Genes encoding the *Vibrio harveyi* haemolysin (VHH)/thermolabile haemolysin (TLH) are widespread in Vibrios

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Abstract: *V. harveyi* VHH haemolysin, which shows high homology to the TLH haemolysin (the identities of their deduced amino acid sequences are up to 85.6%), is a putative virulence factor to marine cultured fish. A VHH probe, which is specific to *V. harveyi vhhA* haemolysin gene, was used to screen *Eco*R Ⅰ digests of total DNA from 57 vibrio strains, including 26 vibrio type strains, 20 *V. harveyi* isolates and 11 *V. parahaemolyticus* isolates. As a result, 1 strong hybridisation band was detected in 13 type strains, including 2 of *Vibrio alginolyticus*, 2 of *V. harveyi*, and 1 strain each of *Grimontia hollisae*, *V. campbellii*, *V. cincinnatiensis*, *V. fischeri*, *V. mimicus*, *V. natriegens*, *V. parahaemolyticus*, *V. proteolyticus* and *V. logei*. Also, 1 weak band was detected in 6 type strains, including *V. anguillarum*, *V. aestuarianus*, *Photobacterium damselae* subsp. *damselae*, *V. fluvialis*, *V. furnissii* and *V. vulnificus*. There was not any hybridization signal in other type strains. Also, *vhh*/*tlh* was present in all isolates of *V. harveyi* and *V. parahaemolyticus*. Moreover, 3 isolates of *V. harveyi*, i.e. VIB 645, VIB 648 and SF1, had duplicated *vhh* genes. The data indicates that *vhh*/*tlh* is widespread in vibrios, especially in *V. harveyi* related species and *V. fischeri* related species. To support this conclusion, the *vhh*/*tlh* homologue genes in *V. anguillarum* VIB 72, *V. campbellii* VIB 285, *V. natriegens* VIB 299 and *V. harveyi* VIB 647 were cloned and sequenced, and the deduced amino acid sequences showed high degree of identities to VHH (67% ~ 99%) and TLH haemolysin (69% ~ 91%). This study will help us to identify the role of *vhh*/*tlh* haemolysin gene in the pathogenicity of vibrios.

Keywords: Vibrios; *V. harveyi*; *V. parahaemolyticus*; haemolysin gene; *vhh*; *tlh*
CLC number: Q933 **Document code**: A **Article ID**: 0001-6209(2007)05-0874-08

1 INTRODUCTION

Vibrio harveyi is one of the main bacterial pathogens of cultured shrimp and fish[1~3]. However, little is known about the pathogenicity mechanisms of the organism. Liu *et al*.[4] considered that proteases, phospholipases, or haemolysins might be important for pathogenicity; cysteine protease being reported as the major exotoxin to penaeid shrimp[5~7]. In a previous study, Zhang and Austin[3] determined that the most pathogenic isolates of *V. harveyi*, VIB 645, contained 2 closely related haemolysin genes (designated *vhhA* and *vhhB*), which were cloned and sequenced. Conversely, the majority (19/20) of other *V. harveyi* cultures possessed only single genes, or none at all[8]. Sequence analysis revealed that the nucleotide sequence identities of *vhhA* and *vhhB* were 77.5% and 77.2%, respectively, to the thermolabile haemolysin (TLH) gene (*tlh*) of *V. parahaemolyticus*[8~10]. TLH, also referred the lecithin-dependent haemolysin (LDH), was one of the haemolysins produced by *V. parahaemolyticus*[9~11]. The TLH gene (*tlh*) contained an open reading frame (ORF) of 1254 bp[10]. Moreover, the *tlh* gene has been found in genomes of all *V. parahaemolyticus* isolates examined, regardless of whether they have been derived from clinical or environmental sources[9,12,13]. In particular, *tlh* was thought to be species-specific to *V. parahemolyticus*[9,12].

Haemolysin is an exotoxin that lyses erythrocytes membranes with the liberation of haemoglobin. There is not standardization in literature of what would really be a haemolysin gene. Generally, any gene with haemolytic activity is considered a haemolysin gene. So far, at least 6 types of haemolysin gene families in the vibrios genomes, including TDH, HlyA, VHH/TLH, δ-VPH, HLX and HLYIII haemolysin genes, have been characterized[11], and there are no gene sequence and protein sequence similarity found among the different families. Some haemolysins, for example, TDH of *Vibrio parahaemolyticus* and HlyA of *Vibrio cholerae* have been studied extensively. However, the role of VHH/TLH haemolysin is unclear, and awaits the out come of further research. In this study, we sought to investigate the distribution the genes encoding VHH/TLH

Foundation item: National Natural Science Foundation of China (30371119); Program for New Century Excellent Talents in University (NCET-04-0645)
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Received: 5 December 2006/Accepted: 8 January 2007/Revised: 25 January 2007

haemolysin among different vibrios.

2 MATERIALS AND METHODS

2.1 Bacterial strains and plasmid

26 vibrio type strains, 20 *V. harveyi* isolates and 11 *V. parahaemolyticus* isolates from a diverse range of hosts and geographical locations, were used (Table 1). Authenticity was verified after the characteristics in Bergey's Manual of Systematic Bacteriology[14]. The bacteria were cultured on Zobell's 2216E medium (Difco) at 28℃, overnight, with sub-culturing every 7 ~ 14 days. *Escherichia coli* JM109, which was employed for bacterial transformations and plasmid isolation, was grown on LB medium[15] at 37℃. *E. coli* transformants were maintained on LB-Ap (LB supplemented with 100μg/mL of ampicillin) agar. pUCm-T (Sangon, China) was used as a vector for cloning *Taq*-amplified PCR products, including blue/white screening and ampicillin resistance as the selectable marker.

Table 1 Bacterial strains and Southern hybridization results

Lab codes	Species name	Strain or source	Country (year of isolation)	*vhh*/*tlh* genes as revealed by Southern hybridization
VIB 314	*Grimontia hollisae*	LMG 17719^T	USA	+
VIB 289	*Photobacterium damselae* subsp. *damselae*	LMG 7892^T	USA	(+)
VIB 288	*Salinivibrio costicola* subsp. *costicola*	LMG 6460^T	Australia	−
VIB 72	*Vibrio anguillarum*	LMG 4437^T	Norway	(+)
VIB 281	*V. aestuarianus*	LMG 7909^T	USA	(+)
VIB 283	*V. alginolyticus*	LMG 4408^T		+
VIB 284	*V. alginolyticus*	LMG 4409^T	Japan	+
VIB 285	*V. campbellii*	LMG 11216^T	USA	+
VIB 286	*V. harveyi*	LMG 7890^T	USA (1982)	+
VIB 287	*V. cincinatiensis*	LMG 7891^T	USA	+
VIB 290	*V. diazotrophicus*	LMG 7893^T	Canada	−
VIB 291	*V. fischeri*	LMG 4414^T	USA (1933)	+
VIB 292	*V. fluvialis*	LMG 7894^T	Bangladesh	(+)
VIB 293	*V. furnissii*	LMG 7910^T		(+)
VIB 294	*V. gazogenes*	LMG 13541^T	USA	−
VIB 295	*V. harveyi*	LMG 4044^T	USA (1935)	+
VIB 296	*V. mediterranei*	LMG 11258^T	Spain	−
VIB 298	*V. mimicus*	LMG 7896^T		+
VIB 299	*V. natriegens*	LMG 7896^T	USA	+
VIB 301	*V. nereis*	LMG 13543^T	USA	−
VIB 304	*V. parahaemolyticus*	LMG 2850^T		+
VIB 305	*V. pelagius*	LMG 3897^T	USA	−
VIB 306	*V. proteolyticus*	LMG 3772^T	USA	+
VIB 309	*V. tubiashii*	LMG 10936^T		−
VIB 310	*V. vulnificus*	LMG 13545^T		(+)
VIB 414	*V. logei*	LMG 19806^T	USA	+
VIB 351	*V. harveyi*	Shark	Bahamas	+
VIB 391	*V. harveyi*	Shrimp	Thailand (1990)	+
VIB 395	*V. harveyi*	LMG 11225		+
VIB 400	*V. harveyi*	LMG 11659		+
VIB 410	*V. harveyi*	ATCC 14126		+
VIB 571	*V. harveyi*	Sea bass	Spain (1990)	+
VIB 572	*V. harveyi*	Sea bream	Spain (1990)	+
VIB 642	*V. harveyi*	Sea bream	Spain (1990)	+
VIB 645	*V. harveyi*	Sea bass	Tunisia (1993)	+
VIB 646	*V. harveyi*	Shark tank water	Denmark (1993)	+
VIB 647	*V. harveyi*	Sea bream	Greece (1992)	+
VIB 648	*V. harveyi*	Shark liver	Denmark	+
VIB 649	*V. harveyi*	Sea bream	Malta (1993)	+
VIB 651	*V. harveyi*	Shark tank water	Denmark (1994)	+
VIB 652	*V. harveyi*	Sea bass	Italy	+
VIB 653	*V. harveyi*	Sea bass	Turkey	+
VIB 658	*V. harveyi*	Sea bream	France (1990)	+
VIB 659	*V. harveyi*	Sea bass	Tunisia	+
SF1	*V. harveyi*	sea perch	China (2002)	+
VBA 642	*V. harveyi*	prawn	Australia (1990)	+

Continue to the table 1

Lab codes	Species name	Strain or source	Country (year of isolation)	*vhh*/*tlh* genes as revealed by Southern hybridization
VIB 457	*V. parahaemolyticus*	LMG 12093		+
VIB 458	*V. parahaemolyticus*	LMG 12094		+
VIB 459	*V. parahaemolyticus*	shrimp	Thailand	+
VIB 461	*V. parahaemolyticus*	shrimp	Thailand	+
VIB 462	*V. parahaemolyticus*	shrimp	Thailand	+
VIB 463	*V. parahaemolyticus*	shrimp	Thailand	+
VIB 611	*V. parahaemolyticus*	ATCC 33844		+
VIB 612	*V. parahaemolyticus*	ATCC 17803		+
VIB 797	*V. parahaemolyticus*	shrimp	China	+
VIB 799	*V. parahaemolyticus*	Thailand, shrimp		+
VIB 800	*V. parahaemolyticus*	shrimp	Thailand	+

LMG, culture collection of the Laboratorium voor Microbiologie, University of Gent, Belgium; T, type strain; PRC, Peoples Republic of China; +, with strong hybridisation band; (+), with weak hybridisation band; –, with no hybridisation signal.

2.2 Isolation of DNA

Total DNA was prepared by the procedure of Ausubel *et al*.[16]. Plasmid DNA, which used for rapid plasmid screening and restriction endonuclease analysis, was isolated by the rapid alkaline extraction method of Birnboim and Doly[17].

2.3 Preparation of digoxigenin (DIG)-labelled DNA probes by PCR

The primers used to synthesize the DIG-labelled VHH DNA probes were VHF2 and VHR2 (Table 2). The VHH probe is specific to the central portion (nucleotides 132 to 1190) of the *V. harveyi vhhA* haemolysin gene[8], and was prepared by PCR amplification from total DNA of *V. harveyi* VIB 645. The design of the primers and resulting PCR was based on the nucleotide sequence of the *vhhA* gene (GenBank AF293430).

Table 2 Primers designed to synthesize the VHH DIG-labeled DNA probe and to amplify the *vhh*/*tlh* genes of Vibrio species

Primer	Sequence (5′→3′)	Annealing site*
VHF1	ATCATGAATAAAACTATTACGTTACT	–3→23
VHR1	GAAAGGATGGTTTGACAAT	1236→1254
VHF2	CACTTATGTCCGCTGCTGGT	132→151
VHR2	GCTGTGGTCGGGTGTGTTAC	1171→1190
VHR3	CCAGAAGACGCACACTCAGA	1126→1145

* Nucleotide positions are relative to the first nucleotide of the translational start codon of the *vhhA* gene of *V. harveyi*.

2.4 Southern blotting and hybridization

DNA samples (100 ng) digested with restriction endonucleases were subjected to electrophoresis through a 1% (W/V) agarose gel and transferred onto a nylon membrane (Amersham) using a modified method[8] originally described by Southern[18].

2.5 Cloning the *vhh*/*tlh* homologue genes in 4 vibrio strains

Nucleotide sequences of *vhh*/*tlh* genes from *V. parahaemolyticus* and *V harveyi* were aligned using ClustalW (http://www.ebi.ac.uk/clustalW/), and 2 pairs of PCR primers were designed from the conserved region to amplify the *vhh*/*tlh* homologue genes in 4 other Vibrio strains (Table 2). While the PCR primers for *V. anguillarum* VIB 72, were VHF2 and VHR2, the PCR primers for *V. campbellii* VIB 285 and *V. natriegens* VIB 299 were VHF2 and VHR3 (Table 2). The expected PCR products were 1059 and 1014 bp, respectively. The PCR primers for the amplification of the full length of the *vhh* gene in *V. harveyi* VIB 647 were VHF1 and VHR1 (Table 2). The chromosomal DNAs of the 4 vibrio strains were used as template. TaKaRa Ex *Taq* DNA polymerase with proof reading activity (TaKaRa Biotech, China) was used for PCR amplification. The PCR conditions involved denaturation at 94℃ for 5 min followed by 30 cycles at 94℃ for 1 min, 50℃ for 1 min and 72℃ for 90 s including a final extension of 10 min at 72℃ with PCR Mastercycler (Eppendorf). The PCR products were cloned into pUCm-T vector (Sangon, China) following the manufacture's instruction. The recombinant plasmids containing the insert of expected size were sequenced by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd, China. Homology searches were performed using BLAST. MegAlign expert sequence analysis software from DNASTAR Inc. was used to construct phylogenic trees. The *vhh*/*tlh* haemolysin gene sequences in *V. harveyi* VIB 647 (designated *vhhC*), *V. natriegens* VIB 299, *V. campbellii* VIB 285, *V. anguillarum* VIB 72, were deposited to GenBank with nucleotide accession numbers as DQ224369, DQ663483, DQ663484 and DQ663485.

3 RESULTS

3.1 Distribution of *vhh* haemolysin gene among different *Vibrio* species

One strong hybridization band was detected in 13 type strains, including 2 of *V. alginolyticus*, 2 of *V. harveyi*, and 1 each of *Grimontia hollisae*, *V. campbellii*, *V. cincinnatiensis*, *V. fischeri*, *V. mimicus*, *V. natriegens*, *V. parahaemolyticus*, *V. proteolyticus* and *V. logei* (Fig. 1). One weak hybridization band was detected in 6 type strains, including *V. anguillarum*, *V. aestuarianus*, *Ph. damselae*, *V. fluvialis*, *V. furnissii* and *V. vulnificus* (Fig. 1). This suggested that these species contain a haemolysin gene with similarity to the *vhh* gene of *V. harveyi*. However, there was not any hybridization signal in *Salinivibrio costicola*, *V. diazotrophicus*, *V. gazogenes*, *V. mediterranei*, *V. nereis*, *V. pelagius* and *V. tubiashii* (Fig. 1). Moreover, at least 1 band was detected in all the cultures of *V. harveyi*. In particular, 2 *Eco*R Ⅰ fragments were identified in 3 isolates of *V. harveyi*, i.e. VIB 645, VIB 648 and SF-1, and 1 band was observed in all other cultures (Fig. 1). Also, 1 band was detected in the 11 *V. parahaemolyticus* cultures.

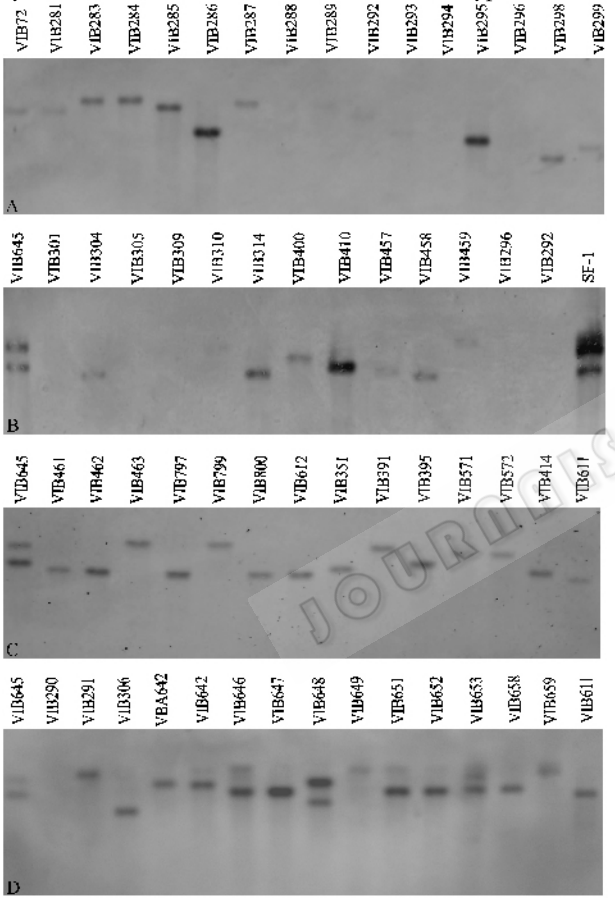

Fig. 1 Detection of *vhh/tlh* haemolysin genes in different *Vibrio* strains. Total DNA from each strain was digested with EcoRI and probed with VHH. A: Line 1-16, strains VIB 72, VIB 281, VIB 283, VIB 284, VIB 285, VIB 286, VIB 287, VIB 288, VIB 289, VIB 292, VIB 293, VIB 294, VIB 295, VIB 296, VIB 298 and VIB 299. B: Line 1-15, strains VIB 645, VIB 301, VIB 304, VIB 305, VIB 309, VIB 310, VIB 314, VIB 400, VIB 410, VIB 457, VIB 458, VIB 459, VIB 460, VIB 292 and SF-1. C: Line 1-15, strains VIB 645, VIB 461, VIB 462, VIB 463, VIB 797, VIB 799, VIB 800, VIB 612, VIB 351, VIB 391, VIB 395, VIB 571, VIB 572, VIB 414 and VIB 611. D: Line 1-16, strains VIB 645, VIB 290, VIB 291, VIB 306, VBA 642, VIB 642, VIB 646, VIB 647, VIB 648, VIB 649, VIB 651, VIB 652, VIB 653, VIB 658, VIB 659 and VIB 611.

3.2 Duplication of *vhh* haemolysin genes in three isolates of *V. harveyi*

The VHH probe was used to examine total DNA from VIB 648 and SF-1 separately digested with 5 further restriction enzymes (Fig. 2). In every case, except *Bam*H Ⅰ, 2 fragments were detected with the probe. This suggested that VIB 648 and SF-1 have 2 very similar, or possibly identical, copies of the haemolysin genes, as is the case for VIB 645[8].

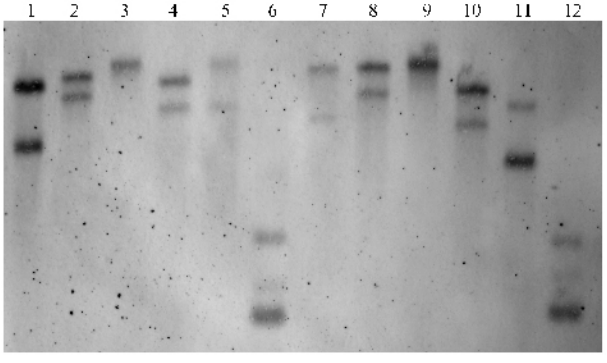

Fig. 2 Southern hybridization analysis of restriction enzyme digested DNA from *V. harveyi* SF-1 and VIB 648 probed with VHH. Lanes: 1-6, SF-1; 7-12, VIB 648. 1, 7, *Xba* Ⅰ; 2, 8, *Pst* Ⅰ; 3, 9, *Bam*H Ⅰ; 4, 10, *Eco*R Ⅰ; 5, 11, *Kpn* Ⅰ; 6, 12, *Hin*d Ⅲ.

3.3 Characterization of the cloned *vhh/tlh* homologue genes in 4 vibrio strains

The *vhh/tlh* homologue genes in *V. anguillarum* VIB 72, *V. campbellii* VIB 285, *V. natriegens* VIB 299 and *V. harveyi* VIB 647 were cloned into pUCm-T vector and sequenced. Analysis of the deduced amino acid sequences of *vhh/tlh* homologue genes of the 4 vibrio strains to VHH and TLH haemolysin revealed high degree of similarity to both VHH (67% ~ 99%) and TLH haemolysin (69% ~ 91%) (Fig. 3). The haemolysin

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VHH:     TYVRCWYRTSYSKDEPATDWEWAENPDGSYFTLDGYWWSSVSFKNMFYTDTPQSVIKQRCEQTLDLANENADITFFAADN 80
TLH:     TYVRCWYRTSYSKDDPATDWEWAKNEDGSYFTIDGYWWSSVSFKNMFYTNTSQNVIRQRCEATLDLANENADITFFAADN 80
VIB647:  TYVRCWYRTSYSKDEPATNWEWAENPDGSYFTLDGYWWSSVSFKNMFYTDTPQSVIKQRCEQTLDLANENADITFFAADN 80
VIB299:  TYVRCWYRTSYSKDDVATDWEWAKNEDGSYFTIDGYWWSAVSFKNMFYTNTTQNVIRQRCESTLDLANENADITFFAADN 80
VIB285:  TYVRCWYRTSYSHDDPETDWEWAENPDGSYFTIEGYWWNALSFKNMFYTNTSQSVIKQRCEQTLDLANENADITYFAADN 80
VIB72    TYVRCWYRPAGNHDDPATDWEWALDEQGNDYTISGYWWSSVRFKNMFYTDTSQYEIKQRCEETLGVSHDSADITYFAADN 80
         ********.: .:*:  *:**** : :*. :*:.****.:: *******:*.*  *:**** **.:::::.****:*****

VHH:     RFSYNHTIWSNDPVMQPDQINKVVALGDSLSDTGNIFNASQWRFPNPNSWFLGHFSNGFVWTEYIAQAKNLPLYNWAVGG 160
TLH:     RFSYNHTIWSNDAAMQPDQINKVVALGDSLSDTGNIFNASQWRFPNPNSWFLGHFSNGFVWTEYIAKAKNLPLYNWAVGG 160
VIB647:  RFSYNHTIWSNDPVMQPDQINKVVALGDSLSDTGNIFNASQWRFPNPNSWFLGHFSNGFVWTEYIAQAKNLPLYNWAVGG 160
VIB299:  RFSYNHTIWSNDAAMQPDQINKVVALGDSLSDTGNIFNASQWRFPNPNSWFLGHFSNGFVWTEYIAKAKNLPLYNWAVGG 160
VIB285:  RWSYNHSIWSNDPVMQPDQINKVVTLGDSLSDTGNIFNASQWRFPNPNSWFLGHFSNGFVWTEYIAQAKNLPLYNWAVGG 160
VIB72:   RFSYNHSIWTNDKQNAKPVINEMVTIGDSLSDTGNIFNASQWKFPNPNSWFLGHFSNGLVWTEYLAKMHNLPVYNWAIGG 160
         *:****:**:**       **::*::****************:***************:*****:*: :***:****:**

VHH:     AAGENQYIALTGVGEQVSSYLAYAKLAKNYKPANTLFTLEFGLNDFMNYNRSVPEVKSDYAEALIKLTDAGAKNLLLMTL 240
TLH:     AAGENQYIALTGVGDQVSSYLTYAKLAKNYKPANTLFTLEFGLNDFMNYNRGVPEVKADYAEALIRLTDAGAKNFMLMTL 240
VIB647:  AAGENQYIALTGVGEQVSSYLAYAKLAKNYKPANTLFTLEFGLNDFMNYNRSVPEVKSDYAEALIKLTDAGAKNLLLMTL 240
VIB299:  AAGENQYIALTGVGEQVSSYLTYAKLAKNYNPANTLFTLEFGLNDFMNYDRSVAEVKSDYAEALIRLTDAGANNFLLMTL 240
VIB285:  AAGENQYIALTGVGKQVSSYLAYMQLAKNYNPANTLFTLEFGLNDFMNYNRSVPEVKADYSEALIKLSDAGARNFLLMTL 240
VIB72:   AAGRNQYIALTGVNDQVRSYLNYMTMAKNYQPENTLFTLEFGLNDFMNYNREVYEVKADFSSAMIRLVDSGAKNIILLTL 240
         ***.*********.**  *** *  :****:* ****************:* * ***:*:::.*:*:* *:**.*::*:**

VHH:     PDATRAPQFTYSTQEEINKIRAKIVEMNEFIKAQAAYYTAQGYNVTLYDTHALFESLTANPEQHGFVNASQACQDINRSS 300
TLH:     PDATKAPQFKYSTQEEIDKIRAKVLEMNEFIKAQAMYYKAQGYNITLFDTHALFETLTSAPEEHGFVNASDPCLDINRSS 300
VIB647:  PDATRAPQFTYSTQEEINKIRAKIVEMNEFIKAQAAYYTAQGYNVTLYDTHALFESLTANPEQHGFVNASQACQDINRSS 300
VIB299:  PDATKAPQFKYSTPEEIEKIRAKVLAMNEFIKEQEKYYKAQGYNITLFDTYALFEALTSAPEDHGFVNATEPCLDINRSS 300
VIB285:  PDATYAPQFKYSSQEEIDTIRAKILETNEFIKAQAAYYTAQGLNITLHDTHALFASLTANPEQHGFSNATEACQDINRSS 300
VIB72:   PDATKAPQFKYSTAEEIAKVSAKITTFNAFIQQQALYYQMQGVNIILFDAHSLFENLTANPQQYGFENATDACLNINRNS 300
         **** ****.**: *** .: **:   * **: *  **  ** *: *.*:::**  **: *:::** **::.* :***.*

VHH:     SVDYLYHHSLRSECASS 337
TLH:     SVDYMYTHALRSECAAS 337
VIB647:  SVDYLYHHALRSECASS 337
VIB299:  SMDYLYSHALRSECASS 337
VIB285:  AADYLYKHSLRSECASS 337
VIB72:   ALDYLYRHSLTNDCAYH 337
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Fig.3 Alignment of the deduced amino acid sequences of *vhh*/*tlh* haemolysin genes cloned from 4 Vibrio strains with VHH and TLH. VHH, haemolysin from *V. harveyi* VIB 645[8] ; TLH, TL haemolysin from *V. parahaemolyticus*[9] ; VIB 647, VIB 72, VIB 299, and VIB 285, VHH/TLH haemolysins from *V. harveyi* VIB 647, *V. anguillarum* VIB 72, *V. natriegens* VIB 299 and *V. campbellii* VIB 285. Symbol: *, identical residues in all sequences; :, conserved substitutions; ., semiconserved substitutions. Numbers on the right refer to the amino acid residue at the end of each line.

gene on *V. harveyi* VIB 647 was designated *vhhC*. As *vhhA* and *vhhB*[8], the ORF of *vhhC* was 1254 nucleotide in length, and encoded polypeptides composed of 418 amino acid residues. The nucleotide sequences of *vhhC* showed high similarity to *vhhA* (99.3%) and *vhhB* (98.4%) of *V. harveyi* VIB 645[8] and the haemolysin genes from *V. harveyi* SF-1 (99.4%, AY487572) and CW-2 (99.3%, AY487571) (Fig. 4). Also, high similarity was revealed to the *tlh* haemolysin gene of *V. parahaemolyticus* (AY289609), the *vpl* phospholipase gene of *V. vulnificus* (AF291424), the *lec* lethinase gene of *V. cholerae* (AC108721), the *phl* lethinase gene of *V. mimicus* (AF035162) and the *phl* lethinase gene of *V. anguillarum* (DQ008059) (Fig. 3).

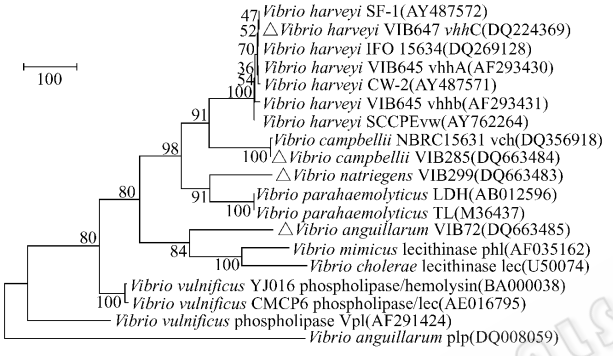

Fig. 4 Phylogenic tree of *vhh/tlh* gene sequences *V. harveyi* VIB 647, *V. anguillarum* VIB 72, *V. campbellii* VIB 285, *V. natriegens* VIB 299, and other *Vibrio* strains already described in GenBank by using MegAlign expert sequence analysis software from DNASTAR Inc.

4 DISCUSSION

It is recognized that haemolysin in the extracellular product of *V. harveyi* is involved in pathogenesis in salmonids[3]. The most pathogenic isolate, *V. harveyi* VIB 645, was found to contain 2 closely related haemolysin genes, which were designated *vhhA* and *vhhB*[8]. The nucleotide sequences of *vhhA* and *vhhB* shows high similarity to *tlh* haemolysin gene of *V. parahaemolyticus*. It will be interesting to determine whether *vhh/tlh* haemolysin genes are also present in other *Vibrio* species, and also if duplications of haemolysin genes are common in *Vibrio* isolates.

The VHH probe, which is specific to *V. harveyi vhhA* haemolysin gene, was used to screen *Eco*R Ⅰ digests of total DNA from 57 *Vibrionaceae* type strains. The data indicates that *vhh/tlh* is widespread in vibrios, especially in *V. harveyi* related species (including *V. harveyi*, *V. campbellii*, *V. alginolyticus*, *V. parahaemolyticus* and *V. natriegens*) and *V. fischeri* related species (including *V. fischeri* and *V. logei*)[19]. To support this conclusion, the *vhh/tlh* homologue genes in *V. anguillarum* VIB 72, *V. campbellii* VIB 285, *V. natriegens* VIB 299 and *V. harveyi* VIB 647 were cloned, and the deduced amino acid sequences of these 4 genes showed high degree of similarity to VHH (67% ~ 99%) and TLH haemolysin (69% ~ 91%).

Previously, we found that a 922 bp *tlh* gene probe of *V. parahaemolyticus* could hybrid with the *vhh* gene of *V. harveyi* in Southern blot[8], and the hybridization pattern was the same when the VHH probe was used (Fig. 1). Therefore, the DNA probes for *tlh* and *vhh* could be used interchangeably. This is in contrast with the result of an earlier study, in which the DNA probe for *V. parahaemolyticus tlh* (450bp) could only hybridise to DNA from *V. parahaemolyticus* but not to DNA from *V. alginolyticus*, *V. mimicus* and *G. hollisae*[12]. The reason for this difference may reflect that the TLH probe (450 bp) that they used is more specific to the *tlh* gene in *V. parahaemolyticus* than in other *Vibrio* species. It was found that there are about 200 bp in common between the TLH probe (450 bp) that they used and the probes that we used (922 bp TLH probe or 1059 bp VHH probe). The reason why *vhh/tlh* was present in some *Vibrio* species but not in others is unclear. Of course, it is possible that the probe we used did not hybridise with the target gene of some *Vibrio* species because of low similarity. It is also possible that this gene was obtained through lateral gene transfer from other species. However, the G + C content of both *V. harveyi vhh* (46%) and *V. parahaemolyticus tlh* (47.6%) are similar to their genomic DNA (46% ~ 48% for *V. harveyi* and 46% ~ 47% for *V. parahaemolyticus*, respectively). Interspecies relatedness has also been reported with the thermostable direct haemolysin originally identified in *V. parahaemolyticus*[20]. Thus, sequence homology with the gene encoding this haemolysin has been found in *V. cholerae* non-O1, *V. mimicus* and *G. hollisae*[21].

That *vhh/tlh* was found in isolates of *V. parahaemolyticus* is consistent with the finding of others[9,12]. *vhh/tlh* was also present in all isolates of

V. *harveyi*, 3 of which (i.e. VIB 645, VIB 648 and SF-1) had duplicate *vhh* genes. Thus, it appears that the duplication of *vhh* in *V*. *harveyi* is not rare. Coincidentally, VIB 645, VIB 648 and SF-1 originated from diseased fish, and were very pathogenic in laboratory-based (fish) infectivity experiments[8,22]. This suggested that the pathogenicity of *V*. *harveyi* to fish may well be related with the duplication of *vhh*.

Previous studies have found that many vibrios were pathogenic to cultured fish and shrimp. In the 24 *Vibrio* species that we have studied, 20 species (including *V*. *anguillarum*, *V*. *harveyi*, *V*. *parahaemolyticus*, *V*. *alginolyticus*, *V*. *fischeri*, *V*. *furnissii*, *V*. *vulnificus*, *Photobacterium damselae* subsp. *damselae*, *V*. *mimicus*, *V*. *campbellii*, *Grimontia hollisae*, *V*. *furnissii*, *V*. *natriegens*, *V*. *logei*, *V*. *aestuarianus*, *V*. *cincinatiensis*, *V*. *proteolyticus*, *V*. *tubiashii*, *V*. *pelagius* and *V*. *gazogenes*) were pathogenic to marine cultured animals[23~26], and 17 of them possess *vhh*/*tlh* genes. Four species, including *Salinivibrio costicola* subsp. *costicola*, *V*. *diazotrophicus*, *V*. *mediterranei* and *V*. *nereis*, which do not possess *vhh*/*tlh* genes, demonstrate either non- or low virulence in the animal models[27,28]. This suggests that VHH/TLH haemolysin may serve a very important role in the virulence of vibrios. Of course, VHH/TLH haemolysin is not the sole virulence factor in vibrios, and the pathogenicity of vibrios may be also caused by other virulence factors.

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VHH/TLH 溶血素基因在海洋弧菌中分布的研究

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摘　要　哈维氏弧菌(*V. harveyi*)的 VHH 溶血素是对海水养殖鱼类的潜在致病因子。哈维氏弧菌的 VHH 溶血素基因与副溶血弧菌(*V. parahaemolyticus*)的 TLH 热不稳定性溶血素基因具有高度相似性,其氨基酸序列的相似性达到 85.6%。根据哈维氏弧菌 *vhhA* 溶血素基因序列,合成一个地高辛标记的 VHH 基因探针,利用其进行 Southern Blot,检测 VHH 溶血素基因在 57 株弧菌(包括 26 株国际标准菌株,20 株哈维氏弧菌,11 株副溶血弧菌)中的分布情况。结果显示,VHH 基因探针与 13 株弧菌标准菌株有强杂交信号,包括 2 株溶藻胶弧菌(*V. alginolyticus*),2 株哈维氏弧菌以及 1 株霍氏格里蒙菌(*Grimontia hollisae*),坎贝氏弧菌(*V. campbellii*),辛辛那提弧菌(*V. cincinatiensis*),费氏弧菌(*V. fischeri*),拟态弧菌(*V. mimicus*),飘浮弧菌(*V. natriegens*),副溶血弧菌,解蛋白弧菌(*V. proteolyticus*)和火神弧菌(*V. logei*)。与 6 株弧菌标准菌株有弱杂交信号,包括鳗弧菌(*V. anguillarum*),河口弧菌(*V. aestuarianus*),美人鱼发光杆菌(*Photobacterium damselae subsp. damselae*),河弧菌(*V. fluvialis*),弗尼斯弧菌(*V. furnissii*)和创伤弧菌(*V. vulnificus*),而另外 7 株弧菌标准菌株中无杂交信号。所有的哈维氏弧菌菌株至少含有一条杂交带,其中菌株 VIB 645, VIB 648 和 SF-1 分别含有 2 条杂交带。11 株副溶血弧菌中均含有一条杂交带。上述数据表明,*vhh/tlh* 溶血素基因广泛分布于弧菌中,尤其是哈维氏弧菌相关菌株和费氏弧菌相关菌株中。另外对鳗弧菌 VIB 72, 坎贝氏弧菌 VIB 285, 飘浮弧菌 VIB 299 和哈维氏弧菌 VIB 647 的 *vhh/tlh* 溶血素基因进行克隆并测序,其氨基酸序列与 VHH 溶血素和 TLH 溶血素氨基酸序列的同源性分别为 67% ~ 99%和 69% ~ 91%。对 *vhh/tlh* 溶血素基因在弧菌中的分布研究,将有助于进一步确定这类溶血素基因在病原弧菌致病性中的作用。

关键词　弧菌　哈维氏弧菌　副溶血弧菌　溶血素基因　*vhh*　*tlh*

中图分类号：Q933　　**文献标识码**：A　　**文章编号**：0001-6209(2007)05-0881-08

基金项目　国家自然科学基金(30371119)　教育部新世纪优秀人才支持计划(NCET-04-0645)

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收稿日期：2006-12-05　**接受日期**：2007-01-08　**修回日期**：2007-01-25