Deleya halophila, a New Species of Moderately Halophilic Bacteria

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Deleya halophila sp. nov., which was isolated from hypersaline soils, is proposed. Each of 38 isolates, together with 8 reference strains, was examined in 97 phenotypic tests, and the data were analyzed by numerical taxonomy procedures. The 38 isolates formed a distinct group at a 76% similarity value, as determined by an analysis performed with the simple matching coefficient and unweighted average linkage clustering. Characteristically, the isolates were aerobic, gram-negative rods that were motile by one to eight peritrichous flagella. They grew optimally in the presence of 7.5% (wt/vol) marine salts. The distinguishing features of the new species are its salt requirement, biochemical features, and nutritional versatility. The guanine-plus-cytosine content of the deoxyribonucleic acid is 66.7 mol%. The type strain of this species is strain F5-7 (= CCM 3662).

Moderately halophilic bacteria are defined as those bacteria which grow optimally at NaCl concentrations between 3 and 15% (wt/vol) (20). Most authors recognize that only a few types of organisms have this property, and, in fact, the majority of the studies have been done with *Vibrio costicola* (20, 21). However, recently we have found a wide variety of moderately halophilic microorganisms in both hypersaline waters (37) and saline soils (24, 25).

In a previous study (24), we isolated and characterized a group of microorganisms which predominated in hypersaline soils and were related to marine species belonging to the genus *Alcaligenes*. Since the marine *Alcaligenes* species have been assigned recently to the new genus *Deleya* (3), in this paper we describe in detail the characteristics of our isolates and propose that they represent a new species, *Deleya halophila*.

MATERIALS AND METHODS

Bacterial strains. The 38 strains studied were isolated from hypersaline soils located near Alicante in southeast Spain, as described previously (24). The composition of the isolation medium (MH medium) was as follows: yeast extract (Difco Laboratories, Detroit, Mich.), 1% (wt/vol); Difco Proteose Peptone no. 3, 0.5% (wt/vol); glucose, 0.1% (wt/vol); and Bacto Agar (Difco), 2% (wt/vol). This medium was supplemented with a balanced mixture of sea salts (33) to give final concentrations of 0.5, 5, 10, 20, and 25% (wt/vol). The pH was adjusted to 7.2 with 1 M KOH. A 1-g soil sample was suspended in 10 ml of a salts solution similar to the solutions used for the culture media; 10-fold dilutions were made, and 0.1 ml of each dilution was spread-plated onto the isolation medium. Incubation was at 32°C for 15 days in sealed plastic bags.

The following reference strains were also included: the moderately halophilic bacteria *Flavobacterium halmephilum* CCM 2831, *Vibrio costicola* NCMB 701^T (T = type strain), and "*Pseudomonas halosaccharolytica*" CCM 2851 and the marine bacteria *Deleya aesta* NCMB 1980, *Deleya cupida* NCMB 1978, *Deleya pacifica* NCMB 1977, *Deleya venusta* NCMB 1979, and *Alcaligenes aquamarinus* NCMB 557. Both the new isolates and the reference strains were grown and maintained on slants of MH medium supplemented with 7.5% (wt/vol) marine salts.

Phenotypic tests. A total of 97 characteristics were deter-

mined for each strain. A basal medium containing 7.5% (wt/vol) marine salts at pH 7.2 was used. Incubation was at 32°C for up to 7 days in sealed containers.

Gram staining was done by the method of Dussault (10). Motility was determined from wet mounts, and flagellum arrangement was assessed by the technique of Rhodes (26). Colony morphology and size and production of diffusible and nondiffusible pigments were examined on MH medium after 4 days of incubation. Growth in liquid MH medium was determined after 2 days of incubation.

The growth of the isolates at different salt concentrations was determined on MH medium containing 0.5, 2, 5, 7.5, 10, 12.5, 15, 20, 25, or 30% (wt/vol) marine salts. The optimum concentration was considered to be the concentration at which visible growth first appeared. Thus, the number of colony-forming units was determined by spreading 0.1-ml samples of 10-fold serial dilutions in salts solution on fresh medium (five replicates for each dilution). Incubation was at 32°C for 96 h. The pH range enabling growth was determined in a similar way on media in which the pH values were adjusted to 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 with HCl and KOH. The temperature range of growth was determined at 5, 15, 25, 37, and 45°C. A result was considered positive if there was visible growth after 7 days, except at 5°C, when the incubation time was 2 weeks. The ability to grow anaerobically was evaluated on plates of MH medium which were inoculated and incubated in jars with the GasPak Anaerobic System (BBL Microbiology Systems, Cockeysville, Md.).

Cultures grown for 18 h on MH medium were tested for catalase by adding 3% (wt/vol) H₂O₂ and for cytochrome oxidase production by using Kovacs reagent (19). Acid production from L-arabinose, D-galactose, D-glucose, glycerol, meso-inositol, lactose, maltose, D-mannitol, sucrose, D-trehalose, and D-xylose was detected in the basal medium with the final sugar concentrations described previously (37).

H₂S production was tested in MH medium containing 0.01% (wt/vol) cysteine with strips of lead acetate-impregnated paper in the mouths of tubes as indicators. For the nitrate and nitrite reduction tests, liquid MH medium containing 0.2% (wt/vol) KNO₃ was used; nitrite was detected with α -naphthylamine-sulfanilic acid reagent (28), and residual nitrate was detected with zinc dust. Nitrite reduction to gaseous compounds was detected in Durham tubes (28).

Gelatin hydrolysis was tested on MH medium containing

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1% (wt/vol) gelatin by flooding the cultures with Frazier acid-HgCl₂ reagent (11). Casein hydrolysis was determined from zones of clearing on plates made by mixing double-strength MH medium with equal amounts of skim milk (6). Starch hydrolysis was tested with cultures on MH medium containing 1% (wt/vol) potato starch by flooding them with Lugol iodine. Lipase activity was detected on a modified Sierra medium (27), as described previously (37). Urea hydrolysis was detected in Christensen medium (5) supplemented with 7.5% (wt/vol) marine salts. Deoxyribonuclease activity was tested by the method of Jeffries et al. (15) on deoxyribonuclease agar (Oxoid Ltd., London, England) containing the salts of MH medium. Phosphatase activity was tested by adding a 1% (wt/vol) aqueous phenolphthalein diphosphate solution to MH medium. Esculin hydrolysis was determined by adding 0.1% (wt/vol) esculin (Merck & Co., Inc., Rahway, N.J.) and 0.05% (wt/vol) ferric citrate to MH medium (6). Hemolysis was tested by aseptically adding 5% (vol/vol) sterile human blood to MH medium. Phenylalanine deamination was assessed on phenylalanine agar (Difco) prepared with a 7.5% (wt/vol) marine salts solution (6). Indole production was tested in liquid MH medium; the presence of indole was shown with Kovacs reagent (18). The methyl red and Voges-Proskauer tests were done in the medium and under the conditions described previously (37).

The nutritional tests were done in the basal medium by

using the substrate concentrations and methodology described previously (37). The following organic compounds were tested as sole sources of carbon and energy or as sole sources of carbon, nitrogen, and energy: L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannose, L-rhamnose, salicin, sucrose, D-trehalose, D-xylose, adonitol, DL-glycerol, *meso*-inositol, D-mannitol, D-sorbitol, acetate, benzoate, caprylate, citrate, fumarate, D-gluconate, hippurate, DL-lactate, DL-malate, pyruvate, propionate, succinate, D-tartrate, L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, L-histidine, L-isoleucine, L-leucine, L-lysine, L-ornithine, L-serine, L-tryptophan, and L-valine.

Antibiotic susceptibility was tested by spreading bacterial suspensions on plates of MH medium and applying antibiotic disks (BBL Microbiology Systems) (bacitracin, 10 U; cephalothin, 30 µg; chloramphenicol, 30 µg; epicillin, 10 µg; lincomycin, 2 µg; neomycin, 30 µg; novobiocin, 30 µg; polymyxin B, 300 U; rifampin A, 30 µg; tetracycline, 30 µg). Zones of inhibition were measured, and susceptibility was determined compared with standard inhibition zones (BBL Microbiology Systems).

Electron microscopy. Representative strains were grown for 18 h on the surfaces of MH agar plates covered with liquid medium. Samples of the liquid cultures were negatively stained with a 2% (wt/vol) solution of phosphotungstic

FIG. 1. Simplified dendrogram showing clustering of the 38 strains of *Deleya halophila* sp. nov. and eight reference strains, based on the simple matching coefficient and unweighted average linkage clustering methods of numerical taxonomy.

acid (pH 7.0). Electron micrographs were obtained with a Carl Zeiss model EM-9S-2 transmission electron microscope.

Numerical analysis. Differential features were used for a numerical analysis. Positive and negative results were coded as 1 and 0, respectively; noncompatible or missing data were coded as 9. The data were examined by using the simple matching coefficient (32) and the Jaccard coefficient (14), and clustering was achieved by unweighted average linkage (31), using the MINT program of F. J. Rolf (Department of Ecology and Evolution, State University of New York at Stony Brook, Stony Brook, N.Y.). Cophenetic values were also obtained (31). The test error was evaluated by examining four strains in duplicate (30). Computations were carried out with a UNIVAC model 1108 computer in the Computer Centre, University of Seville, Seville, Spain.

DNA composition. The deoxyribonucleic acid (DNA) of strain CCM 3662^T was purified by the method of Marmur (22). The guanine-plus-cytosine (G+C) content of the DNA was determined from the midpoint value of the thermal denaturation profile (23) obtained with a Gilford model 250 spectrophotometer at 260 nm; this instrument was programmed for temperature increases of 1.0°C/min. The G+C content was calculated from the thermal denaturation temperature by using the equation of Johnson (16). The G+C content of reference DNA from *Escherichia coli* B was taken as 51 mol%.

RESULTS

Numerical analyses. By using the simple matching coefficient and the unweighted average-linkage clustering algorithm, we grouped the 38 isolates studied in a single phenon at a similarity level of 76% (Fig. 1). The type culture was located near the center of this phenon. The cophenetic value was 0.968, and the estimated test error was less than 2%. The reference strains did not cluster with the environmental isolates, an observation in agreement with the different salt requirements for growth of marine and moderately halophilic bacteria and the numerous differences in phenotypic characteristics. Our isolates joined the *D. pacifica* strain tested at a similarity level of 62% and other *Deleya* species at similarity levels ranging from 58 to 72%. It is clear that the 38 strains studied represent a new group of bacteria isolated from a habitat (hypersaline soil) not well studied in earlier work. The reference strain of *Alcaligenes aquamarinus* clustered with the environmental isolates at a similarity level of only 26%.

The dendrogram obtained by using the Jaccard coefficient was in agreement with the order of strains obtained when the simple matching coefficient was used, although the strains clustered at somewhat different similarity levels. The 38 environmental isolates clustered into one group at a similarity level of 73%. Thus, our numerical analyses indicate that the 38 environmental isolates constitute a new group of bacteria clearly distinct from other halophilic bacteria (Table 1).

Electron microscopy. The morphology, capsular slime formation, and peritrichous flagella of this new species, as represented by strain F5-7^T (= CCM 3662^T), are shown in Fig. 2.

DNA composition. The G+C content of the DNA of strain CCM 3662^T was 66.7 mol%.

DISCUSSION

The genus *Alcaligenes* is very heterogeneous, has not been well characterized, and includes species found in a

wide variety of habitats (terrestrial, freshwater, and seawater) (4, 13, 35). In fact, several seawater species of *Alcaligenes* have been described, but only five marine species were included on the Approved Lists of Bacterial Names

TABLE 1. Characteristics for which the 38 strains of *D. halophila* differ from one another

Characteristic	No. of strains positive	Reaction of strain CCM 3662 ^T
Oxidase production	36	+
Nitrate reduction	22	+
H ₂ S production	31	+
Urease	19	—
Esculin hydrolysis	33	+
Phosphatase	30	+
Deoxyribonuclease	2	—
Growth at:		
pH 5	34	+
pH 10	34	+
4°C	7	+
Acid production from:		
D-Galactose	37	+
D-Glucose	35	+
Maltose	28	+
Sucrose	24	—
D-Trehalose	11	—
D-Xylose	8	+
Growth on: ^b		
D-Cellobiose	36	+
D-Galactose	37	+
Lactose	9	—
Maltose	34	+
D-Mannose	34	+
L-Rhamnose	5	+
Salicin	33	+
Sucrose	37	+
D-Trehalose	36	+
D-Xylose	33	+
Adonitol	1	—
DL-Glycerol	36	—
D-Mannitol	13	—
D-Sorbitol	12	—
Acetate	37	+
Benzoate	2	—
Caprylate	1	—
Fumarate	37	+
D-Gluconate	36	+
Hippurate	4	—
Propionate	35	—
Pyruvate	37	+
D-Tartrate	1	—
Growth on: ^c		
L-Alanine	37	+
L-Arginine	37	+
L-Aspartic acid	36	+
L-Glutamic acid	37	+
L-Histidine	6	—
L-Isoleucine	7	+
L-Leucine	9	+
L-Lysine	29	+
L-Ornithine	23	—
L-Serine	37	+
L-Tryptophan	10	—
L-Valine	1	—
Antibiotic susceptibility		
Bacitracin (10 U)	2	—
Epicillin (10 µg)	18	—
Neomycin (30 µg)	4	—
Polymyxin B (300 U)	35	+

^a +, Positive; —, negative.

^b When supplied as the sole source of carbon and energy.

^c When supplied as the sole source of carbon, nitrogen, and energy.

FIG. 2. Electron micrograph of *Deleya halophila* strain F5-7^T (= CCM 3662^T), showing peritrichously arranged flagella after staining with phosphotungstic acid. Bar = 1 μ m.

(29); these were *Alcaligenes aestus*, *Alcaligenes cupidus*, *Alcaligenes pacificus*, *Alcaligenes venustus* (2), and *Alcaligenes aquamarinus* (12) (*Alcaligenes aquamarinus* was previously described as *Achromobacter aquamarinus* [38]). Recently, the genus has been described as including a new group of shellfish pathogens, *Alcaligenes faecalis* subsp. *homari* (1). However, no moderately halophilic species of this genus has been described. The recent DNA-ribosomal ribonucleic acid hybridization studies of De Ley et al. (7-9, 17, 34, 36) have shown that the marine species of *Alcaligenes* are a distinct lineage that is not related to the type species, *Alcaligenes faecalis*, and that they constitute a sublineage in an evolutionary branch which includes the *Pseudomonas fluorescens* ribosomal ribonucleic acid branch and some *Alteromonas* species. These results, together with variations in phenotypic features from classical species of *Alcaligenes* (K. Kersters, P. Segers, J. P. Gayral, and J. De Ley, Abstr. IV Int. Conf. Culture Collections, p. 34, 1981), suggest that the marine species of *Alcaligenes* should be removed from this genus. Very recently, these species have been placed in a new genus, *Deleya*, as *D. aesta*, *D. cupida*, *D. pacifica*, and *D. venusta*; this genus also includes "*Pseudomonas marina*" as *Deleya marina* (3). The new genus *Deleya* was defined by Baumann et al. (3) as consisting of straight rod-shaped organisms that are strictly aerobic and without proteolytic, lipolytic, or chitinolytic activities. These bacteria are found in marine habitats, and the G+C contents of their DNAs range from 52 to 68 mol%. The primary difference between *Deleya* species and terrestrial *Alcaligenes* or *Pseudomonas* species is "their specific Na⁺ requirement, requiring at least 75 mM NaCl for optimal growth rate and yield" (3).

In a previous study (24), we described a group of 38 strains whose characteristics suggested inclusion in the genus *Alcaligenes*, although they were phenotypically more closely related to the marine species of *Alcaligenes*, especially "*Alcaligenes pacificus*." These strains differed from the

marine *Alcaligenes* strains in their ability to grow in medium containing between 2 and 30% (wt/vol) salt (with optimal growth at 7.5% salt) and in some biochemical and nutritional tests (Table 2). Since the features of this new group are in close accord with the taxonomic description of the genus *Deleya*, and since the data presented in this paper separate this organism from other *Deleya* species, we propose designation of these strains as *Deleya halophila* sp. nov. We also propose to expand the original description of the genus *Deleya* to include not only marine species but also those organisms with moderate halophilic properties. The major distinctions between *D. halophila* and other species of *Deleya* are shown in Table 2. The new species can also be differentiated from *Alcaligenes faecalis* subsp. *homari* (1) by the ability of *D. halophila* strains to grow in different salt concentrations, by their optimum growth at 7.5% salt, and by a number of distinct biochemical activities (ability to hydrolyze starch and esculin and ability to utilize xylose). In addition, *D. halophila* can be differentiated from *Alcaligenes aquamarinus* (12) by a number of important biochemical markers possessed by the new species, including H₂S production, phosphatase, starch and esculin hydrolysis, and their different ability to grow on the following substrates: L-arabinose, D-cellobiose, D-galactose, D-mannose, salicin, D-xylose, *meso*-inositol, benzoate, caprylate, hippurate, and L-histidine.

A description of this new species is given below.

Deleya halophila sp. nov. *Deleya halophila* (hal.o.phi'la. Gr. n. *halos* salt; Gr. adj. *philus* loving; M.L. adj. *halophila* salt loving) cells are gram-negative, nonsporeforming rods 0.5 to 0.7 by 1.5 to 2.0 μ m. Cells are motile by means of one to eight peritrichous flagella. Strict aerobes.

Circular, low convex, smooth, opaque, and cream-pigmented colonies develop on 7.5% (wt/vol) marine salts solid medium after 4 days at 32°C. No pigments. Broth cultures are uniformly turbid.

The optimal marine salts concentration for growth is 7.5%

TABLE 2. Characteristics useful in distinguishing strains of *D. halophila* from phenotypically similar species of *Deleya*^a

Characteristic	Reaction of:				
	<i>D. halophila</i>	<i>D. aesta</i>	<i>D. cupida</i>	<i>D. pacifica</i>	<i>D. venusta</i>
Oxidase production	+	+	—	+	+
H ₂ S production	+	+	—	—	—
Deoxyribonuclease	—	+	+	—	—
Phosphatase production	+	—	+	—	+
Starch hydrolysis	—	+	—	—	—
Esculin hydrolysis	+	—	+	+	—
Growth at:					
pH 5	+	—	+	—	—
pH 10	+	—	+	+	+
4°C	+	—	—	—	+
Acid from D-galactose	+	+	+	—	—
Growth on: ^b					
L-Arabinose	+	—	+	—	—
D-Cellobiose	+	—	+	—	—
D-Galactose	+	+	+	—	—
Maltose	+	—	+	—	+
D-Mannose	+	—	+	—	—
Salicin	+	—	+	—	—
Sucrose	+	—	+	—	+
D-Trehalose	+	—	+	—	+
D-Xylose	+	—	+	—	—
Adonitol	—	—	+	—	—
meso-Inositol	—	+	+	—	+
Benzoate	—	—	+	+	+
Caprylate	—	+	+	—	+
Hippurate	—	—	+	+	+
L-Histidine	—	—	+	+	+
L-Isoleucine	+	+	—	—	+
L-Serine	+	—	+	+	—
Susceptibility to:					
Bacitracin (10 U)	—	+	—	—	—
Cephalothin (30 µg)	+	+	+	+	—
Chloramphenicol (30 µg)	+	+	—	+	+
Neomycin (30 µg)	—	—	+	+	—
Polymyxin B (300 U)	+	+	+	—	+
Rifampin A (30 µg)	+	+	—	+	+
G+C content (mol%)	66.7	57-58	60-63	67-68	52-54

^a Data from this study and references 2 and 3. +, Positive; —, negative.^b When supplied as sole sources of carbon and energy or as sole sources of carbon, nitrogen, and energy (amino acids).

(wt/vol) at 32°C. Able to grow at marine salts concentrations between 2 and 30% (wt/vol). No growth in the absence of sodium chloride.

Growth at 15 to 45°C (optimal growth at 30 to 37°C) and between pH 5 and 10 (optimal growth at pH 7).

Catalase and oxidase are produced.

Acids are produced from D-glucose and D-galactose. Acids are not produced from L-arabinose, lactose, glycerol, meso-inositol, or D-mannitol.

Esculin is hydrolyzed; gelatin, casein, starch, Tween 80, and blood are not hydrolyzed.

Nitrates reduced to nitrites; no reduction of nitrite.

Indole, acetylmethylcarbinol, phenylalanine deaminase, and deoxyribonuclease are not produced.

Phosphatase and hydrogen sulfide are produced.

Methyl red test is negative.

The following compounds are utilized as sole carbon and energy sources: L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, maltose, D-mannose, salicin, sucrose, D-trehalose, D-xylose, DL-glycerol, acetate, citrate, fumarate, D-gluconate, DL-lactate, DL-malate, pyruvate, and succinate.

The following compounds are utilized as sole carbon, nitrogen, and energy sources: L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, and L-serine.

The following compounds are not utilized as sole carbon, nitrogen, and energy sources: meso-inositol, D-tartrate, L-histidine, and L-valine.

Susceptible to cephalothin, chloramphenicol, polymyxin B, and rifampin A.

Not susceptible to bacitracin, lincomycin, neomycin, and tetracycline.

Source: isolated from saline soils near Alicante in south-east Spain.

G+C content of the DNA: 66.7 mol% (thermal denaturation midpoint estimation).

The type strain is strain F5-7; a culture of this strain has been deposited with the Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia, as strain CCM 3662. The description of the type strain is the same as that given above for the species, with exception of those characteristics shown in Table 1.

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