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Vibrio alginolyticus gyrB sequence analysis and *gyrB*-targeted PCR identification in environmental isolates

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ABSTRACT: *gyrB* fragments (about 1.2 kb) of 9 *Vibrio alginolyticus* strains were sequenced, and their phylogenetic relationship with other closely related *Vibrio* species was analyzed. All the *V. alginolyticus* strains grouped into one strongly supported cluster in the phylogenetic tree. There were 54 base variations among the 1167 bp mutual *gyrB* regions of 11 *V. alginolyticus* strains; all the *V. alginolyticus* strains shared the same amino acid sequence except *V. alginolyticus* ATCC 17749. Based on the *gyrB* sequences, we designed 2 primers for specific PCR identification of *V. alginolyticus*. Fifty-two bacterial strains from 12 genera were used to test the PCR specificity, and only *V. alginolyticus* strains produced the predicted 568 bp amplification fragment. In addition, PCR screening of 50 randomly selected environmental strains, grown on thiosulfate citrate bile salts-sucrose (TCBS) medium, gave rise to a positive amplification result for *V. alginolyticus* from 37 of them. To further confirm accuracy of PCR identification, biochemical identification of the 50 strains was carried out. Strains giving positive PCR amplification were biochemically identified as *V. alginolyticus*, while strains that gave negative results were biochemically identified as other *Vibrio* or non-*Vibrio* species. Using the basic local alignment search tool (BLAST), *gyrB* sequences obtained from 2 randomly selected strains (YJ0666 and YJ167B) of the 37 PCR-positive strains showed highest identity values with *V. alginolyticus* strains (>96%). Thus, our results demonstrated that *gyrB* is a good marker for molecular identification of *V. alginolyticus*, and a *gyrB*-based PCR method was successfully developed.

KEY WORDS: *gyrB* · *Vibrio alginolyticus* · PCR identification

INTRODUCTION

Vibrio alginolyticus, of the family *Vibrionaceae*, is ubiquitous in marine and estuarine environments. *V. alginolyticus* has acquired increasing importance as some strains are pathogenic to humans as well as aquatic animals and have caused huge economic losses (Balebona et al. 1998, Daniels & Shafaie 2000, Villamil et al. 2003, Liu et al. 2004). Conventionally, screening and identification of *V. alginolyticus* from environmental bacterial isolates depend on biochemical methods, and commercial identification systems are available. However, due to similar biochemical characteristics and phenotypic flexibility, misidentification of *Vibrio* species including *V. alginolyticus* has often been reported (Robert-Pillot et al. 2002, O'Hara et al. 2003, Thompson et al. 2004). Moreover, identification based on biochemical tests is time consuming and resource intensive. For these reasons, molecular methods are required in screening and routine identification of *V. alginolyticus*. PCR is a rapid and sensitive method for this goal.

Liu et al. (2004) have developed a 16S rRNA gene-based PCR method for identification of *Vibrio alginolyticus*; however, high homology of the 16S rRNA gene in *Vibrio* spp. makes it difficult to reliably differentiate and detect members of *Vibrio* through targeting of this gene (Kita-Tsukamoto et al. 1993, Ruimy et al. 1994). For instance, 16S rRNA gene sequences revealed 99.7% homology between *V. parahaemolyti-*

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cus and *V. alginolyticus* (Ruimy et al. 1994). We have theoretically tested specificity of 4 primers designed by Liu et al. (2004) using the basic local alignment search tool (BLAST, Altschul et al. 1990) in GenBank, and found that the primers also completely matched with 16S rRNA gene regions of many other *Vibrio* species. Therefore, the 16S rRNA gene is not suitable for PCR identification of *V. alginolyticus*. Subsequently, researchers established collagenase and *dnaJ* gene-targeted multiplex-PCR methods for simultaneous identification of several *Vibrio* species including *V. alginolyticus* (Di Pinto et al. 2005, Nhung et al. 2007). In addition, a real-time PCR method has been developed for quantitative detection of *V. alginolyticus* (Zhou et al. 2007). However, multiplex-PCR requires strict reaction PCR conditions, and real-time PCR requires relatively expensive equipment and reagents. If the objective is just identification or screening of one bacterial species, traditional singleplex PCR is enough. Therefore, establishment of a reliable routine PCR method for extensive identification or screening of *V. alginolyticus* from environmental isolates is desirable.

The *gyrB* gene that encodes the B subunit protein of bacterial DNA gyrase has some superiorities for phylogenetic analysis and molecular identification: (1) it does not transfer horizontally among bacterial species; (2) the molecular evolution rate is higher than that of the 16S rRNA gene; (3) it has been found in most of bacterial species; and (4) it has both conserved regions and variable regions suitable for development of PCR primers (Yamamoto & Harayama 1995, Venkateswaran et al. 1998, Fukushima et al. 2002). *gyrB*-targeted PCR methods have been developed for identification of bacteria from various genera including *Vibrio* (Kasai et al. 1998, Venkateswaran et al. 1998, Vuddhakul et al. 2000, Kumar et al. 2006, Thaithongnum et al. 2006, Zhou et al. 2007), *Bacillus* (Yamada et al. 1999), *Pseudomonas* (Yamamoto & Harayama 1995, Izumi et al. 2007), *Leptospira* (Slack et al. 2006), *Flavobacterium* (Izumi et al. 2005), and *Aeromonas* (Sen 2005). These studies demonstrate the preferred choice of the *gyrB* gene for molecular identification of closely related genomic species.

However, the high rate of molecular evolution of the *gyrB* gene also brings reservations about sequence heterogeneity of the gene among conspecific strains. There is no evidence that the same species always has highly similar or identical *gyrB* sequences. A report on molecular typing of *Yersinia frederiksenii* based on *gyrB* sequence showed the existence of *gyrB* sequence variability within the same species (Demarta et al. 2004). We compared the 2 *gyrB* sequences of *Vibrio alginolyticus* published in GenBank, and found that they were very different. Thus, it is necessary to compare multiple *gyrB* sequences from *V. alginolyticus* before establishing a *gyrB*-targeted PCR method.

In the present study, we sequenced and analyzed partial *gyrB* sequences of 9 *Vibrio alginolyticus* strains from different origins, and based on these sequences, we developed a conventional PCR method for identification or screening of *V. alginolyticus* from environmental bacterial isolates.

MATERIALS AND METHODS

Bacterial strains, cultivation and DNA extraction. Bacterial strains were retrieved through purchase, donation or isolation from various environments (see Table 1). All *Vibrio* strains were overnight cultured in alkaline peptone water (APW) medium at 30°C, and other bacterial strains were overnight cultured in the media recommended by the sellers or donators. Genomic DNA from the strains was extracted with the MiniBEST Bacterial Genomic DNA Extraction Kit (Takara Biotechnology) following the method provided by the manufacturer.

Sequencing and analysis of *gyrB* sequences of *Vibrio alginolyticus*. Fragments (about 1.2 kb) of *gyrB* gene from *V. alginolyticus* ZJ0403, ZJ0428, ZJ0476, ZJ0479, ZJ0486, NA0419, NA0425, NA0426, and DX0406 were amplified with universal primers (UP1 and UP2r) as reported previously (Yamamoto & Harayama 1995). Among them, *V. alginolyticus* ZJ0403, ZJ0428, ZJ0479, and ZJ0486 were isolated from juvenile or parent shrimp *Litopenaeus vannamei* in Zhanjiang, China; *V. alginolyticus* ZJ0476 was isolated from bay scallop *Argopecten irradians concentricus* in Zhanjiang, China; *V. alginolyticus* NA0419, NA0425, and NA0426 were isolated from different tissues of red drum *Sciaenops ocellatus* in Shenzhen, China; and *V. alginolyticus* DX0406 was isolated from seawater in Dongxing, China. PCR products were confirmed by agarose electrophoresis, purified, and directly sequenced using an Applied Biosystems 3730 Automatic Sequencer. Nine *gyrB* sequences retrieved and 2 *gyrB* sequences of *V. alginolyticus* (strains no. ATCC 17749 and 12G01) derived from GenBank (AF007288 and AAPS01000042) were aligned with the CLUSTAL-W program in the BioEdit package (Wang et al. 2007). Deduced amino acid sequences of *gyrB* gene were also aligned with the CLUSTAL-W program in the BioEdit package. A neighbor-joining phylogenetic tree was constructed based on nucleic acid sequences using MEGA 3.1 software with a bootstrap test of 1000 replicates.

Species-specific primer design and PCR assay of *Vibrio alginolyticus*. A consensus *gyrB* sequence of the 11 *V. alginolyticus* strains mentioned above was es-

tablished after alignment with CLUSTAL-W program (see Fig. 1). Primer pairs that completely matched with the conservative region of the *gyrB* sequences were designed, and the specificity of primers was theoretically tested by BLAST searches of the GenBank database. Finally, a pair of specific primers, AlgF1 and AlgR1, was established. The primer sequences were 5′-TCA GAG AAA GTT GAG CTA ACG ATT-3′ (AlgF1, forward) and 5′-CAT CGT CGC CTG AAG TCG CTG T -3′ (AlgR1, reverse). The AlgF1 and AlgR1 primers were predicted to amplify a 568 bp fragment from *V. alginolyticus gyrB* gene. The PCR reaction was performed in 25 µl volume containing 1 µl genomic DNA, 0.4 µM each primer, 2.5 µl 10× PCR buffer, 2.0 mM Mg^{2+}, 0.2 mM dNTP, and 1 U Taq DNA polymerase (Takara Biotechnology). The amplification program consisted of an initial denaturation at 94°C for 4 min, 32 cycles of denaturation at 94°C for 30 s, annealing at 64°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 8 min. After PCR amplification, 4 µl of each product was added into a 1.0% agarose gel, electrophoresed, and followed by visualization under UV light.

Specificity of PCR for *gyrB* amplification of *Vibrio alginolyticus*. To test PCR specificity, 52 bacterial strains (including 16 *V. alginolyticus* strains and 22 other *Vibrio* strains) from 12 genera were used (Table 1). PCR and electrophoresis were carried out as described above.

Strain identification from environmental bacterial isolates. Fifty bacterial strains isolated from farming seawater and marine animals in coastal areas of South China (see Table 2), grown on thiosulfate citrate bile salts-sucrose (TCBS) medium, were randomly chosen and overnight cultured in APW followed by DNA extraction. The aforementioned PCR was carried out with DNA templates from the 50 bacterial strains. For further confirmation of PCR specificity, biochemical identification of all 50 environmental bacterial strains was carried out by referring to the descriptions of the species listed in *Bergey's Manual of Systematic Bacteriology* (Brenner et al. 2005). In addition, fragments of *gyrB* gene of 2 randomly selected PCR-positive strains, YJ0666 and YJ167B, were amplified and sequenced according to the method described above. Similarity searches against GenBank entries were performed with the sequences acquired using the basic local alignment search tool (BLAST) algorithm in GenBank.

Nucleotide sequence accession numbers. The *gyrB* sequences of 9 original *Vibrio alginolyticus* strains and the 2 additional strains isolated from environmental samples, YJ0666 and YJ167B, were deposited in the GenBank database under the following accession numbers: EF579668-EF579676 and EF542801-EF542802.

Table 1. Bacterial strains used in the present study. EN: isolated from the environment by our laboratory; APCCC: Aquatic Pathogen Collection Centre of China; CGMCC: Centre for General Microorganisms Collection of China; FJCDC: Fujian Centre for Disease Control and Prevention; ZSU: Zhongshan University; ATCC: American Type Culture Collection; SCSFI: South China Sea Fisheries Institute; BJCDC: Beijing Centre for Disease Control and Prevention; GDIM: Guangdong Institute of Microbiology

Bacterial species	Strain no.	Source
Vibrio alginolyticus	ZJ0403	EN
Vibrio alginolyticus	ZJ0428	EN
Vibrio alginolyticus	ZJ0476	EN
Vibrio alginolyticus	ZJ0479	EN
Vibrio alginolyticus	ZJ0486	EN
Vibrio alginolyticus	ZJ0451	EN
Vibrio alginolyticus	DX0406	EN
Vibrio alginolyticus	NA0419	EN
Vibrio alginolyticus	NA0425	EN
Vibrio alginolyticus	NA0426	EN
Vibrio alginolyticus	0391	APCCC
Vibrio alginolyticus	1.1587	CGMCC
Vibrio alginolyticus	HK8031	FJCDC
Vibrio alginolyticus	Z0504	ZSU
Vibrio alginolyticus	Z0505	ZSU
Vibrio alginolyticus	Z0515	ZSU
Vibrio parahaemolyticus	17802	ATCC
Vibrio parahaemolyticus	0302	SCSFI
Vibrio parahaemolyticus	Z0508	ZSU
Vibrio parahaemolyticus	1.1614	CGMCC
Vibrio mimicus	1.1969	CGMCC
Vibrio fluvialis	1.1609	CGMCC
Vibrio nereis	1.1623	CGMCC
Vibrio splendidus	1.1606	CGMCC
Vibrio pelagius	1.1588	CGMCC
Vibrio vulnificus	1.1758	CGMCC
Vibrio vulnificus	D0683	EN
Vibrio natriegens	1.1594	CGMCC
Vibrio proteolyticus	1.1826	CGMCC
Vibrio campbellii	1.1597	CGMCC
Vibrio campbellii	1.1598	CGMCC
Vibrio campbellii	DX0520	EN
Vibrio harveyi	1.1593	CGMCC
Vibrio harveyi	NH0503	SCSFI
Vibrio harveyi	SD0613	EN
Non-O1 *Vibrio cholerae*	06153	BJCDC
Non-O1 *Vibrio cholerae*	SC0312	EN
Vibrio furnissii	33841	ATCC
Listonella anguillarum	0387	APCCC
Listonella anguillarum	Z0512	ZSU
Pseudomonas fluorescens	10646	ATCC
Pseudomonas aeruginosa	1.50	APCCC
Aeromonas sobria	0398	APCCC
Aeromonas hydrophila	0388	APCCC
Edtardsiella tarda	S1	APCCC
Pseudoalteromonas sp.	GS0432	EN
Shewanella algae	YJ06114	EN
Bacillus subtilis	Bs-1	ZSU
Lactobacillus plantarum	8014	ATCC
Staphyloccocus aureus	6538	ATCC
Salmonella typhimurium	50115	GDIM
Rhodococcus sp.	NH0578	EN

RESULTS

Sequence analysis of *gyrB* gene from *Vibrio alginolyticus*

The partial *gyrB* sequences obtained from *Vibrio alginolyticus* were aligned, and one consensus sequence was created. There were 54 base substitutions among the 1167 bp homologous *gyrB* regions of 11 *V. alginolyticus* strains (Fig. 1). These base substitutions were randomly distributed in the sequences; thus, no obviously variable or conserved regions were observed within the sequences. The sequence heterogeneity was mainly caused by higher base divergence in *V. alginolyticus* ATCC 17749. The *gyrB* sequences of all *V. alginolyticus* strains shared between 96 and 100% homology. It should be noted that the strains derived from the same marine environment or animals (e.g. *V. alginolyticus* NA0419, NA0425, and NA0426) still had different *gyrB* sequences. The *gyrB* sequence variation within the *V. alginolyticus* strains, to some degree, indicated the rich genomic polymorphism in *V. alginolyticus*.

Although the *gyrB* sequences of *Vibrio alginolyticus* strains were different from each other (but *V. alginolyticus* NA0419 is identical to *V. alginolyticus* DX0406, and *V. alginolyticus* ZJ0428 is identical to *V. alginolyticus* ZJ0479), the deduced *gyrB* amino acid sequences of 9 *V. alginolyticus* strains isolated were identical with the sequence of *V. alginolyticus* 12G01 (AAPS01000042) released in GenBank. However, compared with other strains, *V. alginolyticus* ATCC 17749 had 21 amino acid variations among the 388 amino acids predicted from the *gyrB* partial sequences (Fig. 2).

All the *gyrB* sequences of *Vibrio alginolyticus* identified by BLAST searches (including the sequences released by us) and some related sequences were used for construction of a phylogenetic tree (Fig. 3). All the *V. alginolyticus* strains were grouped into one strongly supported cluster. The *gyrB*-based phylogenetic tree indicated that *V. alginolyticus* and *V. harveyi* had the closest phylogenetic relationship, but the similarity values were still lower than 88.4%. Additionally, all sequences from the same species were grouped into one branch, except for *V. harveyi* LB5 and *V. campbel-*

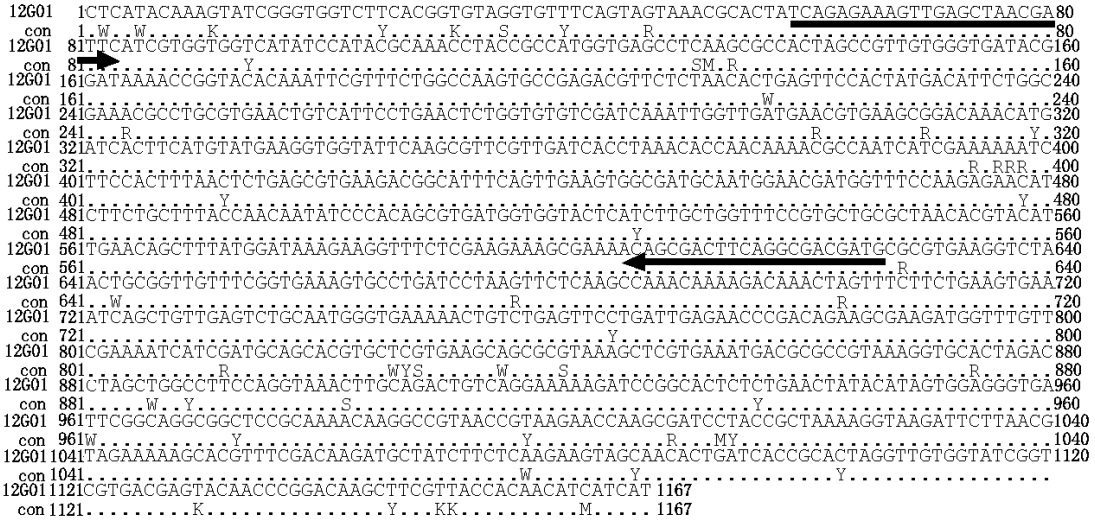

Fig. 1. *Vibrio alginolyticus. gyrB* sequence comparison of *V. alginolyticus* 12G01 and consensus sequence (con) derived from alignment of 11 *V. alginolyticus* strains. Variable base sites are shown with letters in consensus sequence. Primer AlgF1: forward black arrow; primer AlgR1: reverse black arrow

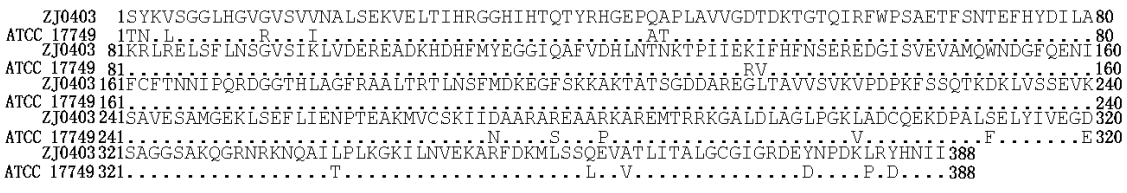

Fig. 2. *Vibrio alginolyticus.* Comparison of *gyrB* amino acid sequences from *V. alginolyticus* ZJ0403 and ATCC 17749. Different amino acid sites are shown with letters in the sequence of *V. alginolyticus* ATCC 17749

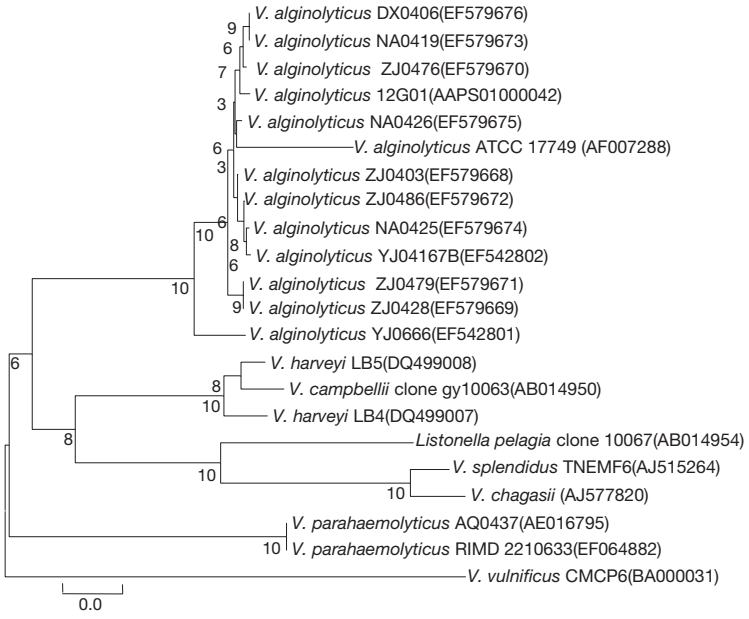

Fig. 3. *gyrB* sequence-based phylogenetic tree constructed by neighbor-joining method. Bootstrap values were obtained after 1000 repetitions. Scale bar indicates 1% sequence dissimilarity. *V*: *Vibrio*

lii clone gy10063 (Fig. 3). The higher divergence of *gyrB* and the agreement between phenotype and genotype, based on *gyrB* sequence, demonstrate that the *gyrB* gene might be an alternative marker to the 16S rRNA gene for phylogeny or molecular identification in *Vibrio* species.

PCR assay for primer specificity

By BLAST searches, we found there were no complete matches (especially at 3′ end of each primer) with other *Vibrio* species for both primers AlgF1 and AlgR1. Of all 52 bacterial strains tested, only *V. alginolyticus* strains gave rise to positive amplification and produced a predicted 568 bp fragment (Figs. 4 & 5). No non-specific fragments were amplified, which was demonstrated by electrophoresis of PCR products.

PCR identification of *Vibrio alginolyticus* from environmental isolates

Fifty environmental bacterial strains (randomly selected) were screened using *Vibrio alginolyticus*-specific PCR. Thirty-seven of those strains produced predicted 568 bp PCR fragments (74% occurrence rate), indicating that they were likely to be *V. alginolyticus*. Subsequent biochemical identification of these 50 strains demonstrated that the strains that gave positive amplification were biochemically identified as *V. alginolyticus*, while the strains that gave no amplification were biochemically identified as other *Vibrio* or non-*Vibrio* species (Table 2).

Two PCR-positive strains, YJ0666 and YJ167B, which were biochemically identified as *Vibrio alginolyticus*, were randomly selected for *gyrB* sequencing. BLAST searches showed that *gyrB* sequences of both strains had highest identity values with *V. alginolyticus* strains (>96%). They were grouped into the cluster on the phylogenetic tree containing all the other *V. alginolyticus* strains, which were obviously distinguishable from the clusters formed by other *Vibrio* species (Fig. 3). In this cluster, the *gyrB* sequences of the strain YJ0666 and *V. alginolyticus* ATCC 17749 had the lowest similarity value, but was still above 96%. According to Thompson & Swings (2006), in *Vibrio* species, the strains of the same species would have >94% *gyrB* similarity. Thus, based on *gyrB* sequences, 11 sequenced strains (including the strains YJ0666 and YJ167B) should belong to *V. alginolyticus*. The above results confirmed the identification accuracy of *V. alginolyticus*-specific PCR. Additionally, the fact that 37 of 50 bacterial strains were identified as *V. alginolyticus* by PCR suggests the prevalence of this species in the marine farming environment is high.

DISCUSSION

Comparison of 16S rRNA gene sequences has been the 'gold standard' in bacterial molecular systematics (Woese 1987, Vandamme et al. 1996), and many species-specific PCR identifications were developed

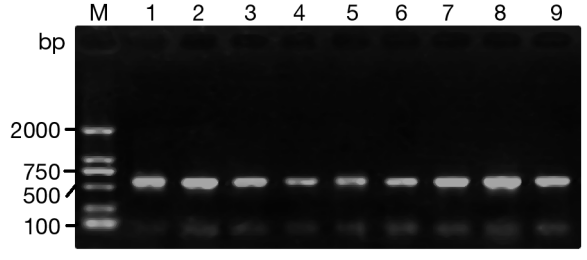

Fig. 4. *Vibrio alginolyticus.* Agarose electrophoresis of 9 *V. alginolyticus* strains used for *gyrB* sequencing and primer design. Predicted amplification fragments were 568 bp in length. Lane M: molecular marker; lane 1: *V. alginolyticus* ZJ0403; lane 2: ZJ0428; lane 3: ZJ0476; lane 4: ZJ0479; lane 5: ZJ0486; lane 6: DX0406; lane 7: NA0419; lane 8: NA0425; lane 9: NA0426

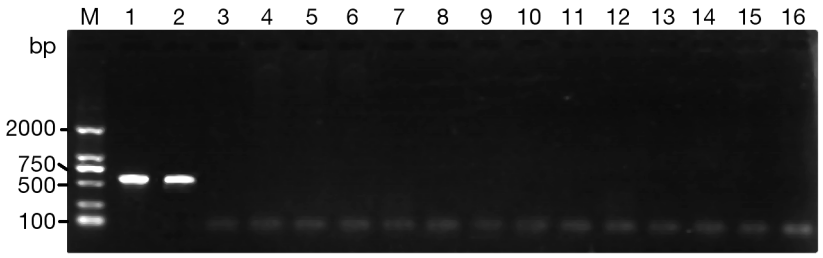

Fig. 5. Typical agarose electrophoresis of PCR products from *Vibrio alginolyticus* and other bacteria. Lane M: molecular marker; lane 1: *V. alginolyticus* 0391; lane 2: *V. alginolyticus* 1.1587; lane 3: *V. parahaemolyticus* ATCC 17802; lane 4: *V. mimicus* 1.1969; lane 5: *V. fluvialis* 1.1609; lane 6: *V. nereis* 1.1623; lane 7: *V. splendidus* 1.1606; lane 8: *V. pelagius*1.1588; lane 9: *V. vulnificus* 1.1758; lane 10: *V. natriegens* 1.1594; lane 11: *V. campbellii* 1.5997; lane 12: *V. harveyi* 1.1593; lane 13: Non-O1 *V. cholerae* 06153; lane 14: *Listonella anguillarum* 0387; lane 15: *Shewanella algae* YJ06114; lane 16: *Pseudomonas fluorescens* ATCC 10646

based on this gene. However, for closely related bacterial species, such as those from the genera *Vibrio* (Kita-Tsukamoto et al. 1993, Ruimy et al. 1994, Thompson & Swings 2006), *Pandoraea* (Coenye & LiPuma 2002), *Bacillus* (Yamada et al. 1999, La Duc et al. 2004), *Aeromonas* (Tacão et al. 2005), *Gordonia* (Shen et al. 2006), and *Mycobacterium* (Goh et al. 2006), it lacks sufficient differentiation ability. Therefore, alternative markers for phylogeny and molecular identification have been exploited; specifically, *gyrB* has shown superiority in this level of species differentiation (Tacão et al. 2005, La Duc et al. 2004, Demarta et al. 2004, Kumar et al. 2006). In the present study, high *gyrB* homology within *V. alginolyticus* and obvious genetic distances among different *Vibrio* species also revealed that *gyrB* could be a better phylogenetic marker for *Vibrio*. The *gyrB* gene has also been used for PCR identification of *Vibrio* species (Kasai et al. 1998, Venkateswaran et al. 1998, Vuddhakul et al. 2000, Kumar et al. 2006, Thaithongnum et al. 2006). The *gyrB*-based PCR identification of *V. alginolyticus* in the present study and the PCR methods for identification of other *Vibrio* species indicate *gyrB* could be a potential candidate for molecular identification of *Vibrio* species.

Although many base variations in *gyrB* sequences of *Vibrio alginolyticus* strains exist, deduced amino acid sequences of all the strains isolated were completely consistent with that of *V. alginolyticus* 12G01 (AAPS01000042) released in GenBank with the excep-

Table 2. *Vibrio alginolyticus*-specific PCR and biochemical identification of 50 environmental bacterial isolates. Positive (+) and negative (–) amplification by specific PCR

Isolates	Biochemical identification	Specific PCR	Isolates	Biochemical identification	Specific PCR
A0505	*Vibrio* sp.	–	E06125	*V. alginolyticus*	+
A0508	*Photobacterium damselae*	–	E06131	*V. alginolyticus*	+
A0517	*V. alginolyticus*	+	E06159	*Shewanella* sp.	–
A0529	*V. alginolyticus*	+	E06161	*V. alginolyticus*	+
A0532	*V. alginolyticus*	+	E06165	*V. parahaemolyticus*	–
A0548	*V. alginolyticus*	+	YJ167B	*V. alginolyticus*	+
E06001	*V. alginolyticus*	+	E06169	*V. alginolyticus*	+
E06011	*V. parahaemolyticus*	–	E06172	*V. campbellii*	–
E06012	*V. alginolyticus*	+	E06180	*V. alginolyticus*	+
E06019	*V. alginolyticus*	+	E06181	*V. alginolyticus*	+
E06020	*V. alginolyticus*	+	E06197	*V. alginolyticus*	+
E06024	*Shewanella* sp.	–	E06206	*V. cholerae*	–
E06041	*V. alginolyticus*	+	E06208	*V. alginolyticus*	+
E06045	*V. alginolyticus*	+	E06214	*Aeromonas hydrophila*	–
E06047	*V. alginolyticus*	+	E06229	*V. alginolyticus*	+
E06052	*V. alginolyticus*	+	E06234	*V. alginolyticus*	+
E06057	*V. alginolyticus*	+	E06237	*V. campbellii*	–
E06064	*V. alginolyticus*	+	E06249	*V. alginolyticus*	+
YJ0666	*V. alginolyticus*	+	E06256	*V. alginolyticus*	+
E06074	*V. alginolyticus*	+	E06263	*V. alginolyticus*	+
E06075	*V. alginolyticus*	+	E06266	*V. alginolyticus*	+
E06090	*V. alginolyticus*	+	E06268	*V. alginolyticus*	+
E06096	*V. alginolyticus*	+	E06274	*V. alginolyticus*	+
E06117	*V. harveyi*	–	E06280	*V. alginolyticus*	+
E06119	*Proteus mirabilis*	–	E06286	*V. harveyi*	–

tion of *V. alginolyticus* ATCC 17749, whose amino acid sequence has 21 variable sites, compared with other *V. alginolyticus* strains. This might reflect true differences in amino acid sequences in the same species or sequencing mistakes due to unperfected technology at that time (the *gyrB* sequence of *V. alginolyticus* ATCC 17749 was published in 1998).

The relatively high heterogeneity of *gyrB* sequences within *Vibrio alginolyticus* strains indicates that when we design *gyrB*-based species-specific primers, multiple sequences within the same species should be available and aligned to allow design of primers to the conserved regions of specific species. Otherwise, false negative results may occur in follow-up PCR identification. On the other hand, the relatively high variation of *gyrB* sequences among *V. alginolyticus* strains (even isolated from the same animal) laterally reflect the genomic polymorphism of *V. alginolyticus*, which was previously confirmed by several studies (Vandenberghe et al. 1999, Zanetti et al. 1999, Chen et al. 2002, Sudheesh et al. 2002).

A high frequency of occurrence (74%) of *Vibrio alginolyticus* among isolated bacterial strains, as determined by the screening of *V. alginolyticus* using PCR following biochemical identification, demonstrates the high prevalence of *V. alginolyticus* in the marine farming environment. The prevalence level found here is in accordance with other investigations (Barbieri et al. 1999, Vandenberghe et al. 1999, Baffone et al. 2000); thus, there is a potential hazard of a vibriosis outbreak caused by *V. alginolyticus*, though we currently know little about the pathogenesis of the bacterium.

In summary, we sequenced and analyzed fragments of *gyrB* gene from *Vibrio alginolyticus*, and developed a *gyrB*-based PCR method for rapid identification and screening of *V. alginolyticus* from environmental bacterial isolates. Results indicate that the *gyrB* gene is a good marker for molecular identification of *V. alginolyticus*.

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