Halomonas elongata, a New Genus and Species of Extremely Salt-Tolerant Bacteria

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The morphological, biochemical, and physiological characteristics of nine bacterial strains isolated from a solar salt facility located on Bonaire, Netherlands Antilles are described. The bacteria were gram-negative rods which produce white, opaque colonies on solid media. During the log phase of growth, the cultures consisted of single and paired cells with polar flagella predominating. Older cultures characteristically produced highly elongated, flexible rods. All of these strains reduced NO₃ to NO₂, grew anaerobically in the presence of NO₃, and fermented glucose but oxidized sucrose, glycerol, mannose, and cellobiose. All strains were ornithine and lysine decarboxylase positive, catalase positive, and cytochrome oxidase negative. Eight of the nine strains grew in a complex Casamino Acids liquid medium containing from 0 to 32% (wt/vol) solar salt at temperatures from 23 to 37°C; the ninth strain was restricted in its growth to 0 to 20% solar salt. The guanine plus cytosine content of the deoxyribonucleic acid was 61 ± 1 mol%. This combination of morphology, salt tolerance, and guanine plus cytosine content supports the establishment of a new genus, *Halomonas*, in Family II (*Vibrionaceae*) of part 8, Gram-Negative Facultatively Anaerobic Bacteria, of *Bergey's Manual* (8th edition). The type species of this genus is *H. elongata*, the type strain of which is isolate 1H9 (= ATCC 33173). Strain 1H15 is regarded as belonging to a biovar of *H. elongata* on the basis of its production of lophotrichous cells and its inability to grow at 37°C in the presence of 32% solar salt.

Since the mid-nineteenth century, it has been recognized that bacteria are able to grow in high salt concentrations and are responsible for the reddening of salted fish. These bacteria were found to require high salt concentrations, and later Gibbons noted that the extremely halophilic bacteria could only survive in solutions containing more than 15% (wt/vol) NaCl (3). He suggested that this requirement for salt and the production of red pigment could be used as taxonomic characteristics to classify certain bacteria as extreme halophiles (3). Later, Larsen described a classification system which differentiated slight, moderate, and extreme halophiles on the basis of their upper and lower limits of NaCl requirements (9). Most of the studies that led to this classification dealt with the red halophilic bacteria.

In 1919, though, LeFevre and Round reported the isolation from cucumber fermentation brines of a group of 21 nonpigmented halotolerant bac-

teria. One of their five groups grew in 0 to 15% NaCl concentrations, whereas the other four groups of rods exhibited growth over the range of 5 to 25% NaCl (10). Recently, Matheson et al. investigated the growth characteristics and internal solute concentration of a halotolerant bacterium that arose as a contaminant on an agar medium originally prepared for extremely halophilic bacteria (13). This isolate has not been further characterized. Meanwhile, Colwell et al. (2) compared the relationship of a group of freshly isolated halophilic bacteria and halotolerant bacteria to a large number of laboratory strains of *Halobacterium*. The authors found that the freshly isolated and the laboratory strains of *Halobacterium* were very closely related. The halotolerant bacterial strains, although closely related to one another, showed very little similarity to the halophilic cultures. This situation prompted a more detailed examination of this group of halotolerant bacteria.

It is the purpose of this paper to describe these halotolerant bacteria further and to define their taxonomic position.

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MATERIALS AND METHODS

Bacterial strains. A list of the strains studied, their dates of isolation, and the environmental salt concentrations of the source materials are shown in Table 1. All isolates were from salterns containing more than 10% salt and were obtained by direct surface spread plating on the isolation medium. However, media routinely used with the halotolerant isolates contained 8% (wt/vol) solar salt to distinguish these organisms from the obligate halophiles. This salt concentration has been found to be the optimal salt concentration for the growth of these bacteria regardless of the kind of medium or the growth temperature (Vreeland and Martin, submitted for publication).

Culture maintenance. The medium of Abram and Gibbons (3) was modified as follows: 1,000 ml of distilled water, 1.0 g of yeast extract (Yeast Products, Inc., Paterson, N.J.), 7.5 g of Casamino Acids with vitamins (Difco Laboratories, Detroit, Mich.), 5.0 g of proteose peptone no. 3, 3.0 g of sodium citrate, 20.0 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.005 g of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, and 7.5 g of K_2HPO_4 , supplemented with 80 g of solar salt produced at the study site (Antilles International Salt Co., Bonaire, Netherlands Antilles). The pH was adjusted with NaOH (1 N) to 8.0 ± 0.1 before autoclaving and was 7.5 after autoclaving at 121°C for 20 min. When required, agar (Fisher Chemical Co., Springfield, N.J.) was added at 2% (wt/vol) concentration. The incubation temperature was routinely 30°C , and stock cultures were stored at 4°C .

Colonial morphology. Colonial morphology was described after growth for 7 to 14 days on the casein medium described previously (2).

Gram reaction and motility. Cultures from broth and solid media were examined both by normal procedures and by a modification of these procedures (14). A 20% (wt/vol) solar salt solution was used to wash the smears between each step, and decolorization was accomplished with acetone-ethanol (50:50, vol/vol). Wet mounts were prepared for motility determi-

nation from various growth stages and were examined by light microscopy and phase-contrast microscopy. The cultures used for staining varied from early log through the stationary growth phase and were consistent with respect to their Gram reaction.

Electron microscopy: flagellar arrangement and fine structure. The flagellar arrangement of the seven viable strains was examined by staining with 2% uranyl acetate. Log-phase cells in broth culture were transferred onto Formvar-coated grids by dipping the grids into the cell suspension. The grids were then dipped rapidly into the stain, excess stain was removed with filter paper, and the grids were air-dried overnight.

The fine structure and type of cell division of strain 1H9 were examined via transmission electron microscopy. The cells were harvested from both mid-log and stationary-phase cultures. For the initial fixation, the cell pellet was suspended in 3% glutaraldehyde in phosphate buffer for 2 h at room temperature. The cells were then suspended in 2% ion agar and postfixed in OsO_4 for 1 h followed by 2% uranyl acetate for 1 h. The cell preparations were then dehydrated with acetone and embedded in Epon 812 resin. Thin sections were made with an LKB ultramicrotome (LKB Co., Rockville, Md.) fitted with a diamond knife. The sections were stained with uranyl acetate and lead citrate solutions. Micrographs were obtained from a Phillips EM 201 (Phillips Co., Eindhoven, Holland) transmission electron microscope with Kodak electron microscop film 4489 (Estar thick base).

Carbohydrate reactions. The oxidative or fermentative utilization of various carbohydrates was studied with the bromothymol blue medium of Holding and Collee (5). Basal bromothymol blue medium was prepared with 8% solar salt (wt/vol) and supplemented before autoclaving with 0.5% sucrose, glycerol, mannose, cellobiose, gluconate, glucose, or lactose. Representative strains (1H15, 1H11, and 1H9) were also tested for their ability to use the same sugars as sole carbon sources when 0.5% (wt/vol) of the filter-sterilized solutions was added to sterile chemically defined medium composed of 5.3 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.8 g of KCl, 4.0 g of $(\text{NH}_4)_2\text{SO}_4$, 0.6 g of $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 0.04 g of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, and 80 g of NaCl in 1,000 ml of distilled water, pH 7.0, after autoclaving. The oxidative or fermentative metabolism of glucose was also determined in the medium of Hugh and Leifson (6) to which 8% solar salt was added in the tubes.

Biochemical tests. The ability of the strains to hydrolyze starch was detected on 0.5% (wt/vol) corn starch medium, which, after growth had occurred, was flooded with Gram iodine solution; clearing around the growth indicated the hydrolysis of starch. Proteolytic capability was examined using 12.0% (wt/vol) nutrient gelatin (Difco) and the casein medium previously described (2). Casein hydrolysis was presumed when clear zones appeared around the colonies. Protein hydrolysis was confirmed when the clear zones persisted after flooding the plate with 0.01% amido black in 7.5% acetic acid. Gelatin liquefaction was considered positive if the inoculated medium failed to solidify after sufficient incubation followed by cooling to 4°C . The tests performed with the Patho-Tec Rapid

TABLE 1. List of salt-tolerant strains used in this study^a

Strain no.	Date isolated	Environmental salt concn ^b
1H3	June 1974	12.8
1H6	June 1974	18.6
1H9	June 1974	25.6
1H11	June 1974	18.6
1H12	June 1974	18.6
1H14	June 1974	18.6
1H15	June 1974	15.4
1H19	June 1974	15.4
2H17	Oct. 1974	21.0

^a All strains were isolated on complex casein medium (2) containing 25% solar salt (wt/vol).

^b Samples were obtained from condensers at the Antilles International Salt Co. facility on Bonaire, Netherlands Antilles.

ID System (Warner Lambert, Morris Plains, N.J.) were for cytochrome oxidase, reduction of nitrate to nitrite, phenylalanine deaminase, lysine decarboxylase, production of H_2S from cysteine, production of indole from tryptophan, acetylmethylcarbinol (Voges-Proskauer test), urease production, esculin hydrolysis, malonate utilization, ornithine decarboxylase, and production of β -galactosidase. The use of the test strips was adopted after their comparison with traditional media showed them to be less susceptible to interference from the high salt concentrations. Methyl red tests were performed after growth of the test organism in methyl red-Voges-Proskauer medium (Difco) rehydrated with the appropriate salt solution. The results of all tests involving media and indicators were considered negative if no reaction or growth had occurred by 14 days after the time required to grow the organisms under optimal conditions.

Oxygen requirements. The oxygen requirements of the organisms were determined with thioglycollate medium (Difco) rehydrated with 8% solar salt solution (wt/vol) and by the growth on the casein medium in a candle jar. Additional studies included incubation on maintenance medium prepared with and without 0.1% KNO_3 in anaerobic jars (BBL Microbiology Systems, Baltimore, Md.), where anaerobic conditions were created with CO_2 and H_2 and were monitored with standard BBL indicator strips.

Nitrogen requirements. The nitrogen requirements were determined in media containing KNO_3 , $(NH_4)_2SO_4$, Casamino Acids, or casein.

pH range. The pH range for growth of each isolate was tested at 30°C in broth maintenance medium at the usual salt concentration. The test pH values, before sterilization, were: 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0; they remained at ± 0.1 pH unit after autoclaving.

Antibiotic susceptibility. The antibiotic susceptibility of all strains was determined on surface-seeded maintenance medium and with susceptibility disks obtained from Difco except for those containing the Vibriostat agent, 0/129, and $HgCl_2$, which were prepared in our laboratory. The following antibiotics were used: streptomycin (10 μg), penicillin (10 U), ampicillin (30 U), chloramphenicol (30 U), tetracycline (30 U), novobiocin (30 U), neomycin (30 U), bacitracin (10 U), and nalidixic acid (30 U). In addition, susceptibility to mercurials was similarly tested with $HgCl_2$ (1:5,000).

DNA base composition. The guanine plus cytosine (G+C) content of the deoxyribonucleic acid (DNA) was determined by the thermal denaturation procedure of Mandel et al. (11). Log-phase cells of 1H9 and 1H15 were ruptured, and the DNA was recovered by the method of Marmur (12). Stationary-phase cells were highly resistant to breakage procedures, even those which included various surfactants.

Salt and temperature ranges for growth. The isolates were tested for their ability to grow in broth maintenance medium at different temperatures and salt concentrations. The temperatures were: 4, 15, 23, 30, 37, and 45°C, and the solar salt concentrations tested were 0, 3.5, 8.0, 15.0, 20.0, and 32.0% (wt/vol). After 3 weeks, all of the tubes without visible growth, along with those in which growth was questionable, were placed for an additional 4 weeks at 30°C to determine whether the apparent inhibition had ac-

tually been lethal to the bacterium. Organisms which were able to resume active growth during this period were considered to have survived the exposure to the inhibitory salt and temperature conditions, whereas those organisms that did not show a return to active growth were considered killed by that individual salt or temperature condition or both.

Computer analysis. Both S_I and S_M single-linkage analyses of the strain data were performed at the University of Maryland; the programs used were developed by R. R. Colwell.

RESULTS

Morphology. The isolates were rod-shaped organisms which occurred both singly and in pairs during the log phase of growth (Fig. 1). Transmission electron microscopy of negatively stained cells (Fig. 2) showed that these cells have a typical gram-negative cell wall structure and that both curved and straight forms were present in all preparations (Fig. 1 and 2). Stationary-phase cells from the maintenance medium produced elongated, flexuous filaments of varying lengths (Fig. 1 and 2). This elongation appears to be the result of the faster growth rates obtained in shake flasks and not, as originally thought, of increased oxygen tension (Vreeland, Martin, and Litchfield, unpublished data). When isolates were grown in a chemically defined, amino acid-vitamin medium, only about 25% of the cells showed elongation.

Cell division occurred via fission (Fig. 2) and appeared to be apical and transverse in the elongated cells (Fig. 1c).

The short cells were motile and were either polarly flagellated or peritrichous (Fig. 3). Negative staining with uranyl acetate showed the position of attachment and position of the flagella on 1H9, 1H11, and 1H15 when viewed by transmission electron microscopy (Fig. 3).

Colonial morphology. On a complex solid medium, these bacteria produced smooth, glistening, opaque, white colonies about 2 mm in diameter. After 24 h the colonies began to spread, apparently as the numbers of the long, flexuous filaments increased, but they still were less than 4 mm in diameter.

Cultural and physiological characteristics. The isolates were gram negative by conventional and by modified Gram-staining techniques. The specific biochemical and physiological characteristics of the strains are listed in Table 2. They reduced NO_3 to NO_2 and were able to grow anaerobically in the presence of NO_3 (Table 2). They were catalase positive, Kovac oxidase positive, and ornithine and lysine decarboxylase positive; utilized malonate; fermented glucose; and oxidized glycerol, sucrose, mannose, and cellobiose. Most of the strains also oxidized lactose and gluconate. None of the

FIG. 1. Morphology of selected cells of the salt-tolerant isolate 1H9. Scanning electron photomicrographs of cells grown in the complex casein medium (2): (A) with shaking ($\times 4,300$); (B) from the same medium but under stationary conditions ($\times 4,300$). (C) An enlargement of an elongated cell from the same culture undergoing two divisions ($\times 15,650$).

strains produced H_2S from cysteine, hydrolyzed starch or casein, or liquefied agar. Phenylalanine deaminase and cytochrome oxidase were not produced, according to the tests employed (Table 2).

All of the strains were susceptible to $HgCl_2$ and chloromycetin and showed only slight susceptibility to penicillin, streptomycin, tetracycline, ampicillin, vibriostat O/129, novobiocin, neomycin, bacitracin, or nalidixic acid (Table 2).

pH range for growth. The broth maintenance medium was adjusted to pH 4 to 9 before autoclaving and was checked afterwards to assure the same pH levels. All nine isolates were able to grow over the pH range 5 to 9, but none grew at pH 4 (Table 2).

Salt and temperature tolerance. The salt and temperature tolerance tests performed on the nine isolates (Table 3) showed that these organisms were able to grow over a wide range

of temperature and salt concentrations. All nine isolates consistently grew, or at least survived, in salt concentrations from 3.5 to 20% added solar salt and at temperatures ranging from 4 to 45°C. Only one strain, 1H15, was unable to survive in 32% solar salt at any temperature. At 32% solar salt and 45°C, most of the strains were killed. The only strain which survived under this condition was strain 1H9. The best growth and the greatest tolerance to various salt levels were achieved at 30°C. This temperature allowed all nine strains to grow well in the maintenance medium containing no added salt as well as in maintenance medium containing 20% solar salt. Eight strains also grew well in maintenance medium with 32% solar salt and at 30°C.

When the other growth-promoting temperatures were examined in terms of their ability to promote salt tolerance, the following order was noted: 30°C > 23°C, 37°C > 15°C > 45°C >

FIG. 2. Thin-section transmission electron photomicrographs of strain 1H9 showing cell elongation (A and B) and division of the short form (C to E) found during growth on the complex casein medium under conditions of shaking. (A) One large cell ($\times 22,300$); (B) An enlargement of the marked area in A ($70,400\times$). (C to E) The sequential division of the short forms of 1H9 ($\times 15,800$).

FIG. 3. Transmission electron photomicrographs of uranyl acetate-stained cells showing flagellar arrangements and attachment. (A) strain 1H9; (B) 1H11; (C) 1H15—all at $\times 17,200$. (D) An enlargement of the flagellar attachments of a cell of strain 1H15 ($\times 103,700$).

4°C. The lowest temperature tested, 4°C, was found to inhibit growth of seven strains, but when the cultures were returned to 30°C for an additional 28-day incubation, all had survived the low temperature at the 0 or 3.5% solar salt levels (Table 3).

At the lowest temperature tested, 4°C, none of the isolates grew with 0 or 3.5% solar salt, but 1H14 and 1H9 grew at this temperature when

salt concentrations were 8 to 20%. Further research on the salt tolerance of strain 1H9 and its relationship to temperature and type of medium will be described elsewhere (Vreeland and Martin, submitted for publication).

Carbon source tests. Representative strains were selected for further examination of the individual carbon sources which could be metabolized. Strains 1H9, 1H11, and 1H15 were able

TABLE 2. Biochemical and morphological characteristics of the halotolerant bacteria

Characteristic	Strain designation ^a								
	1H15	1H14	1H12	1H9	2H17	1H6	1H3	1H19	1H11
Cells rod shaped	+	+	+	+	—	—	—	+	+
Cells curved rods	+	+	—	—	—	—	—	+	+
Cells long, flexuous rods	+	+	+	+	+	+	+	+	+
Squared ends	—	—	—	—	—	—	—	—	+
Rounded ends	+	+	+	+	—	+	+	+	—
Gram negative	+	+	+	+	+	+	+	+	+
Motile (flagellar)	+	+	+	+	+	+	+	d	+
Motile (flexing rods)	+	+	+	+	+	+	+	+	+
Single cells	+	+	+	+	+	+	+	+	+
Paired cells	+	+	+	+	+	+	+	+	+
Short chains	+	+	—	+	—	+	+	+	+
Colony <2 mm (diam)	+	+	+	+	+	+	+	+	+
Raised colonies	+	—	—	—	—	+	+	+	—
Convex colonies	—	+	+	+	+	—	—	—	—
Entire edges	+	+	+	—	+	+	—	—	+
Crenated edges	—	—	—	+	—	—	+	+	—
Opaque colonies	+	+	+	+	+	+	+	+	+
White colonies	+	+	+	+	+	+	+	+	+
Growth in thioglycollate (surface)	+	+	+	+	—	+	+	+	+
Lycine decarboxylase	+	+	+	+	+	+	+	+	+
Ornithine decarboxylase	+	+	+	+	+	+	+	+	+
Urease produced	+	+	—	+	+	+	—	—	+
NO ₃ reduced to NO ₂	+	+	+	+	+	+	+	+	+
Gelatin liquefied	+	+	—	+	—	—	—	+	—
Catalase positive	+	+	+	+	+	+	+	+	+
Cytochrome oxidase	—	—	—	—	—	—	—	—	—
Kovac's oxidase	+	+	+	+	d	+	+	+	+
Esculin hydrolyzed	+	+	+	+	—	—	+	—	—
Malonate utilized	+	+	+	+	+	+	+	+	+
β-Galactosidase produced	+	—	—	—	+	—	—	—	—
Susceptibility to:									
Penicillin	—	—	—	—	d	+	—	+	—
Streptomycin	—	—	—	—	+	—	—	—	—
HgCl ₂	+	+	+	+	+	+	+	+	+
Tetracycline	—	—	—	—	d	—	+	—	—
Ampicillin	—	—	+	—	d	—	—	+	+
Nalidixic acid	—	—	—	—	d	—	—	—	—
Bacitracin	—	—	—	—	d	+	—	+	—
Novobiocin	—	—	—	—	d	—	+	+	—
Neomycin	—	—	+	—	d	—	—	—	—
Chloromycetin	+	+	—	+	+	+	+	+	+
Glucose fermented	+	+	+	+	+	+	+	+	+
Lactose oxidized	+	+	+	+	+	—	—	—	+
Lactose alkaline	—	—	—	—	—	+	+	+	—
Glycerol oxidized	+	+	+	+	+	+	+	+	+
Sucrose oxidized	+	+	+	+	+	+	+	+	+
Mannose oxidized	+	+	+	+	+	+	+	+	+
Cellobiose oxidized	+	+	+	+	+	+	+	+	+
Gluconate oxidized	—	—	+	+	+	+	+	+	+
Growth pH 5.0–9.0	+	+	+	+	+	+	+	+	+

^a +, Positive or present for the organism listed; —, negative or absent for the organism listed; d, test not done.

to utilize glucose, gluconate, glycerol, lactose, mannose, and sucrose as their sole sources of carbon (Table 4). Additionally, 1H9 was tested and was found to grow with cellobiose, succinate,

mannitol, alanine, and glutamine as sole carbon sources.

DNA base composition. The G+C contents of the DNAs of strains 1H9 and 1H11 were 60.5

TABLE 3. *Tolerance of salt-tolerant strains to various temperature and salt conditions*

Incubation temp (°C) ^a	% Solar salt (wt/vol) ^b	% Growth Survival	
		Growth	Survival
4	0	0	100
	3.5	0	100
	8.0	22.2	77.8
	15.0	22.2	77.8
	20.0	11.1	88.8
	32.0	0	66.6 ^c
15	0	44.4	55.6
	3.5	100	NA
	8.0	100	NA
	15.0	100	NA
	20.0	100	NA
	32.0	0	66.6 ^c
23	0	100	NA
	3.5	100	NA
	8.0	100	NA
	15.0	100	NA
	20.0	100	NA
	32.0	77.8 ^c	0
30	0	100	NA
	3.5	100	NA
	8.0	100	NA
	15.0	100	NA
	20.0	100	NA
	32.0	88.9 ^c	0
37	0	100	NA
	3.5	100	NA
	8.0	100	NA
	15.0	100	NA
	20.0	100	NA
	32.0	66.6	11.1 ^c
45	0	0	88.9 ^c
	3.5	88.9 ^c	0
	8.0	88.9 ^c	0
	15.0	100	NA
	20.0	88.9	11.1
	32.0	0	11.1

^a Incubation was under stationary conditions.^b The solar salt was incorporated in the broth maintenance medium.^c No growth after 28 days after transfer to 30°C; culture assumed to be dead. Abbreviation: NA, Not applicable.

± 0.5 mol% as determined by buoyant density or thermal denaturation curves or both (11, 12).

Taxonomic interrelationships. Feature-frequency analysis of all of the tests performed on these bacteria indicated that the following characteristics are distinctive to all members of this group (Table 5). All are gram-negative, rod-shaped cells with flexuous filaments during the stationary growth phase, and all produce small white colonies on solid media. They produce a heavy, even turbidity in broth at pH 5 to 9 and can grow over the salt range of 0 to 32% solar salt at a temperature of 30°C. They are catalase and Kovac's oxidase positive and are able to

reduce nitrate and grow anaerobically when NO₃ is present. They ferment glucose and oxidize other sugars. Ornithine and lysine are decarboxylated, but the metabolism of other substrates differs widely among the strains. The only antimicrobial compound to which all are susceptible is mercuric chloride.

Cluster analysis on the basis of positive matches resulted in the matrix shown in Fig. 4. The overall similarity was greater than 80%, with strains 1H14 and 1H15 demonstrating 100% similarity to each other, whereas 1H19 was 100% similar to 1H15. When examined via the *S_M* matching coefficient, the level of similarity of these three strains decreased somewhat due to differences in urease production, gluconate oxidation, and susceptibility to ampicillin.

A dendrogram (Fig. 5) depicting the interrelatedness of the strains emphasizes their intra-group homogeneity. Further analysis revealed that strain 1H9 possessed 96.5% similarity to the computer-determined hypothetical mean organism (HMO), and this strain was therefore considered to be most representative of the group.

DISCUSSION

Based on the natural environment of the halotolerant bacteria, their ability to grow well in high salt concentrations, and a demonstrated requirement for sodium ions (Vreeland and Martin, submitted for publication), it would seem advantageous to place these organisms in a taxonomic position close to that of the *Halobacteriaceae*. However, in a major study of the taxonomy of the obligately halophilic bacteria, Colwell et al. (2) showed clearly that there is little similarity between these two groups of bacteria,

TABLE 4. *Utilization^a of carbon sources by representative strains*

Carbon source ^b	Strain		
	1H9	1H11	1H15
Glucose	+	+	+
Gluconate	+	+	+
Glycerol	+	+	+
Lactose	+	±	+
Mannose	+	+	+
Sucrose	+	+	+
Cellobiose	+	±	+
Succinate	+	NT	NT
Mannitol	+	NT	NT
Alanine	+	NT	NT
Glutamine	+	NT	NT
No additional carbon source	—	—	—

^a Growth was estimated visually.^b Basal medium contained 8% NaCl, 0.4% (NH₄)₂SO₄ as nitrogen source, and other compounds, as described in the text. Symbols: —, No visible growth; ±, slight growth; +, excellent growth; NT, not tested.

TABLE 5. Feature frequency of the biochemical and morphological characteristics of the nine halotolerant strains studied

Characteristic	%
Morphological	
Rod shaped	100
Flexuous filaments formed in late log-stationary phase	100
Single cells	100
Paired cells	100
Chains (fewer than five cells)	77.8
Growth on agar	
Colony <2 mm (24 h)	100
Opaque colonies	100
White colony	100
Glistening colony	100
Smooth colony	100
Entire edges	66.6
Raised colonies	44.4
Convex colonies	44.4
Crenated edges	33.3
Growth in broth	
Heavy, even turbidity	100
Growth at pH 5.0 to 9.0 in 8% NaCl	100
Physiological	
Gram-negative	100
NO ₃ reduced to NO ₂	100
Growth anaerobically with NO ₃	100
Growth anaerobically without NO ₃	0
Surface growth in thioglycollate	88.9
Biochemical	
Catalase-positive	100
Kovac's oxidase present	100
Ornithine decarboxylase present	100
Lysine decarboxylase present	100
Glucose fermented	100
Glycerol, sucrose, mannose, and cellobiose oxidized	100
Malonate utilized	100
Gluconate oxidized	77.8
Lactose oxidized	66.6
Urease present	66.5
Esculin hydrolyzed	55.5
Lactose alkaline	44.4
Gelatin liquefied	44.4
β -Galactosidase present	33.3
Indole produced	22
Methyl red positive	11
Voges-Proskauer positive	11
H ₂ S produced from cysteine	0
Casein hydrolyzed	0
Starch hydrolyzed	0
Agar liquefied	0
Cytochrome oxidase present	0
Phenylalanine deaminase present	0
Susceptible to:	
HgCl ₂ (1:5,000)	100
Chloromycetin (30 U)	88.8
Ampicillin (30 U)	50
Penicillin G (10 U)	25
Bacitracin (10 U)	25
Novobiocin (30 U)	25
Neomycin (30 U)	12.5
Tetracycline (30 U)	12.5
Nalidixic acid (30 U)	0
Streptomycin (10 μ g)	0
Vibriostat O/129	0

FIG. 4. Similarity matrix based on positive matches for the nine halotolerant strains studied.

FIG. 5. Dendrogram showing the interrelatedness of the halotolerant isolates based upon the S_j matching coefficient. The asterisk designates the type strain, 1H9, selected on the basis of its similarity to the hypothetical mean organism.

less than 40%. A further restriction to such placement is that the organisms described in this paper have a typical gram-negative cell wall-membrane complex (Fig. 2), and, unlike the halobacteria (9), they do not contain carotenoid pigments but possess fatty acids and diacylphospholipids instead of glyceryl-ether lipids (Kiefer and Litchfield, unpublished data). Unusual cell wall and lipid compositions are all requirements for inclusion with the *Halobacteriaceae* (1).

The variety of irregularly spaced bends and convolutions present in the cells of this unusual group of bacteria (Fig. 1 and 2) would indicate that these strains are flexible. This flexibility, plus the high degree of motility, a characteristic spinning motion, and the high G+C value might argue that these isolates are related to such genera as *Spirillum*, *Aquaspirillum*, *Oceanospirillum* (7), and *Serpens* (4). Certainly, it seems possible that they could be included in this group. However, the fermentative attack on glucose, anaerobic growth on nitrate, presence of urease, and especially the lack of cytochrome oxidase would all seem to argue against the

inclusion of the isolates described here as members of any of these genera (8).

There is one other group of bacteria which are known to have curved cells, variable flagellation, and mixed attacks on different carbohydrates: the *Vibrionaceae*, Family II, Part 8 of *Bergey's Manual* (1). Besides the already mentioned characteristics, the family is also described as facultatively anaerobic, with a nonexacting nutrition, often isolated from freshwater and seawater, and with G+C values ranging from 39 to 63 mol% (1). However, the group of halotolerant isolates described here cannot be adequately accommodated in any of the existing genera in this family. Justification for the establishment of a new genus for these organisms, therefore, is based on the following characteristics distinctive to this group: nonsusceptibility to the vibriostat agent (O/129), negative cytochrome oxidase test, extreme salt tolerance, distinctive morphology cycle, glucose fermentation, flexible motility, and high G+C content of the DNA. We therefore propose for these organisms the establishment of a new genus, *Halomonas*, in the family *Vibrionaceae*.

Halomonas gen. nov. (Gr. n. hals, halos salt, the sea; Gr. monas small rod; M. L. fem. n. *Halomonas* the salt short rods).

Cells are rod shaped and occur singly or in pairs in the logarithmic phase of growth. Elongated, flexuous rods are produced during the stationary growth phase in complex medium. In chemically defined media, shorter flexuous rods are observed in approximately 25% of the cultures.

Gram negative. Motile. Flagellar arrangement depends on the strain, but it is either lateral or polar. On solid, complex media containing 8% salt, colonies are white to cream, opaque, and less than 2 mm in diameter; spreading may occur after extended incubation.

Facultatively anaerobic. NO_3 is reduced to NO_2 . Catalase positive; generally Kovac oxidase positive, but all strains are cytochrome oxidase negative. Ornithine and lysine are decarboxylated, but phenylalanine is not deaminated; H_2S is not produced from cysteine; and casein and starch are not usually hydrolyzed.

Glucose is fermented without gas production. Glycerol, sucrose, mannose, and cellobiose are oxidized. These carbohydrates can also serve as sole carbon sources with $(\text{NH}_4)_2\text{SO}_4$ as the nitrogen source.

All cultures are susceptible to HgCl_2 (1:5,000), and most are susceptible to chloromycetin (30 U). Generally resistant to penicillin (10 U), ampicillin (30 U), tetracycline (30 U), 2,4-diamino-6,7-diisopropyl pteridine (Vibriostat O/129), no-

vobiocin (30 U), streptomycin (10 µg), bacitracin (10 U), nalidixic acid (30 U), and neomycin (30 U).

Growth occurs from pH 5 to 9 in Casamino Acid medium containing 8% solar salt. The strains grow in Casamino Acid medium or nutrient broth without added salt or in similar media containing up to 32% solar salt when incubated at temperatures of 23 to 37°C. Growth can also occur in media with 3.5 to 20% solar salt at 15 and 45°C. Optimum conditions for growth on complex media are 3.5 to 8% solar salt (wt/vol) at 30 or 37°C.

The G+C content of the DNA is 60.5 ± 0.5 mol%.

The type species is *H. elongata* sp. nov.

Halomonas elongata sp. nov. (e-lon-ga'ta. L. fem. part. adj. *elongata* elongated, stretched out).

Inasmuch as the genus currently contains only one species, the description of this species is the same as that given above for the genus.

The type strain of *H. elongata* is 1H9, a culture of which has been deposited in the American Type Culture Collection (ATCC 33173). It, along with most of the strains, possesses urease and liquefies gelatin. The only distinguishing characteristics noted, so far, are that it is able to produce slight visible growth at 4°C and survives 45°C in 8 to 20% solar salt.

Strain 1H15 (ATCC 33174) is regarded as belonging to a biovar of this species because its cells are lophotrichous and it is unable to grow at 37°C in the presence of 32% solar salt.

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