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Taxonomic Study and Amended Description of *Vibrio costicola*

M. T. GARCIA,[1] A. VENTOSA,[1]* F. RUIZ-BERRAQUERO,[1] AND M. KOCUR[2]

Departamento de Microbiología, Facultad de Farmacia, Universidad de Sevilla, Seville, Spain,[1] and Czechoslovak Collection of Microorganisms, J. E. Purkyne University, Brno, Czechoslovakia[2]

A total of 54 moderately halophilic vibrios, which were isolated from several salterns located in different areas of Spain, were examined by using a wide range of morphological, physiological, biochemical, and nutritional tests. The resulting data, together with data for four reference *Vibrio costicola* strains including the type strain *V. costicola* NCMB 701, and other marine species that were similarly examined, were compared by using several numerical taxonomic methods. There was a strong similarity between the 54 isolates and four reference strains of *V. costicola* that were isolated from cured meats. On the basis of these and other molecular data, including guanine-plus-cytosine content of the deoxyribonucleic acid and the plasmid content, we propose an amended description of this species.

Vibrio costicola is a bacterium which is able to grow over a wide range of salt concentrations and thus is included in the moderately halophilic category (11). This species was isolated and described for the first time by Smith (24), as "*V. costicolus*," from rib bones in certain Australian bacon, suggesting the name "*costicolus*" (rib dweller). At present, *V. costicola* is included as a valid species in the Approved Lists of Bacterial Names (23).

From a physiological point of view, many studies have been carried out with this species, and in fact, it has been considered as an organism representative of moderately halophilic bacteria. However, only a few taxonomic studies have been carried out, and all of them have studied organisms isolated from cured meats and curing brines (7, 24, 29; J. Robinson, Ph.D. thesis, McGill University, 1950).

In the *Bergey's Manual of Determinative Bacteriology* (8th ed.), this species was included as a member of the genus *Vibrio* and named correctly as *V. costicola*, but the taxonomic description was not very exhaustive (22). The recent edition of *Bergey's Manual of Systematic Bacteriology* also includes this species in the genus *Vibrio* and provides more phenotypic characteristics, especially nutritional tests, but is based only upon two strains isolated from cured meats (1).

In a taxonomic study of gram-negative rods isolated from solar salterns, Ventosa et al. (28) showed that microorganisms with phenotypic characteristics similar to those of *V. costicola* were present in high proportions in some ponds of the salterns. In the present work we isolated and characterized 54 vibrios from several hypersaline habitats and studied them in depth, together with the type strain *V. costicola* NCMB 701^T as well as some other strains of this species isolated from cured meats and other reference strains.

MATERIALS AND METHODS

Bacterial strains. The 54 strains studied were isolated from several solar salterns in Spain, located near Alicante, Huelva, and Cadiz, in the southeast, south, and southwest of Spain, respectively, and from the Canary Islands (Spain) in the Atlantic Ocean. The isolation medium, as well as the methodology, has been previously described (28). The selection of the strains was made on the basis of the Gram stain, aerobic and anaerobic growth, salt response, and oxidase test. The 54 strains selected were moderately halophilic, gram-negative, curved rods; they were facultatively anaerobic and oxidase positive.

The reference strains used in this study included four strains of *V. costicola* isolated from cured meat: *V. costicola* NCMB 701^T (type strain), *V. costicola* CCM 2811, *V. costicola* 6, and *V. costicola* AV3; the two latter strains were kindly supplied by G. A. Gardner, Ulster Curers' Association, Belfast, Ireland. Also included were four marine vibrios (*V. alginolyticus* CECT 521, *V. natriegens* CECT 526, *V. parahaemolyticus* CECT 511^T, and *V. pelagius* CECT 527^T) and two moderately halophilic bacteria (*Deleya halophila* CCM 3662^T and *Halomonas elongata* ATCC 33173^T).

The maintenance medium was complex medium supplemented with 10% (wt/vol) salts, with the following percent composition (wt/vol): NaCl, 8.1; $MgCl_2$, 0.7; $MgSO_4$, 0.96; $CaCl_2$, 0.036; KCl, 0.2; $NaHCO_3$, 0.006; NaBr, 0.0026; supplemented with 0.5% (wt/vol) Proteose-Peptone no. 3 (Difco Laboratories), 1% (wt/vol) yeast extract (Difco), 0.1% (wt/vol) glucose. This medium was solidified with 2% (wt/vol) agar (Difco).

Phenotypic characterization. Tests for total of 151 characteristics, including morphological, physiological, biochemical, and nutritional tests (Table 1), were carried out. The methodology used has been previously described (19, 28). Unless otherwise indicated, the tests were carried out with 10% (wt/vol) salts (pH 7.5), and the incubation was at 37°C in sealed containers. The *ortho*-nitrophenyl galactoside (ONPG) test as well as tests for the presence of arginine, ornithine, and lysine decarboxylase, using the Møller decarboxylase broth, were carried out as described by Koneman et al. (12). Tyrosine hydrolysis was detected by inoculating plates with the maintenance medium containing 0.5% (wt/vol) L-tyrosine and examining them for up to 7 days until the appearance of clear zones around colonies (3). Citrate utilization was tested in Simmons citrate, and lecithinase production was tested in egg yolk medium (3).

Electron microscopy. Representative strains were grown for 18 h on the surface of maintenance medium plates covered with liquid medium. Samples of the liquid cultures were negatively stained with a 1% (wt/vol) solution of phosphotungstic acid (pH 7.0).

Numerical analysis. The 119 differential features obtained were used for the numerical analysis. Positive and negative results were coded as 1 and 0, respectively; noncompatible or missing data were coded as 9. Strain similarities were

* Corresponding author.

TABLE 1. Phenotypic characteristics of 58 strains of *V. costicola* and the type strain *V. costicola* NCMB 701[T]

Characteristic[a]	No. of strains positive	Reaction of strain NCMB 701[T]
Growth at % saline:		
0.5	55	+
15	57	+
20	57	+
25	2	−
Growth at temp:		
5°C	57	+
50°C	24	−
Catalase	53	+
Indole production	1	−
Methyl red	31	−
Voges-Proskauer	52	+
Nitrate reduction	7	−
Citrate	12	−
H_2S production	32	−
Phenylanine deaminase	19	−
Arginine decarboxylase	53	+
Hydrolysis of:		
Esculin	41	+
Casein	54	−
Starch	5	−
Tyrosine	14	−
Tween 80	30	−
Phosphatase	18	−
Deoxyribonuclease	48	−
Lecithinase	49	+
Hemolysis	49	+
Acid production from:		
Glycerol	47	+
D-Glucose	57	+
m-Inositol	5	−
Maltose	56	+
D-Mannitol	35	+
Mannose	27	−
Salicin	12	−
Sucrose	50	+
D-Trehalose	57	+
D-Xylose	4	+
Carbohydrate utilization:		
Amygdalin	6	−
D-Cellobiose	5	−
D-Fructose	40	−
D-Fucose	32	+
D-Galactose	12	−
D-Galactosamine	5	−
D-Gluconolactone	3	−
D-Glucose	44	+
D-Glucosamine	38	−
Inulin	10	−
Lactose	2	−
Maltose	46	−
D-Mannose	12	−
D-Melibiose	5	−
D-Raffinose	11	−
L-Rhamnose	2	−
D-Ribose	41	−
Salicin	13	−
Starch	49	−
Sucrose	50	+
D-Trehalose	57	+
D-Xylose	4	+
Alcohol utilization:		
Adonitol	7	−
Dulcitol	7	−
Erythritol	1	−
Ethanol	46	−
D-Mannitol	39	+
m-Inositol	27	−
Propanol	8	−
D-Sorbitol	18	−
Carboxylic acid utilization:		
N-Acetylglucosamine	56	+
DL-α-Aminobutyrate	4	−
δ-Aminovalerate	9	−
Benzoate	1	−
Butyrate	50	−
Caprylate	42	−
α-Cetoglutarate	24	−
Citrate	14	−
DL-Glycerate	38	−
D-Gluconate	44	+
D-Glucuronate	12	−
Glutamate	57	+
Hippurate	5	−
Lactate	56	+
DL-Malate	52	−
Oxalate	12	−
Propionate	53	−
Quinate	1	−
D-Saccharate	8	−
Succinate	57	+
D-Tartrate	1	−
Amino acid utilization:		
L-Alanine	56	+
DL-Arginine	49	−
L-Asparagine	55	+
Phenylalanine	28	−
Glycine	51	−
Glutamic acid	12	−
L-Glutamine	54	−
L-Histidine	36	+
L-Isoleucine	2	−
L-Leucine	15	−
DL-Lysine	10	−
L-Ornithine	54	+
L-Proline	57	+
L-Serine	57	+
L-Threonine	47	+
L-Valine	4	−

[a] All strains were gram-negative curved rods, motile by polar flagellum. They were facultative anaerobes and grew in 3, 5, and 10% (wt/vol) salts and at 15, 25, 37, and 45°C and pH 5, 6, 7, 8, 9, and 10; were oxidase positive and hydrolyzed gelatin, and grew on glycerol, acetate, fumarate, and pyruvate as the sole source of carbon and energy. None grew at 0 and 30% (wt/vol) salts, at pH 4, 11, and 12, reduced nitrites, produced acid from D-arabinose or lactose, produced β-galactosidase, or decarboxylated lysine or ornithine. None grew on esculin, D-arabinose, *cis*-aconitate, *p*-hydroxybenzoate, malonate, salicylate, or suberate as the sole source of carbon and energy. None grew on allantoin, aspartic acid, betaine, creatine, ethionine, L-methionine, putrescine, sarcosine, or L-tryptophan as the sole source of carbon, nitrogen, and energy.

estimated with both simple matching (S_{SM}) (27) and Jaccard coefficients (S_J) (8), and cluster analysis was achieved by using the unweighted pair group method of averages (UPGMA), single linkage, complete linkage, and centroid linkage algorithms (26). Cophenetic correlation was also obtained in each method (26). The test error was evaluated by examining 10 strains in duplicate (25). These computations were performed with the MINT program of F. J. Rolf, State University of New York, Stony Brook, N.Y., using a UNIVAC 1108 computer in the Computer Centre, University of Seville, Spain.

DNA base composition. Exponential-phase cells of some representative strains were ruptured, and the deoxyribonucleic acid (DNA) was purified by using the method of Marmur (12). The guanine-plus-cytosine (G+C) content of the DNA was determined from the midpoint value (T_m) of the thermal denaturation profile (13) obtained with a Perkin-Elmer UV-Vis 551S spectrophotometer at 260 nm; this instrument was programmed for temperature increases of 1.0°C/min. The T_m was determined by the graphic method described by Ferragut and Leclerc (6), and G+C content was calculated from this temperature by using the equation of Owen and Hill (16), using standard saline citrate (SSC). The T_m value of reference DNA from *Escherichia coli* NCTC 9001 was taken as 74.6°C in 0.1× SSC (17).

Plasmid analysis. Plasmid profile analysis was performed by horizontal gel electrophoresis on 0.5% agarose gels in Tris borate buffer containing tris(hydroxymethyl)aminomethane base (89 mM), disodium ethylenediaminetetraacetate (2.5 mM), and boric acid (8.9 mM), pH 8.2 (14). The method used was a modification of the Eckhardt procedure (5) described by Rosenberg et al. (21). After electrophoresis for 1 h at 1.5 V/cm followed by 3 h at 7 V/cm, the gels were stained for 20 min with ethidium bromide (5 μg/ml) in electrophoresis buffer at room temperature. The bands from the washed gels were then visualized by a shortwave ultraviolet light (254 nm) transilluminator. Photographs were taken with a Nikon camera equipped with a red filter (Wratten no. 23A) and Kodak Tri-X-Pan film. The molecular weights of the plasmids were estimated from a double-logarithmic plot constructed on the basis of the seven plasmids of *Rhizobium leguminosarum* T3. This strain was obtained from J. E. Beringer.

RESULTS

Reproducibility of results. The inclusion of the pairs of randomly chosen duplicate strains in the analysis enabled experimental test error to be calculated. The probability (P) of an erroneous result averaged 3.5%, equal to an observed S_{SM} value of about 93% between duplicate cultures. A small number of tests were responsible for most of the test error.

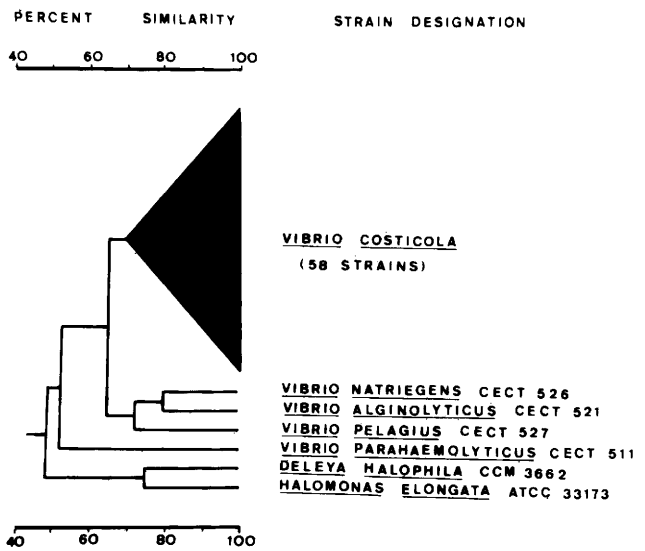

FIG. 1. Simplified dendrogram showing clustering of the 58 strains of *V. costicola* and 6 reference strains, based on the simple matching coefficient and unweighted average linkage clustering methods of numerical taxonomy.

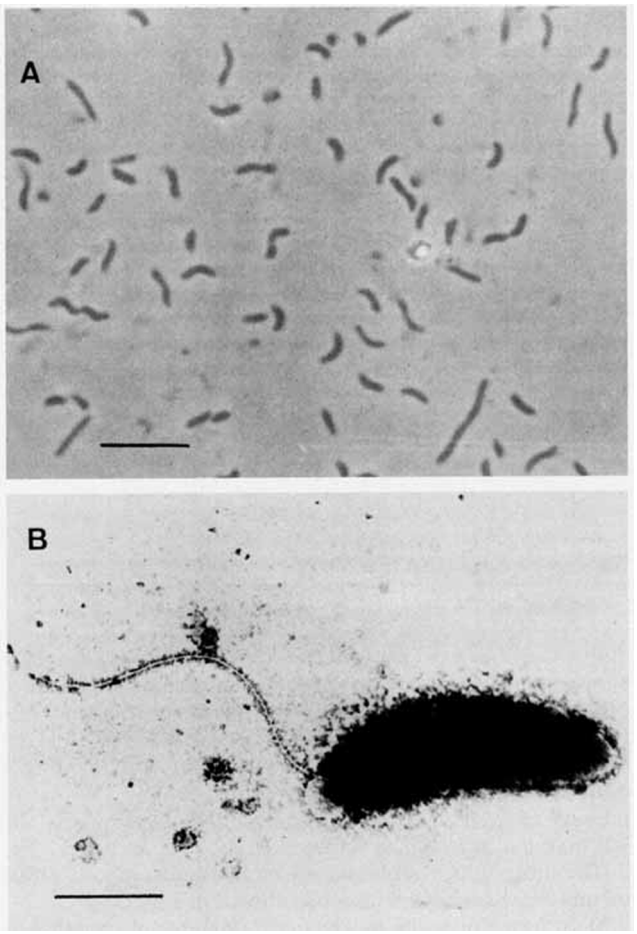

FIG. 2. (A) Phase-contrast micrograph of *V. costicola* NCMB 701^T. Bar, 10 μm. (B) Negatively stained *V. costicola* A-514 cell showing the polar monotrichous flagellum. Bar, 1 μm.

These tests, with a test variance of 0.1, were acid production from trehalose, arginine decarboxylase, utilization of dulcitol, fucose, inulin, mannitol, *m*-inositol, and raffinose as sole sources of carbon and energy, and utilization of glutamic acid as the sole source of carbon, nitrogen, energy. The utilization of D-mannose as the sole source of carbon and energy was found to be insufficiently reproducible (test variance = 0.2), and the result of this test was not included in the data matrix.

Numerical analysis. The results of the numerical study of the characteristics of the strains grouped by means of the S_{SM} coefficient and UPGMA clustering yielded the dendrogram shown in Fig. 1. Cluster composition was not markedly affected by either the coefficient or the clustering method when the S_J coefficient and single linkage and centroid linkage algorithms were used but were very distorted when the complete linkage algorithm was used. Cophenetic values were 0.924, 0.852, 0.896, and 0.082 for the UPGMA, single linkage, centroid linkage, and complete linkage, respectively.

In the dendrogram obtained by the S_{SM} coefficient and the UPGMA clustering, at a 70% similarity level all the isolates from hypersaline habitats grouped in a single phenon together with the *V. costicola* reference strains. The other reference strains were not grouped. In the *V. costicola* phenon, the four reference strains were grouped in a

TABLE 2. Plasmids detected in representative strains of *V. costicola* and other reference strains

Microorganism	No. of plasmids	Approx mol wt (10^6)
V. costicola NCMB 701	1	ND[a]
V. costicola CCM 2811	1	ND
V. costicola 6	1	255
V. costicola AV3	3	310, 440, 520
A-514	0	
E-359	0	
E-379	1	260
GC-19	2	255, 555
H-105	2	255, 555
H-490	1	165
V-11	1	310
V-16	0	
SF-18	1	ND
V. alginolyticus CECT 521	0	
V. natriegens CECT 526	0	
V. parahaemolyticus CECT 511	0	
V. pelagius CECT 527	0	
Deleya halophila CCM 3662	0	
Halomonas elongata ATCC 33170	0	

[a] ND, Not determined.

subphenon with a similarity level of 80% and joined to the other subphenon, constituted by the majority of isolates from natural habitats, at a 73.5% similarity level. Only two strains isolated from a solar saltern were not included in either subphenon. The results obtained for the *V. costicola* phenon as well as for the type strain *V. costicola* NCMB 701^T are summarized in Table 1.

The morphology and flagellation of representative strains of this *V. costicola* phenon are shown in Fig. 2.

DNA base composition. The G+C contents of the DNAs of 10 representative strains of *V. costicola* ranged from 49.4 to 50.5 mol% (mean value = 49.9 mol% ± 0.55 standard deviation). The G+C content of the DNA of the type strain *V. costicola* NCMB 701^T was 49.9 mol%, and the values obtained for *V. alginolyticus* CECT 501, *V. natriegens* CECT 526, *V. parahaemolyticus* CECT 511, and *V. pelagius* CECT 527 were 47.0, 47.2, 47.6, and 45.7 mol%, respectively.

Plasmid profiles. Of the 58 strains included in the *V. costicola* phenon, in 22 strains extrachromosomal DNA bands were not detected, in 29 strains only one plasmid was detected, in 6 strains two plasmids were detected, and in 1 strain there were three different plasmids. The plasmid contents and the approximate molecular weights in some representative strains of *V. costicola* and other marine vibrios and moderately halophilic bacteria are summarized in Table 2.

DISCUSSION

V. costicola is a species of the genus *Vibrio* that was first described by Smith (24) and then included in the editions of *Bergey's Manual* (1, 2, 22) and the Approved Lists of Bacterial Names (23).

The description of this species has been based on strains isolated from cured foods since this organism can be present in foods preserved with brines. Recent studies of solar salterns revealed that microorganisms phenotypically similar to this species are present in ponds and predominate in ponds with 10 to 25% (wt/vol) salts (20). However, in similar studies carried out with hypersaline soils, they were not isolated (18). Their presence in hypersaline lakes and in seawater has not been reported, probably because studies approaching this matter have not been carried out.

In the present study, we compared the characteristics of some strains of *V. costicola* isolated from cured meats together with a large number of isolates from several solar salterns covering a wide geographical area and ponds with different salinities. All the isolates were grouped together with the *V. costicola* reference strains (Fig. 1), indicating that they have very similar phenotypic characteristics (Table 1). The fact that the four strains from culture collections clustered separately in a subphenon is mainly because of their different nutritional capabilities, being less versatile than fresh isolates, which could be justified because the organisms have been maintained in culture collections for long periods.

Comparing our results with those of Smith (26), we found discrepancies in three tests for a total of 21 common characters: nitrate reduction, casein hydrolysis, and acid production from maltose. Gardner (7) studied 17 strains of *V. costicola* isolated from cured meats, and in the 10 characteristics common with our study there are discrepancies in the following tests: nitrate reduction and gelatin and casein hydrolyses. Hence, among the small number of tests that can be compared, it is clear that differences in nitrate reduction and proteolytic activity of strains isolated from cured meats or natural habitats can be found. In a recent overview, West and Colwell (29) mentioned some features useful in the identification of species of the genus *Vibrio*, including *V. costicola*. There is a good agreement between our results and those obtained in this broad study.

With respect to the recent description of this species in *Bergey's Manual* (1), in which a broad screening of nutritional tests is included, this description is based only on the results obtained with two strains; compared with our data, in 34 tests the results are similar, in 6 tests (Voges-Proskauer, gelatin hydrolysis, and utilization of glutamate, malate, ornithine, and proline) different results were obtained, and in 15 tests there were differences between different strains.

On the basis of those results and the description of other marine vibrios, we chose the tests that may be useful in the

TABLE 3. Phenotypic characteristics that differentiate *V. costicola* from other *Vibrio* species of marine origin

Characteristic	Reaction[a]				
	V. costicola	*V. alginolyticus*	*V. natriegens*	*V. parahaemolyticus*	*V. pelagius*
Lateral flagella	−	+	−	+	−
Growth in 20% (wt/vol) salts	+	−	−	−	−
Arginine decarboxylase	+	−	−	−	−
Voges-Proskauer	+	+	−	−	−
Gelatin hydrolysis	+	+	d	+	−
Starch hydrolysis	−	+	d	+	−
Growth on:					
cis-Aconitate	−	+	+	+	+
Ornithine	+	−	+	−	+

[a] +, 90% or more of strains are positive; −, 90% or more are negative; d, 11 to 89% are positive.

differentiation of *V. costicola* from other related halophilic vibrios from marine orgin (Table 3).

The G+C contents of the 10 representative strains of *V. costicola* ranged from 49.4 to 50.5 mol%. This range is very narrow and includes strains from culture collections as well as fresh isolates from different sampling sites, in agreement with the criteria of De Ley (4), which mentioned that the G+C contents of strains belonging to the same species should have a range of about 1% mol. For the type strain *V. costicola* NCMB 701^T, the G+C content obtained by us by means of the T_m determination was 49.9 mol%; this value is very near the 50.0 mol% obtained by a CsCl gradient (1). Thus, the G+C content of this species is included in the range that is currently accepted for the genus *Vibrio*, i.e., 38 to 51 mol% (1). The G+C contents obtained for the marine vibrios used as reference strains were in the corresponding range given for each species (1).

It is well known that halobacteria have a large amount of extrachromosomal DNA which can constitute in some strains up to 36% of the total DNA (9, 15). In a recent study, two to five high-molecular-weight plasmids were detected in the majority of halobacteria examined (Gutierrez et al., submitted for publication). To know if moderately halophilic bacteria could also have such a high proportion of large plasmids, we thought it would be interesting to investigate their presence in the strains studied in the present work. By means of agarose gel electrophoresis, no plasmids were detected in 22 of the strains of *V. costicola* examined. However, in the other 29 strains, one to three different bands of extrachromosomal DNA were detected. We conclude that in *V. costicola* the number of plasmids detected is not as high as that found in halobacteria, but the high molecular weight of some of the megaplasmids detected in some strains is surprising. As in the case of halobacteria, the possible relationships between the plasmid content and the phenotypic characteristics that they can codify remain unknown.

Since it seems justified that the strains isolated from hypersaline habitats should be included in the species *V. costicola*, on the basis of our results as well as those reported in *Bergey's Manual* (1), the following formal proposal for the amended description of this species is made.

Amended description of *Vibrio costicola* (Smith 1938). *Vibrio costicola* (cos.ti'co.la. L. n. *costa*, rib; L. subst. *cola*, dweller; M. L. n. *costicola*, rib dweller) curved rods are gram negative, nonsporeforming, 0.5 by 1.5 to 3.2 μm. Cell motile by one polar flagellum. Facultative anaerobes.

Circular, convex, opaque, cream-colored pigmented colonies (2 to 3 mm) develop on 10% (wt/vol) marine salts solid medium after 2 days of incubation at 37°C. No pigments. Broth cultures are uniformly turbid.

The optimal marine salts concentration for growth is 10% (wt/vol) at 37°C; grows at marine salts concentrations between 0.5 and 20% (wt/vol). No growth in the absence of NaCl.

Growth at 5 to 45°C and pH 5 to 10 (optimal growth at 37°C and pH 7.5).

Catalase and oxidase are produced. Acid is produced from D-glucose, maltose, and D-trehalose; acid is not produced from D-arabinose, inositol, lactose, or D-xylose. Gelatin and casein are hydrolyzed; starch is not hydrolyzed.

Voges-Proskauer and arginine decarboxylase tests are positive. Indole, β-galactosidase, lysine, and ornithine decarboxylase tests are negative. Nitrates usually not reduced to nitrites; no reduction of nitrite.

The following compounds are utilized as sole carbon and energy sources: D-trehalose, glycerol, acetate, *N*-acetylglucosamine, fumarate, glutamate, lactate, DL-malate, pyruvate, propionate, or succinate.

The following compounds are not utilized as sole carbon and energy sources: amygdalin, D-arabinose, D-cellobiose, esculin, D-galactosamine, D-gluconolactone, lactose, D-melibiose, L-rhamnose, D-xylose, erythritol, *cis*-aconitate, benzoate, *p*-hydroxybenzoate, hippurate, malonate, quinate, salicylate, suberate, and D-tartrate.

The following compounds are utilized as sole carbon, nitrogen, and energy sources: L-alanine, L-asparagine, L-glutamine, L-ornithine, L-proline, and L-serine.

The following compounds are not utilized as sole carbon, nitrogen, and energy sources: L-allantoin, L-aspartic acid, betaine, creatine, ethionine, L-isoleucine, L-methionine, putrescine, sarcosine, L-tryptophan, and L-valine.

Isolated from hypersaline habitats and from salted food.

G+C content of the DNA is between 49.4 and 50.5 mol%.

The type strain is NCMB 701^T. The description of this strain is the same as that given above for the species except that acid is produced from D-xylose, casein is hydrolyzed, and DL-malate and propionate are utilized as sole sources of carbon and energy. G+C content of the DNA of this strain is 50.0 (CsCl) or 49.9 (T_m) mol%, respectively.

Six of the newly isolated strains have been deposited with the Czechoslovak Collection of Microorganisms under the following numbers: CCM 3523 (strain A-514), CCM 3939 (strain E-357), CCM 3935 (strain GC-19), CCM 3938 (strain H-490), CCM 3936 (strain V-11), and CCM 3937 (strain SF-18).

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