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Biochemical and Molecular Taxonomy of a Mild Halophilic Strain of *Citrobacter* Isolated from Hypersaline Environment

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ABSTRACT

A strain of *Citrobacter* was isolated from the hypersaline Dead Sea of Jordan. The strain (DS1) was identified by molecular and biochemical methods. Based on 16S rRNA gene analysis, the isolate is most related to *Citrobacter freundii* and *Citrobacter murliniae* (maximum similarity is 94%). Relative to other citrobacters, the strain has a relatively high GC content (55%). The biochemical tests showed that the strain is Gram-negative, oxidase-negative and ferment lactose as other enteric bacteria. DS1 utilizes citrate as sole carbon source as other citrobacters. However, the main differences between our strain and other citrobacters is that the isolate is mild halophilic (optimum NaCl concentration in the growth medium is 4%) and o-nitrophenyl-β-galactosidase (ONPG)-negative. Based on the differences in the physiological and biochemical properties, as well as low 16S rRNA sequence similarity of this novel isolate, DS1 is suggested as new species of *Citrobacter* in the family Enterobacteriaceae.

Key words: Halophilic, *Citrobacter*, Dead Sea, rRNA sequence

INTRODUCTION

Citrobacter is a genus of Gram-negative, facultative anaerobic, non-spore forming rods belonging to Enterobacteriaceae family. As their name indicates, they usually utilize citrate as sole carbon source (Borenshtein and Schauer, 2006). On the basis of their 16S rDNA sequences, *Citrobacter* species clusters within the Gammaproteobacteria, with *Escherichia*, *Erwinia*, *Salmonella* and *Serratia* species as their closest relatives (Frederiksen, 2005). The first *Citrobacter* species was isolated in 1928 under the name *Bacterium freundii* and then named as *Citrobacter freundii* in 1932 (Borenshtein and Schauer, 2006), as a result, another ten species were isolated and validly described in the literature. At present and according to Bergey's Manual of Systematic Bacteriology, eleven species are reported in the genus including: *C. freundii*, *C. koseri*, *C. amalonaticus*, *C. farmeri*, *C. youngae*, *C. braakii*, *C. werkmanii*, *C. sedlakii*, *C. rodentium*, *C. gillenii* and *C. murliniae* (Frederiksen, 2005). Citrobacters are disseminated in different habitats in nature. In addition for being part of the fecal flora of human, citrobacters have been isolated from the intestinal tract of dogs, cats, horses, cows, birds and tortoises (Janda and Abbott, 2006). Citrobacters were also recovered from sea water (Dufour and Cabelli, 1975; Janda and Abbott, 2006; Kakizaki *et al*., 2009), sewage and soil (Borenshtein and Schauer, 2006). From a medical point of view, members of the genus *Citrobacter* are associated with different human infections (Janda and Abbott, 2006) especially in neonates, young infants, aged,

immunocompromised and depilated patients (Wang *et al*., 2000; Doran, 1999). The most prominent disease linked to this genus involves infections of the Central Nervous System (CNS) such as neonatal meningitis (Janda and Abbott, 2006). *C. koseri* is a species best known as the cause of sepsis and meningitis leading to central nervous system abscesses in neonates and young infants (Doran, 1999). Also, *C. sedlakii* was isolated from human cerebrospinal fluids (Dyer *et al*., 1997). Some *Citrobacter* species were also linked to bacteremia, septicemia and urinary tract infection, which is a common site from which citrobacters are isolated in clinical laboratory (Janda and Abbott, 2006). Citrobacters without acquired antibiotic resistance are normally susceptible to sulfonamides, trimethoprim, aminoglycosides, chloramphenicol, tetracycline, nalidixic acid and among others. Citrobacters are generally resistant to erythromycin, lincosamides, fusidic acid, vancomycin and most importantly β-lactams such as ampicillin and carbenicillin (Borenshtein and Schauer, 2006). Until now, extensive studies on susceptibility, acquired resistance, pathogenesis and virulence of the genus *Citrobacter* are still very few (Janda and Abbott, 2006). In this study, we isolated a Gram-negative rod-shaped bacterium from recreational site in the hypersaline Dead Sea area. The isolate belong to the genus in the family Enterobacteriaceae in the class *Gammaproteobacteria*. Different molecular, physiological and biochemical tests were carried out to further identify and describe this isolate.

MATERIALS AND METHODS

Sampling of dead sea water: Our isolate originate from coastal surface water sample obtained from recreational area located in Dead Sea at the following coordinates (N 31°44'39.7", E 35°35'30.4") in September, 2010. The *in situ* temperature and pH of water sample were measured by a portable pH meter (Microcomputer pH meter T19000, Trans Instruments). Dead Sea water sample was collected in a clean sterile glass bottle leaving enough head space in the bottle and transported immediately to the lab.

Isolation: Water sample was enriched in a modified artificial sea water solution (Rodriguez-Valera *et al*., 1980). The medium contains the following ingredients per liter distilled water: $MgCl_2.6H_2O$: 5.67 g, $MgSO_4.7H_2O$: 6.8 g, $NaHCO_3$: 0.19 g, $CalCl_2.2H_2O$: 1.47 g, KCl: 0.72 g, KH_2PO_4: 0.5 g. The enrichment cultures were carried out with modified artificial sea water supplemented with peptone: 10 g, glycerol: 3 g, yeast extract: 2 g and NaCl: 25-45 g. Agar (18 g L^{-1}) was added if a solid medium was needed for isolation. Ten milliliter of the Dead Sea water were transferred to 250 mL Erlenmeyer flask containing 100 mL of the high salinity medium and incubated overnight in dark with shaking (100 rpm) at 30°C. The enriched bacteria were sub-cultured two times by streaking plate method on solid high salinity medium. Then, the separated colonies were transferred to get pure cultures. Glycerol stocks of the isolates were also prepared and stored at -20°C. Among the isolates is the isolate DS1 which was further analyzed by different molecular and biochemical methods.

Cell size measurement: The cell size of strain DS1 was measured using a calibrated stage micrometer at highest magnification using a compound light microscope as indicated earlier by Cappuccino and Sherman (2008).

Determining optimum salinity: Strain DS1 was cultivated in high salinity medium different in NaCl concentration (35, 40 and 45%). Experiments were done in triplicates. The growth was monitored as increase in Optical Density (OD). OD was measured spectrophotometrically at 540 nm.

Molecular identification

Genomic DNA preparation: Cells from freshly prepared culture were centrifuged at 7000 rpm for 5 minutes in 1.5 mL tube. One milliliter of cell lysis solution (Qiagen, Valencia, CA, USA) was added to cell pellet. Proteins were precipitated from the mixture by adding 0.5 mL protein precipitation solution (Qiagen, Valencia, CA, USA). The mixture was then vortexed vigorously and centrifuged at 13000 rpm for 5 min. Cold isopropanol (3 mL) was added to 15 mL tube and the supernatant was carefully poured on cold isopropanol. The mixture was inverted several times. The solution was then centrifuged 13000 rpm for 1 min. Finally, the pellet was air-dried and re-suspended in 250 μL Hydration solutions and stored in refrigerator for 16S rRNA gene sequencing.

16S rRNA gene sequencing: 16S rRNA gene sequence amplification and sequencing was carried out by Macrogen Inc., Seoul, Korea. According to Macrogen Inc., PCR was performed as following: 1 μL of template DNA was mixed with 20 μL of PCR reaction solution. The following primers: 27F/1492R primers (27F: 5'-AGA GTT TGA TCM TGG CTC AG-3', 1492R: 5'-TAC GGY TAC CTT GTT ACG ACT T-3') were used. Then, 35 amplification cycles were carried out at 94°C for 45 sec, 55°C for 60 sec and 72°C for 60 sec. Unincorporated PCR primers and dNTPs were removed from PCR products by Montage PCR Clean up kit (Millipore). The purified PCR products of approximately 1,400 bp were sequenced by 518F/800R primers (518F: 5'-CCA GCA GCC GCG GTA ATA CG-3', 800R: 5'-TAC CAG GGT ATC TAA TCC-3'). Sequencing was performed by using Big Dye terminator cycle sequencing kit v.3.1 (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730 XL automated DNA sequencing system (Applied BioSystems, USA) at the Macrogen, Inc., Seoul, Korea.

Sequence analysis and phylogenetic affiliation: A BLAST search (http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi) was carried out against the complete GenBank database. Sequences and their closest relatives were used to construct a phylogenetic tree. The sequence alignments and the phylogenetic tree construction were conducted in MEGA5 (Tamura *et al*., 2004, 2011). GC content and melting Temperature (Tm) of the sequence were calculated by Oligo Calculator (http://mbcf.dfci.harvard.edu/docs/oligocalc.htmL).

Biochemical identification: DS1 isolate was Gram-stained and tested for oxidase using an aqueous solution (1%) of N,N,N',N'-tetramethyl-p-phenylenediamine. Based on the test, the qualitative RapID™ One System (Remel, USA) was applied to identify the strain. The biochemical tests included in the system are: hydrolysis of urea, hydrolysis of arginine, hydrolysis of ornithine, hydrolysis of lysine, utilization of aliphatic thiol, hydrolysis of the fatty acid ester, utilization of sugar aldehyde, utilization sorbitol, hydrolysis of ρ-nitrophenyl-β,D-glucuronide, hydrolysis of ρ-nitrophenyl-β,D-galactoside, hydrolysis of ρ-nitrophenyl-β,D-glucoside, ρ-nitrophenyl-β,D-xyloside, ρ-nitrophenyl-nacetyl-β,D-glucosaminide, utilization of malonate, hydrolysis of proline-β-naphthylamide, hydrolysis of γ-glutamyl-β-naphthylamide, hydrolysis of pyrrolidonyl-β-naphthylamide, utilization of adonitol and utilization of tryptophane. Catalase test was done by addition H_2O_2 on a sample from young culture of the strain where bubble formation is used as indicator for positive result. Additionally, a qualitative test for the following exoenzymes was carried out: gelatinase, amylase and protease. The ability of the isolate to grow with citrate as sole carbon and energy was tested by cultivating the strain in Simmon-Citrate agar and their ability to ferment lactose was tested on MacConkey agar and Eosin Methylene Blue (EMB) agar.

RESULTS

Isolation of strain DS1: Strain DS1 was isolated from Dead Sea water. The colonies of strain DS1 are white and filamentous in form and rough in appearance (Fig. 1a). It can be seen that the cells of the DS1 isolate appear to be a Gram-negative, short (cell size is about 3.5×1.8 μm) and rod-shaped (Fig. 1b).

Optimum salinity: The strain was found to be tolerant to high concentrations of NaCl (3-4.5%). However, the growth rate decreased as salt concentration increased. The optimum NaCl concentrations was 3.5% as shown in Fig. 2.

Biochemical properties of strain DS1: The DS1 strain was tested for several biochemical activities. Regarding the exoenzymes: amylase, gelatinase and protease, the strain was found to be negative for the above mentioned enzymes. In respect to endoenzymes, the results showed that the strain is oxidase-negative and catalase-positive. Other biochemical tests are shown in Table 1. When results of the biochemical tests were applied to ERIC software (Remel, USA), which contains *Citrobacter* species in its database, to identify the strain, "unknown" result was obtained. The strain was found to grow with citrate as sole energy and carbon source. The strain was also able to grow on EMB and MacConkey agar and produced dark metallic sheen on the EMB and pink colonies on MacConkey agar indicating that the strain is able to ferment lactose.

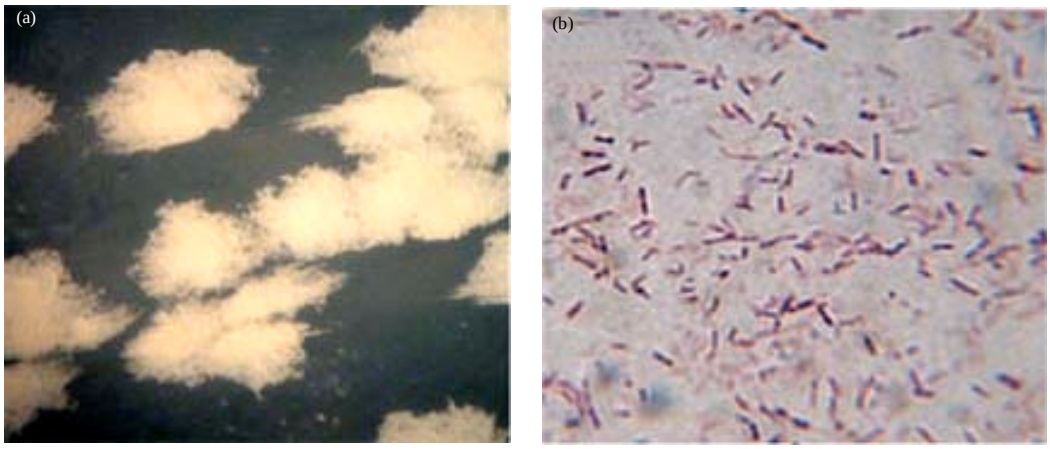

Fig. 1(a-b): Isolated strain DS1, (a) Colonies and (b) Cells, Cells are Gram-stained and 1000x magnified

Table 1: Qualitative biochemical tests of the isolated strains and their identification results using ERIC software

Test	URE	ADH	ODS	LDC	TET	LIP	KSF	SBL	GUR	ONPG	BGLU	BXYL	NAG	MAL	PRO	GGT	PYR	ADON	IND
Strain DS1	+	+	-	-	-	+	-	+	-	-	+	-	-	-	-	-	+	-	-

URE: Hydrolysis of urea, ADH: Hydrolysis of arginine, ODS: Hydrolysis of ornithine, LDC: Hydrolysis of lysine, TET: Utilization of aliphatic thiol, LIP: Hydrolysis of the fatty acid ester, KSF: Utilization of sugar aldehyde, SBL: Utilization sorbitol, GUR: Hydrolysis of ρ-nitrophenyl-β,D-glucuronide, ONPG: Hydrolysis of ρ-nitrophenyl-β,D-galactoside, BGLU: Hydrolysis of ρ-nitrophenyl-β,D-glucoside, BXYL: ρ-nitrophenyl-β,D-xyloside, NAG: ρ-nitrophenyl-nacetyl-β,D-glucosaminide, MAL: Utilization of malonate, PRO: Hydrolysis of proline-β-naphthylamide, GGT: Hydrolysis of γ-glutamyl-β-naphthylamide, PYR: Hydrolysis of pyrrolidonyl-β-naphthylamide, ADON: Utilization of adonitol and IND: Utilization of tryptophane, +: Positive reaction, -: Negative reaction

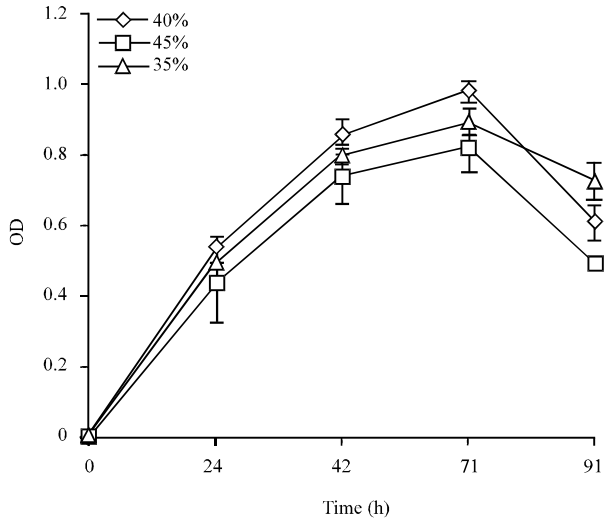

Fig. 2: Growth behavior of strain DS1 under different salinity concentrations (3.5, 4 and 4.5 g L^{-1})

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NNNNNNNGNTCGCACTCAGCGTCAGTCTTTGCCAGAGGAGGCCGCCTTCCGCCACTCGGTGATT
CCTCCACGATCTCTACGCATTTCACCGCTACACCTGGAATTCTACCCCCCTCTACAGGACTCTAG
CCTGCCAGTTTCGGATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCCGACTTGACAGACC
GCCTGCGAGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTG
CTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGAGTAACGTCAATTGCTGACGTTATTAACCACAA
CACCTTCCTCCTCGCTGAAAGTACTTTACAACCCGAAGACCTTCATCATACACGCGGCATTGCTG
CATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGT
CTCAGTTCCAGTGTGGCTGATCATCCTCTCAGACCAGCTACGGATCGTCGCCTTGGTGAGCCATT
ACCCCACCTACTAGCTAATGCCATCTGGGCACATCCGATGGCGAGAGGCCCGAAGGTCCCTCTC
TTTGGTCTTGCGGGGTTATGCGATATTAGCTGCCGTTTCTACTATTTATCCCACTCCATCGGGCAT
TTTCCGAGACATTACTCACCCGTCCGCCACTCGTCGCCGAAGGAGCACGCTCGTCTGCGCTACTC
TTCGACCTGCATGTGCTAGGCCTGCCGCCGGCGTTCAATCTCACNATANNCAAATNTAAAATTN
AACTAAACCTTTTTCCAATTTGTCGGTTGCGCCCTGTGAGGCTTGCCCCCCTGAAGCTAACACGT
AAGCCTACCGCCTGTGGAGTACCGACGCAGGGTCAAACTCGTATGACTTGAGAGGTGTCAGCAC
ACGCGGTGGAGCATGTGGTTTAATTCTAAGCTACGAGATGAGCG
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Fig. 3: The DNA sequence for 16S rRNA gene of strain DS1

GC content and melting point: To determine the GC content and melting Temperature (Tm) of the 16S rRNA sequence of this strain, the Oligo Calculator program was used. This analysis revealed that the percent GC content of the 16S rRNA sequence was 55% and melting point was found to be 87°C.

Molecular identification of strain DS1: Because biochemical tests failed to identify the name of this isolate, we decided to use the 16S rRNA gene sequence analysis. Recently, the 16S rRNA sequence analysis as molecular identification of unknown strain is widely used. The DNA sequence for 16S rRNA gene is presented in Fig. 3. The resulting 16S rRNA sequence showed relatively low similarity to known *Citrobacter* spp. The closest relatives are *Citrobacter freundii* and *Citrobacter murliniae*. The sequence coverage was 97% but the similarity did not exceed 94% as compared to the above mentioned strains. This data suggests that DS1 belong to the genus *Citrobacter*, family Enterobacteriaceae, order Enterobacteriales of the class Gammaproteobacteria. The phylogenetic tree based on 16S rRNA gene sequence of the isolate DS1 and the closest relatives is depicted in Fig. 4.

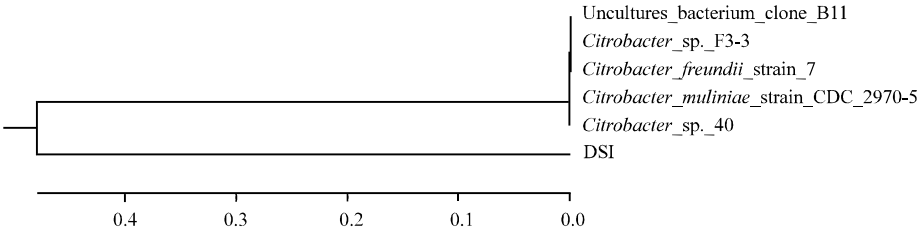

Fig. 4: 16S rRNA gene-based phylogenetic tree of DS1 and its closest relatives, The sequences were retrieved from NCBI website and the tree was constructed by MEGA5 software (Tamura *et al.*, 2011), The evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al.*, 2004)

DISCUSSION

In this study, we describe a new species of *Citrobacter* from recreational hypersaline environment, the Dead Sea of Jordan. The isolate, DS1, was identified based on different criteria, namely, colonial and cell morphology, physiology, biochemical activity and molecular aspects. The strain was found to be Gram-negative, oxidase-negative and ferment lactose. These characteristics are among the main characteristics that characterize members of the family Enterobacteriaceae and coliforms. There are a molecular and biochemical hints place the strain in the genera *Citrobacter* but not enough to place it in any of the previously described species. At the molecular level, our strain represents a new species based on 16S rRNA gene sequence analysis. Comparing the 16S rRNA sequence with the public database revealed only 94% similarity to *Citrobacter freundii* and *Citrobacter murliniae* despite the high coverage percentage (97%). Similarity value is below the conventional similarity percentage that the species of the same genus must share, i.e., 97% 16S rRNA gene similarity (Rossello-Mora and Amann, 2001). If we consider the GC content, we find that our strain have a relatively higher percent GC content (55%) as compared to other citrobacters which generally have an average of 50-52% (Frederiksen, 2005). At the physiological level, strain DS1 is unique in its source, the Dead Sea. Dead Sea is will known for its high overall salt concentration which reaches 340 gL^{-1} (Ma'or *et al.*, 2006). On the other hand, when the water of Dead Sea is compared to the water of oceans, it has a relatively lower sodium chloride content. The most frequently sources of isolation of citrobacters are the gastrointestinal tracts and hands of hospital staff members, in addition to soil, sewage and water (Doran, 1999; Borenshtein and Schauer, 2006). However, environmental sources are not always considered to be an important reservoirs of the organisms (Doran, 1999). Available literature indicated that there are a number of citrobacters have been isolated from marine environments but no report, thus far, indicated their isolation from extreme environments like Dead Sea. For instance, pure cultures of *Citrobacter* were previously isolated from various marine waters in the northeast part of the Unites States (Dufour and Cabelli, 1975) and, recently, *Citrobacter* was also detected in blood from cadavers retrieved from the aquatic environments (Kakizaki *et al.*, 2009). The most prominent character of our strain is growth at high salinity. When the growth of the strain in presence of different NaCl concentrations was tested, 4% NaCl concentration was found to be the optimum concentration for its growth. This falls in the optimum NaCl range of mild halophiles (1-6%) (Madigan and Martinko, 2006). Therefore, the isolate can be defined as mild halophilic. Encountering such microorganism in the Dead Sea is not actually unexpected since different bacterial and archaeal

strains have been isolated from Dead Sea starting from the year 1932 when Volcani published his report describing the native microorganisms living in Dead Sea (Oren and Ventosa, 1999).

Among the prominent biochemical properties of our isolated strain is being O-nitrophenyl-β-galactosidase negative (ONPG-negative). This is an important key characteristics that differentiate our strain from other *Citrobacter* strain, because all reported strains of *Citrobacter* species are ONPG-positive (Frederiksen, 2005). Another important biochemical property of strain DS1 is that the strain can utilize citrate as sole carbon source. This fully agrees with the name of the genus (*Citrobacter* = citrate-utilizing rods). However, there are a number of *Citrobacter* species cannot utilize citrate as sole carbon source like *C. farmeri* (De la Miza *et al*., 2004).

The isolated strain is lysine decarboxylase-negative. All validly described citrobacters and strain DS1 are lysine decarboxylase-negative. This property is very useful to differentiate lactose negative citrobacters from *Salmonella* (Frederiksen, 2005). Indole test (utilization of tryptophane) is another biochemical activity that is used to differentiate citrobacters. Our strain DS1, was found to be indole negative. The indole negative strains of *Citrobacter* are include *C. amalonaticus*, *C. gillenii*, *C. rodentium* and *C. werkmanii* (Frederiksen, 2005). Moreover, our strain cannot utilize adonitol. Similarly, all strains of *Citrobacter* cannot utilize adonitol except *C. koseri*. Our strain is also urease positive like other *Citrobacter* sp. which can hydrolyze urea except *C. gillenii* (Borenshtein and Schauer, 2006). Urea hydrolysis is a property of many enteric except *E. coli* and *Salmonella*.

CONCLUSION

A new strain of *Citrobacter* was isolated for the first time from Dead Sea. Based on its physiological and biochemical properties as well as its molecular taxonomy, the strain belongs to family Enterobacteriaceae and represents a new species of *Citrobacter* and differ primarily from other *Citrobacter* sp. by being mild halophilic.

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