JOURNAL OF BACTERIOLOGY, Jan. 2005, p. 752–757 Vol. 187, No. 2
0021-9193/05/$08.00+0 doi:10.1128/JB.187.2.752–757.2005

Vibrios Commonly Possess Two Chromosomes

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Received 2 July 2004/Accepted 1 October 2004

The prevalence of the two-chromosome configuration was investigated in 34 species of vibrios and closely related species. Pulsed-field gel electrophoresis of undigested genomic DNA suggested that vibrios commonly have two chromosomes. The size of the large chromosome is predominantly within a narrow range (3.0 to 3.3 Mb), whereas the size of the small chromosome varies considerably among the vibrios (0.8 to 2.4 Mb). This fact suggests that the structure of the small chromosome is more flexible than that of the large chromosome during the evolution of vibrios.

Vibrios, which are gram-negative halophilic bacteria that include more than 60 species, comprise the major culturable bacteria in marine and estuarine environments (10, 40). Although many marine bacteria are known to be difficult to grow in artificial media containing rich nutrients, most vibrios grow well on ordinary peptone-containing media. Vibrios have high degradative ability (e.g., chitin digestion and production of various extracellular proteases) (1, 28). Several *Vibrio* species are devastating pathogens for fish, shellfish, coral, and mammals (10, 30, 40). The best documented example is *Vibrio cholerae*, the etiological agent of cholera. When exposed to certain conditions, some vibrios become nonculturable without losing their respiratory activity. This phenomenon is called the "viable but nonculturable" state (5, 9). Nonpathogenic vibrios are also known. For example, *V. mediterranei* plays a role as the first colonizer of the gut of turbot larvae and may prevent the colonization of the gut by opportunistic bacteria (17). *V. fischeri* plays an important role in the development of the light organ of juvenile squid of the species *Euprymna scolopes* (44). *V. natriegens*, which produces nitrogenase, plays an important role in providing fixed nitrogen de novo to marine ecosystems (6). The optimal temperatures for growth of *V. salmonicida*, *V. logei*, and *V. wodanis* are as low as those for the growth of psychrophilic bacteria (12, 26). Thus, vibrios with various features and lifestyles exist.

Previously, we reported that some species of the genus *Vibrio*, including *V. parahaemolyticus*, *V. cholerae*, *V. anguillarum*, *V. fluvialis*, and *V. vulnificus*, which cause illness in humans, fish, and other animals, possess two chromosomes (39, 46). Trucksis et al. also reported that *V. cholerae* and *V. mimicus* possess two chromosomes (42). Recently, the whole-genome sequences of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* were published; these studies confirmed that their genome structures consist of two circular chromosomes (2, 16, 24). In this study, we extensively investigated the genome structure of vibrios, including pathogenic and nonpathogenic strains, and of species closely related to the genus *Vibrio* to evaluate the prevalence of the two-chromosome structure among these bacteria.

The strains we examined are listed in Table 1. The vibrios were grown in Luria-Bertani broth (34) or Difco marine broth 2216. Bacteria of the genera *Plesiomonas* and *Aeromonas* were grown in tryptic soy broth (Difco). The culture temperatures were 15°C for *V. logei* and *V. salmonicida* and 10°C for *V. wodanis* and *Photobacterium profundum*. Other bacteria were cultured at room temperature. The preparation of genomic DNA embedded in agarose gels and the protocol for pulsed-field gel electrophoresis (PFGE) have been described previously (18). PFGE was performed with a CHEF DRIII or CHEF Mapper XA system (Bio-Rad). PFGE was run on 0.8% pulsed-field certified agarose (Bio-Rad) in 1× TAE (40 mM Tris-acetate, 1 mM EDTA [pH 8.0]) at 14°C. Run conditions were generated by the auto-algorithm mode of the CHEF Mapper XA system, with a size range of 0.5 to 4 Mb (for block 1, 72 h, initial pulse time of 20 min 0 s, final pulse time of 29 min 45 s, angle of 106°, at 2 V/cm; and for block 2, 8 h 40 min, initial pulse time of 40.85 s, final pulse time of 2 min 25.98 s, angle of 120°, at 6 V/cm). Thiourea was added to the gel buffer when DNA degradation occurred (50 or 500 μM thiourea) (29). PFGE was performed at least three times, and chromosome sizes were estimated for each sample based on the comparison of the motility with that of other samples and the molecular weight markers. We analyzed 36 species of vibrios by PFGE and then described the 34 species that showed clear DNA bands on PFGE gels. Using the PFGE results, we estimated the numbers and sizes of the chromosomes for each vibrio and closely related species, except for some strains for which the genomic DNA was degraded during preparation.

16S rRNA gene sequences of the strains used were obtained from the GenBank, EMBL, and DDBJ databases. The sequences were aligned by using the CLUSTAL X program (version 1.8) (41). A neighbor-joining tree was constructed from the distance matrix estimated by the algorithm of Kimura's two-parameter model (20, 32) by using the CLUSTAL X program. Bootstrap confidence values were calculated with 1,000 resamplings. Maximum-likelihood and maximum-parsimony analyses were performed by using PAUP software (version 4.0) (11, 38).

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TABLE 1. Bacterial strains used in this study and the results of PFGE[a]

Family, genus, and species	Estimated chromosome sizes (Mb)	Total size (Mb)	Strain no. or reference[b]
Vibrionaceae			
Vibrio nigripulchritudo	4.0, 2.4	6.4	ATCC 27043^T
Vibrio mediterranei	3.6, 2.3	5.9	IFO15635^T
Vibrio harveyi	3.5, 2.4	5.9	ATCC 14126^T
Vibrio alginolyticus	3.3, 2.0	5.3	ATCC 17749^T
Vibrio salmonicida	3.3, 2.0	5.3	NC1M B2262^T
Vibrio tubiashii	3.3, 1.9	5.2	ATCC 19109^T
Vibrio parahaemolyticus	3.3, 1.9	5.2	RIMD 2210633
Vibrio campbellii	3.3, 1.9	5.2	ATCC 25920^T
Vibrio natriegens	3.3, 1.9	5.2	ATCC 14048^T
Vibrio nereis	3.3, 1.9	5.2	IAM14407^T
Vibrio carchariae	3.3, 1.9	5.2	ATCC 35084^T
Vibrio fluvialis	3.3, 1.9	5.2	ATCC 33809^T
Vibrio fischeri	3.2, 1.8	5.0	NCIMB1281^T
Vibrio vulnificus	3.2, 1.8	5.0	RIMD2219025
Vibrio splendidus	3.2, 1.8	5.0	ATCC 33125^T
Vibrio orientalis	3.3, 1.7	5.0	ATCC 33934^T
Vibrio aestuarianus	3.2, 1.8	5.0	ATCC 35048^T
Vibrio pelagius	3.2, 1.7	4.9	NBRC15639^T
Vibrio wodanis	3.3, 1.6	4.9	NCIMB13582^T
Vibrio furnissii	3.2, 1.7	4.9	ATCC 35016^T
Vibrio proteolyticus	3.2, 1.7	4.9	ATCC 15338^T
Vibrio ichthyoenteri	3.2, 1.4	4.6	F-2^T
Vibrio pectenicida	3.2, 1.4	4.6	CIP105190^T
Vibrio logei	3.0, 1.5	4.5	ATCC 15382
Vibrio mimicus	3.0, 1.5	4.5	ATCC 33653^T
Vibrio mytili	3.0, 1.5	4.5	NCIMB13275^T
Vibrio rumoiensis	3.0, 1.3	4.3	ATCC 35023^T
Vibrio anguillarum	3.0, 1.2	4.2	RIMD2202001
Vibrio gazogenes	3.0, 1.2	4.2	ATCC 29988^T
Vibrio cholerae (EI Tor)	3.0, 1.1	4.1	RIMD2203320
Vibrio halioticoli	3.0, 1.1	4.1	IAM14598
Vibrio hollisae	3.2, 0.8	4.0	JCM1284
Vibrio ordalii	3.0, 0.9	3.9	CIP103205^T
Vibrio metschnikovii	3.0, 0.9	3.9	IAM1039
Photobacterium leiognathi	3.3, 1.6	4.9	NCIMB2193^T
Photobacterium damselae subsp. *damselae*	3.2, 1.4	4.6	ATCC 33539^T
Photobacterium angustum	3.2, 1.8	5.0	CIP75.10^T
Salinivibrio costicola	3.2, 1.3	4.5	DSM11403^T
Salinivibrio costicola subsp. *vallismortis*	3.2, 1.0	4.2	DSM8285^T
Aeromonadaceae			
Aeromonas hydrophila		5	RIMD0111027
Aeromonas sobria		5	RIMD0111003
Aeromonas salmonicida		4.7	20
Enterobacteriaceae			
Plesiomonas shigelloides		3.5	RIMD0112016

[a] Strain designations, numbers, and sizes of chromosomes in the bacterial strains classified in the genus *Vibrio* and the species closely related to *Vibrio*.

[b] Sources for the strains are as follows: American Type Culture Collection (ATCC), Rockville, Md.; Collection de Bacteries de l'Institut Pasteur (CIP), Paris, France; Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM), Braunschweig, Germany; IAM Culture Collection (IAM), Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo, Japan; Institute for Fermentation (IFO), Osaka, Japan; Japan Collection of Microorganisms (JCM), RIKEN, Saitama, Japan; NITE Biological Resource Center (NBRC), National Institute of Technology and Evaluation, Chiba, Japan; National Collections of Industrial and Marine Bacteria, Ltd. (NCIMB), Aberdeen, United Kingdom; Research Institute for Microbial Diseases (RIMD), Osaka University, Osaka, Japan.

We investigated the chromosomal configurations of 34 species of the genus *Vibrio* by using PFGE of intact (undigested) genomic DNA, as described previously (46). Figure 1 shows the PFGE patterns of chromosomes from some of our samples. The results for all strains are listed in Table 1. Two chromosomes were observed in all the vibrios examined (Table 1).

We were interested in the prevalence of two chromosomes in species other than the genus *Vibrio* (Fig. 2). Species closely related to the genus *Vibrio* were also investigated. PFGE results for strains of the genera *Photobacterium* and *Salinivibrio*, members of the family *Vibrionaceae*, revealed that they also possess two chromosomes. Therefore, we suggest that this feature is common among the *Vibrionaceae*. In contrast, strains of the genera *Aeromonas* and *Plesiomonas*, which were included in the family *Vibrionaceae* until recently but have now been reclassified into the families *Aeromonadaceae* and *Enterobacteriaceae*, respectively, possessed only one chromosome (Table 1) (31). *Aeromonas salmonicida*, a known fish pathogen, has already been shown to possess a single circular chromosome of 4,658 ± 30 kb (43). In this study, *Aeromonas hydrophila* and

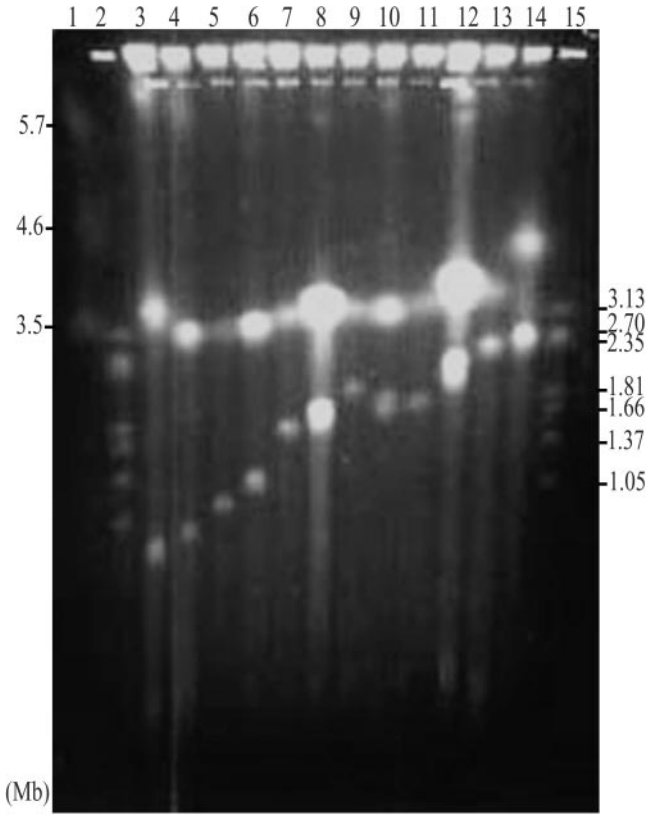

FIG. 1. PFGE of intact genomic DNAs of bacterial species belonging to the genus *Vibrio*. Lanes: 1 and 2, *Schizosaccharomyces pombe* and *Hansenula wingei* PFGE markers, respectively; 3, *V. hollisae*; 4, *V. metschnikovii*; 5, *V. cholerae* (El Tor); 6, *V. anguillarum*; 7, *V. furnissii*; 8, *V. orientalis*; 9, *V. parahaemolyticus* (RIMD2210633); 10, *V. splendidus*; 11, *V. vulnificus*; 12, *V. alginolyticus*; 13, *V. harveyi*; 14, *V. nigripulchritudo*; 15, *H. wingei* PFGE marker.

Aeromonas sobria were also shown to possess only one chromosome of 5 Mb. *Plesiomonas shigelloides* also possessed a single chromosome of 3.5 Mb (Table 1). This result suggests that the possession of two chromosomes, a common feature among the *Vibrionaceae*, is not beyond the family. According to these results, the origin of the possession of two chromosomes dates back to the diversification of the *Vibrionaceae*. Recently, Egan and Waldor reported that the *rctB* gene, which is essential for the replication of the small chromosome in *V. cholerae*, is present in the genera *Vibrio* and *Photobacterium* of the family *Vibrionaceae* (8). From their results, Egan and Waldor proposed that an ancestor of the small chromosome must have been acquired before the diversification of this family, which is consistent with our present data.

PFGE showed not only the number of chromosomes but also the size of each chromosome (Table 1). Estimated sizes of the chromosomes of *V. cholerae* El Tor and *V. parahaemolyticus* RIMD2210633 agreed well with the results of whole-genome sequencing (Fig. 1, lanes 5 and 9, respectively) (16, 24). The sizes of the large chromosomes of the vibrios, except for a few strains, clustered in a relatively narrow range of 3.0 to 3.3 Mb, whereas the sizes of the small chromosomes varied considerably (from 0.8 to 2.4 Mb) (Fig. 1; Table 1). These results suggest that the large chromosomes of the vibrios are relatively constant in size, whereas the small chromosomes are structurally more flexible. Whole-genome sequences of three *Vibrio* species, *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, confirmed these chromosomal trends (2, 16, 24). In these three vibrios, most of the essential genes required for growth and viability occur on the large chromosome. The small chromosome seems to contain more genes related to transcriptional regulation and the transport of various substrates than does the large chromosome. Genes classified in these categories have a role in the bacterial response to environmental changes. The features of the large and small chromosomes of these three vibrios may extend to other vibrios. We speculate that the ancestral vibrio diversified into various species and maintained most essential genes on the large chromosome. This view is supported by the observed low frequency of interchromosomal rearrangements (24).

In addition to those of the family *Vibrionaceae*, some eubacteria with multiple chromosomes are distributed in alpha-proteobacteria (genera *Agrobacterium*, *Sinorhizobium*, *Rhizobium*, *Rhodobacter*, and *Brucella*) and beta-proteobacteria (genera *Burkholderia* and *Ralstonia*, etc.) (19, 21, 36, 37). However, some features of the chromosomes of these bacteria differ from those of the vibrios. In the genus *Agrobacterium*, strains of the three species *A. tumefaciens*, *A. rubi*, and *A. rhizogenes* possess two chromosomes (7, 15, 19). Some *Agrobacterium* strains, however, possess small linear chromosomes, whereas vibrios commonly possess two circular chromosomes. Furthermore, genome size seems to be more variable in strains of the genus *Agrobacterium* than in vibrios (19). *Agrobacterium* species also tend to possess three or more large replicons. A few strains of the genera *Sinorhizobium* and *Rhizobium* also possess three or more large replicons (13, 19). Members of the *Burkholderia cepacia* complex possess complicated genomes (3, 4, 22, 23). Almost all *Burkholderia cepacia* complex strains examined so far possess three large (>600-kb) replicons (probably chromosomes) with total genome sizes of more than 7 Mb. The genomes of some strains show frequent and large-scale rearrangements, including the deletion of part of one chromosome or its translocation to another chromosome. Whole-genome sequences of strains of the genera *Ralstonia*, *Rhodobacter*, and *Brucella* have been determined (27, 33, 47). Although these strains also possess multiple chromosomes, the prevalence of multichromosome structures of the genera *Ralstonia* and *Rhodobacter* has not yet been extensively investigated (33, 35). In the genus *Brucella*, *B. suis*, *B. abortus*, *B. ovis*, *B. neotomae*, and *B. melitensis* have multiple chromosomes, but these species are now considered to be one species, *B. melitensis* (14, 25).

In this study, we found that all the vibrios examined possess two chromosomes. This investigation clearly demonstrates the features of the vibrio genomes. First, the size of the large chromosome is relatively constant among the vibrios. Second, the size of the small chromosome is variable. Third, unlike members of the *Rhizobiaceae* and *Burkholderiaceae*, no vibrios possess large replicons (>0.3 Mb) apart from their two chromosomes. Finally, we found no vibrio with only one chromosome. Thus, the genome structure of vibrios appears to be more constant and more stable than those of other bacteria with multiple chromosomes.

Why has the small chromosome of vibrios not been integrated into the large chromosome? Heidelberg et al. suggested

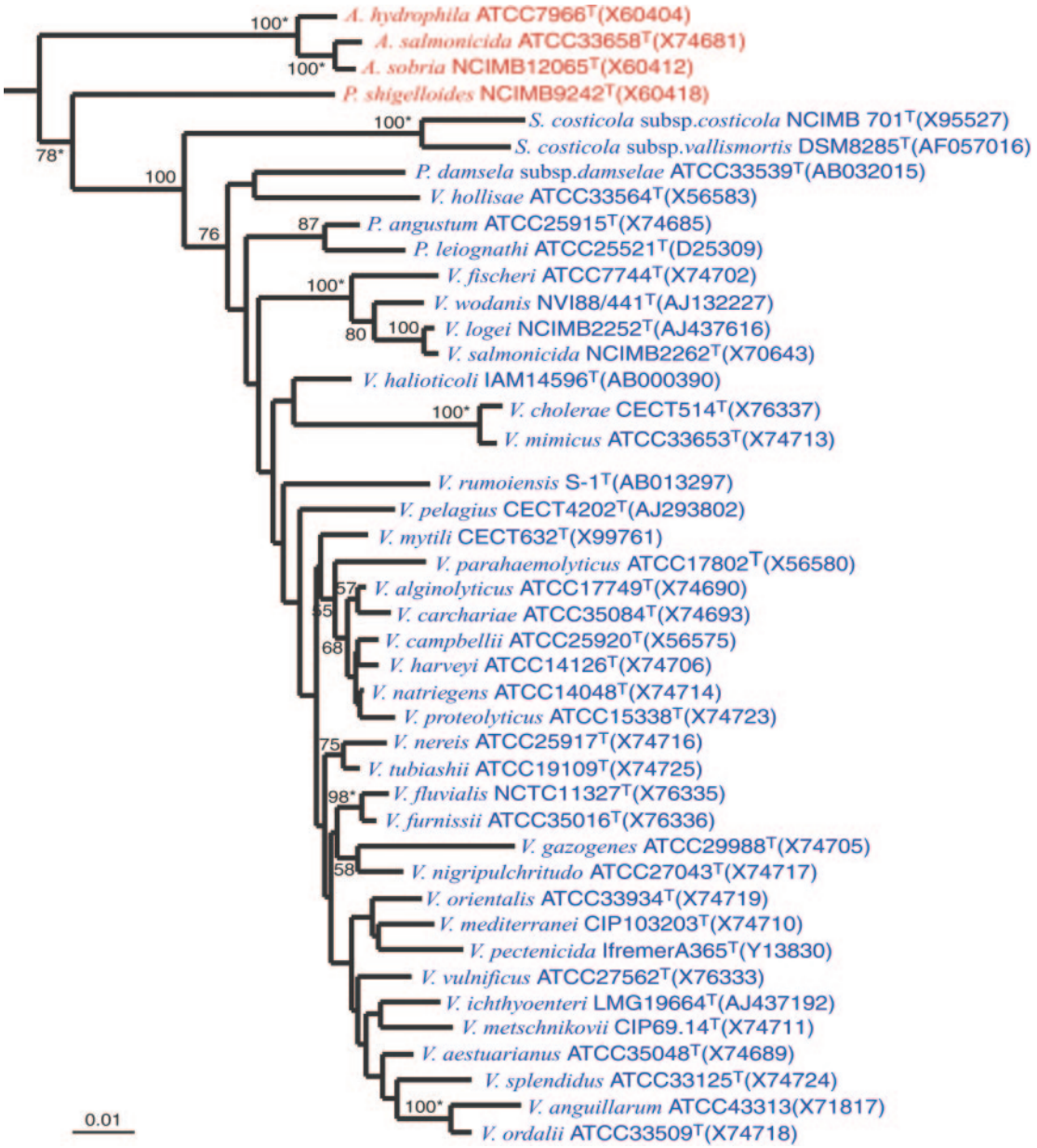

FIG. 2. Phylogenetic tree based on 16S rRNA genes showing the relationships of the strains listed in Table 1. Strains possessing one chromosome are indicated in red, and strains possessing two chromosomes are shown in blue. This tree is based on a 1,152-bp alignment of 16S rRNA gene sequences and was constructed by using the neighbor-joining method. Accession numbers for the sequences are given in parentheses. To estimate the root position of the tree, *Halomonas elongata* ATCC 33173^T (accession no. X67023) and *Pseudomonas aeruginosa* PAO1 (accession no. NC002516) were used as an outgroup. Numbers at the nodes indicate the levels of bootstrap support (percentages) based on a neighbor-joining analysis of 1,000 resampled data sets; only values above 50% are shown. Asterisks indicate branches that were also recovered by using the maximum-parsimony and maximum-likelihood methods. Bar, 0.01 substitution per nucleotide position.

that the small chromosome may play an important specialized function that applies the evolutionary selective pressure necessary to suppress such integration events when they do occur (16). For example, if there is a difference in copy number between the large and small chromosomes under certain environmental conditions, then the small chromosome may have accumulated genes that are better expressed at a higher or lower copy number than the genes on the large chromosome. A second possibility suggested by Heidelberg et al. is that one chromosome may partition into daughter cells in the absence of the other chromosome (aberrant segregation) in response to environmental cues (16). Such single-chromosome-containing cells ("drone" cells) would be replication defective but would maintain metabolic activity, and they would therefore constitute a potential source of the viable but nonculturable cells that are observed in *V. cholerae*. Such drone cells may also play a role in *V. cholerae* biofilms by, for example, producing extracellular chitinases, proteases, and other degradative enzymes

that enhance the survival, in the biofilm, of the cells retaining two chromosomes, without directly competing with these cells for nutrients.

Xu et al. reported an interesting experimental study of the role of the small chromosome. They compared the genomic transcriptional pattern (i.e., the transcriptome) of *V. cholerae* in cells grown in the upper intestines of rabbits with that of cells grown aerobically under laboratory conditions (45). Under both conditions, the genes showing the highest levels of expression occurred primarily on the large chromosomes. However, many genes on the small chromosome were expressed during in vivo growth. In the rabbit's upper intestine, iron limitation, anaerobiosis, and nutrient limitation are prominent environmental conditions encountered by *V. cholerae* during growth. The expression of 24 or more genes involved in iron transport or storage was increased in vivo. Xu et al. proposed that the role of the small chromosome in the bacterium's adaptation to host-related nutritional stresses may partly explain the phenomenal success of members of the genus *Vibrio* as environmentally diverse organisms. Thus, it is possible that a genome structure consisting of two chromosomes is advantageous to vibrios under the specific environmental conditions that they encounter in their life cycles.

In this study, we have demonstrated that vibrios commonly possess two chromosomes and that this genome structure is stable among species of the family *Vibrionaceae*, despite their various niches. Vibrios seem to have maintained relatively flexible small chromosomes while retaining most essential genes on the large chromosome. This might be a survival strategy acquired by the vibrios that has facilitated their adaptation to various niches in the process of evolution.

We thank K. Ishimaru for the gift of *Vibrio ichthyoenteri* strain F-2.

This work was supported by the Research for the Future program (grant 00L01411) of the Japan Society for the Promotion of Science and by Grants-in-Aid for Scientific Research on Priority Areas and Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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