Genomic Diversity of Clinical and Environmental *Vibrio cholerae* Strains Isolated in Brazil between 1991 and 2001 as Revealed by Fluorescent Amplified Fragment Length Polymorphism Analysis

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Vibrio cholerae* is a ubiquitous and abundant organism in aquatic environments, particularly in coastal areas, estuaries, and rivers. This organism was the cause of a considerable number of deaths in Brazil during the last decade. In this study we applied the genomic fingerprinting technique fluorescent amplified fragment length polymorphism (FAFLP) to analyze 106 *V. cholerae* O1 and non-O1 and non-O139 strains isolated from clinical specimens and the environment between 1991 and 2001. Numerical analysis of the FAFLP patterns disclosed seven main groups of genomes, all of them originated from a variety of different places in different years, suggesting that *V. cholerae* is a very diverse species. O1 and non-O1 and non-O139 strains were distinguishable by FAFLP, although clinical and environmental strains clustered together in a few cases. The persistence of some strains of highly related genomes during several years and in completely different geographical regions suggests that these strains are highly successful in adapting to changing environmental conditions.

Between 1991 and 1998 at least 1,296 deaths due to cholera occurred in Brazil, mostly in the northern and northeastern regions of the country (25). The lack of a water network supply and sanitation as well as poverty were concluded to be the main reasons for this high rate of mortality. The study of the *Vibrio cholerae* population structure and cholera molecular epidemiology may help us to understand the spread of this disease. Molecular typing techniques, i.e., fluorescent amplified fragment length polymorphism (FAFLP), repetitive extragenic palindrome-PCR, multilocus enzyme electrophoresis (MLEE), multilocus sequence typing (MLST), randomly amplified polymorphic DNA, and ribotyping, have been extensively applied in the study of *V. cholerae* (5). Although ribotyping has been perhaps the most used technique in the study of the diversity, epidemiology, and evolution of *V. cholerae* (24), Lan and Reeves (14) have recently highlighted the fact that most of the variation in ribotyping is due to recombination events which take place at a very high rate (10^{-5} per cell per generation). In these cases, ribotyping the similarity between isolates does not reflect their origin from the same ancestor. It has been suggested that a robust and reproducible alternative is the use of FAFLP fingerprinting technique (11, 15). FAFLP analysis is performed basically in four steps (5, 10). (i) The digestion of total genomic DNA is accomplished with two restriction enzymes, a 4-base cutter (e.g., *Taq*I) which digests the DNA frequently and a 6-base cutter (e.g., *Apa*I) which digests the DNA less frequently. Subsequent ligation of the restriction halfsite-specific adaptors to all restriction fragments

is performed by using a DNA ligase. (ii) Selective amplification of these fragments is done with two PCR primers that have corresponding adaptor and restriction site sequences as their target sites. Selective bases that flank the restriction site sequences are included in the primers to reduce the number of fragments amplified. The final steps are (iii) electrophoretic separation of the PCR products on polyacrylamide gels with selective detection of fragments which contain the fluorescently labeled primer and (iv) computer-assisted numerical analysis of the band patterns. FAFLP indexes variation in the whole genome and thus is considered to give useful information on the short- and long-term evolution of bacterial strains. In the present study we applied FAFLP analysis to uncover the genomic diversity of clinical and environmental *V. cholerae* O1 and non-O1 and non-O139 isolates from Brazil during a 11-year sampling period, corresponding to the beginning of the seventh pandemic in Latin America.

MATERIALS AND METHODS

Bacterial strains, growth condition, and DNA extraction. A total of 106 *V. cholerae* strains, i.e., 10 reference strains and 96 isolates originated from different Brazilian states were examined in this study (Fig. 1). Eighty-seven strains originated from clinical specimens, and 20 strains were isolated from the environment. Strains were grown aerobically on tryptone soy agar (Oxoid) supplemented with 2% (wt/vol) NaCl for 24 h at 28°C. DNA was extracted following the method described by Pitcher et al. (16a). All strains included in this study are deposited in the BCCM/LMG bacterial collection at Ghent University (Ghent, Belgium) and in the Oswaldo Cruz Institute Collection (Rio de Janeiro, Brazil).

FAFLP template preparation and PCRs. Template preparation was carried out essentially as described previously (11, 22). Briefly, 1 μg of high-molecular-mass DNA was digested with *Taq*I and *Apa*I (Amersham Pharmacia Biotech, Uppsala, Sweden) followed by ligation of restriction half-site-specific adapters to all restriction fragments with T4 ligase (Amersham Pharmacia Biotech). An aliquot of 2.5 μl of template was mixed with 0.8 μl of A01-6FAM primer (5′-GACTGCGTACAGGCCCA-3′; 1 μM), 0.8 μl of T01-ABI (5′-CGATGAG

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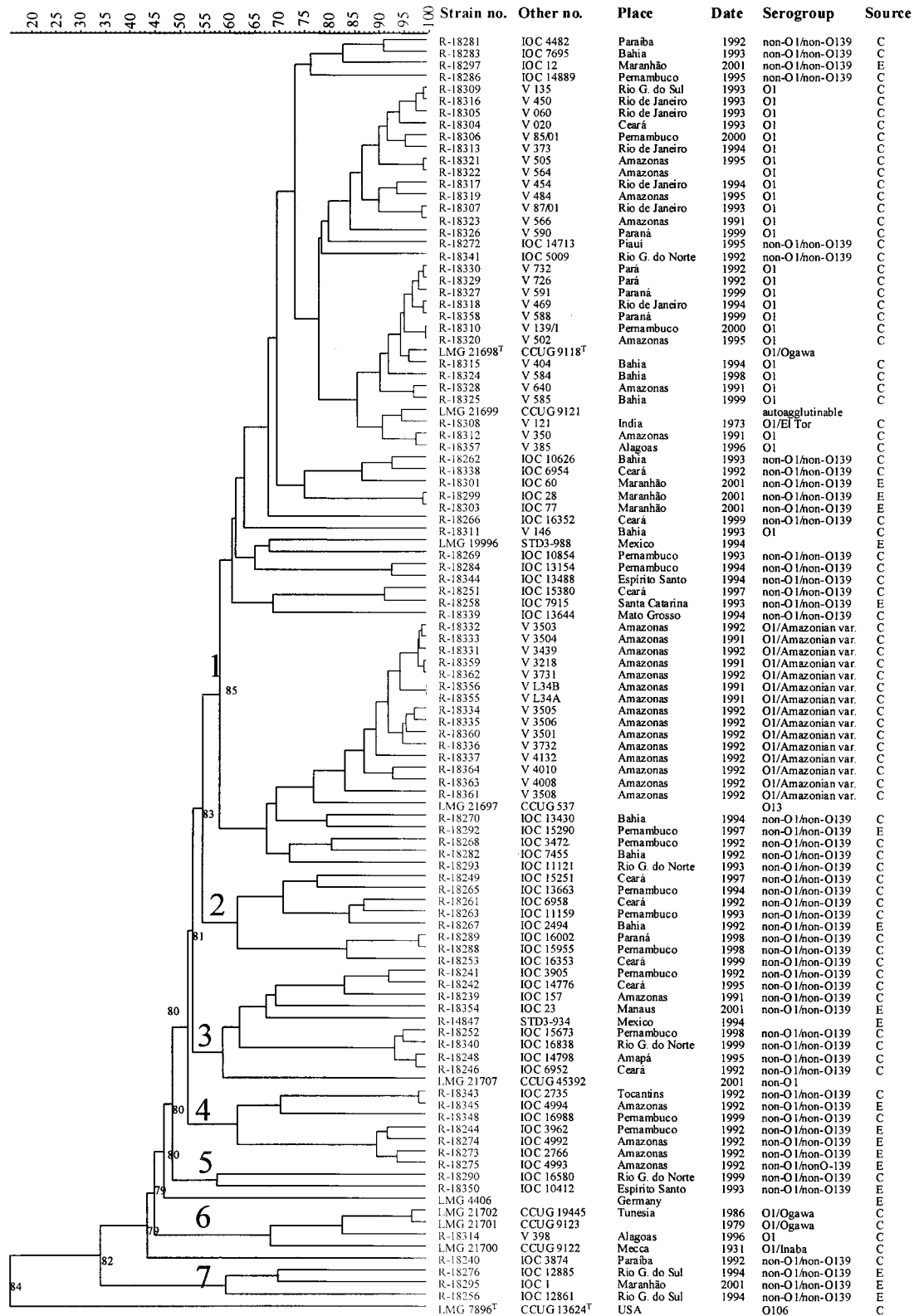
Strain no.	Other no.	Place	Date	Serogroup	Source
R-18281	IOC 4482	Paraíba	1992	non-O1/non-O139	C
R-18283	IOC 7695	Bahia	1993	non-O1/non-O139	C
R-18297	IOC 12	Maranhão	2001	non-O1/non-O139	E
R-18286	IOC 14889	Pernambuco	1995	non-O1/non-O139	C
R-18309	V 135	Rio G. do Sul	1993	O1	C
R-18316	V 450	Rio de Janeiro	1993	O1	C
R-18305	V 060	Rio de Janeiro	1993	O1	C
R-18304	V 020	Ceará	1993	O1	C
R-18306	V 85/01	Pernambuco	2000	O1	C
R-18313	V 373	Rio de Janeiro	1994	O1	C
R-18321	V 505	Amazonas	1995	O1	C
R-18322	V 564	Amazonas		O1	C
R-18317	V 454	Rio de Janeiro	1994	O1	C
R-18319	V 484	Amazonas	1995	O1	C
R-18307	V 87/01	Rio de Janeiro	1993	O1	C
R-18323	V 566	Amazonas	1991	O1	C
R-18326	V 590	Paraná	1999	O1	C
R-18272	IOC 14713	Piauí	1995	non-O1/non-O139	C
R-18341	IOC 5009	Rio G. do Norte	1992	non-O1/non-O139	C
R-18330	V 732	Pará	1992	O1	C
R-18329	V 726	Pará	1992	O1	C
R-18327	V 591	Paraná	1999	O1	C
R-18318	V 469	Rio de Janeiro	1994	O1	C
R-18358	V 588	Paraná	1999	O1	C
R-18310	V 139/1	Pernambuco	2000	O1	C
R-18320	V 502	Amazonas	1995	O1	C
LMG 21698^T	CCUG 9118^T			O1/Ogawa	
R-18315	V 404	Bahia	1994	O1	C
R-18324	V 584	Bahia	1998	O1	C
R-18328	V 640	Amazonas	1991	O1	C
R-18325	V 585	Bahia	1999	O1	C
LMG 21699	CCUG 9121			autoagglutinable	
R-18308	V 121	India	1973	O1/El Tor	C
R-18312	V 350	Amazonas	1991	O1	C
R-18357	V 385	Alagoas	1996	O1	C
R-18262	IOC 10626	Bahia	1993	non-O1/non-O139	C
R-18338	IOC 6954	Ceará	1992	non-O1/non-O139	C
R-18301	IOC 60	Maranhão	2001	non-O1/non-O139	E
R-18299	IOC 28	Maranhão	2001	non-O1/non-O139	E
R-18303	IOC 77	Maranhão	2001	non-O1/non-O139	E
R-18266	IOC 16352	Ceará	1999	non-O1/non-O139	C
R-18311	V 146	Bahia	1993	O1	C
LMG 19996	STD3-988	Mexico	1994		E
R-18269	IOC 10854	Pernambuco	1993	non-O1/non-O139	C
R-18284	IOC 13154	Pernambuco	1994	non-O1/non-O139	C
R-18344	IOC 13488	Espírito Santo	1994	non-O1/non-O139	C
R-18251	IOC 15380	Ceará	1997	non-O1/non-O139	C
R-18258	IOC 7915	Santa Catarina	1993	non-O1/non-O139	E
R-18339	IOC 13644	Mato Grosso	1994	non-O1/non-O139	C
R-18332	V 3503	Amazonas	1992	O1/Amazonian var.	C
R-18333	V 3504	Amazonas	1991	O1/Amazonian var.	C
R-18331	V 3439	Amazonas	1992	O1/Amazonian var.	C
R-18359	V 3218	Amazonas	1991	O1/Amazonian var.	C
R-18362	V 3731	Amazonas	1992	O1/Amazonian var.	C
R-18356	V L34B	Amazonas	1991	O1/Amazonian var.	C
R-18355	V L34A	Amazonas	1991	O1/Amazonian var.	C
R-18334	V 3505	Amazonas	1992	O1/Amazonian var.	C
R-18335	V 3506	Amazonas	1992	O1/Amazonian var.	C
R-18360	V 3501	Amazonas	1992	O1/Amazonian var.	C
R-18336	V 3732	Amazonas	1992	O1/Amazonian var.	C
R-18337	V 4132	Amazonas	1992	O1/Amazonian var.	C
R-18364	V 4010	Amazonas	1992	O1/Amazonian var.	C
R-18363	V 4008	Amazonas	1992	O1/Amazonian var.	C
R-18361	V 3508	Amazonas	1992	O1/Amazonian var.	C
LMG 21697	CCUG 537			O13	
R-18270	IOC 13430	Bahia	1994	non-O1/non-O139	C
R-18292	IOC 15290	Pernambuco	1997	non-O1/non-O139	E
R-18268	IOC 3472	Pernambuco	1992	non-O1/non-O139	C
R-18282	IOC 7455	Bahia	1992	non-O1/non-O139	C
R-18293	IOC 11121	Rio G. do Norte	1993	non-O1/non-O139	C
R-18249	IOC 15251	Ceará	1997	non-O1/non-O139	C
R-18265	IOC 13663	Pernambuco	1994	non-O1/non-O139	C
R-18261	IOC 6958	Ceará	1992	non-O1/non-O139	C
R-18263	IOC 11159	Pernambuco	1993	non-O1/non-O139	C
R-18267	IOC 2494	Bahia	1992	non-O1/non-O139	E
R-18289	IOC 16002	Paraná	1998	non-O1/non-O139	C
R-18288	IOC 15955	Pernambuco	1998	non-O1/non-O139	C
R-18253	IOC 16353	Ceará	1999	non-O1/non-O139	C
R-18241	IOC 3905	Pernambuco	1992	non-O1/non-O139	C
R-18242	IOC 14776	Ceará	1995	non-O1/non-O139	C
R-18239	IOC 157	Amazonas	1991	non-O1/non-O139	C
R-18354	IOC 23	Manaus	2001	non-O1/non-O139	E
R-14847	STD3-934	Mexico	1994		E
R-18252	IOC 15673	Pernambuco	1998	non-O1/non-O139	C
R-18340	IOC 16838	Rio G. do Norte	1999	non-O1/non-O139	C
R-18248	IOC 14798	Amapá	1995	non-O1/non-O139	C
R-18246	IOC 6952	Ceará	1992	non-O1/non-O139	C
LMG 21707	CCUG 45392		2001	non-O1	
R-18343	IOC 2735	Tocantins	1992	non-O1/non-O139	C
R-18345	IOC 4994	Amazonas	1992	non-O1/non-O139	E
R-18348	IOC 16988	Pernambuco	1999	non-O1/non-O139	C
R-18244	IOC 3962	Pernambuco	1992	non-O1/non-O139	E
R-18274	IOC 4992	Amazonas	1992	non-O1/non-O139	E
R-18273	IOC 2766	Amazonas	1992	non-O1/non-O139	E
R-18275	IOC 4993	Amazonas	1992	non-O1/nonO-139	E
R-18290	IOC 16580	Rio G. do Norte	1999	non-O1/non-O139	E
R-18350	IOC 10412	Espírito Santo	1993	non-O1/non-O139	E
LMG 4406		Germany			E
LMG 21702	CCUG 19445	Tunesia	1986	O1/Ogawa	C
LMG 21701	CCUG 9123		1979	O1/Ogawa	C
R-18314	V 398	Alagoas	1996	O1	C
LMG 21700	CCUG 9122	Mecca	1931	O1/Inaba	C
R-18240	IOC 3874	Paraíba	1992	non-O1/non-O139	C
R-18276	IOC 12885	Rio G. do Sul	1994	non-O1/non-O139	E
R-18295	IOC 1	Maranhão	2001	non-O1/non-O139	E
R-18256	IOC 12861	Rio G. do Sul	1994	non-O1/non-O139	E
LMG 7896^T	CCUG 13624^T	USA		O106	C

FIG. 1. Dendrogram derived from FAFLP patterns of 106 *V. cholerae* strains with the *Apa*I/*Taq*I enzyme combination. The dendrogram was constructed by using the Pearson coefficient and the unweighted pair group method of arithmetic averages. *V. mimicus* LMG 7896^T was included as an out-group. The year, place, and source of isolation of strains are listed. C and E mean clinical and environmental isolates, respectively. Cophenetic correlation values are shown on the left.

TCCTGACCGAA-3′; 5 μM), and 12 μl of amplification core mix (Applied Biosystems). The A01-6FAM primer is fluorescently labeled, and the selective bases (adenosine) at the 3′ end are underlined.

The amplification reactions were performed in a GeneAmp PCR System 9600 thermocycler (Applied Biosystems) as described previously (22).

Capillary electrophoresis and numerical analysis. Six microliters of the selective PCR product was mixed with 24 μl of deionized formamide, 1 μl of GeneScan-500 TAMRA size standard, and 1 μl of GeneScan-2500 TAMRA size standard and heated at 95°C for 3 min. Subsequently, the mix was chilled on ice for a few minutes. Separation of the selective PCR products was generated in an ABI 310 genetic analyzer (Applied Biosystems). The time and voltage of sample injection were 16 s and 15 kV, respectively. Each run was performed at 60°C for 28 min at 10 mA and 15 kV. Normalization of the patterns was performed by the GeneScan 3.1 software (Applied Biosystems). Normalized tables of peaks, containing fragments of 50 to 536 bp, were transferred into BioNumerics 2.5 software (Applied Maths, Sint-Martens-Latem, Belgium) for numerical analyses. Pattern similarity was calculated by using the Pearson coefficient, and dendrograms were obtained by unweighted pair group method of arithmetic averages (21). Cophenetic correlation and a cluster cutoff algorithm based on the Point-Biserial correlation (12) were applied in order to evaluate the robustness of clusters. Additionally, principal component analysis (PCA), a nonhierarchical technique, was performed on binarized FAFLP data in order to obtain the relatedness among strains in a three-dimensional space.

DNA hybridization. DNA-DNA hybridization was performed under stringent conditions by using the microplate technique with photobiotin-labeled DNA at a temperature of 40°C for 3 h as described previously (26). DNA similarity values are means of reciprocal and nonreciprocal reactions, each of which are performed in quadruplicates.

RESULTS AND DISCUSSION

The results depicted in Fig. 1 clearly denote that *V. cholerae* is a diverse species. The strains examined consist of seven main groups of genomes, not correlated to particular dates or places. This grouping was supported by cophenetic correlation and a cluster cutoff algorithm. Cluster 1 ($n = 70$) was the largest group, including the type strain of *V. cholerae*, LMG 21698^T, and other established reference strains such as LMG 19996 (22), LMG 21699, El Tor R-18308, and Amazon variant strains (3). Cluster 1 was composed of O1 and non-O1 and non-O139 strains isolated from patients and the environment. Overall isolates from the different serogroups formed subgroups, whereas some clinical and environmental strains (e.g., R-18301 and R-18338; R-18251 and R18258) were highly related. Some clonal groups of strains (i.e., strains having about 95% pattern similarity) were correlated by either the year or the region of isolation. For instance, strains R-18309, R-18316, R-18305, and R-18304 were all isolated in 1993 but in states at least 1,500 km away from each other. Strains R-18331, R-18332, R-18333, R-18355, R-18356, R-18359, and R-18362 were all isolated in Amazonas state in 1991 to 1992 and were related to the majority of the cases of cholera in that region (3). These strains have been referred to as a new variant of *V. cholerae* O1, the Amazon variant. According to many phenotypic and genotypic techniques, this variant is completely distinct from other *V. cholerae* O1 strains (3).

Cluster 2 ($n = 8$) consisted of non-O1 and non-O139 strains, with all strains but one (R-18267) from patients mostly in northeastern Brazil between 1992 and 1999. Strains R-18288 and R-18289, isolated at very distant places in 1998, were clearly a single clone. Cluster 3 ($n = 10$) harbored clinical and environmental non-O1 strains, including the reference strain R-14847 analyzed in a previous study (22). A conspicuous subgroup of highly related strains, i.e., R-18246, R-18248, R-18252, and R-18340, which were all isolated in northeastern
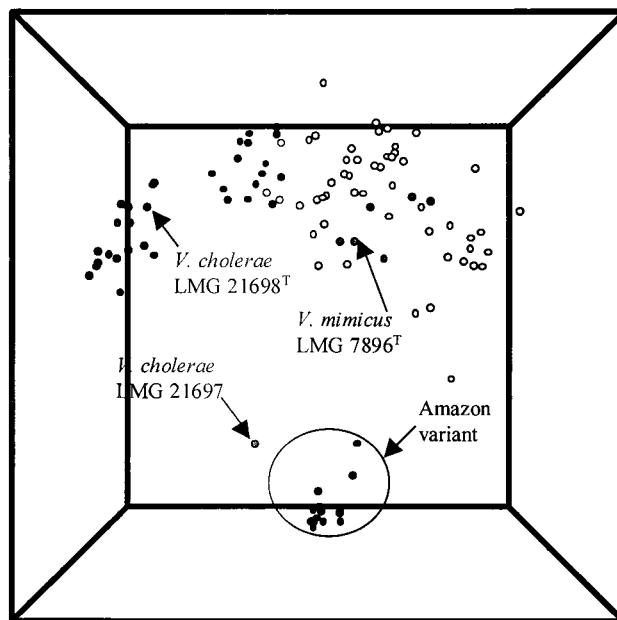

FIG. 2. Three-dimensional representation of PCA derived from the FAFLP patterns (24 ± 4 bands) of 106 *V. cholerae* strains. Black dots indicate O1 strains while white and grey dots indicate non-O1 strains and strains of other serogroups, respectively.

Brazil was disclosed. Cluster 4 ($n = 7$) consisted mostly of environmental non-O1 and non-O-139 strains isolated in 1992 while cluster 5 ($n = 2$) was composed of two less-related strains. Cluster 6 ($n = 4$) harbored four reference O1 strains from clinical specimens isolated over a span of 65 years. Cluster 7 ($n = 3$) consisted of three environmental non-O1 and non-O139 strains. A few strains were unclustered; this was the case for the former *Vibrio albensis* type strain LMG 4406 and for R-18240.

V. cholerae O1 and non-O1 were distinguishable but yet closely related by FAFLP analysis (Fig. 1 and 2). Although certain overlap between O1 and non-O1 strains was observed, most O1 and non-O1 strains were clustering apart (Fig. 2). The high genome plasticity of *V. cholerae* O1 and non-O1 as revealed by our FAFLP analysis is in agreement with previous detailed investigations. When MLEE analysis of 15 housekeeping enzyme loci was applied to a collection of 107 clinical and environmental *V. cholerae* strains, 99 electrophoretic types were disclosed and no significant clustering between serogroups, biotype, and country of isolation were observed, suggesting that *V. cholerae* strains share a common genetic background regardless of phenotype and/or geographical distribution (6). Subsequently, the same workers applied MLST of six housekeeping enzyme loci on a subset of 31 *V. cholerae* serogroup O139 strains (7). Among this subset they found four groups of strains, clearly proving that *V. cholerae* serogroup O139 consists of multiple epidemic lineages.

Because the Amazon O1 population was so apart from the other *V. cholerae* strains as revealed by PCA (Fig. 2), we decided to perform DNA-DNA hybridization experiments to check whether this population would not be in fact another species. *V. cholerae* strains, including the Amazon variant, were highly related, i.e., ≥94% similarity, despite their long span of

TABLE 1. DNA-DNA similarity values

Strain	% Similarity to strain:							
	R-18333	R-18337	R-18355	R-18306	R-18308	R-18325	LMG 21698[T]	LMG 7896[T]
V. cholerae Amazon variant R-18333	100							
V. cholerae Amazon variant R-18337	98	100						
V. cholerae Amazon variant R-18355	102	103	100					
V. cholerae R-18306	97	96	98	100				
V. cholerae R-18308	96	97	98	99	100			
V. cholerae R-18325	94	97	97	97	101	100		
V. cholerae LMG 21698[T]	96	96	101	99	99	99	100	
V. mimicus LMG 7896[T]	73	73	73	74	75	77	79	100

isolation (Table 1). This similarity level corresponded to FAFLP pair-wise pattern similarities of about 75%, indicating the higher discriminatory power of the latter technique. Our careful experiments not only proved that the Amazon variant belongs to the species *V. cholerae* but also showed that *Vibrio mimicus* LMG 7896[T] is highly related to *V. cholerae* (Table 1). The species *V. mimicus* was proposed by Davis et al. in 1981 (4) to encompass biochemically atypical *V. cholerae* strains. These authors reported high DNA-DNA similarity (i.e., 67%) among *V. cholerae* and *V. mimicus*. Nevertheless, these two species can be clearly differentiated on the basis of AFLP (22) and MLEE (23).

Another interesting aspect of this study is the diversity of non-O1 and non-O139 strains within the epidemics of cholera in Brazil. These strains, which were isolated mostly from patients with watery diarrhea, showed a remarkably high genome diversity as they appeared in different FAFLP clusters and they were also related to O1 strains. Serogroup O1 strains were supposed to be the only strains responsible for epidemic cholera, but in 1993 serogroup O139 was found to be the cause of epidemics of cholera in Bangladesh and India (8). Although currently most outbreaks throughout the world are caused by serogroup O1 or O139, in Brazil, cholera cases due to non-O1 and non-O139 strains have been observed. A typical example of such a successful lineage is the group represented by the strains R-18246, R-18248, R-18252, and R-18340, which were isolated in northeastern Brazil during 1992 to 1999 (Fig. 1, cluster 3).

While the precise role of non-O1 and non-O139 strains in the cholera outbreaks in Brazil is unknown at present, our results suggest that strains of different serogroups are highly related and may be important in the evolution of *V. cholerae* and cholera. Recent studies on the distribution of virulence genes in *V. cholerae* strains isolated in Brazil and worldwide have demonstrated that both clinical and environmental strains of *V. cholerae* of different serogroups may harbor virulence genes, i.e., *ctxAB* and *tcpA* genes, and are thus potentially pathogenic (1, 2, 17). Lateral gene transfer of both virulence and antibiotic resistance genes is an important and ancient process that has been taking place in the evolution of gram-negative bacterial pathogens (8, 13, 18, 19). Bearing this process in mind and taking into consideration the high abundance of vibrios (i.e., $\sim 10^8$ cells $\cdot$ liter^{-1} or up to 4% of *Bacteria*) in aquatic environments (9), an important aspect to consider in the study of the evolution of cholera would be the role of other *Vibrio* species (currently more than 55) as potential reservoirs of virulence genes (16, 20). MLST of housekeeping and viru-

lence genes is under way in order to better understand the population biology of vibrios, including *V. cholerae*.

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