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The *fur* Gene as a New Phylogenetic Marker for *Vibrionaceae* Species Identification

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Microbial taxonomy is essential in all areas of microbial science. The 16S rRNA gene sequence is one of the main phylogenetic species markers; however, it does not provide discrimination in the family *Vibrionaceae*, where other molecular techniques allow better interspecies resolution. Although multilocus sequence analysis (MLSA) has been used successfully in the identification of *Vibrio* species, the technique has several limitations. They include the fact that several locus amplifications and sequencing have to be performed, which still sometimes lead to doubtful identifications. Using an *in silico* approach based on genomes from 103 *Vibrionaceae* strains, we demonstrate here the high resolution of the *fur* gene in the identification of *Vibrionaceae* species and its usefulness as a phylogenetic marker. The *fur* gene showed within-species similarity higher than 95%, and the relationships inferred from its use were in agreement with those observed for 16S rRNA analysis and MLSA. Furthermore, we developed a *fur* PCR sequencing-based method that allowed identification of *Vibrio* species. The discovery of the phylogenetic power of the *fur* gene and the development of a PCR method that can be used in amplification and sequencing of the gene are of general interest whether for use alone or together with the previously suggested loci in an MLSA.

In microbial science, rapid identification of isolates to the genus or species level is essential in many areas, for instance, when diagnosing an infection or unraveling the microbial diversity in different niches. Therefore, identification and classification must be reliable, reproducible, and informative and at the same time fast and user friendly. Hence, tools that allow identification should be easy and affordable for the average user (1).

Identification of microbial species was originally based on phenotypic assays, due to both the influence of other biology disciplines, such as botany, and the technological limitations at the time (2). While phenotypic identification has been an important tool, the approach was of more limited use for several microbial groups, including the family *Vibrionaceae*, where some species had indistinguishable phenotypes and other species could have divergent phenotypes among strains of the same species (1). More recently, genetically based methods, in particular, have been developed for species identification and phylogeny, with the "molecular clock" approach introduced by Carl Woese proving a strong tool (3). The most common genetic marker has been the 16S rRNA gene, but the sequences of a range of housekeeping genes are also being used. In the future, genome sequences (gene sequences or single-nucleotide polymorphisms [SNP]) will be used in phylogeny and identification.

The family *Vibrionaceae* comprises 159 species in 6 genera, of which the genus *Vibrio* is the largest and most extensively characterized (4, 5). For many genera, the common approach of using 16S rRNA gene similarity as the main phylogenetic species marker has failed, due to its low interspecies resolution (6).

Due to the limitations of the 16S rRNA gene phylogeny in *Vibrio* classification and to the development of molecular techniques, such as fluorescent amplified fragment length polymorphism (FALP) and multilocus sequence analysis (MLSA), the classification of *Vibrionaceae* soon evolved from using a single gene to using several gene sequences for identification and phylogeny. In particular, the introduction of an MLSA scheme using nine gene sequences (*ftsZ*, *gapA*, *gyrB*, *mreB*, *pyrH*, *recA*, *rpoA*, *topA*, and the 16S rRNA gene) has provided much higher resolution for *Vibrionaceae* identification and taxonomy, and this has allowed the identification of new *Vibrio* species previously misidentified (6–11).

While the use of MLSA as described above has improved the resolution of *Vibrionaceae* phylogeny, it has been hampered by requiring several gene sequences. With the explosion of genome sequencing, this will become easier; however, for a great number of laboratories, the use of a single gene sequence for identification and phylogeny will be preferable for years to come. The use of the *fur* gene as a phylogenetic marker in bacteria has been suggested in several studies (12, 13). Also, one study (14) suggested the use of the *fur* gene as a discriminative phylogenetic marker between the species *Alliivibrio salmonicida* and *Alliivibrio logei* (previously *Vibrio salmonicida* and *Vibrio logei*, respectively); however, to our knowledge, no further work has addressed this possible marker. The *fur* gene encodes a ferric uptake regulator (Fur), which in most bacterial species is the major system for maintenance of iron homeostasis. Fur senses excess intracellular Fe^{2+} and binds to the promoter regions of the genes involved in iron acquisition,

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TABLE 1 *Vibrionaceae* strains used in this study

Species	Strain	WGS/*fur* GenBank accession no.
Strains used in the *in silico* study[a]		
Aliivibrio fischeri	ZF-211	AJYI01
Aliivibrio logei	5S-186	AJYJ01
Aliivibrio logei	ATCC 35077	ASAH01
Enterovibrio calviensis	DSM 14347^T	JHZA01
Enterovibrio norvegicus	FF-33	AJYD01
Grimontia hollisae	CIP 101886^T	ADAQ01
Grimontia sp.	AK16	ANFM02
Photobacterium aphoticum	JCM 19237	BBMN01
Photobacterium angustum	S14	AAOJ01
Photobacterium damselae subsp. *damselae*	CIP 102761^T	ADBS01
Photobacterium damselae subsp. *piscicida*	DI21	AKYG01
Photobacterium halotolerans	S2753	JMIB01
Photobacterium halotolerans	DSM 18316^T	AULG01
Photobacterium leiognathi	lrivu.4.1	BANQ01
Photobacterium leiognathi subsp. *mandapamensis*	svers.1.1.	BACE01
Photobacterium phosphoreum	ANT220	CCAR01
Photobacterium profundum	3TCK	AAPH01
Photobacterium sp.	SKA34	AAOU01
Photobacterium sp.	AK15	AMZO01
Salinivibrio costicola subsp. *costicola*	ATCC 33508^T = LMG 11651^T	ASAI01
Shewanella xiamenensis	BC01	JAEC01
Vibrio albensis = *V. cholerae*	VL426	ACHV01
Vibrio anguillarum	96F	AEZA01
Vibrio anguillarum	RV22	AEZB01
Vibrio azureus	NBRC 104587^T	BATL01
Vibrio brasiliensis	LMG 20546^T	AEVS01
Vibrio breoganii	ZF-55	AJYL01
Vibrio campbellii	CAIM 519^T = NBRC 15631^T	AMDG01
Vibrio caribbeanicus	ATCC BAA-2122^T	AEIU01
Vibrio cholerae	MO10	AAKF03
Vibrio cholerae	CP110	AMWF01
Vibrio cholerae	TM 11079-80	ACHW01
Vibrio coralliilyticus	P1	AEQS01
Vibrio coralliilyticus	ATCC BAA-450^T	ACZN01
Vibrio coralliilyticus	OCN008	AVOO01
Vibrio cyclitrophicus	ZF14	AIDH01
Vibrio cyclitrophicus	FF75	ATLT01
Vibrio diazotrophicus	NBRC 103148^T	BBJY01
Vibrio ezurae	NBRC 102218^T	BATM01
Vibrio fortis	Dailan14	JFFR01
Vibrio fluvialis	PG41	ASXS01
Vibrio fluvialis	I21563	ASXT01
Vibrio halioticoli	NBRC 102217^T	BAUJ01
Vibrio harveyi	CAIM 1792	AHHQ01
Vibrio harveyi	NBRC 15634 = ATCC 14126^T	BAOD01
Vibrio harveyi	ZJ0603	AKIH01
Vibrio harveyi	AOD131	AOMR01
Vibrio harveyi	E385	AYKI01
Vibrio harveyi	VHJR4	CAUN01
Vibrio harveyi	VHJR7	CAUO01
Vibrio ichthyoenteri	ATCC 700023^T	AFWF01
Vibrio jasicida	090810c	BAOC01
Vibrio kanaloae	5S-149	AJYX01
Vibrio litoralis	DSM 17657^T	AUFZ01
Vibrio maritimus	JCM 19240	BBMT01
Vibrio maritimus	JCM 19235	BBMR01
Vibrio metschnikovii	CIP 69.14^T	ACZO01
Vibrio mimicus	VM223	ADAJ01
Vibrio mimicus	MB451	ADAF01
Vibrio mimicus	CAIM 602^T	AOMO01
Vibrio mimicus	SX-4	ADOO01
Vibrio natriegens	NBRC 15636^T = ATCC 14048^T = DSM 759^T	ATFJ01
Vibrio nigripulchritudo	ATCC 27043^T	AFWJ01
Vibrio nigripulchritudo	FTn2	CANW01
Vibrio nigripulchritudo	SOn1	CAOF01
Vibrio ordalii	FS-238	AJYS01
Vibrio ordalii	12B09	AJYV01
Vibrio ordalii	FF-93	AJYT01
Vibrio ordalii	FS-144	AJYU01
Vibrio orientalis	CIP 102891^T = ATCC 33934^T	ACZV01
Vibrio owensii	ATCC 25919	BANZ01
Vibrio owensii	CAIM 1854^T	BAOH01
Vibrio pacinii	DSM 19139^T	JONH01
Vibrio parahaemolyticus	NIHCB0603	AVOM01
Vibrio parahaemolyticus	IDH02189	JAHD01
Vibrio proteolyticus	NBRC 13287^T	BATJ01
Vibrio rhizosphaerae	DSM 18581^T	JONG01
Vibrio rotiferianus	DAT722	AFAJ01
Vibrio rumoiensis	1S-45	AJYK01
Vibrio sagamiensis	NBRC 104589^T	BAOJ01
Vibrio scophthalmi	LMG 19158^T	AFWE01
Vibrio shilonii = *V. mediterranei*	AK1^T	ABCH01
Vibrio sinaloensis	DSM 21326	AEVT01
Vibrio sp.	PPCK-2014	JJMN01
Vibrio splendidus	ZS-139	AJZE01
Vibrio splendidus	FF-6	AJZI01
Vibrio splendidus	ATCC 33789	AFWG01
Vibrio splendidus	12B01	AAMR01
Vibrio splendidus	12E03	AJZD01
Vibrio splendidus	ZF-90	AJZF01
Vibrio splendidus	5S-101	AJZG01
Vibrio splendidus	FF-500	AJZH01
Vibrio splendidus	1F-157	AJZJ01
Vibrio splendidus	1S-124	AJZL01
Vibrio tasmaniensis	1F-187	AJZM01
Vibrio tasmaniensis	1F-155	AJZN01
Vibrio tasmaniensis	5F-79	AJZP01
Vibrio tasmaniensis	ZS-17	AJZQ01
Vibrio tubiashii	ATCC 19109^T	AFWI01
Vibrio tubiashii	NCIMB 1337 = ATCC 19106	AHHF01
Vibrio variabilis	JCM 19239	BBMS01
Vibrio vulnificus	BAA87	JDSE01
Vibrio vulnificus	NBRC 15645^T = ATCC 27562^T	AMQV01
Culture collection strains used in PCR sequencing method development		
Aliivibrio fischeri	DSM 2168	KP721366

(Continued on following page)

TABLE 1 (Continued)

Species	Strain	WGS/*fur* GenBank accession no.
Enterovibrio calviensis	DSM 14347^T	KP721381
Grimontia hollisae	DSM 15132^T	KP721382
Photobacterium damselae	ATCC 33539^T	KP721367
Photobacterium halotolerans	LMG 22194^T	KP721368
Photobacterium angustum	S14	KP721369
Photobacterium rosenbergii	LMG 22223^T	KP721370
Vibrio coralliilyticus	ATCC BAA-450^T = DSM 19607^T	KP721371
Vibrio fluvialis	NCTC 11327^T	KP721372
Vibrio harveyi	DSM 19623^T = ATCC 14126^T	KP721373
Vibrio nigripulchritudo	ATCC 27043^T	KP721374
Vibrio owensii	DY05^T	KP721375
Vibrio parahaemolyticus	ATCC 17802^T	KP721376
Vibrio splendidus	ATCC 33125^T	KP721377
Vibrio vulnificus	ATCC 27562^T = DSM 10143^T	KP721378
Vibrio anguillarum	DSM 21597^T	KP721379
Vibrio ponticus	DSM 16217^T	KP721383
Vibrio chagasii	DSM 17138^T	KP721384
Vibrio brasiliensis	DSM 17184^T	KP721385
Vibrio porteresiae	DSM 19223^T	KP721386
Vibrio pectenicida	DSM 19585^T	KP721387
Salinivibrio costicola subsp. *costicola*	DSM 11403^T	KP721380
Isolates used in the testing of the PCR sequencing method		
Photobacterium halotolerans	S2753	KP721398
Vibrio anguillarum	775	KP721388
Vibrio coralliilyticus	S2043	KP721394
Vibrio coralliilyticus	S2052	KP721395
Vibrio nigripulchritudo	S2604	KP721397
Vibrio neptunius	S2394	KP721396
Vibrio parahaemolyticus	V2	KP721401
Vibrio sp.	S188	KP721389
Vibrio sp.	S203	KP721390
Vibrio sp.	S344	KP721391
Vibrio sp.	S787	KP721392
Vibrio sp.	S1110	KP721393
Vibrio sp.	S2757	KP721399
Vibrio sp.	S4497	KP721400
Vibrio sp.	VibAn	KP721402

[a] Genomes from GenBank.

thereby blocking their transcription. In contrast, when iron availability is limited, derepression of the corresponding genes occurs. In addition to iron transport, Fur controls a range of other processes, such as redox stress resistance, energy metabolism, flagellar chemotaxis, and metabolic pathways (15–17).

The purpose of the present study was to determine if the *fur* gene could be used as a new phylogenetic marker in the identification of *Vibrionaceae* species. The availability of several fully genome-sequenced strains allowed us to address this in an *in silico* analysis. To facilitate broader use of *fur* as a phylogenetic marker, despite highly variable regions outside the *fur* gene, we developed a PCR sequencing-based method for the analysis of the *fur* gene in *Vibrionaceae* species.

MATERIALS AND METHODS

***In silico* analysis of *fur* sequences.** The whole-genome sequences (WGS) from 104 strains were used in this study: 83 *Vibrio* strains representing 44 species, 3 *Aliivibrio* strains representing 2 species, 12 *Photobacterium* strains representing 7 species, 2 *Enterovibrio* strains representing 2 species, 2 *Grimontia* strains representing 1 species, 1 *Salinivibrio costicola* strain, and 1 sequence from *Shewanella xiamenensis* BC01 as an unrelated Gram-negative bacillus (Table 1). The genome sequences were used in the *in silico* analysis of the *fur* gene. The genomes were all analyzed using CLC Main Workbench version 7 (CLC, Aarhus, Denmark). For the genomes annotated by NCBI, an annotation-based search was performed for the *fur* genes. Those not annotated were submitted to a BLAST search against the annotated *fur* genes and manually curated if necessary.

Phylogenetic-data analysis. The *fur* sequences isolated *in silico* or PCR amplified and sequenced in this study were aligned using the alignment tools in CLC Main Workbench version 7 (CLC, Aarhus, Denmark). The Gap cost settings were as follows: gap open cost of 10 points and gap extension cost of 1 point, and end gaps were treated like any other gap. The alignments obtained were used to perform a pairwise comparison of the number of differences and the percent identity using CLC Main Workbench version 7 (CLC, Aarhus, Denmark). Furthermore, maximum-likelihood phylogeny trees were also generated using the CLC Main Workbench version 7 (CLC, Aarhus, Denmark) tools. Neighbor-joining was the tree construction method used, with the Jukes-Cantor nucleotide distance measure. The design of the trees was finalized using MEGA 6 (18).

Bacterial strains and genomic-DNA extraction. The bacterial strains used for development of the PCR method (Table 1) were grown in Marine Broth (Difco; catalog no. 279110) overnight at 25°C, and genomic DNA was isolated using the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany). The quality of the genomic DNA was checked by 1% agarose gel electrophoresis and quantified by absorbance using DeNovix DS-11.

Primer design. The degenerate primers fur_AP _fw (5′-CCWCCATAYTGDGWMCGRTTNGCATTCCWCCATAYTGDGWMCGRTTNGCATT-3′) and fur_AP_rv (5′-ACWGTHGGYYTWCGTGATACWTGGG-3′) were designed according to the alignments done using the *fur* regions of several *Vibrio*, *Aliivibrio*, and *Photobacterium* strains. Also, an extra set of primers were designed for amplification in other *Vibrio* species where the AP primers did not work: fur_V_fw (5′-TAACCYYTTGAASTTGAASTTCG-3′), fur_TS_rv (5′-CGWAYDGGHTAYTTCTGTGYDGAT-3′), and fur_OM_rv (5′-GTGGCRGATAAYGTKMGHAAAGG-3′). These primers were then used to amplify the whole *fur* gene. Due to the different sizes of the fragments, internal primers were also designed to confirm the presence of the *fur* gene in the amplified fragment: fur_Sp_internal_fw (5′-CACCAYTTYGAAGGCGGYAAGTC-3′) and fur_Sp_internal_rv (5′-ATYTCTTTYTGKCGYTCTTCRAT-3′).

PCR amplification and sequencing. Amplification reaction mixtures contained 1× *Pfu*X7 buffer [20 mM Tris-HCl, pH 8.8, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1 mg/ml bovine serum albumin (BSA), 0.1% Triton X-100], a 200 μM deoxynucleoside triphosphate (dNTP) mixture, 0.4 μM each primer, 1 μl of a 10-fold dilution of genomic DNA as the template, and 1 μl of *Pfu*X7 polymerase (19) in a final reaction volume of 25 μl. The PCR amplification was carried out in a thermal cycler (Veriti 96-well thermal cycler; Applied Biosystems) as follows: a 2-min initial denaturation step at 98°C, followed by 30 cycles of 98°C for 20 s, 52°C for 20 s, and 72°C for 25 s, with a final extension step of 2 min at 72°C. The amplified products were visualized by agarose gel electrophoresis (1 or 1.5%) and ethidium bromide staining. The PCR products were enzymatically purified by treatment with exonuclease I (ExoI) (Thermo Scientific) and FastAP thermosensitive alkaline phosphatase (Thermo Scientific) before sequencing at GATC Biotech (Cologne, Germany) or Macrogen (Amsterdam, The Netherlands). The sequences were analyzed using CLC Main Workbench version 7 (CLC, Aarhus, Denmark).

Nucleotide sequence accession numbers. The sequence data generated in this study were deposited in GenBank under the accession numbers provided in Table 1.

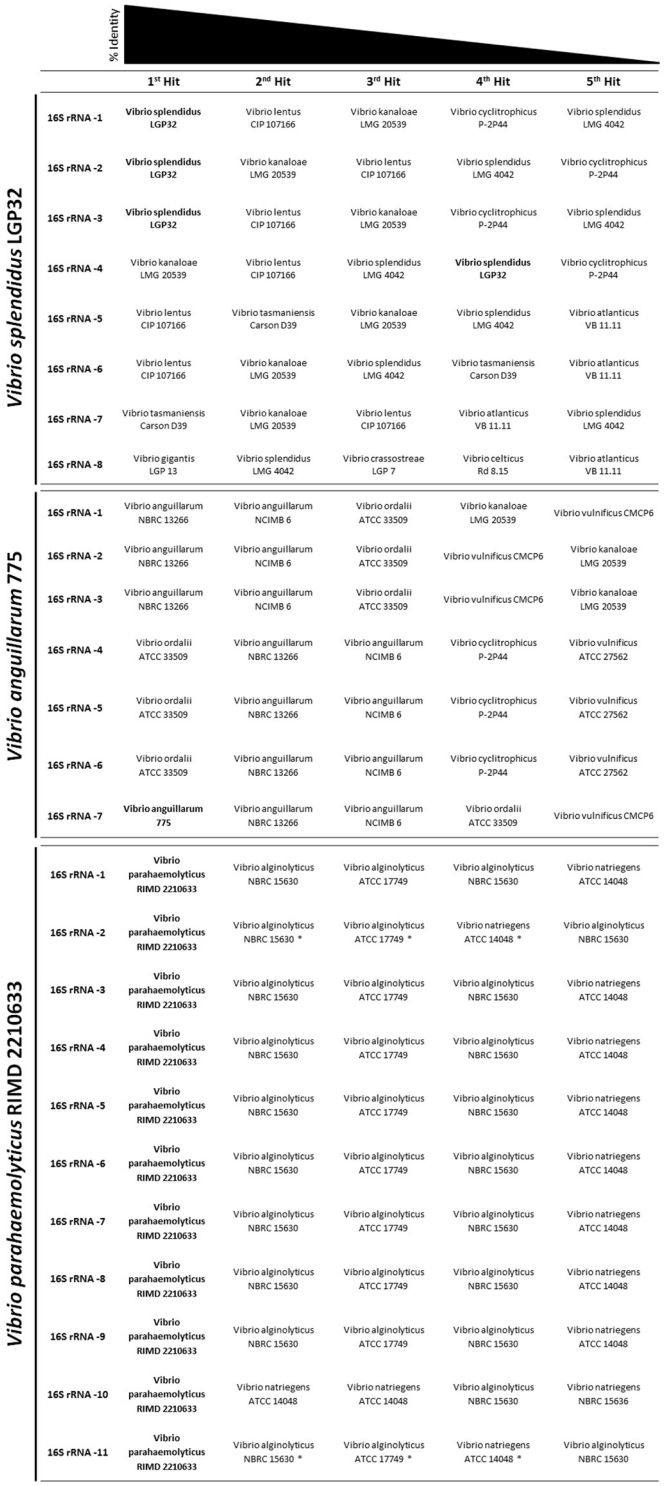

FIG 1 BLAST analyses and closest hits to the different 16S rRNA alleles in three different *Vibrionaceae* strains. Hits from the same strain are in boldface.

RESULTS AND DISCUSSION

16S rRNA gene limitations. 16S rRNA gene sequences have long been used for distinguishing and classifying new strains at the genus and species levels. This approach has been very successful in several bacterial groups, but not in the *Vibrionaceae*, due to low

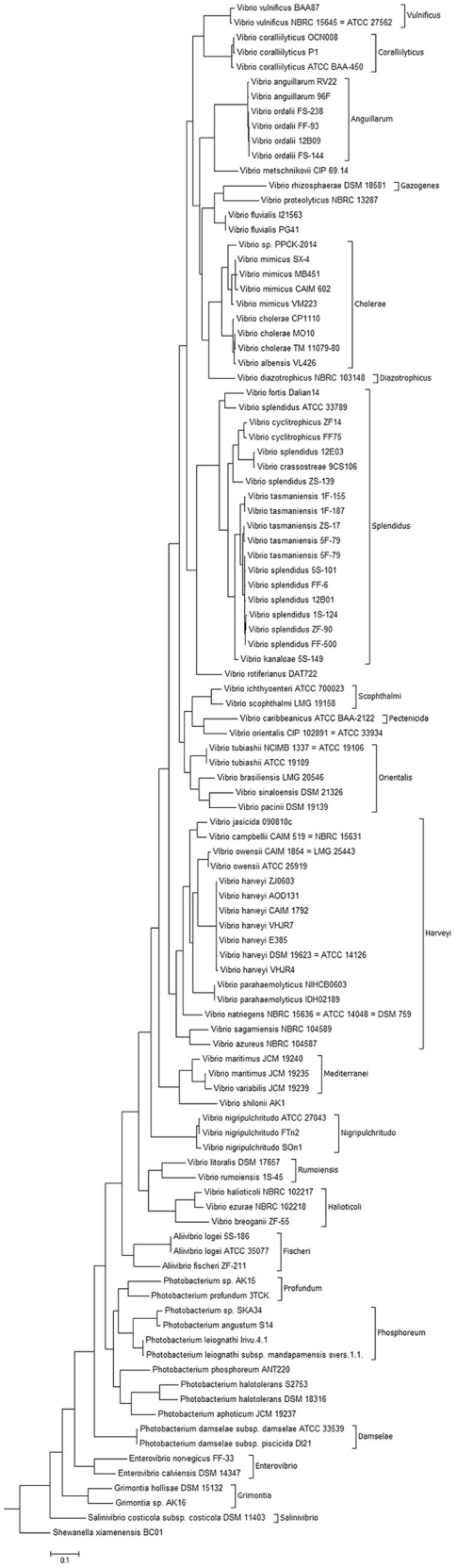

FIG 2 Phylogenetic tree of 103 *Vibrionaceae* strains. The tree is based on *in silico* analyses using the complete *fur* gene sequences and was constructed by the neighbor-joining method. *S. xiamenensis* BC01 was used as the outlier.

FIG 3 Phylogenetic tree of 140 *Vibrionaceae* isolates (including strains shown in Fig. 1). The tree is based on the PCR-amplified and sequenced complete *fur* gene sequences of representatives of each species and the *fur* sequences of the type strains and of the isolates used in the development and testing of the PCR sequencing-based method and was constructed using the neighbor-joining method. The circles indicate *fur* genes sequenced in the development and testing of the PCR sequencing-based method: the solid circles are the type strains used in the development of the method, and the open circles are the environmental strains used to test the method. *S. xiamenensis* BC01 was used as the outlier.

interspecies resolution achieved using the gene (6). This is in part caused by the many alleles of the 16S rRNA gene that, when cloned and sequenced individually, can identify a strain as belonging to several different species (20). To confirm this observation, we used three closed genomes as examples and used the different 16S rRNA alleles in a BLAST search in order to identify the species (Fig. 1). Not only did the number of alleles seem to be variable among vibrios, but the identification drawn from each allele pointed to a different species, and thus, the identifications made using this approach in vibrios are very questionable. Furthermore, when using next-generation sequencing techniques, such as Illumina, the length of the reads obtained does not allow differentiation of the different alleles, resulting in genome assemblies with only one 16S rRNA allele.

***In silico* analysis of *fur* sequences.** For all 103 strains analyzed, only one copy of the *fur* gene was identified in the whole genome. The *fur* sequences varied in size between 441 and 456 bp, with the exception of *Shewanella xiamenensis* BC01, where the *fur* gene was only 432 bp. The *fur* genes with 441 bp were from *Vibrio halioticoli* and *Vibrio ezurae*, and only *Vibrio nigripulchritudo* SOn1 had a 456-bp *fur* sequence, making the variance of the gene size mostly between 444 and 453 bp (see Fig. S1 in the supplemental material).

The maximum-likelihood phylogeny tree constructed with the alignment of the *fur* genes (Fig. 2) confirmed the clustering of the different species in a manner similar to what has been observed for both 16S rRNA gene- and MLSA-based trees (4, 6, 8, 21–24). The clades recently emended by Sawabe et al. (4) could also be identified (Fig. 2), although a few differences arose. Within the *Vibrio* species, the major differences observed were the clustering of *Vibrio rotiferianus* DAT722 within the *Splendidus* clade rather than within the *Harveyi* clade, as previously described (4). Other species, such as *Vibrio metschnikovii* and *Vibrio fluvialis*, did not cluster in the *Cholerae* clade but were clearly closely related. A similar observation was made for the species *Vibrio orientalis* and *Photobacterium phosphoreum*.

At the species level, some of the strains previously identified as *Vibrio splendidus*, namely, *V. splendidus* 12E03, *V. splendidus* ZS-139, and *V. splendidus* ATCC 33789, clustered separately from the other *V. splendidus* strains. In fact, *V. splendidus* 12E03, *V. splendidus* ZS-139, and *V. splendidus* ATCC 33789 clustered in the *Vibrio cyclitrophicus* and the *Vibrio fortis* branches of the phylogenetic tree (Fig. 2). This could be explained by the previously demonstrated genetic diversity and polyphyletic nature of *V. splendidus* (7, 8, 25), or it could be that these strains were misidentified, which is a recurrent problem within the genus *Vibrio* (22, 26, 27). The second hypothesis seems to be consistent with the genomic data index (ANI) at EzGenome (http://www.ezbiocloud.net/ezgenome/hierarchy?n=Vibrionales&d=2#), where ANI-based trees locate these strains closer to other named *Vibrio* species.

A comparison of the 103 sequences revealed a percent similarity in the same species of more than 97%, with the exception of two species pairs, *V. splendidus* and *Vibrio tasmaniensis*, as well as

FIG 4 Analysis of the variability downstream of the *fur* gene by alignment of the *fur* regions from six whole-genome-sequenced *Vibrio* species.

Vibrio anguillarum and *Vibrio ordalii*, where the above-mentioned species threshold did not apply (see Fig. S2 in the supplemental material). This is similar to what has been shown by others using the genes *rpoA*, *recA*, and *pyrH*, where 98%, 94%, and 94% similarities within the same species were reported (8). Among these genes, *pyrH* has been described as the most discriminatory (28); however, this study was performed in a small selection of strains, focusing mostly on the *Harveyi*, *Campbellii*, and *Rotiferianus* groups, making it difficult to do meaningful comparison between the discriminatory powers of this gene and the *fur* gene described here. Another gene reported as a possible good phylogenetic marker in the family *Vibrionaceae* is *atpA* (22). Compared with this gene, *fur* presents itself as a better phylogeny discriminator. The *atpA* gene phylogeny showed very high homology between species within the main identified groups, which could vary between 94 and 99%, while in the *fur* gene phylogeny, only the two previously described pairs (*V. splendidus*-*V. tasmaniensis* and *V. anguillarum*-*V. ordalii*) show that limitation.

Development of a PCR sequencing-based method. The above-described *in silico* analysis confirmed the value of the *fur* sequence as a phylogenetic marker in the family *Vibrionaceae*, allowing the distinction of most of the strains at species level. Therefore, a PCR-based method that could be widely used by researchers working with *Vibrionaceae* genera would be extremely valuable as a simpler tool in the classification of *Vibrio* isolates or as an extra differentiation marker added to an MLSA.

To develop and validate the PCR method and the designed primers, 22 type strains obtained from several culture collections (Fig. 3) were used in the establishment of a general protocol for the amplification of *fur*. They included 1 *Aliivibrio* type strain, 4 *Photobacterium* type strains, 1 *Enterovibrio* type strain, 1 *Salinivibrio* type strain, 1 *Grimontia* type strain, and 14 *Vibrio* type strains. The degenerate fur_AP primers (see Materials and Methods) amplified the *fur* gene in most *Vibrio* species and in all the *Photobacterium*, *Aliivibrio*, *Enterovibrio*, *Grimontia*, and *Salinivibrio* species tested. The main challenge was the design of a reverse primer suitable for all the *Vibrio* strains, since the variation in sequence downstream of the *fur* gene is significant (Fig. 4). Therefore, we designed an extra set of primers, including one forward and two distinct reverse primers, allowing us to amplify the *fur* genes from the *Vibrio* strains on which the above-mentioned set of primers (AP) did not work. The primers developed here did allow amplification of all the tested strains of *Vibrio* species and the other genera of the family *Vibrionaceae*. The differences between the flanking regions of the *fur* gene have been described previously (13), and the conserved genetic organization of the region upstream from the *fur* gene was not reflected in the downstream region, where there is higher variability between species (13). We also noted in our amplification process that there was a species-dependent fragment size of the amplicon. The relationship between the fragment size amplified and the species needs further investigation, although it could possibly expedite the attribution of a provisional clade or even species at an earlier stage in the classification process.

The limited number of genomes available from *Grimontia*, *Salinivibrio*, *Enterovibrio*, *Photobacterium*, and *Aliivibrio* species hampers the design of more universal primers, and once more WGS from strains belonging to these genera are available, a more in-depth analysis of the *fur* gene flanking regions might provide enough information for the design of primers suitable for all the strains belonging to these species. The so-called "primer problems" have been previously reported in several MLSA analyses, both for strains from the genus *Vibrio* and for strains of the genus

Photobacterium (4, 6). This likely reflects the large genomic variability between members of the family *Vibrionaceae*.

After amplification and sequencing of the amplicons, the sequences were used in two approaches: (i) the *fur* sequences were extracted from the consensus sequence and subjected to BLAST searches against the NCBI database or (ii) the whole consensus sequence was used in the BLAST analysis. Both approaches showed a high level of identification, since the first BLAST hits in both cases corresponded to strains of the same species as the tested strains. Nevertheless, this approach using the NCBI database directly presented some limitations. Because few *fur* sequences are available for the *Vibrio* species, the hits that were obtained corresponded to whole-genome-sequenced strains, limiting the analysis to such strains. In fact, the results were more expressive and clearer when the extracted *fur* sequences were subjected to BLAST searches against a database created with the sequences used in the *in silico* analysis. This shows the importance of creating a database with more *fur* sequences in order to obtain more accurate identifications and to bring this method to its full potential.

Identification of *Vibrio* strains using the *fur* gene sequence. To test the method developed, a collection of previously isolated *Vibrio* species was used. The strains have been categorized in our laboratory by conventional 16S rRNA gene sequence analyses (29), and some also by sequencing of *recA*, *toxR*, and *rpoA* genes (30). PCR, as has been described, worked well for all the tested strains (Fig. 4), so the amplicons were purified and sequenced. The *fur* genes were identified in all the sequenced amplicons.

The isolated *fur* sequences were incorporated in the phylogenetic tree, giving an overview of the distribution of the isolates between the different species and clades (Fig. 3). Of the tested isolates, strains S2757 and S2394 could not be identified with high certainty, although it is obvious that strain S2757 clustered closely with *Vibrio tubiashii* (Fig. 3). This inconclusive identification could indicate that strain S2757 belongs to a new species; further attempts using MLSA were also inconclusive (data not shown). With respect to strain S2394, the fact that no WGS or *fur* sequences from *Vibrio neptunius* are available led to a close association of the strain with *Vibrio coralliilyticus*, which makes sense, given its close phylogenetic proximity (4) (Fig. 3).

It is evident that whole-genome sequencing and bioinformatics will drive identification and taxonomy in the future. Nevertheless, it may be a while until the average laboratory can afford the whole-genome sequencing of their strains and can master the bioinformatics needed to correctly identify them. Therefore, we believe that the discovery of the phylogenetic power of the *fur* gene and the development of a PCR method that can be used in amplification and sequencing of the gene is of general interest, whether for use alone or together with the previously suggested loci in an MLSA.

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