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Nitrogen Fixation by *Vibrio parahaemolyticus* and Its Implications for a New Ecological Niche▽

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A *Vibrio parahaemolyticus* strain isolated from the rhizosphere of the ecosystem dominant estuarine grass, *Spartina alterniflora*, was characterized and shown to carry *nifH*, the gene encoding the nitrogenase iron protein, and to fix N_2. Nitrogen fixation may contribute substantially to the adaptability, niche breadth, and ecological significance of *V. parahaemolyticus*.

The family *Vibrionaceae* includes opportunistic pathogens of humans and animals (e.g., see references 10 and 28), as well as many less-pernicious species that are free-living chemoheterotrophs and/or commensals of marine fauna (e.g., see references 14 and 15). Outbreaks of *Vibrio* food poisonings, particularly those produced by *Vibrio parahaemolyticus*, are common in coastal areas during warmer months and are generally correlated with ingestion of raw or undercooked seafood. Maintenance of vibrios in estuarine environments during the winter months is often attributed to association with sediment or zooplankton (e.g., see references 17 to 19). However, the potential for other ecological niches, resulting in new reservoirs for *V. parahaemolyticus*, certainly exists.

Estuaries are frequently nitrogen limited, making the ability to reduce ("fix") atmospheric N_2 to ammonia highly advantageous. Only three *Vibrio* species, *V. diazotrophicus*, *V. natriegens*, and the opportunistic human pathogen *Vibrio cincinnatiensis* (5), have been shown to fix atmospheric N_2 (30). Although *V. parahaemolyticus* has been recovered from the roots of salt marsh plants (1, 2), environments supporting high rates of N_2 fixation (31), this organism was not previously shown to fix N_2 or to have any other activity that might promote a stable interaction with plant hosts. Here we present evidence of N_2 fixation by a strain of *V. parahaemolyticus* isolated from the rhizosphere of *Spartina alterniflora*, the dominant plant in salt marshes along the Atlantic and northern Gulf coasts of temperate North America. These results expand the niche breadth of this important potential human pathogen and contribute to understanding its maintenance in estuarine systems.

V. parahaemolyticus 22702 was isolated from *S. alterniflora* rhizosphere sediment collected from Sapelo Island, GA (8). Identification of this strain was accomplished using thiosulfate citrate bile salt sucrose agar (20) (BD Diagnostic Systems, Sparks, MD) and β-galactosidase activity (12) and confirmed by recovery and analysis of phylogenetically informative genes. DNA was extracted from overnight cultures using Prepman Ultra (ABI, Foster City, CA). Primers for PCR amplification of *recA* and *rpoA* were designed using the *V. parahaemolyticus* RIMD 2210633 sequences and Primer3 (25), *recA* 33F (5′-TGCGCTAGGTCAAATTGAAA-3′), *recA* 1008R (5′-AGCAGGTGCTTCTGGTTGAG-3′), *rpoA* 58F (5′-AGCTCGACTCACGCAAAAGT-3′), and *rpoA* 958R (5′-CTAGGCGCATACCCAGAGAC-3′). An internal *recA* primer, 622R (5′-TCGTTTCAGGGTTACCGAAC-3′), was also used in sequencing. The PCR program was 98°C for 30 s, followed by 35 cycles of 98°C for 10 s, 58°C for 30 s, and 72°C for 40 s and a final extension at 72°C for 10 min. Sequencing of amplicons was performed using an ABI Prism 3730 DNA analyzer, and sequences were edited and assembled using BioEdit version 7.0.5.2 (16). The 16S rRNA gene was amplified using primers 27F and 1492R (22). The following primers were employed for sequencing reactions: 519F (5′-CAGCAGCCGCGGTAA-3′), 529R (5′-CGCGGCTGCTGGCAC-3′), 907R (5′-CCCCGTCAATTCCTTTGAGTTT-3′), 1099F (5′-GCAACGAGCGCAACCC-3′), and 1240R (5′-CCATTGTAGCACGTGT-3′). The sequencing program was 34 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min, and sequences were determined using an ABI 3100 DNA analyzer. The 16S rRNA gene, *recA*, and *rpoA* sequences produced in this study or obtained from GenBank were joined (27) and then aligned using ClustalW (29). The concatenated sequences represented 87% of the full-length 16S rRNA gene and 71% and 78% of the coding regions for *recA* and *rpoA*, respectively. A neighbor-joining tree was constructed using the Jukes-Cantor correction (MEGA v. 3.1 [21]) (Fig. 1). This analysis placed 22702 with other *V. parahaemolyticus* strains and clearly differentiated it from species closely related to *V. parahaemolyticus*, such as *Vibrio campbellii*.

Strain 22702 maintained growth in the absence of combined nitrogen sources over several transfers and was subsequently tested for the presence of the nitrogenase iron protein gene (*nifH*), a widely used marker for diazotrophic bacteria. The *nifH* gene of strain 22702 was amplified using primers *nifH* F [5′-TACGG(P/K)AAKGG(P/G)GG(P/K)ATPGG-3′] and *nifH* R [5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG(G/C)ACGATGTAGATPTCCTG-3′] as described previously (23). Amplicons were cloned into pGEM-T (9) and sequenced using the Sp6 and T7 primers and the methods described above. Partial *nifH* nucleotide sequences were

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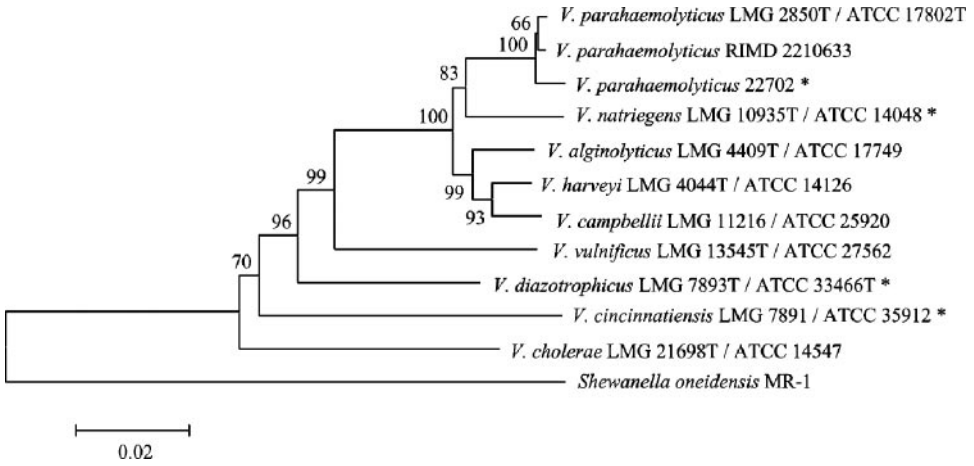

FIG. 1. Neighbor-joining phylogeny of concatenated sequences of 16S rRNA, *recA*, and *rpoA* genes from species of *Vibrio*. A neighbor-joining tree was constructed using the Jukes-Cantor correction. Bootstrap values represent 1,000 replications, and values of <50% are not shown. Diazotrophic strains are marked with asterisks.

aligned, and a neighbor-joining phylogeny was constructed in MEGA using pairwise deletion of gaps and missing data and the Kimura two-parameter correction. Phylogenetic analysis showed a clear affiliation of the *V. parahaemolyticus* strain 22702 *nifH* sequence with sequences from other *Vibrio* species (Fig. 2) and also clearly differentiated the strain 22702 *nifH* sequence from those of other N_2-fixing vibrios.

V. parahaemolyticus strain 22702 was tested for its ability to fix N_2 using the acetylene reduction assay. Serum vials containing 10 ml of mineral salts medium (2) and a N_2 headspace were inoculated with strain 22702, acetylene injected (10% of headspace volume), and the vials incubated at 30°C for 15 h. Four randomly selected replicate vials were sacrificed at each of 11 time intervals over the course of this incubation. Acetylene reduction activity was assayed by gas chromatography (1). Cell protein was quantified using the Rose Bengal dye binding assay (11). Negative controls were uninoculated medium, and rates were adjusted to account for "background" ethylene. Protein and ethylene quantities were determined from standard curves, and specific activities were reported as means ± standard deviations ($n = 4$). While the acetylene reduction rate of strain 22702 (1.31 ± 0.40 nmol $C_2H_2 \cdot min^{-1} \cdot mg^{-1}$ protein; this study) is lower than those of previously described diazotrophic vibrios (38, 51, and 17 nmol $C_2H_2 \cdot min^{-1} \cdot mg^{-1}$ protein for *V. diazotrophicus* ATCC 33466, *V. natriegens* ATCC 14048, and *V. cincinnatiensis* ATCC 35912, respectively [30]), it nonetheless provides definitive evidence of N_2 fixation by *V. parahaemolyticus*. This capability enables this organism to maintain viability and growth in culture under strongly nitrogen-limited conditions and is expected to contribute to its capacity to colonize and grow on roots of salt marsh grasses under similarly nitrogen-limited conditions in salt marsh ecosystems.

Vibrionaceae, including *V. parahaemolyticus*, may play a substantial role in N_2 fixation in the salt marsh ecosystem. The N_2-fixing bacterial assemblage associated with the rhizosphere of salt marsh plants is a significant contributor of utilizable nitrogen to the system (reviewed in reference 24). Salt marsh vibrios can participate in N_2 fixation, as indicated by recovery from *S. alterniflora* rhizosphere samples of a *nifH* cDNA sequence allied with *nifH* sequences from *Vibrio* species (6). Previous studies (2, 3, 23) recovered numerous diazotrophs from the roots of *Spartina alterniflora*, *Juncus roemerianus*, and *Salicornia virginica*. Vibrios comprised more than half of this culture collection, and about 14% of the *Vibrio* strains were identified as *V. parahaemolyticus*. These findings imply that this potential human pathogen could be a substantial rhizosphere resident and a contributing member of the salt marsh diazotroph community. *Vibrio* species are abundant on the roots of salt marsh plants, and cultivation-independent quantification of these organisms is ongoing (J. D. Criminger and C. R. Lovell, unpublished data).

The significance of *V. parahaemolyticus* and other vibrios in salt marsh ecology requires further study, but the implications

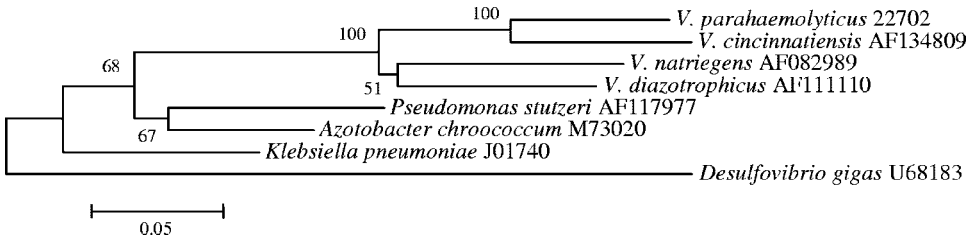

FIG. 2. Neighbor-joining phylogeny of *Vibrio* and reference *nifH* sequences. A neighbor-joining tree was constructed using the Kimura two-parameter correction. Bootstrap values represent 1,000 replications, and values of <50% are not shown.

of N_2 fixation by potential human pathogens are of immediate interest. *Vibrio cholerae*, *V. parahaemolyticus*, and *Vibrio vulnificus* are potent human pathogens (4, 7, 13, 32, 33), and prior to this study, none of these species were known to fix N_2. The capability of some *V. parahaemolyticus* strains to fix N_2 may support their maintenance in nitrogen-limited coastal marine environments, contributing to a broader distribution of pathogenic strains. This key function may also define a new niche for *V. parahaemolyticus* as a rhizosphere-associated heterotrophic diazotroph. Finally, association with plant roots may provide a particularly important advantage to this organism as a refugium from cooler, growth-limiting temperatures and from predation (26). Disturbance of the rhizosphere by infaunal burrowing, severe weather events, and human activities may then facilitate transfer of this organism to the overlying waters, mobilizing pathogenic strains and potentially contributing to infectious outbreaks. Ultimately, maintenance and propagation of this organism is of significant human health concern, and identifying natural habitats and functions that may broaden its distribution is essential to predicting epidemic outbreaks of *V. parahaemolyticus*.

Nucleotide sequence accession numbers. GenBank accession numbers for sequences of *V. parahaemolyticus* strain 22702 determined in this work are as follows: for *recA* and *rpoA*, EU018456 and EU018455, respectively; for the 16S rRNA gene, EF203421; and for *nifH*, EF203422.

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