Genomic Diversity and Homologous Recombination in *Vibrio parahaemolyticus* as Revealed by Amplified Fragment Length Polymorphism (AFLP) and Multilocus Sequence Analysis (MLSA)

FABIANO L. THOMPSON¹*, ILSE CLEENWERCK², JEAN SWINGS², JUNKO MATSUYAMA³, and TETSUYA IIDA⁴

¹ Department of Genetics, Institute of Biology, Federal University of Rio de Janeiro (UFRJ), CEP 21944–970, RJ, Brazil

² Laboratory of Microbiology and BCCMTM/LMG Bacteria Collection, Ghent University, K.L. Ledeganckstraat 35, Ghent 9000, Belgium

³ Pathogenic Microbes Repository Unit, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565–0871, Japan

⁴ Laboratory of Genomic Research on Pathogenic Bacteria, International Research Center for Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565–0871, Japan

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We examined the genomic diversity of 29 representative *Vibrio parahaemolyticus* strains based on amplified fragment length polymorphism (AFLP) and multilocus sequence analysis (MLSA). Several strains had indistinguishable AFLP patterns, suggesting high genomic similarity. Recombination detection methods and phylogenetic reconstructions based on the gene sequences of recombination/repair protein (*recA*), DNA gyrase beta subunit (*gyrB*), and phosphoglucosyltransferase (*pgm*) revealed that recombination has occurred within the species *V. parahaemolyticus*. We suggest that homologous recombination is an important force in the evolution of *V. parahaemolyticus*.

Key words: diversity, homologous recombination, *Vibrio parahaemolyticus*, AFLP, MLSA

The species *Vibrio parahaemolyticus* was described in the 1950s during food-borne gastroenteritis outbreaks in Japan that lead to hundreds of cases of diarrhoea associated with the consumption of raw fish. In 1980, *V. alginolyticus* was proposed as a new species to encompass biotype two of *V. parahaemolyticus*. Phenotypically these two species share many features in common. Of an array of more than 50 tests, only utilization of D-glucosamine, growth at 10% NaCl, and Voges-Proskauer differ between these species¹. *V. alginolyticus* and *V. parahaemolyticus* share 67% DNA-DNA hybridization similarity (DDH), and 57% with the pair *V. harveyi* and *V. campbellii*^{5,21}. In spite of the close relatedness between these species, genomic fingerprinting analyses, such as amplified fragment length polymorphism

(AFLP) and rep-PCR, and multilocus sequence analysis (MLSA) clearly discriminate the bacterial strains and species^{5,11,18,20}. Currently, researchers are able to identify their isolates through the internet using curated MLSA data of vibrios (<http://www.taxvibrio.lncc.br/>)^{22,24}. Because *V. parahaemolyticus* includes both human and marine animal pathogenic strains⁷, identification at the strain level is crucial for pinpointing the widespread successful clones associated with human and animal health worldwide.

Since 1990s pandemic strains of *V. parahaemolyticus* have emerged from a single clone (serotype O3:K6), associated with hundreds of cases worldwide. Previous pulsed field gel electrophoresis (PFGE) data indicated that *V. parahaemolyticus* strains have a non-clonal population structure, possibly with recombination between strains giving rise to new epidemic clones^{9,16,25,26}. However, it is not clear yet if recombination occurs in housekeeping genes of *V. para-*

* Corresponding author. E-mail address: fabiano.thompson@biologia.ufrj.br; Tel./Fax: +55–21–25626382.

Table 1. Strain List

Strain	Synonym	Source	Place of isolation			Year of Isolation	PCR		Serotype
							<i>tdh</i>	<i>trh</i>	
vp1	RIMD 2210554	Patient	Japan	Osaka	Osaka aq*	1983	—	+	O4:K11
vp2	RIMD 2210633	Patient	Japan	Osaka	Kansai aq	1996	+	—	O3:K6
vp3	RIMD 2210883	Patient	Japan	Osaka	Osaka aq	1992	+	—	O4:K11
vp4	RIMD 2212470	Environment	Japan	Toyama		2001	—	—	O5:KUT
vp5	RIMD 2212490	Environment	Japan	Toyama		2001	—	—	O5:KUT
vp6	RIMD 2210086	Patient	Japan	Osaka		1968	+	—	O4:K12
vp7	RIMD 2210536	Patient	Japan	Osaka	Osaka aq	1985	—	+	O3:K6
vp8	RIMD 2210580	Environment	Japan	Naha	Naha aq	1991	—	—	O5:KUT
vp9	RIMD 2210856	Patient	Japan	Osaka	Osaka aq	1991	+	+	O10:KUT
vp10	RIMD 2211770	Environment	Thailand	Bangkok		1994	—	—	O10:K61
vp11	RIMD 2212201	Environment	Japan	Osaka		1999	—	—	O3:K20
vp12	RIMD 2212475	Environment	Japan	Toyama		1999	—	—	O5:KUT
vp13	RIMD 2212673	Patient	Japan	Osaka	Kansai aq	2000	—	+	O1:K56
vp14	RIMD 21212686	Patient	Japan	Osaka	Kansai aq	2001	+	—	O3:K6
vp15	RIMD 2212819	Patient	Japan	Osaka	Kansai aq	2001	+	+	O8:K74
vp16	RIMD 2212924	Patient	Japan	Osaka	Kansai aq	2001	+	—	O4:K68
vp17	RIMD 22121044	Patient	Japan	Osaka	Kansai aq	2001	+	—	O4:K4
vp18	RIMD 2210001	Patient	Japan	Osaka		1950	—	+	O1:K1
vp19	RIMD 2210050	Patient	Japan	Kobe		1972	+	+	O4:K12
vp20	RIMD 2210056	Patient	Japan	Kobe		1972	+	—	O4:K8
vp21	RIMD 2210384	Environment	Japan	Osaka		1976	—	—	O4:KUT
vp22	RIMD 2210600	Patient	Japan	Osaka	Kansai aq	1996	+	—	O3:K5
vp23	RIMD 2210706	Patient	Japan	Osaka	Osaka aq	1993	+	—	O4:K12
vp24	RIMD 2210877	Patient	Japan	Osaka	Osaka aq	1992	+	—	O1:KUT
vp25	RIMD 2210884	Patient	Japan	Osaka	Osaka aq	1992	+	—	O4:K8
vp26	RIMD 2210986	Environment	Thailand	Bangkok		1994	—	—	O1:KUT
vp27	RIMD 2211424	Patient	Japan	Osaka	Osaka aq	1989	+	+	O6:K18
vp28	RIMD 2211499	Patient	Japan	Osaka	Osaka aq	1994	+	—	O2:K3
vp29	RIMD 2211932	Patient	Japan	Osaka	Osaka aq	1990	+	—	O8:KUT

aq, airport quarantine station. vp, *Vibrio parahaemolyticus*. RIMD, Research Institute of Microbial Diseases, Osaka University.

Table 2. PCR and sequencing primers

Gene name	Primer name	Primer sequence (5'→3')	Binding primer position (nt)
Phosphoglucosyltransferase (<i>pgm</i>)	pgm_F	AAAGATACCCACGCGTTGTC	253
	pgm_R	GCTAAGAAATGGTTCGGGTTTCAT	977
Recombination/repair protein (<i>recA</i>)	recA_F	TCGGTAAAGGCTCTATCATG	59
	recA_R	TTAAAGCCTTGCCATACAT	803
DNA gyrase beta subunit (<i>gyrB</i>)	gyrB_F	GAAGGTGGTATTCAAGCG	655
	gyrB_R	AGAGTCACCCCTCCACAATGTA	1284

F, forward; R, reverse. The standard sequence used for primer design is the strain *V. parahaemolyticus* vp 2 (RIMD2210633).

haemolyticus and its potential effects in the whole genome of this bacterium. Homologous recombination may act as a cohesion force that purges genome wide diversity among

conspecific strains⁴). If this hypothesis is true, one should be able to detect homologous recombination among strains with highly related genomes. The aim of this study was to

access the genetic similarity of a representative collection of clinical and environmental *V. parahaemolyticus* strains by means of amplified fragment length polymorphism (AFLP) and multilocus sequence analysis (MLSA) approaches. In addition, different types of recombination detection analyses were performed in order to check if recombination may have occurred between closely related *V. parahaemolyticus* strains.

Materials and Methods

Bacterial strains

The 29 representative strains used in this study represent well characterized strains from both clinical and environmental sources, including strains with different serotypes carrying thermostable directed hemolysin (*tdh*) and thermostable related hemolysin (*trh*) genes (Table 1). Strains were grown on Trypton Soy Agar supplemented with 2% NaCl at 27°C for 24 h. DNA was extracted as described previously²⁰.

AFLP analysis

Template preparation, PCR, and electrophoresis were carried out essentially as described previously²⁰. Band patterns of 50 to 536 base pairs, were transferred into BioNumerics 2.0 software (Applied Maths, St-Martens-Latem, Belgium) for numerical analysis. Clustering of the patterns was done using Dice and UPGMA¹⁹. For the comparison of the fragments, a band position tolerance value of 0.5% was allowed to compensate for the misalignment of homologous bands due to technical imperfections.

Gene sequence analysis

The primers and the respective amplified positions of the three genetic loci (*recA*, *gyrB*, and *pgm*) examined in this study are listed in Table 2. The amplification and sequencing of fragments of these genes were performed as described previously²². The annealing temperature was 55°C for all three loci. Two runs were generated for each gene. Sequences were assembled with Kodon (Applied Biosystems, St-Martens-Latem, Belgium).

Numerical analyses

The sequences were aligned using ClustalX and phylogenetic trees were constructed by the neighbor-joining method (MEGA ver. 4.0)¹⁸. The robustness of each topology was checked by 500 bootstrap replications. Trees were drawn by using MEGA version 4.0¹².

Recombination was analyzed in each gene by using

SplitsTree version 4⁸, the Phi statistical test², Sawyers¹⁷, the Index of Association (*Ia*)¹³, and Homoplasy¹⁴ that are implemented in the software package START (<http://pubmlst.org/software/analysis/start/>)¹⁰. The GC content and the ratio of mean synonymous substitutions per synonymous site/mean non-synonymous substitutions per non-synonymous site (*ds/dn*) were also calculated using START. The gene sequences have been deposited in our own online database (<http://www.taxvibrio.lncc.br/>) and in GenBank under the accession nos. EF601726–EF601812.

Fig. 1. Dendrogram based on the AFLP band patterns using the Dice band-based similarity and Ward dendrogram building algorithm. A band position of 0.5% was allowed. LMG 2850^T and LMG 11670 originated from a diseased individual in Japan and Water of Yellow Sea (China), respectively.

Results and Discussion

The AFLP band patterns consisted of around 90 fragments ranging from 75 to 500 bp in size (Fig. 1). The highly heterogeneous strains vp20, vp24, and vp25 appeared at the outskirts of the cluster, but these strains clearly belong to the species *V. parahaemolyticus* because they share >65% AFLP pattern similarity with LMG 2850^T. It is well known that AFLP pairwise similarity above 60% corresponds to DDH similarity above 70% which circumscribes the species level^{21,23}. Some pairs of strains had nearly identical AFLP patterns (i.e. >90% similarity, equivalent to the level of reproducibility of this technique), e.g. vp6 and vp23, vp2 and vp14, vp3 and vp17. These pairs are indistinguishable by AFLP and, thus, they may be considered clonal. Strains vp6 and vp23 were isolated 25 years apart each other, yet they showed a remarkable similarity of genomes, including the presence of a *tdh* gene. Possibly these strains have highly successful genomes in the environment. The pan-

demic strains vp2, vp14, vp7, and vp16 had highly related genomes with more than 80% mutual similarity. These strains belong to serotypes associated with pandemics (O3:O6, O4:K68). The AFLP analysis revealed that both clinical and environmental isolates of *V. parahaemolyticus* share a common genetic background, suggesting that pandemic strains may arise from environmental strains via e.g. transduction or transformation^{13–15}. However, environmental strains did not possess *tdh* and *trh* genes.

The grouping shown by the phylogenetic trees based on the gene sequences of *recA*, *gyrB*, and *pgm* was in agreement with the AFLP dendrogram (Fig. 2). For instance, strains with nearly identical AFLP patterns always had identical gene sequences. However, the comparison of the three phylogenetic revealed that each gene possibly had a slightly different evolutionary story. According to *gyrB* gene sequences strain vp5 seems to be an earlier ancestor of the species from which diversification might have occurred. Two groups of highly related strains (i.e. vp16, vp3, vp17,

Fig. 2. Phylogenetic trees based on the Neighbour Joining method with the Kimura-Two parameter model and the fragments of the *recA*, *gyrB*, and *pgm* genes. Bootstrap values after 500 repetitions are shown. The scale bar is indicated (2% sequence difference). The trees were rooted with *Photobacterium profundum* SS9 gene sequences.

Table 3. Summary of gene features

Gene name	Length (nt)	dn/ds	% GC (Mean±SD)	No. alleles	Recombination* Phi	Sawyers	Homoplasy**
<i>RecA</i> protein (<i>recA</i>)	633	0.002	46.01±0.43	20	<0.001	0.00	0.004
<i>DNA gyrase, subunit B</i> (<i>gyrB</i>)	510	0.000	48.80±0.26	22	0.1	0.02	<0.001
<i>Phosphoglucomutase</i> (<i>pgm</i>)	588	0.101	46.13±0.20	24	<0.001	<0.001	<0.001

Phi recombination statistics (Bruen *et al.*, 2006). * P value, statistical significance. ** The ratio of true homoplasies/expected homoplasies if clonal for *gyrB*, *recA*, and *pgm* were 14/2.9, 151/6.1, and 14/3.7.

Fig. 3. Splits decomposition tree based on the concatenated gene sequences of *recA*, *gyrB*, and *pgm* (1731 nt). Bootstrap values after 500 repetitions are shown. The scale bar is indicated (0.1% sequence difference). Fit=91.5; fit of the data into the model of reticulate evolution.

vp22, vp14, vp2, and vp13; vp28, vp8, vp12, vp21, vp24, vp26, vp15, vp1, vp10, and vp25) on the basis of *recA* gene sequences appeared scattered in the other two trees. Recombination has been deemed one of the causes of incongruence in the topology of phylogenetic trees^{3,6}.

The three genetic loci examined in this study showed low *dn/ds* typical of housekeeping phylogenetically informative genes (Table 3). The splits decomposition analysis and the Phi test based on the concatenated sequences of the three

genes did point out to a reticulate evolution possibly due to recombination (Table 3, Figure 3). The linkage disequilibrium was evaluated using the allelic profiles of each strain (Table 4). The observed value of variance (0.147) was higher than the expected variance (0.099) if the strains were experiencing free recombination with an Index of association ($I_a=0.482$). This analysis suggested that the population was in linkage disequilibrium, but this does not preclude that recombination between strains did indeed take place³.

Table 4. Summary of allelic profiles

Strain	<i>gyrB</i>	<i>pgm</i>	<i>recA</i>
vp1	6	16	12
vp2	7	22	7
vp3	11	13	6
vp4	12	10	10
vp5	2	19	11
vp6	1	12	20
vp7	7	21	18
vp8	5	8	16
vp9	8	2	19
vp10	9	23	12
vp11	4	17	5
vp12	3	9	17
vp13	10	18	8
vp14	7	22	6
vp15	7	4	12
vp16	7	22	6
vp17	11	13	6
vp18	13	3	1
vp19	14	1	3
vp20	15	8	4
vp21	16	15	14
vp22	17	5	6
vp23	1	11	20
vp24	18	16	12
vp25	15	8	13
vp26	19	24	12
vp27	20	14	9
vp28	21	6	15
vp29	22	20	2

The *Ia* alone is not a good predictor of recombination if the collection of strains is small and/or biased through clinical isolates where the clonal spread of a successful strain is very common. Ideally different recombination detection tests should be applied in order to confidently find recombination events. In this study, the tests Phi, Sawyers, and Homoplasy did point to recombination (Table 3).

The Phi recombination test takes polymorphic sites in an alignment with opposing incongruent phylogenetic signals as breakpoints of recombination, while the Homoplasy test points out that excess Homoplasy (that is, shared similarity in different branches of a phylogenetic tree not inherited directly from an ancestor) is likely to have occurred due to recombination. The most parsimonious explanation for finding identical substitutions in different branches of a tree is recombination. Several events of mutation would be necessary to explain Homoplasies. According to Sawyer

(1989)¹⁷, a gene conversion event will increase the value of the sum of the squares of condensed fragments (SSCF) because it results in an identical region within two sequences and may produce an unusually long fragment.

In this study we analyzed the gene sequences of *V. parahaemolyticus* by means of several recombination detection methods. Overall, our analysis points out that homologous recombination between conspecific strains of *Vibrio parahaemolyticus* is an important force possibly purging diversification of this species. Work is under way to unravel the impact of genome-wide recombination in the evolution of *Vibrio parahaemolyticus* genomes. Recombination rates may well vary in different portions of the genomes, with hypervariable (e.g. the superintegron region) and conserved regions (e.g. the *oriC* regions), playing distinct roles in the dynamics of vibrios genomes and their biology.

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