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A NEW STRAIN OF *Shewanella putrefaciens* ISOLATED FROM THE DEAD SEA OF JORDAN

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ABSTRACT

Halophilic bacteria flourish in environments with high salinities such as the Dead Sea of Jordan. In this current study, a new strain, assigned as DS4, was isolated from the Dead Sea of Jordan. The strain was identified based on biochemical and physiological characteristics, and it was further identified by 16S rDNA sequence analysis. DS4 was shown to be Gram-negative, oxidase-positive, non-lactose fermenting, and sulfide-producing on TSI medium. The strain was found to lack the following exoenzymes: amylase, gelatinase, and protease. Strain DS4 was able to grow at high salinity range of 3.4–4.5% NaCl and optimally at 4% NaCl. Furthermore, the 16S rRNA gene analysis revealed that the gene was 99% identical with the genes of several strains of *Shewanella putrefaciens* deposited in the public nucleotide database. Moreover, the antibiogram of this new strain was determined by well diffusion method. The strain was found to be sensitive to various antibiotics (at concentration of 10 mg ml^{-1}) including cloxacillin, chloramphenicol, tetracycline, penicillin, and cyclohexamide. In view of all the above characteristics of strain DS4, it can be concluded that this isolate represents a new mild halophilic strain of *Shewanella putrefaciens* from the Dead Sea of Jordan.

ABSTRAK

Bakteria halofilik hidup dalam persekitaran yang bergaram tinggi seperti Dead Sea di Jordan. Dalam kajian ini, satu strain baru yang dinamakan sebagai DS4, telah dipencil dari Dead Sea. Strain tersebut telah dikenalpasti berdasarkan cirri biokimia dan fisiologi, serta jujukan 16S rDNA. DS4 merupakan bacteria Gram-negatif, positif oksidase, tidak berupaya untuk fermentasi laktosa dan menghasilkan sulfida apabila dikulturkan dalam media TSI. Strain ini juga didapati tidak mempunyai eksoenzim berikut: amilase, gelatinase dan protease. Strain DS4 dapat hidup pada julat saliniti yang tinggi iaitu di antara 3.4–4.5% NaCl dan optimum pada 4% NaCl. Analisis jujukan16S rRNA menunjukkan 99% identiti dengan jujukan gen yang sama daripada beberapa strain *Shewanella putrefaciens* yang telah dideposit ke dalam pangkalan data nukleotida awam. Selain dariapad aitu, antibiogram terhadap strain baru ini telah ditentukan dengan kaedah sebaran cakera. Strain ini didapati sensitif terhadap pelbagai antibiotik (pada kepekatan 10 mg ml^{-1}) termasuklah kloksasilin, kloramfenikol, tetrasiklin, penisilin, dansikloheksamid. Berdasarkan kesemua ciri DS54 di atas, dapat disimpulkan bahawa pencilan tersebut adalah *Shewanella putrefaciens* strain halofilik sederhana daripada Dead Sea, Jordan.

Keywords: *Shewanella putrefaciens,* Dead Sea, 16S rDNA sequence, phylogenetic analysis

INTRODUCTION

The first species of the genus *Shewanella* was isolated in 1931 as contaminating microorganisms responsible for butter putrefaction but under the name *Achromobacter putrefaciens* (Hau and Gralnick, 2007). After many years, the taxonomy of *A. putrefaciens* was revised several times. In 1985, MacDonell and Colwell, established the new genus *Shewanlla* and the strain *S. putrefaciens* was born (MacDonelland Colwell, 1985; Hau and Gralnick, 2007). *Shewanlla putrefaciens* is also a type species of the genus according to Bergey's Manual of Systematic Bacteriology (Bowman, 2005). Based on 16S rRNA sequences and DNA:DNA hybridization, the genus *Shewanella* consists of at least 40 species (Hau and Gralnick, 2007). In addition to distinct molecular characteristics of the genus, the species of *Shewanella* are characterized by several biochemical and physiological characteristics. Species of *Shewanella* are Gram-negative, oxidase- and catalase-positive rod-shaped bacteria, and

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currently belonging to the class Gamma-proteobacteria (Bowman, 2005; Sharma and Kalawat, 2010).

At the physiological level, *Shewanella* spp. are highly diverse and therefore living in a wide range of habitats from which they can be isolated (Bowman, 2005). For example, *Shewanella* spp. have been isolated from marine environments (Ivanova *et al.*, 2001; Bozal *et al.*, 2002), dairy, poultry, beef, seafood products (Bowman, 2005), clinical samples (Brink *et al*., 1995), sediment, soil, ponds and lakes, sewage, and subsurface groundwater (Bowman, 2005).

Recently, we carried out a bacteriological study to explore the halophilic bacteria inhabiting the Dead Sea and their seasonal variation (Jacob, 2012; Jacob and Irshaid, 2012). Dead Sea is a well known unique and extreme environment. For example, the Dead Sea is among the most saline natural environment in the world (Avriel *et al*., 2011; Jacob, 2012), the highest in barometric pressure (88 mm hg) due to low geographic location (more than 420 m below sea level), and unique UV radiation (Avriel *et al*., 2011). Several studies have reported new bacterial strains and species in this unique environment (Oren A., 1983; Oren A., 2006; Jacob, 2012; Jacob and Irshaid, 2012). Some of those bacteria possess potential application(s) in biotechnology and applied microbiology (Oren, 2006). Recent data indicate that *Shewanella* species are not common human pathogens, however, *S. putrefaciens* and *S. algae* were found to be involved in some medical conditions (Holt *et al*., 2005). For instance, *S. putrefaciens* was found to be associated with various human infections such as bacteremia, cellulitis, ear infection, cerebellar abscess, and peritonitis (Soon *et al*., 2008).

In this study, a new strain, designated as DS4, was isolated from a recreational area in the Dead Sea of Jordan. The strain was identified based on 16S rRNA gene as *S. putrefaciens*. In addition, some key biochemical and physiological characteristics of this strain were also investigated and the sensitivity of the strain towards various antibiotics was also evaluated.

MATERIALS AND METHODS

Isolationand cultivation of strain DS4

A water sample was collected from the surface water of a recreational area in the Dead Sea in clean and sterile glass bottles, and transported to the lab for enrichment and isolation in September, 2010. Dead Sea water sample (10 ml) was transferred to high salinity medium and incubated overnight in dark with shaking (100 rpm) at 30ºCas described earlier (Jacob, 2012). Solid high salinity medium was also prepared by adding agar (18 g/l) to high salinity medium (Jacob, 2012). Streak plate method was then applied to obtain single colonies on the solid medium. The single colonies were sub-cultured several times to get pure cultures. One of these cultures was assigned as DS4 and glycerol stock (30%, glycerol: liquid culture) of the new isolate was prepared and stored at -20ºC. Halotolerance of the strain DS4 was tested by cultivating the strain in high salinity media of different salinities (3.5, 4, and 4.5% NaCl). Growth was measured spectrophotometrically at 600 nm. Cultures were run in triplicates.

Molecular identification

DNA isolation was carried out as previously described by Jacob and Irshaid, (2012). Briefly, cells were centrifuged at 7000 rpm, lysed, and protein was then precipitated. The DNA was precipitated by chilled isopropanol and stored in refrigerator at -20ºC.

16S rDNA amplification and sequencing were carried out by Macrogen Inc., Seoul, Korea as earlier described by Jacob and Irshaid, (2012). The 16S rDNA sequence was analyzed by BLAST (http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi) against the complete nucleotide collection database to identify the strains. The sequence alignments and the phylogenetic tree construction were conducted using MEGA5 (Tamura *et al.*, 2004; Tamura *et al.*, 2011). The GC content of the sequence was calculated by OligoCalsoftware (http://www.basic.northwestern.edu/biotools/oligocalc.html). 16S rDNA sequence was deposited in NCBI GenBank.

Biochemical identification

Cells from pure culture were Gram-stained and then tested for oxidase activity using an aqueous solution (1%) of N, N, N', N'-tetramethyl-p-phenylenediamine (Cappuccino and Sherman, 2008). Catalase test was also performed by adding drops of H_2O_2 on fresh colonies of the strain and observing the bubble formation. The strain was cultivated on triple sugar iron (TSI) agar to infer its ability to produce sulfide, and on MacConkey agar and eosinemethylelene blue (EMB) to infer their lactose fermentation. Additionally, the strain was cultivated on nutrient gelatin, starch agar, and skimmed milk agar to test the presence of gelatinase, amylase, and protease, respectively. In case of starch agar, iodine solution was applied to test for the activity of amylase (Cappuccino and Sherman, 2008). Additional biochemical tests, like indole production, arginine dihydrolase, urease production, were done byRapID^{TM} One System (Remel, USA).

Antibiogram determination

Antibiotic sensitivity testing was carried out by the agar well diffusion method (Parekh and Chanda,

2007). Strain DS4, was freshly cultivated in high salinity medium and incubated overnight at 30ºC. The fresh inoculum (0.1 ml) was used to inoculate Mueller-Hinton agar plates. A sterile cork borer was then used to make equidistant wells (6 mm diameter) on inoculated Mueller-Hinton agar plates. Each well was filled with 50 µl of the antibiotic solutions (10 $mgml^{-1}$). The tested antibiotics were: cloxacillin, chloramphenicol, tetracycline, penicillin, and cyclohexamide. After that, the plates were allowed to stand for few minutes for pre diffusion and incubated at 30ºC for 24 hours. The diameters of inhibition zones formed around the wells were measured in to the nearest millimeter. Experiments were carried out in triplicates.

RESULTS

In this study, a new bacterial strain, designated as DS4, was isolated from the surface water of Dead Sea. Cells of the strain DS4 were Gram-negative and rod-shaped (Fig. 1). The 16S rDNA of the strain DS4 was partially sequenced and about 942 bases long (Fig. 2). BLAST analysis was performed for the partial16S rDNA sequence of the strain DS4, yielding 99% identities to several strains of known *S. putrefaciens*. 16S rDNA sequence was deposited in GenBank with the accession number KC898195. In addition, the phylogenetic tree shows the relationship between our strain DS4 and *S. putrefaciens* and some closely related bacterial

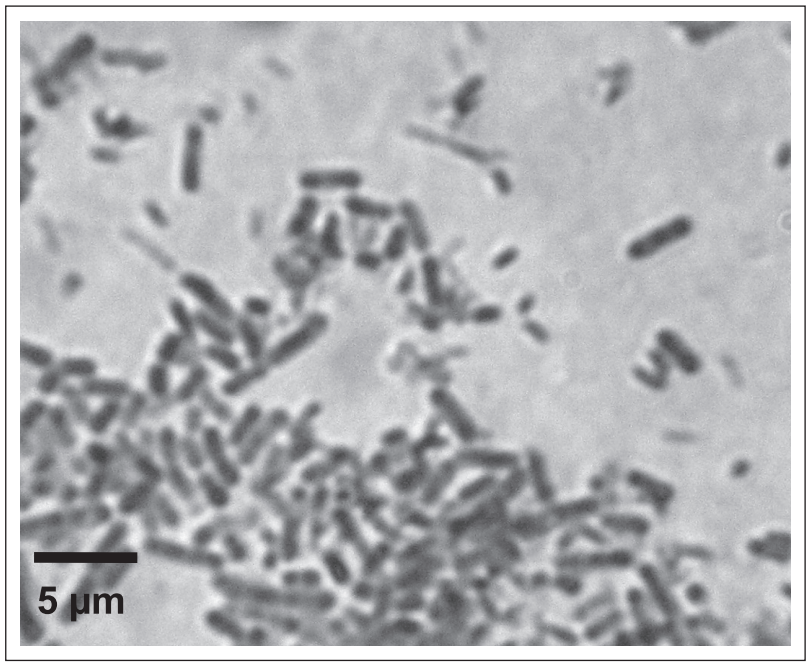

Fig. 1. Light micrograph showing the cells of the new isolate DS4.

NNNNNTNACGNATCGGATTACTGGGCGTAAGCGTGCGCAGGCGGTTTGTTAAGCGAGATGTGAAAGCCCTGGGCTCAACC
TAGGAATAGCATTTCGAACTGGCGAACTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGA
GATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCATGCACGAAAGCGTGGGGAGCAAAC
AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTCGGAGTTTGGTGTCTTGAACACTGGGCTCTCAAGCTA
ACGCATTAAGTAGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGT
GGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCACAGAAGACTGCAGAGATGCGGTT
GTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACG
AGCGCAACCCCTATCCTTATTTGCCAGCACGTAATGGTGGGAACTCTAGGGAGACTGCCGGTGATAAACCGGAGGAAGGT
GGGGACGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGAGTACAGAGGGTTGCAAA
GCCGCGAGGTGGAGCTAATCTCACAAAGCTCGTCGTAGTCCGGATTGCAGTCTGCAACTCGACTCCATGAAGTCGGAATC
GCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGG
GCTGCAAAAGAAGTGGGTAGCTTAACCTTCGGGGGGCGCTCACACTTTGTGATCATGACTGGGTGATCTAN

Fig. 2. The partial nucleotide sequence of the 16S rDNA of the strain DS4.

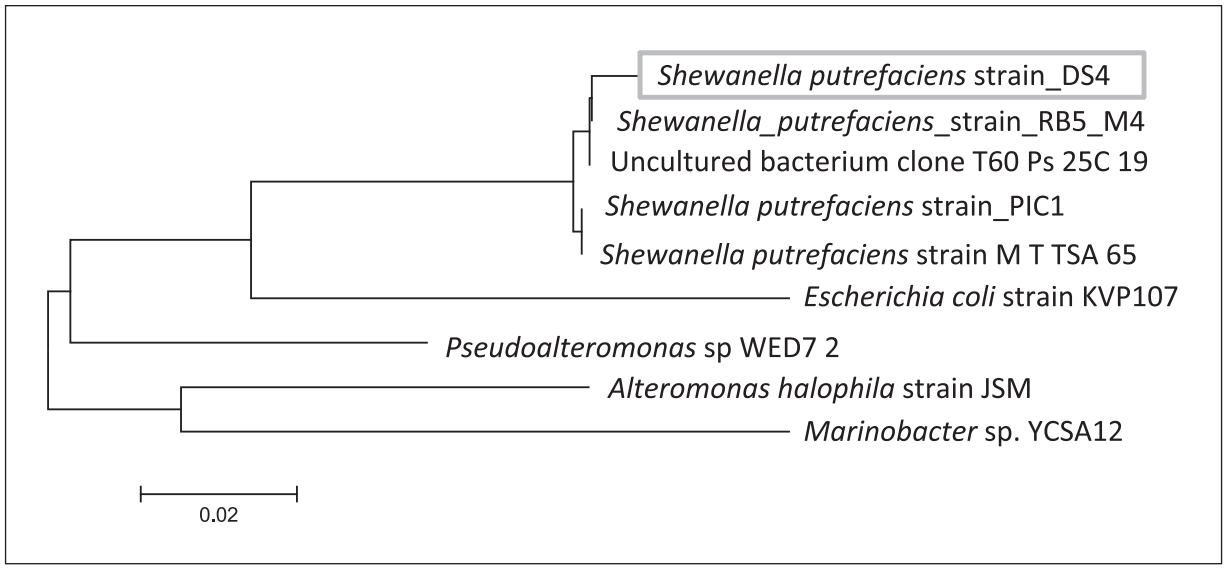

Fig. 3. Phylogenetic relationship of strain DS4 (marked with rectangle) with its closest relatives. The tree was constructed using the neighbor-joining method using MEGA5. The scale bar represents 0.02 nucleotide substitutions per position.

Table 1. Some qualitative biochemical characteristics of strain DS4

Gram Stain	Oxidase	Catalase	Lactose fermentation	Sulfide production on TSI	Indol production	Urease	arginine dihydrolase	Gelatnase	Amylase	Protease
-	+	+	-	+	-	-	-	-	-	-

species (Fig. 3). Thus, based on BLAST and phylogenetic analyses, strain DS4 represents a new strain of the species *S. putrefaciens*, a γ-proteobacterium in the domain bacteria. Analysis of the 16S rDNA sequence also showed a high GC content (54%) of the resulting strain DS4 nucleotide sequence.

The biochemical analysis of the strain DS4 showed that this strain is oxidase- and catalase-positive and produces sulfide in TSI medium. This strain was also not able to ferment lactose. Additionally, this strain was shown to be negative for the following tests: indole production, arginine dihydrolase, and urease (Table 1). Regarding exoenzymes, the strain was found to lack the following exoenzymes: gelatinase, amylase, and protease (Table 1).

The strain DS4 was able to grow at 3.5–4.5% NaCl. The optimum NaCl was found to be 4% (Fig. 4). These data clearly indicate that DS4 strain is a moderately halophilic strain.

To test the susceptibility of the strain DS4 to various antibiotics, agar well diffusion experiments were performed. Data generated from these experiments revealed that this strain is sensitive to different antibiotics. The strain was found to be sensitive to all tested antibitiotics including cloxacillin, chloramphenicol, tetracycline, penicillin, and cyclohexamide. The strain was most sensitive to tetracycline with inhibition zone of (40 mm), and least sensitive tocyclohexamide (inhibition zone of 10 mm) (Fig. 5).

DISCUSSION

The results generated in this current study revealed detail information on the isolation, identification and characterization of the strain DS4 obtained from Dead Sea of Jordan. Recently, for correct identification of newly discovered strain to the species level, several molecular and biochemical

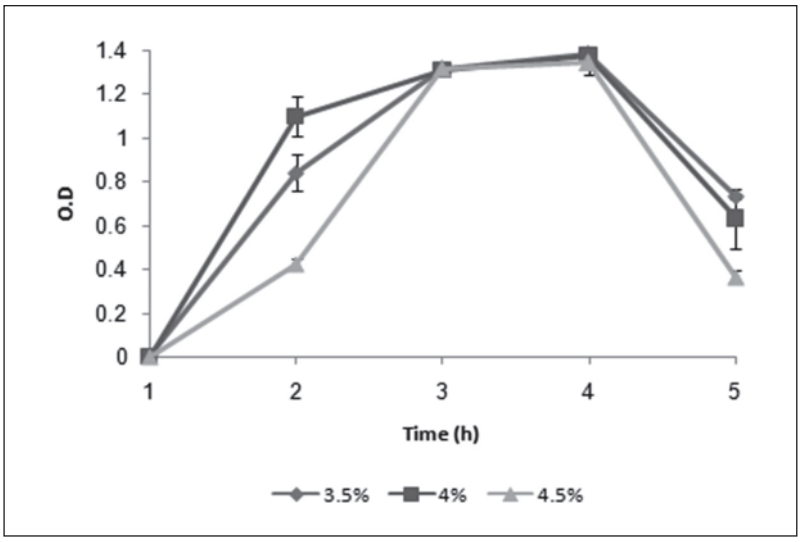

Fig. 4. Growth behavior of strain DS4 at different salinities. The growth was measured at 600 nm. Each point represents the average of three readings.

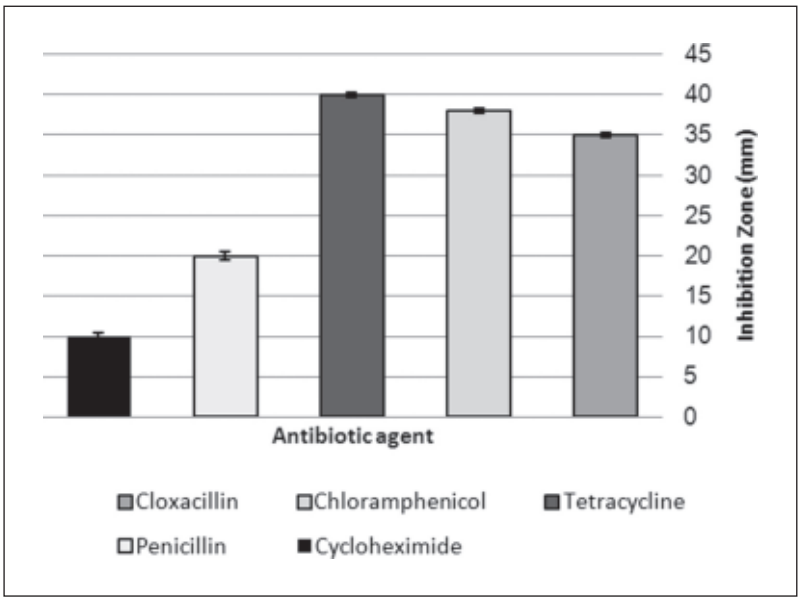

Fig. 5. Antibiotic sensitivity of strain DS4 represented as inhibition zone diameters produced by different antibiotics in Mueller-Hinton agar plates inoculated by strain DS4. Each value is the average of three readings.

techniques have been used. Among the most widely used methods in molecular taxonomy is based on 16S rDNA sequence and DNA:DNA hybridization (Rosselló-Mora and Amann, 2001). Analysis of the 16S rDNA sequence of the isolated strain, indicates that strain DS4 is a new strain of *S. putrefaciens* (99% identity), belonging to the genus *Shewanella*. *Shewanella* is phylogenetically most closely related to the genera *Pseudoalteromonas*, *Alteromonas*, *Ferrimonas* and *Colwellia*. In fact, all of these genera are members of the family Alteromonadaceae in the subdivision γ-proteobacteria of the domain bacteria (Bowman, 2005).

At the biochemical level, characteristics that differentiate *Shewanella* from other members of the Alteromonadaceae include H_2S production, ornithine decarboxylase activity, and halophilicity (Austin and Lee, 1999; Bowman, 2005) and all are major characteristics of the new isolated strain. In addition to the aforementioned biochemical characteristics, *S. putrefaciens* is characterized also by being Gram-negative, oxidase-positive, and

rod-shaped with non-fermenting nature. In clinical laboratories, Gram- negative, oxidase-positive, non-fermenting rods are routinely classified as *Pseudomonas* spp. and no further analysis is made (Sharma and Kalawat, 2010). Therefore, additional molecular and biochemical analyses are essential to correctly identify the strain.

As expected, the strain DS4 was characterized by being mild halophilic strain which grow best at 4% NaCl. The optimum NaCl range of mild halophiles was defined to be 1–6%NaCl, thus, our strain DS4 falls in this range of mild halophilic salinity (Madigan *et al*., 2012). Previously, it has been reported that the growth of some *Shewanella* spp. are characterized by requirement for high concentration of salt, high hydrostatic pressure, and cold temperature (Kato and Nogi, 2001), rendering them typical marine bacteria. Although, *S. putrefaciens* strain was not commonly encountered in Dead Sea environment, this strain was reported to be isolated from other marine environments (Kato and Nogi, 2001; Yang *et al*., 2006). However, strain DS4 originates from a unique aquatic environment, i.e. the Dead Sea of Jordan. This environment is characterized by being among the saltiest environment in the world (Madigan *et al*., 2012). In addition to being the saltiest, the Dead Sea of Jordan is characterized by being the highest in barometric pressure (88 mm hg), and by having a unique UV radiation (Avriel *et al*., 2011). Therefore, only halophilic and halotolerant microorganisms are expected to survive in this salty environment.

Even though, the pathogenicity of strain DS4 was not tested, literature data indicated that human infections with members of the genus *Shewanella* were rare, and the only *Shewanella* spp. found in clinical specimens were *S. putrefaciens* and *S. algae* (Holt *et al*., 2005). Therefore, one potential predisposing factor for infection with *S. putrefaciens* is exposure to seawater or a marine environment with a skin lesion or skin trauma, especially in recreational marine environments, because *Shewanella* spp. are found mainly in marine environments. Recently, Soon *et al*. (2008) reported that *S. putrefaciens* is associated with various human infections such as bacteremia, cellulitis (skin and soft tissue infection), ear infection, cerebellar abscess, wound infection, osteomyelitis, empyema, endocarditis, and peritonitis.

However, the antimicrobial susceptibility tests of strain DS4 indicate that the strain is susceptible to different antibiotics. The antibiotic susceptibility was in the following descending order: tetracycline, chloramphenicol, cloxacillin, penicillin, and finally cyclohexamide. Tetracycline was previously reported as effective antibacterial against *S. putrefaciens* (Holt *et al*., 2005). Moreover, previous studies indicated that *S. putrefaciens*is is susceptible to additional antibiotics like amoxicillin-clavulanic acid, netilmicin, gentamicin, piperacillin, and cefotaxime, penicillin, ampicillin, and tetracycline (Pagani *et al*., 2003; Botelho-Nevers *et al*., 2005).

Taken together, on the basis of the data derived from the physiological, and biochemical analyses, and in view of the 16S rDNA sequence of the strain DS4 in relation to closet strains, it could be stated that the strain DS4 belongs to *Shewanella putrefaciens*. Moreover, its salt requirement for optimum growth reveals that this strain DS4 might be placed in the group of moderately halophilic bacteria. To our knowledge, this is the first report on isolation of a mild halophilic *S. putrefaciens* strain from the Dead Sea of Jordan.

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