novel species, for which the name *Halobacillus blutaparonensis* sp. nov. is proposed.

Key words: *Halobacillus blutaparonensis*, moderately halophilic bacterium, taxonomy, *Blutaparon portulacoides*, new species

Blutaparon portulacoides (St. Hill.) Mears (Amaranthaceae) is a perennial, rhizomatous herb with succulent and frequently shed leaves, which first colonized the embryo dunes and backshores of Southwestern Atlantic Ocean beaches [6]. In Brazil, it is commonly found in the sand parallel to the beach line in Restinga de Jurubatiba, Rio de Janeiro, Brazil, and is able to tolerate well this salt-stressed zone, high temperatures, and exposure to storm tides [3]. *B. portulacoides* is also of great medical interest, owing to the presence of flavonol, irisone B, sitosteryl, vanillic acid, and the steroids stigmasterol, sitosterol, and campesterol [7]. However, little information is available about the microbial population associated with the roots of *B. portulacoides*. Thus, exploring the diversity of this population may represent a potential source for the discovery of novel strains and bioactive compounds. In a previous study, the Gram-positive spore-forming bacterial community associated with the roots of *B. portulacoides* was investigated and revealed members of the genera *Halobacillus*, *Virgibacillus*, and *Oceanobacillus* [2]. Among the isolates, one spore-forming moderately halophilic strain appeared to be related to the genus *Halobacillus*, based on a comparison of part of its 16S rRNA gene sequence with those for other species of the genus. However, different characteristics suggested that it could belong to a new species [2]. Accordingly, this study determined the phenotypic characteristics, DNA-DNA relatedness to other species, and 16S rRNA gene sequence of this novel isolate, and the resulting data strongly suggest that the strain belongs to a novel species within the genus *Halobacillus*, for which the name *Halobacillus blutaparonensis* sp. nov. is proposed.

MATERIALS AND METHODS

Isolation and Maintenance of Strain M9^T

Strain M9^T was isolated from macerated roots of *B. portulacoides* using the method described by Seldin *et al.* [13]. The plants were harvested and the roots shaken to remove the loosely attached soil. One g of roots together

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with the adhering soil was mixed with 9 ml of distilled water, shaken for 10 min, and the water discarded. This procedure was repeated three times, then and the washed roots were macerated, mixed with 9 ml of distilled water, and pasteurized (80°C, 10 min). Two-fold serial dilutions of the root sample were plated onto LB agar (tryptone 1%, yeast extract 0.5%, NaCl 0.5%) supplemented with NaCl to reach 10% (w/v) and incubated for 3–5 days at 32°C. The type strains belonging to different species of *Halobacillus* used in this study were obtained either from the Korean Collection for Type Cultures (KCTC), Korea, or from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Germany. The bacterial strains were stored at room temperature on LB agar or at -80°C in an LB medium containing 20% glycerol.

Morphological, Physiological, and Biochemical Characterization

For the morphological and physiological characterization, strain M9^T was generally cultivated in LB plus 10% NaCl, and the incubation carried out by shaking at 30°C. Most of the biochemical tests were performed using the methods and media (supplemented with 10% NaCl) described by Gordon *et al.* [8]. The catalase activity was determined by bubble production in a 3% (v/v) hydrogen peroxide solution, and the acid production from carbohydrates was determined either as described by Gordon *et al.* [8] or using an API 50 CH kit (bioMérieux). The enzyme activity (arginine dihydrolase, urease, β-galactosidase, hydrolysis of esculin and gelatin) and assimilation tests were determined using an API 20 NE system (bioMérieux). Growth under anaerobic conditions was determined after incubation for 7 days in anaerobic Gaspak jars (BBL) containing an atmosphere of 80% N_2, 10% CO_2, and 10% H_2. Growth under various NaCl concentrations (0–25%), temperatures (up to 50°C), and pHs (5–11) was measured in LB (with the addition of 10% NaCl in the temperature and pH assays). The cellular morphology, form, and position of the spores were observed using an Axioplan 2 microscope (Zeiss). The cellular motility of the novel isolate was observed in fresh wet-mounts of a young bacterial culture in LB broth, and the presence of flagella examined using a transmission electron microscope (FEI Morgagni 268) after negatively staining the cells with 2% (w/v) phosphotungstic acid.

Preparation of Cell Wall and Determination of Peptidoglycan Structure and Fatty Acid Composition

The preparation of the cell wall and determination of the peptidoglycan structure were carried out as described in Yoon *et al.* [19]. The isoprenoid quinones of strain M9^T were extracted from 100 mg of freeze-dried cells according to Collins and Jones [5], and purified by preparative thin-layer chromatography (TLC, silica gel F254; Merck). The ubiquinone fraction was then analyzed by high-performance liquid chromatography (HPLC, Hitachi L-5000) equipped with a reverse-phase column (YMC pack ODS-AM; YMC Co.), as described by Shin *et al.* [14]. The FAMEs were extracted and prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System [12].

DNA Extraction and DNA-DNA Hybridization

The chromosomal DNA was isolated and purified according to methods described previously [17, 19], along with the DNA-DNA hybridization [4, 9].

16S rRNA Gene Phylogenetic Analysis

The 16S rRNA gene sequences were amplified by a PCR using the universal primers and PCR conditions described

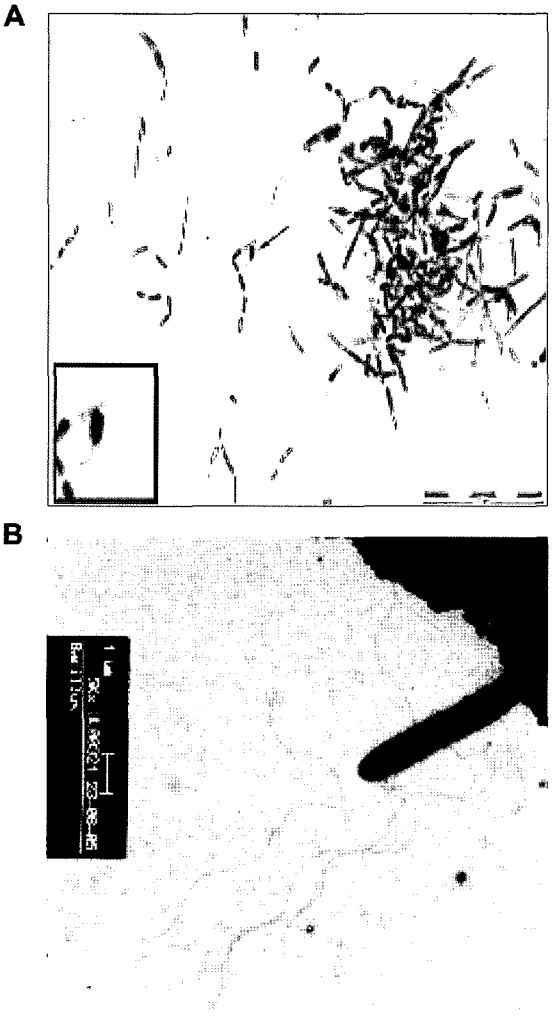

Fig. 1. A. Light micrograph of cells of strain M9^T grown in LB agar for 5 days at 32°C. Bar=20 μm.
The arrow shows a spore than can be observed in detail (left/bottom). **B.** Transmission electron microscopy of strain M9^T. Bar=1 μm.

by Yoon *et al.* [18]. The PCR products were then purified with a QIAquick PCR purification kit (Qiagen) and the sequencing of the purified 16S rDNAs performed in an Applied Biosystems model 377 automatic DNA sequencer using an ABI PRISM BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems), as recommended by the manufacturer. The software Clustal-X [16] was used to align the 16S rRNA gene sequence of strain M9^T with the nine recognized species of the genus *Halobacillus* and two spore-forming Gram-positive bacteria recovered from the GenBank database. A phylogenetic tree was also constructed using the neighbor-joining (NJ) method and the 2-parameter model of Kimura. The software MEGA 3.0 [10] was used to perform the neighbor-joining analysis and to calculate the pair-to-pair *p*-distance values among the 16S rRNA gene sequences for the different species studied here. Alignment gaps and unidentified base positions were not taken into account for the calculations.

RESULTS AND DISCUSSION

Strain M9^T was found to be Gram-positive or Gram-variable (old cultures), and the cells were rod-shaped (measuring 0.5 to 0.7 by 2.1 to 3.0 μm on LB agar, Fig. 1A), single or in short chains, and motile. In high concentrations of salt, a filamentous form appeared. The spores of the cells, which were scarce and more easily observed in LB (0.5% NaCl), were ellipsoidal, distending the sporangia, and located in the subterminal to terminal (predominant) position of the cell. The M9^T cells were also motile, presenting long peritrichous flagella (Fig. 1B). The colonies of the novel

Table 1. Phenotypic characteristics that differentiate *Halobacillus blutaparonensis* from other *Halobacillus* spp.

Characteristic	1	2	3	4	5	6	7	8	9	10
Cell morphology	Rods	Cocci or oval	Rods	Rods	Rods	Rods	Rods	Rods or long filamentous rods	Rods	Rods with club-shaped
Gram staining	V	+	V	+	V	+	+	V	+	+
Spore shape	E	S	E	E/S	E	E/S	E/S	E	E	E
Spore position	ST/T	C/L	C/ST	C/ST	C/ST	C/ST	C/ST	C/ST	C/ST	C/ST
Sporangium swollen	+	-	+	ND	+	ND	+	+	ND	ND
Colony pigmentation	Cream colored-yellow	Orange	Pale orange-yellow	Orange	Light orange-yellow	Orange	White	Light yellow	Orange	Orange
Motility	+	+	+	+	+	+	-	+	+	+
Flagellation	P	S/P	P	P	S	P	Absent	S	P	P
Growth at										
45°C	+	-	+	-	-	-	+	+	+	-
pH 5.5	-	-	+	-	+	-	-	-	+	-
0.5% NaCl	+	-	+	+	-	+	-	+	+	+
25% NaCl	-	-	-	+	-	+	-	-	+	-
Hydrolysis of										
Casein	+	+	+	-	-	-	+	+	+	+
Starch	+	+	-	-	+	-	+	-	+	+
Gelatin	-	+	+	+	-	+	+	+	-	+
Esculin	+	-	+	-	+	-	+	-	-	-
Acid production from										
D-Fructose	+	-	+	+	+	+	+	-	+	+
D-Galactose	w	-	w	-	-	+	-	-	-	-
Maltose	+	-	+	+	-	+	+	+	+	+
Sucrose	+	-	+	+	+	+	-	+	+	+
D-Xylose	-	-	-	+	-	-	-	-	+	-
D-Glucose	+	-	+	+	+	+	+	+	+	+
D-Mannitol	+	-	+	+	-	-	+	+	+	+
D-Trehalose	+	-	+	+	+	+	ND	+	+	+

Species: 1, *Halobacillus blutaparonensis* (M9^T, this study); 2, *H. halophilus* [15]; 3, *H. salinus* [19]; 4, *H. litoralis* [15]; 5, *H. locisalis* [20]; 6, *H. trueperi* [15]; 7, *H. karajaensis* [1]; 8, *H. yeomjeoni* [21]; 9, *H. dabanensis* [11]; 10, *H. aidingensis* [11]. ND, not determined, (+) positive results, (w) weakly positive results, (-) negative results, and (v) variable results. Spore shape and position: E, ellipsoidal; S, spherical; C, central; L, lateral; ST, subterminal; and T, terminal. Flagellation: P, peritrichous; S, single. All species showed negative results for anaerobic growth, Voges-Proskauer test, and nitrate reduction to nitrite, but positive results for catalase.

isolate were cream colored to yellow, 3 to 5 mm in diameter after 3 days on LB agar, smooth, circular to slightly irregular, and a little raised. Strain M9^T was able to grow in temperatures up to 45°C, at a pH up to 9.0, but not lower than 6.0, and in the absence of NaCl or presence of 20% NaCl. No growth was observed in LB supplemented with 25% NaCl. Furthermore, the isolate did not grow under anaerobic conditions, presented a negative Voges-Proskauer test, and showed catalase activity. Nitrate was not reduced to nitrite. Casein, esculin, and starch were hydrolyzed, but gelatin was not. In assays using API 20 NE, arginine dihydrolase and urease were absent, whereas β-glucosidase and β-galactosidase were present. Gluconate, caprate, adipate, citrate, arabinose, and phenyl-acetate were not assimilated by strain M9^T, whereas glucose, mannitol, malate, *N*-acetyl glucosamine, and maltose were. Acid was produced from sucrose, fructose, raffinose, *N*-acetyl glucosamine, salicine, cellobiose, melibiose, starch, mannitol, ribose, glycerol, mannose, lactose, glucose, maltose, and trehalose. A weak reaction was observed with amygdaline, arbutine, and galactose. Acid was not produced from erythritol, D- and L-arabinose, D- and L-xylose, adonitol, β-methyl-xyloside, L-sorbose, rhamnose, dulcitol, inositol, sorbitol, α-methyl D-mannoside, α-methyl D-glucoside, inuline, melezitose, glycogene, xylitol, β-gentiobiose, D-turanose, D-lyxose, D-tagatose, D- and L-fucose, D- and L-arabitol, gluconate, and 2-ceto- and 5-ceto-gluconate. The characteristics that differentiate the new isolate from related species are shown in Table 1. From the results of the cell-wall analysis, strain M9^T presented a peptidoglycan-type L-Orn-D-Asp, which is a distinguishing mark of the genus *Halobacillus* [15, 19, 21]. Strain M9^T did not contain diaminopimelic acid in the cell-wall peptidoglycan, and the predominant menaquinone found was unsaturated menaquinone with seven isoprene units (MK-7). The fatty acids detected in strain M9^T are shown in Table 2. Although there were differences in the proportion of some fatty acids, the profile presented by M9^T was similar to those for the type strains of *Halobacillus* species [1, 11, 19–21].

The almost complete 16S rRNA gene sequence (1,463 nt) for strain M9^T was submitted to BLAST-N and the first hits were closely related to *Halobacillus* sp., with only the sixth hit related to a recognized *Halobacillus* species, *H. trueperi*. In the phylogenetic tree based on the neighbor-joining algorithm, strain M9^T fell within the radiation of the cluster comprising *Halobacillus* species (Fig. 2). A similar tree topology was found for the tree generated using the maximum-parsimony algorithm (data not shown). Strain M9^T exhibited 16S rRNA gene similarity levels of 97.8–99.4% with the type strains of the other nine *Halobacillus* species; the highest value corresponding to the type strain of *H. trueperi*. Furthermore, the levels of 16S rRNA gene similarity between the isolated strain and the type strains of the other genera used (*Bacillus subtilis* and *Brevibacillus brevis*) as outgroups in the phylogenetic tree were less than 93.4%. DNA-DNA hybridization studies were performed to determine the genomic relationship between strain M9^T and the type strains for two of the closest *Halobacillus* species determined in BLAST-N. As a result, strain M9^T showed mean DNA-DNA relatedness levels of 21% and 18% with *H. trueperi* KCTC 3686^T and *H. locisalis* KCTC 3788^T, respectively.

Thus, when considering the phenotypic, phylogenetic, and genotypic characteristics of the isolate, it was concluded that strain M9^T belongs to the genus *Halobacillus*, although it shows differences from the other known *Halobacillus* species described so far. With respect to salt tolerance, enzymatic activities, and fermentation of carbohydrates (Table 1), strain M9^T grew well in the presence of 0.5% NaCl, whereas *H. halophilus*, *H. locisalis*, and *H. karajensis* do not grow under such conditions. Furthermore, strain M9^T was able to hydrolyze casein and starch, and this has not been observed with *H. salinus*, *H. litoralis*, *H. locisalis*, *H. trueperi*, and *H. yeomjeoni* [1, 15, 19–21]. The ability

Table 2. Profiles of cellular fatty acids obtained for strain M9^T and other *Halobacillus* species.

Fatty acid	1	2[a]	3[a]	4[a]	5[b]	6[a]	7[c]	8[d]	9[e]	10[e]
iso-$C_{14:0}$	3.0	12.2	9.4	6.4	11.2	21.7	2.0	3.4	1.10	2.34
iso-$C_{15:0}$	30.5	7.5	26.3	15.8	8.4	7.7	11.3	8.8	7.45	13.47
iso-$C_{16:0}$	9.4	15.2	15.7	5.4	15.9	31.5	6.9	19.3	8.15	7.47
iso-$C_{17:0}$	7.2	1.2	4.2	1.5	1.4	2.1	5.0	3.5	2.22	4.36
$C_{15:0}$	nd	1.5	1.6	1.1	1.1	nd	0.3	nd	2.93	0.26
$C_{16:0}$	5.45	0.9	1.0	0.6	0.9	0.9	1.1	1.6	2.03	2.28
anteiso-$C_{13:0}$	nd	0.4	nd	0.4	0.5	nd	nd	nd	0.07	0.08
anteiso-$C_{15:0}$	36.9	47.3	31.7	57.0	42.0	25.3	42.4	40.4	49.7	42.04
anteiso-$C_{17:0}$	6.9	11.9	6.2	8.2	13.0	6.5	16.0	23.0	18.63	15.68

Species: 1, *Halobacillus blutaparonensis* (M9^T, this study); 2, *H. halophilus*; 3, *H. salinus*; 4, *H. litoralis*; 5, *H. locisalis*; 6, *H. trueperi*; 7, *H. karajaensis*; 8, *H. yeomjeoni*; 9, *H. dabanensis*; 10, *H. aidingensis*.

[a]Data from Yoon *et al.* [19]. [b]Data from Yoon *et al.* [20]. [c]Data from Amoozegar *et al.* [1]. [d]Data from Yoon *et al.* [21]. [e]Data from Liu *et al.* [11]. nd, not detected.

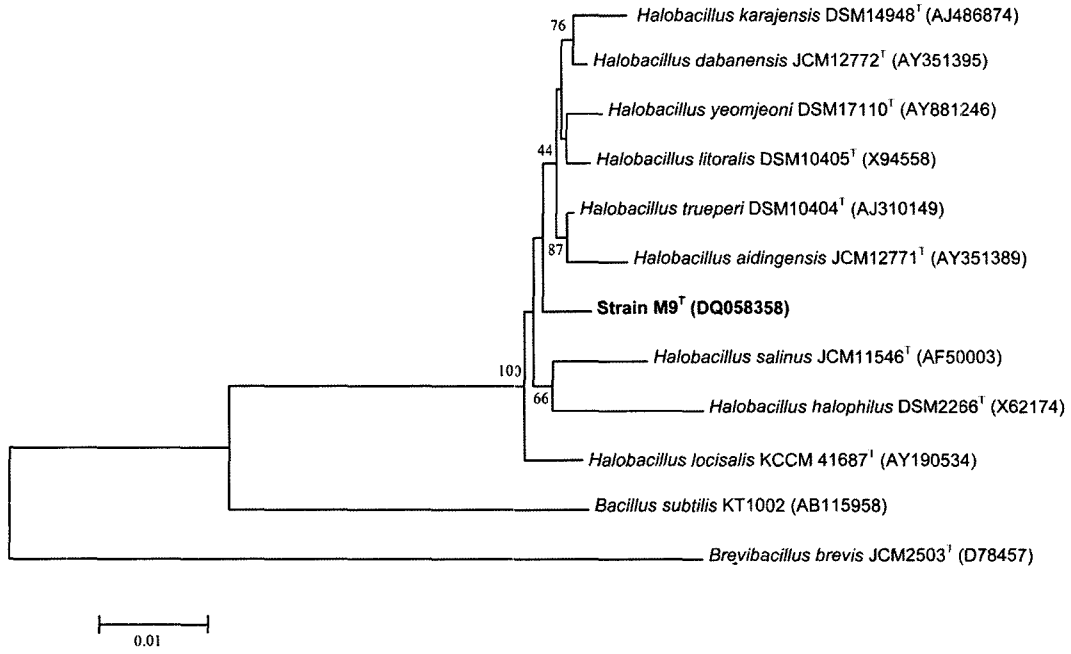

Fig. 2. Consensus phylogenetic tree based on 16S rRNA gene sequences showing relationship between strain M9^T, type strains of different *Halobacillus* species, and other representatives from two related genera.
The tree was constructed based on the neighbor-joining method. Bootstrap analyses were performed with 2,000 repetitions and only values higher than 50% are shown. The GenBank accession number for each species is enclosed in parentheses.

to hydrolyze esculin, presented by strain M9^T, also differentiates it from *H. litoralis*, *H. halophilus*, *H. trueperi*, *H. yeomjeoni*, *H. dabanensis*, and *H. aidingensis*, whereas the hydrolysis of gelatin has been observed for almost all species of *Halobacillus* (negative test only observed for *H. locisalis*, *H. dabanensis*, and strain M9^T; [11, 20]). Also, the growth tests at 45°C and pH 5.5 differentiate strain M9^T from the type strains of *H. dabanensis* and *H. aidingensis* (Table 1, [11]). The phylogenetic and DNA-DNA hybridization data also support the proposal that strain M9^T should be placed as the type strain of a novel species of the genus *Halobacillus*, *Halobacillus blutaparonensis* sp. nov.

Description of *Halobacillus blutaparonensis* sp. nov.

Halobacillus blutaparonensis (blu.ta.pa.ro.nen'sis. NL. masc. adj. *blutaparonensis* referring to the plant genus from where the strain was isolated in association with the roots).

Cells are rods and 0.5 to 0.7 wide by 2.1 to 3.0 μm long in 5-day cultures at 32°C on LB agar. Gram-positive in young cultures or Gram-variable in old cultures. Motile by means of peritrichous flagella. Subterminal to terminal ellipsoidal spores are observed in swollen sporangia. Colonies are cream colored to yellow, measure 3 to 5 mm in diameter, and are smooth, round, or slightly irregular after 5 days on LB agar. Growth occurs in either the absence of NaCl (0%) or presence of 20% (w/v) NaCl and 2–4% (w/v) is optimal for growth. Growth does not occur in the presence of 25% NaCl. Growth occurs up to 45°C (optimum 28–32°C) and the pH range for growth is between 6.0 and 9.0, with the optimal around 8.0. No growth occurs under anaerobic conditions. Catalase positive. Nitrate is not reduced to nitrite. Casein, esculin, and starch are hydrolyzed. Voges-Proskauer test is negative. Acid is produced from sucrose, fructose, raffinose, *N*-acetyl glucosamine, salicine, cellobiose, melibiose, starch, mannitol, ribose, glycerol, mannose, lactose, glucose, maltose, and trehalose. β-glucosidase and β-galactosidase are present. Glucose, mannitol, malate, *N*-acetyl glucosamine, and maltose are assimilated by strain M9^T. The cell wall contains a peptidoglycan of L-Orn-D-Asp. Diaminopimelic acid is not present in the cell-wall peptidoglycan and the predominant menaquinone is MK7. The major fatty acids are anteiso-$C_{15:0}$ and iso-$C_{15:0}$. The type strain, strain M9^T (=ATCC BAA-1217^T, =CIP 108771^T, =KCTC 3980^T), was isolated in association with the roots of *Blutaparon portulacoides* found in the sand parallel to the beach line in Restinga de Jurubatiba, Rio de Janeiro, Brazil.

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