Analysis of 16S rRNA Gene Sequences of *Vibrio costicola* Strains: Description of *Salinivibrio costicola* gen. nov., comb. nov.

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The phylogenetic positions of six *Vibrio costicola* strains were determined by direct sequencing and analysis of their PCR-amplified 16S ribosomal DNAs. A comparative analysis of the sequence data revealed that the moderate halophile *V. costicola* forms a monophyletic branch that is distinct from other *Vibrio* species and from moderately halophilic species of other genera. These results complement phenotypic and genotypic data determined previously. The molecular evidence, together with several phenotypic differences, distinguishes *V. costicola* from species of the genus *Vibrio* and other species belonging to the gamma subclass of the *Proteobacteria* and indicates that *V. costicola* should be placed in a new and separate genus. The name *Salinivibrio costicola* gen. nov., comb. nov. is proposed for this bacterium. The guanine-plus-cytosine content of the DNA is 49.4 to 50.5 mol%. The type strain of *S. costicola* is strain NCIMB 701 (= ATCC 33508).

Vibrio costicola is a moderately halophilic bacterium that was originally isolated from salted foods (29) and grows optimally in media containing 10% (wt/vol) salts (7). This species has been isolated frequently from salted meats and brines (8, 28), and it has been determined only recently that *V. costicola* is a normal inhabitant of hypersaline environments (7, 19, 32). In fact, *V. costicola* is the predominant organism in saltern pond waters that have intermediate salt concentrations (concentrations between 10 and 15%) (26). *V. costicola* has been also isolated from other saline habitats, such as saline soils, but in lower proportions (24). Until recently, only a few moderately halophilic species had been described, and *V. costicola* was widely used by researchers as a model for the study of the physiological features of obligate halophiles. Thus, in many respects, *V. costicola* has been considered the representative model for studies of moderately halophilic bacteria and has been used as the moderately halophilic organism in which osmoregulatory and other physiological mechanisms have been studied (15, 16).

A recent study of isolates obtained from hypersaline habitats and four strains isolated from cured meats demonstrated that *V. costicola* is widely distributed in hypersaline aquatic environments, and an emended description of this species was proposed by Garcia et al. (7). In addition, DNA-DNA homology studies revealed the genotypic homogeneity of representative strains of this species (11), and this species was shown to be not closely related to other *Vibrio* species (3, 11). The data in these studies suggested that *V. costicola* should be placed in a separate genus (3).

Recently, Kita-Tsukamoto et al. (14) compared partial 16S rRNA sequences (approximately 600 nucleotides) of a large number of *Vibrio* species, and the resulting data also demonstrated that *V. costicola* is not related to other *Vibrio* species or related organisms. While the manuscript of this paper was being prepared, Ruimy et al. (27) described the complete 16S rRNA and ribosomal DNA (rDNA) sequences of the type strain of *V. costicola* in their comprehensive study of *Vibrio*, *Photobacterium*, *Aeromonas*, and *Plesiomonas* sequences. The

analysis of these authors also demonstrated that *V. costicola* clusters outside the main body of *Vibrio* species (that is, *Vibrio cholerae* and related species). In this study, we determined the nearly complete 16S rRNA gene sequences of several *V. costicola* strains, including the type strain, and our results confirm that the phylogenetic distances between this species and the other *Vibrio* species are relatively large (14, 27). On the basis of these data and other data, we formally propose that *V. costicola* should be transferred to a new genus as *Salinivibrio costicola* gen. nov., comb. nov.

The moderately halophilic strains which we studied were *V. costicola* NCIMB 701^T (T = type strain), AV3, E-367, V-15, 6, and H-178. The *V. costicola* type strain and strains 6 and AV3 were originally isolated from cured meat (8), while the other *V. costicola* strains were isolated from salterns located in different areas of Spain (7). Cells were grown in a medium containing 0.5% (wt/vol) yeast extract (Difco) and a salt mixture (final salt concentration, 10%) (32). The pH was adjusted to 7.5, and the preparations were incubated at 37°C in an orbital shaker at 200 strokes per min. When necessary, the medium was solidified by adding 20 g of Bacto Agar (Difco) per liter.

Genomic DNA isolation, PCR amplification of the 16S rRNA genes, and direct determination of the PCR-amplified 16S rDNA sequences were carried out by using methods that have been described previously in detail (20). The sequences which we obtained were aligned with reference 16S rRNA and rRNA gene sequences by using the evolutionarily conserved primary sequence and the secondary structure as references (10, 34). Dendrograms were generated from comparisons of pairs of sequences and evolutionary distances (12) by using a pairwise, weighted, least-squares distance method (22). Maximum-likelihood analyses were performed by using fast DNA-ML (18).

The nearly complete primary sequences (1,502 to 1,512 nucleotide positions) of the 16S rRNA genes of six *V. costicola* strains (including the type strain) were determined and deposited in the EMBL data library. The PCR allowed us to amplify all of the gene encoding the 16S rRNAs of these microorganisms except nucleotide positions at the 5' and 3' termini of the gene. After the optimal alignment was determined, the sequences of the six *V. costicola* strains were compared with a database consisting of more than 2,000 prokaryotic sequences (18).

The 16S rDNA sequence comparisons unequivocally showed

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FIG. 1. Unrooted phylogenetic tree derived from an analysis of the complete 16S rRNA sequences of six *V. costicola* strains (strains NCIMB 701^T, E-367, 6, V-15, H-178, and AV-3) and other species of the genus *Vibrio*. The topology was obtained by using an evolutionary distance method. The same topology was obtained when a maximum-likelihood method was used. Bootstrap values (6) that were generated from 100 replications are given for the *V. costicola* branch.

that *V. costicola* clusters in the gamma subclass of the *Proteobacteria*. Although other moderately halophilic species belonging to the genera *Halomonas*, *Deleya*, and *Halovibrio* are also included in this phylogenetic subdivision, our results indicate that these organisms are not related to *V. costicola*. Dobson et al. (4) pointed out that *Halovibrio variabilis* is closely related to species of the genera *Halomonas* and *Deleya*. Our data support this relationship and establish that the genera *Halovibrio*, *Halomonas*, and *Deleya*, which also include moderate halophiles, constitute a phylogenetic branch that is completely different from the *V. costicola* branch.

The levels of sequence similarity for the six *V. costicola* strains ranged from 98.6% (level of sequence similarity between strains E-367 and AV3) to 99.9% (level of sequence similarity between strains NCIMB 701^T and E-367). The high levels of similarity of the *V. costicola* strains which we studied at the molecular level, regardless of their origins or sources of isolation, are remarkable.

An unrooted phylogenetic tree derived from the calculated evolutionary distances obtained for *V. costicola* and other species of the genus *Vibrio* is shown in Fig. 1. The six strains of *V. costicola* are clearly phylogenetically distinct from the other *Vibrio* species, including *V. cholerae*, the type species of the genus *Vibrio*; the levels of sequence similarity between the six *V. costicola* strains and *V. cholerae* ranged from 86.8 to 87.1%. Such sequence similarity values are much lower than the similarity values typically observed for species belonging to the same genus. For example, the average level of 16S rRNA sequence similarity for the other *Vibrio* species for which sequence data exist was 95%, and the values ranged from 90% (level of sequence similarity between *V. cholerae* and *Vibrio hollisae*) to 99.5% (level of sequence similarity between *Vibrio campbellii* and *Vibrio alginolyticus*). These values demonstrate the heterogeneity of the genus *Vibrio* as it is currently defined, and the recommendations of Ruimy et al. (27) for reassignment of some *Vibrio* species to separate genera should be considered. The levels of sequence similarity between *V. cos-*

ticola strains and *V. cholerae* (and other species of this genus) are markedly lower than any other sequence similarity values obtained for *Vibrio* species. It should be pointed out that we determined the 16S rRNA gene sequences of several strains of *V. costicola*, and thus the species is defined phylogenetically not only on the basis of its type strain, but also on the basis of five other strains isolated from very different hypersaline environments or from cured meats.

In addition, it is important to note that the closest genetic relatives of *V. costicola* are not other *Vibrio* species but the species belonging to the genus *Photobacterium*. Figure 2 shows the relationships of *V. costicola* to representative species of genera belonging to the gamma subdivision of the *Proteobacteria*. Gauthier et al. (9) recommended that reassignment of *V. costicola* and *V. hollisae* to the genus *Photobacterium* should be considered on the basis of the results of 16S rRNA gene sequence comparisons and the ability of the organisms to accumulate polyhydroxybutyrate, which is characteristic of the species belonging to the genus *Photobacterium*. However, the levels of sequence similarity between *V. costicola* NCIMB 701^T and *Photobacterium* species (*Photobacterium phosphoreum*, 89.5%; *Photobacterium angustum*, 89.7%; *Photobacterium damsela* subsp. *piscicida*, 91.2%; *Photobacterium leiognathi*, 89.3%) clearly show that *V. costicola* comprises a branch that is evolutionarily distinct from the *Photobacterium* branch. In addition, the primary sequence of the 16S rRNA genes of *V. costicola* has insertions at helices between positions 183 and 193 (*Escherichia coli* 16S rRNA gene sequence numbering) and positions 207 and 214. These differences in the primary sequence, which produce significant secondary structure changes, are found in the sequences of all strains of *V. costicola*, but are not present in the sequences of any *Vibrio* or *Photobacterium* species.

Figure 2 shows the inferred relative phylogenetic relationships of *V. costicola* with other species belonging to the gamma subclass of the *Proteobacteria*, including other moderately halophilic species. *V. costicola* is quite distinct from the other mod-

FIG. 2. Unrooted phylogenetic tree determined by using masked (17) 16S rRNA sequence comparisons of six *V. costicola* strains (strains NCIMB 701^T, E-367, 6, V-15, H-178, and AV-3), other moderately halophilic species, and other members of the gamma subclass of the *Proteobacteria*. The topology was obtained by using an evolutionary distance method. The same topology was obtained when a maximum-likelihood method was used. Bootstrap values (6) that were generated from 100 replications are given for the *V. costicola* branch.

erate halophiles (4, 20), and these phylogenetic data reflect the phenotypic and chemotaxonomic differences between these species and *V. costicola* (7).

In a previous study that was based on a numerical analysis of phenotypic characteristics of 54 isolates obtained from several salterns, we showed that these strains were very similar to the type strain of *V. costicola* and to three other reference strains originally isolated from cured meats. Thus, an emended description of *V. costicola* was proposed (7). Although *V. costicola* has phenotypic features that are characteristic of species of the genus *Vibrio*, the phylogenetic relationships of this species with other *Vibrio* species, as well as with other moderately halophilic gram-negative species, were not known. In this study we estimated the phylogenetic position of *V. costicola* on the basis of the results of a comparison of the nearly complete 16S rDNA sequences of six strains of *V. costicola*, including type strain NCIMB 701, two strains isolated from salted foods, and three new isolates obtained from water samples from salterns in geographically distinct areas. Our sequence data clearly demonstrated that these six strains form a homogeneous cluster that is phylogenetically distinct from the species of the genus *Vibrio* that have been analyzed previously (Fig. 1) and that the calculated evolutionary distance of this cluster from the type species of the genus *Vibrio*, *V. cholerae*, is clearly large enough to warrant placement of *V. costicola* in a separate genus. Our results expand and confirm the results of a study of marine bacteria, mainly members of the family *Vibrionaceae*, that was based on determinations of partial 16S rRNA sequences (14), in which *V. costicola* was tentatively shown to be distinct from other vibrios. While our data are consistent with the data determined previously, we should point out that the regions sequenced in the previous study were not the regions which we found to be the most informative for the six strains of *V. costicola* when they were compared with other *Vibrio* species. Ruimy et al. (27) reported that *V. costicola* was most closely related to *V. hollisae*, *Vibrio damsela*, and species of the genus *Photobacterium* and was not closely related to *V. cholerae* and the other *Vibrio* species which they analyzed.

The sequence of the *V. costicola* strain studied by Ruimy et al. (27) (listed as NCIMB 701^T) was compared with the sequence of the same strain determined in this study. Correspondence between workers in the two laboratories was able to explain most of the differences between the sequences (21). However, there are five nucleotides that are different in the two sequences, and the differences may be attributed to the different methods used to determine the sequence. The 16S rDNA sequence of *V. costicola* NCIMB 701^T determined in this study was used in all of our sequence comparisons and phylogenetic analyses. The only significant differences in the conclusions of the two studies are due to differences in the 16S rRNA sequences of *V. hollisae*. The sequence of *V. hollisae* CIP 101886^T determined and used by Ruimy et al. (27) differs from the previously published sequence of *V. hollisae* ATCC 33564^T (5) by more than 4% (approximately 60 nucleotide positions). We used the earlier published sequence for the analysis in this study. While it is important to explain the difference observed, our analysis of *V. costicola* was not affected significantly.

It is noteworthy that the limited molecular data previously available for *V. costicola* also suggested that this species was not closely related to other *Vibrio* species. The level of DNA-DNA homology of *V. costicola* and *V. cholerae* was found to be as low as 3% (3) or 2% (2). In addition, the low levels of DNA relatedness between *V. costicola* and other *Vibrio* species led previous authors to propose that this species (as well as other *Vibrio* species) should be placed in a separate genus (3). In a recent study, the levels of DNA-DNA homology between *V. costicola* NCIMB 701^T and isolates obtained from hypersaline environments and reference strains obtained from cured meats were determined to be very high (>70%), and the authors concluded that these organisms belong to the same genospecies (11). In contrast, the levels of DNA-DNA relatedness with other type strains of *Vibrio* species were very low, as were the levels of relatedness with other moderately halophilic species, such as *Deleya halophila* and *Halomonas halmophila* (11). These DNA-DNA hybridization results are consistent with the

TABLE 1. Phenotypic characteristics that differentiate the genus *Salinivibrio* from the genus *Vibrio* and other related gram-negative genera^a

Characteristic	<i>Salinivibrio</i>	<i>Vibrio</i>	<i>Halovibrio</i>	<i>Deleya</i>	<i>Halomonas</i>	<i>Chromohalobacter</i>	<i>Volcaniella</i>	<i>Arhodomonas</i>
Morphology	Curved rods	Straight or curved rods	Curved rods	Rods	Rods	Rods	Short rods	Short rods
Pigmentation	None	Cream	Light brown	None	Cream-yellow	Violet	None	None
Flagellation								
Polar	+ ^b	+	+	—	—	—	—	+
Peritrichous	—	—	—	(+)	(+)	+	—	—
Oxidase activity	+	+	+	(+)	+	—	—	—
Growth without NaCl	—	(—)	—	—	(—)	(—)	—	—
Growth in the presence of 20% NaCl	+	—	+	(+)	+	+	+	+
Optimal growth in the presence of 5 to 10% salt	+	—	+	+	+	+	+	+
Anaerobic growth	+	+	—	—	—	—	—	—
Acid production from D-glucose	+	+	—	+	±	+	—	ND
Hydrolysis of:								
Gelatin	+	(+)	—	—	ND	—	+	—
Starch	—	(+)	—	ND	ND	—	—	—
Voges-Proskauer reaction	+	(—)	ND	ND	ND	—	—	—
Indole production	—	ND	—	ND	(—)	—	—	—
β-Galactosidase activity	—	+/-	ND	ND	ND	—	ND	ND
Decarboxylase activities								
Arginine	+	—	—	—	ND	—	ND	—
Lysine	—	+/-	—	ND	ND	—	ND	—
Ornithine	—	+/-	—	ND	ND	—	ND	—
G+C content (mol%)	49.4–50.5	38–51	61	52–68	59–63	62.1–64.9	59.1–65.7	67

^a Data from references 1, 2, 7, 13, 23, 25, 30, 31, and 33.^b +, positive; —, negative; (+), most species are positive; (—), most species are negative; ND, not determined.

data that were obtained in this study and previous studies and were based on 16S rRNA gene sequence comparisons.

Because of the significant phenotypic and genotypic differences between *V. costicola* and other *Vibrio* species, as well as other bacteria belonging to the gamma subclass of the *Proteobacteria*, we propose that *V. costicola* should be transferred to a new genus, the genus *Salinivibrio*, as *Salinivibrio costicola* gen. nov., comb. nov. Table 1 shows useful features for distinguishing the genus *Salinivibrio* from the genus *Vibrio* and other related genera which include moderately halophilic species. More detailed data that can be used to differentiate *S. costicola* from related *Vibrio* species, as well as moderately halophilic species, have been reported elsewhere recently (1, 7, 30). A description of the genus *Salinivibrio*, which is the third genus (along with the genera *Vibrio* and *Photobacterium*) in the family *Vibrionaceae* (9, 27), is given below.

Description of the genus *Salinivibrio* gen. nov. *Salinivibrio* (Sa.li.ni.vib'ri.o. L. adj. *salinus*, saline; L.v. *vibrio*, move rapidly to and from, vibrate; M.L. masc. n. *Salinivibrio*, saline organism which vibrates). Gram negative. Cells are curved rods that are 0.5 µm wide and 1.5 to 3.2 µm long and occur singly or occasionally united by S shapes or spirals. Motile by means of one polar flagellum. Spores are not formed. Colonies are circular, convex, opaque, and cream colored. Broth cultures are uniformly turbid.

Moderately halophilic. Grows in the presence of marine salt concentrations between 0.5 and 20% (wt/vol) and optimally in the presence of 10% (wt/vol) salts at 37°C. No growth occurs in the absence of NaCl. Growth occurs at 5 to 45°C (optimal growth occurs at 37°C) and at pH 5 to 10 (optimal growth occurs at pH 7.5).

Facultatively anaerobic. Catalase and oxidase are produced. Chemoheterotrophic. Acid but no gas is produced from D-glucose. Gelatin is hydrolyzed, but starch is not hydrolyzed.

Voges-Proskauer and arginine decarboxylase tests are positive. Indole, β-galactosidase, lysine decarboxylase, and ornithine decarboxylase tests are negative.

The DNA base composition ranges from 49.4 to 50.5 mol% G+C (as determined by the thermal denaturation method). Isolated from hypersaline environments (salterns, saline soils) and from salted food (cured meats and brines). The type species is *Salinivibrio costicola* (formerly *Vibrio costicola*). The genus *Salinivibrio* is a member of the gamma subclass of the *Proteobacteria*.

The description of *Salinivibrio costicola* comb. nov. is based on the data of García et al. (7). The type strain of this species is strain NCIMB 701 (= ATCC 33508); its G+C content is 50.0 mol% (as determined by the CsCl method or 49.9 mol% (as determined by the thermal denaturation method).

Nucleotide sequence accession numbers. The nearly complete primary sequences of the 16S rRNA genes of *V. costicola* strains which we determined have been deposited in the EMBL data library under the following accession numbers: *V. costicola* NCIMB 701^T, X95527; *V. costicola* AV3, X95528; *V. costicola* 6, X95531; *V. costicola* E-367, X95529; *V. costicola* V-15, X95530; and *V. costicola* H-178, X95532.

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