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Development of a duplex Taqman real-time PCR assay for rapid identification of *Vibrio splendidus* -related and *V. aestuarianus* strains from bacterial cultures

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Abstract :

To enable the rapid and accurate identification of *Vibrio splendidus*-related and *V. aestuarianus* strains associated with Pacific cupped oyster *Crassostrea gigas* mortality, we developed a duplex Taqman real-time PCR assay and evaluated its efficacy. This technique proved to be rapid, sensitive, and specific and will be particularly valuable for epidemiologic studies.

Keywords : Multiplex real-time PCR, Gene sequencing, Molluscs, *Splendidus clade*, *Vibrio aestuarianus*

Since 2008, severe seasonal mortality events of juvenile cultured Pacific oyster *Crassostrea gigas* have been recurrently reported in Europe, especially along the whole French coastline. Mortality rates range from 60 to 90% (Martenot et al., 2011). Diagnostic analysis of abnormal mortality outbreaks in French mollusc aquaculture, based on the scheme edited by the European Council Directive 2006/88/EC on aquatic animal's health, resulted in the positive detection of one virus, herpes virus type 1 (OsHV-1), and two bacteria, *Vibrio splendidus*-related and *V. aestuarianus,* found at a high prevalence of 76% and 51%, respectively, in 2008. These infectious agents were shown to exhibit virulent abilities in experimental infection trials (Goudenège et al., 2015; Luna-Acosta et al., 2011; Saulnier et al., 2009; Schikorski et al., 2011). Recently, it has been shown that these agents can act solely or in concert (polymicrobial disease) in the field (Lemire et al., 2014; Petton et al., 2015). There is an urgent need to rapidly detect *Vibrio splendidus*-related and *V. aestuarianu*s to gain a better understanding of the epidemiological significance of their association with or without other infective agents, such as OsHV-1, and the effect of such interactions in detrimental environmental or host factors.

In the past decade, molecular typing methods have revolutionized bacterial species determination due to high reproducibility and discriminatory power. They encompass whole-genome typing methods, such as partial or complete genome sequencing, DNA-DNA hybridization, DNA fingerprinting methods and the widely used multilocus sequence typing (MLST) analysis (Sawabe et al., 2007, 2013). Previous studies performed by MLST analysis of housekeeping genes have evidenced that *V. splendidus*-related strains belong to the *Splendidus* clade and encompass at least 17 species (Table 2) having a high degree of genetic similarity (Lasa et al., 2013). The recent increase in newly described species isolated from *C. gigas* oyster (Gonzalez-Castillo et al., 2016, 2014) and our phylogenical analysis on other strains isolated from oyster based on the high discriminatory power of the *gyr*B gene observed for *V. splendidus*-related strains (Fig. S1) suggest that other *Vibrio* species not yet described may exist inside this clade. To rapidly identify bacterial colonies obtained by classical culture methods, we developed and validated a Duplex Taqman real time PCR Assay (DTA) for co-determination (in a single reaction tube) of all *V. splendidus*-related strains (rather than a particular *Vibrio* species) and *V. aestuarianus* bacterial isolates, using for the last *Vibrio* species an already developed real time PCR assay (Saulnier et al., 2009).

Sequence alignment comparisons between housekeeping genes available in Genbank consisted of 16S rRNA, *rec*A, *rpo*A, *tox*R and *gyr*B. We selected one gene, 16S rRNA, which displayed a 25 bp long region that was conserved for all *V. splendidus*-related strains, except for *V. chagasii*, *V. fortis, V. gallaecicus, V. hemicentroti* and *V. pelagius,* and was divergent from other *Vibrio*. Taqman probes targeting this region and flanking primers were designed with the aid of Primer 3 software (Table 1). A total of 44 *Vibrio* species reference strains were used to assess the specificity of the oligonucleotide probes and primers (Table 2). All strains were grown for 96 h at 20°C on plates of Marine agar 2216 (Difco) and DNA extracted by a 10 min boiling procedure in ultra-pure water (Sigma) as described in Saulnier et al. (2009). Two biological replicates from distinct colonies were tested for each bacterial strain. Real-time PCR assay was performed, as described by Saulnier et al. (Saulnier et al., 2009). Briefly, we utilized the same reactive concentrations which included 200 nM of each oligonucleotidic probe and the same PCR thermal profile. Only 14 out of 44 tested *Vibrio* strains yielded positive amplification signals, with cycle threshold values ranging from 15 to 25, when selected for either FAM or ROX filters. Moreover, 12 out of 17 strains belonging to the *V. splendidus*-related reference strains were positive using the FAM filter (*Splendidus* clade probe), whereas five strains belonging to *V. chagasii, V. gallaecicus, V. hemicentroti, V. pelagius* and *V. fortis* species were negative (Table 2). Only the two *V. aestuarianus* reference strains yielded a positive amplification signal with ROX filter (*V. aestuarianus* probe). Specificity was determined via agarose gel electrophoresis with 150 and 270-bp long PCR products detected for *V. splendidus*-related strains and *V. aestuarianus*, respectively (not shown). All strains were isolated on Marine Agar plate cultures selecting only dominant bacteria by the plate counting method. Of 39 oysters mortality cases analysed in 2008 through active the French surveillance network, 181 bacterial strains were screened using our DTA. A total of 76 strains (42%) were positive, indicating their affiliation to either *V. aestuarianus* sp. (23 strains) or *Splendidus* clade (53 strains). To confirm these results using a taxonomic-independent approach, sequencing analysis (Genbank accession numbers in Table S1) was performed on randomly selected strains in each oyster batch (1) from the DTA-negative strains (16SrDNA sequencing on 59 of the 105 negative strains) and (2) from the DTA-positive strains (16SrDNA sequencing on 2 of the 23 positive *V. aestuarianus* strains and 8 of the 53 positive *Splendidus* clade strains and *gyr*B sequencing on 15 and 24 *V. aestuarianus* and *Splendidus* clade-positive strains respectively (Le Roux et al., 2004)). Concerning 16SrDNA sequencing analyses (Fig. S2), results of both tests were in agreement for all DTA-negative and positive *V. aestuarianus* (67/67, 2/2) and nearly all *V. splendidus*-related tested strains (50/61, 8/8), respectively, at the level of 97% nucleotide identity with 03/008^T and LMG 4042^T reference strains

(Stackebrandt and Goebel, 1994). However, a mismatch occurred for eleven negative *V. splendidus*-related strains that were tentatively classified as *V. fortis* (n=4) or *V. chagasii* (n=7). All 15 randomly selected DTA-positive *V. aestuarianus* strains were found to be affiliated to *V. aestuarianus* (15/15) in addition to the 24 arbitrarily selected DTA-positive *V. splendidus*-related positive strains, as measured by *gyr*B sequencing, which confirmed the specificity of an already assessed molecular diagnostic test (Saulnier et al., 2009). The specificities of our DTA for *V. aestuarianus* and *Spendidus* clade were 100% (67/67) and 100% (50/50), respectively, and their sensitivities were 100% (15/15) and 82.0% (50/61), respectively. Hence, the use of DTA is a simple and efficient method for rapid (less than two hours), sensitive and specific typing of dominant bacterial strains belonging to either the *V. aestuarianus* (23/181 = 12.7%) or the *V. splendidus* group (53/181 = 29.3%), both bacteria being closely involved in mortality outbreaks of juvenile Pacific cultured oyster *Crassostrea gigas.*

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Figure captions

Figure S1 (supplementary data)

Phylogenetic tree of a partial protein-coding *gyr*B gene from 41 dominant bacterial strains (all DTA positive) isolated on Zobell Agar plates from *Crassotrea gigas* oysters sampled in a context of abnormal mortalities (39 episodes along the French coasts in 2008) through the mission of Repamo's French surveillance network. Analysis was performed using Neighbour-Joining method and Kimura 2 parameter model on 487gap-free sites. The evolutionary distances were computed. Bootstrap values (1000 replicates) greater than 50% are indicated. Genbank accession number of reference type strains is included to identify the strains analysed in this study. DTA-positive Splendidus clade and Vibrio aestuarianus strains are framed by a dashed and solid line respectively.

Figure S2 (supplementary data)

Phylogenetic tree of partial 16S rDNA from Vibrio isolated from C. gigas oysters collected in the context of mortalities (39 episodes in 2008)and Vibrio reference strains. A total of 591gap-free sites were analysed. The evolutionary distances were computed using the Neighbour-Joining method. Bootstrap values (1000 replicates) greater than 50% are indicated. Genbank accession number of reference type strains is included to identify the strains analysed in this study. DTA-positive Splendidus clade and Vibrio aestuarianus strains are framed by a dashed and solid line respectively.

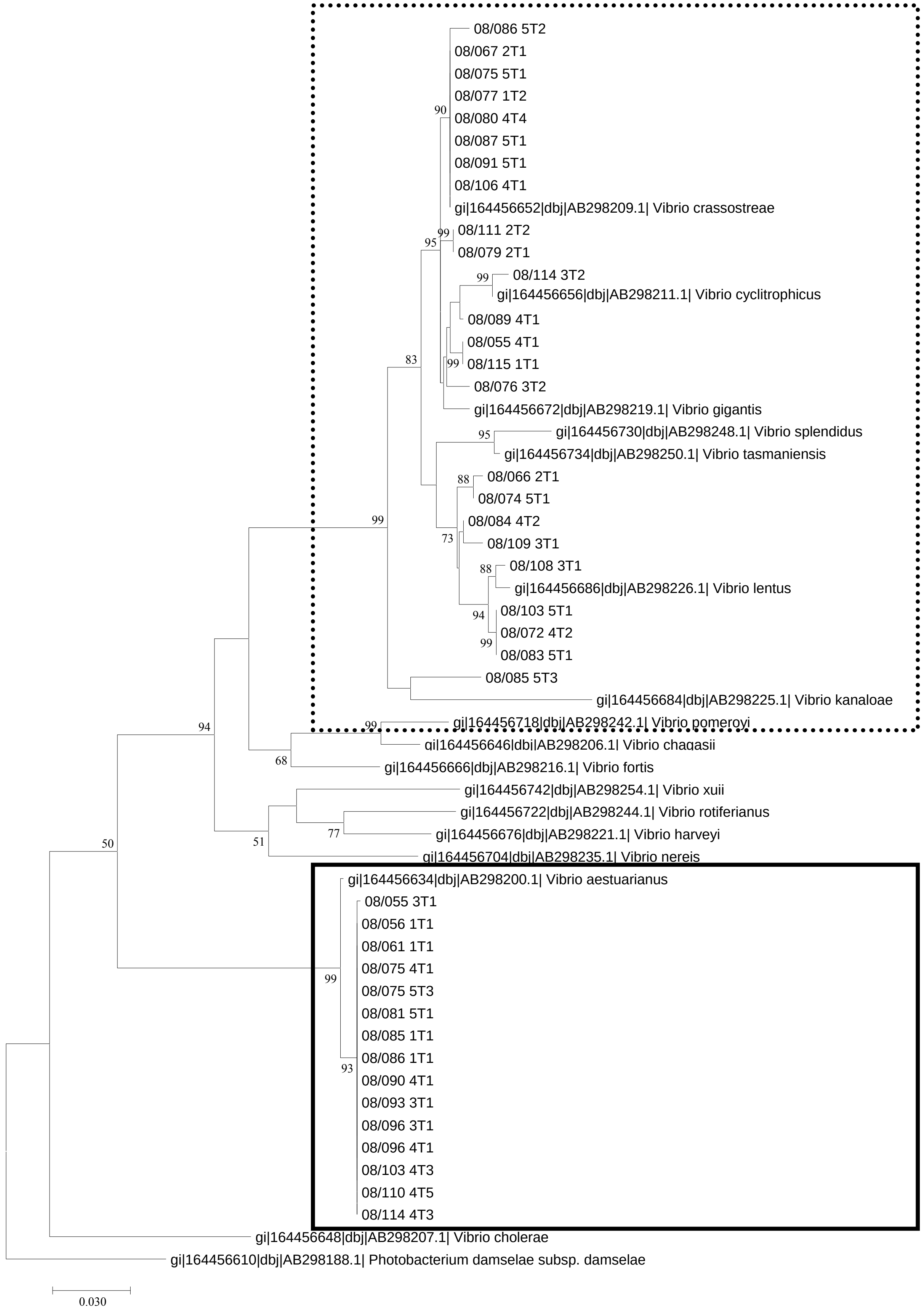

Figure S1 (supplementary data)

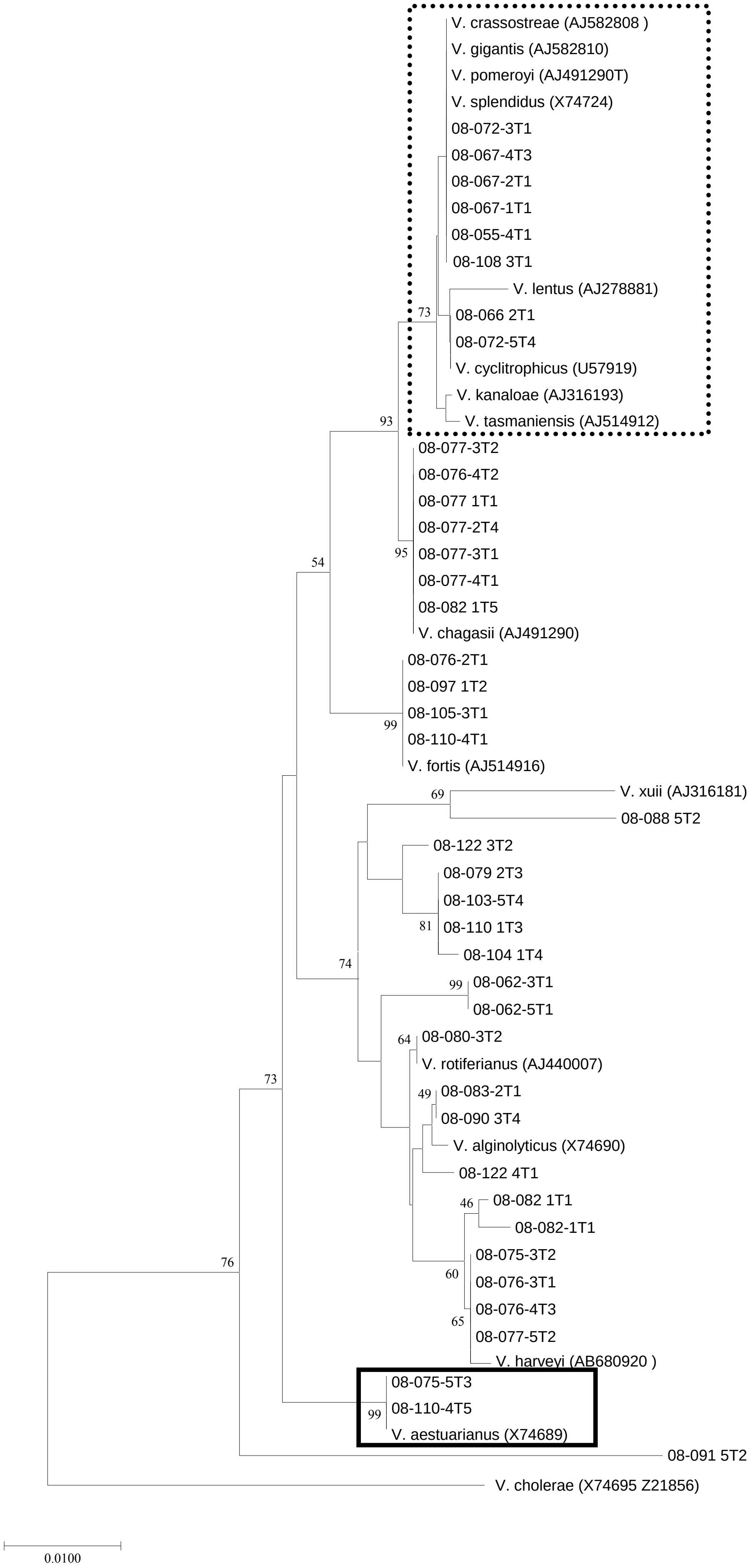

Figure S2 (supplementary data)

Name	Function	Sequence (5′–3′)	Tm (°C)
16S spF2	Forward primer	ATCATGGCTCAGATTGAACG	58
16S spR2	Reverse primer	CAATGGTTATCCCCCACATC	60
16S probe	TaqMan PCR probe	FAM-CCCATTAACGCACCCGAAGGATTG BHQ1	71
DNAj aes F1	Forward primer	GTATGAAATTTTAACTGACCCACAA	58
DNAj aes R1	Reverse primer	CAATTTCTTTCGAACAACCAC	59
DNAj probe	TaqMan PCR probe	Texas Red-TGGTAGCGCAGACTTCGGCGAC-BHQ2	71

Table 1: Nucleotide sequences and melting temperature (Tm) of primers and Taqman probes for real time PCR reaction.

Clade	**Species**	**Name**	**DTA results**
Anguillarum	*Vibrio anguillarum*	LMG 4437 T	-
	Vibrio aestuarianus subsp. *aestuarianus*	03/008 T	+
	Vibrio aestuarianus subsp. *francensis*	02/041 T	+
Coralliilyticus	*Vibrio coralliilyticus*	LMG 19607 T	-
	Vibrio neptunius	LMG 20536 T	-
Halioticoli	*Vibrio ezurae*	DSMZ 17533 T	-
	Vibrio superstes	DSMZ 16383 T	-
	Vibrio gallicus	DSMZ 16639 T	-
Harveyi	*Vibrio harveyi*	LMG 4044 T	-
	Vibrio communis	DSMZ 23055 T	-
	V. inhibens	DSMZ 23440 T	-
	Vibrio rotiferianus	DSMZ 17186 T	-
	Vibrio sagamiensis	DSMZ 25651 T	-
Mediterranei	*Vibrio mediterranei*	DSMZ 19502 T	-
	Vibrio maritimus	DSMZ 26148 T	-
	Vibrio variabilis	DSMZ 26147 T	-
Nereis	*Vibrio xuii*	DSMZ 17185 T	-
Nigripulchritudo	*Vibrio penaeicida*	DSMZ 14398 T	-
orphan	*Vibrio tapetis*	CECT 4600 T	-
Orientalis	*Vibrio europaeus*	07/118T2	-
	Vibrio tubiashii	DSMZ 19142 T	-
	Vibrio hepatarius	LMG 20362 T	-
	Vibrio brasiliensis	DSMZ 17184 T	-
	Vibrio atypicus	DSMZ 25292 T	-
not defined	*Vibrio cortegadensis*	DSMZ 102805 T	-
	Vibrio shilonii	DSMZ 13774 T	-
	Vibrio pacini	LMG 19999 T	-
Splendidus	*Vibrio artabrorum*	DSMZ 26480 T	+
	Vibrio atlanticus	DSMZ 26479 T	+
	Vibrio celticus	DSMZ 26172 T	+
	Vibrio chagasii	LMG 21353 T	-
	Vibrio crassostreae	LGP7 T	+
	Vibrio cyclitrophicus	LMG 21359 T	+
	Vibrio fortis	DSMZ 19133 T	-
	Vibrio gallaecicus	DSMZ 23502 T	-
	Vibrio gigantis	LGP13 T	+
	Vibrio hemicentroti	DSMZ 26178 T	-
	Vibrio kanaloae	LGM 20539 T	+
	Vibrio lentus	CIP 107166 T	+
	Vibrio pelagius	LMG 3897 T	-
	Vibrio pomeroyi	LMG 20537 T	+
	Vibrio splendidus	LMG 4042 T	+
	Vibrio tasmaniensis	LMG 20012 T	+
	Vibrio toranzoniae	DSMZ 28519 T	+

Table 2: Specificity of the Taqman real time PCR assay according to reference bacterial strains.

Strain number	**Mortality batches**	**Sampling period**	**Origin and (department number)**	**Oyster age class**	**DTA identification**	**16s rRNA GenBank number**	***gyr*B GenBank number**
08-055 3T1	08-055	15/05/2008	La Flotte en Ré (17)	spat	*V. aestuarianus*		KY930534
08-056 1T1	08-056	15/05/2008	La Flotte en Ré (17)	adult	*V. aestuarianus*		KY930535
08-061 1T1	08-061	20/05/2008	Thau (34)	juvenile	*V. aestuarianus*		KY930536
08-075 4T1	08-075	30/06/2008	Arcachon (33)	spat	*V. aestuarianus*		KY930537
08-075 5T3	08-075	30/06/2008	Arcachon (33)	spat	*V. aestuarianus*	KY923251	KY930538
08-081 5T1	08-081	30/06/2008	Ronce-Perquis	juvenile	*V. aestuarianus*		KY930539
08-085 1T1	08-085	03/07/2008	Penthièvre (56)	spat	*V. aestuarianus*		KY930540
08-086 1T1	08-086	03/07/2008	Penthièvre (56)	spat	*V. aestuarianus*		KY930541
08-090 4T1	08-090	04/07/2008	Chatelaillon (17)	juvenile	*V. aestuarianus*		KY930542
08-093 3T1	08-093	07/07/2008	Aiguillon sur Mer (17)	juvenile	*V. aestuarianus*		KY930543
08-096 3T1	08-096	09/07/2008	Rivière d'Auray (56)	spat/ juvenile	*V. aestuarianus*		KY930544
08-096 4T1	08-096	09/07/2008	Rivière d'Auray (56)	spat/ juvenile	*V. aestuarianus*		KY930545
08/103 4T3	08/103	17/07/2008	Rivière d'Auray (56)	adult	*V. aestuarianus*		KY930546
08/110 4T5	08/110	20/07/2008	Ronce les bains (17)	adults	*V. aestuarianus*	KY923252	KY930547
08/114 4T3	08/114	21/07/2008	Saint Germain sur Ay (50)	juvenile	*V. aestuarianus*		KY930548
08-055 4T1	08-055	15/05/2008	La Flotte en Ré (17)	spat	*V. splendidus*-related	KY923253	KY930549
08-066 2T1	08-066	28/05/2008	Golfe du Morbihan (56)	spat	*V. splendidus*-related	KY923254	KY930550
08-067 1T1	08-067	05/06/2008	Esnandes-Marsilly (17)	spat	*V. splendidus*-related	KY923255	
08-067 2T1	08-067	05/06/2008	Esnandes-Marsilly (17)	spat	*V. splendidus*-related	KY923256	KY930551
08-067 4T3	08-067	05/06/2008	Esnandes-Marsilly (17)	spat	*V. splendidus*-related	KY923257	
08-072 3T1	08-072	19/06/2008	Baie des Veys (50)	juvenile	*V. splendidus*-related	KY923258	
08-072 4T2	08-072	19/06/2008	Baie des Veys (50)	juvenile	*V. splendidus*-related		KY930552

08-072 5T4	08-072	19/06/2008	Baie des Veys (50)	juvenile	*V. splendidus*-related	KY923259	
08-074 5T1	08-074	01/07/2008	Meuvaine (50)	juvenile	*V. splendidus*-related		KY930553
08-075 5T1	08-075	30/06/2008	Arcachon (33)	spat	*V. splendidus*-related		KY930554
08-076 3T2	08-076	02/07/2008	Marseillan (34)	spat	*V. splendidus*-related		KY930555
08-077 1T2	08-077	02/07/2008	Thau (34)	spat	*V. splendidus*-related		KY930556
08-079 2T1	08-079	02/07/2008	Blainville (50)	spat	*V. splendidus*-related		KY930557
08-080 4T4	08-080	30/06/2008	Ronce-Perquis (17)	spat	*V. splendidus*-related		KY930558
08-083 5T1	08-083	02/07/2008	Blainville (50)	juvenile	*V. splendidus*-related		KY930559
08-084 4T2	08-084	02/07/2008	Gouville (50)	spat	*V. splendidus*-related		KY930560
08-085 5T3	08-085	03/07/2008	Penthièvre (56)	spat	*V. splendidus*-related		KY930561
08-086 5T2	08-086	03/07/2008	Penthièvre (56)	spat	*V. splendidus*-related		KY930562
08-087 5T1	08-087	03/07/2008	Etel (56)	spat	*V. splendidus*-related		KY930563
08-089 4T1	08-089	04/07/2008	Bretagne Nord (29)	spat	*V. splendidus*-related		KY930564
08-091 5T1	08-091	07/07/2008	Cancale (35)	juvenile	*V. splendidus*-related		KY930565
08/103 5T1	08/103	17/07/2008	Rivière dAuray (56)	adult	*V. splendidus*-related		KY930566
08/106 4T1	08/106	17/07/2008	Bonne anse (17)	spat	*V. splendidus*-related		KY930567
08/108 3T1	08/108	21/07/2008	Baie des Veys (50)	spat	*V. splendidus*-related	KY923260	KY930568
08/109 3T1	08/109	21/07/2008	Baie des Veys (50)	juvenile	*V. splendidus*-related		KY930569
08/111 2T2	08/111	21/07/2008	Ile d'aix (17)	adult	*V. splendidus*-related		KY930570
08/114 3T2	08/114	21/07/2008	Saint Germain sur Ay (50)	juvenile	*V. splendidus*-related		KY930571
08/115 1T1	08/115	22/07/2008	Etang prevost (80)	spat	*V. splendidus*-related		KY930572
08-062 3T1	08-062	23/05/2008	Ars en Ré (17)	juvenile	negative	KY923649	
08-062 5T1	08-062	23/05/2008	Ars en Ré (17)	juvenile	negative	KY923650	
08-066 5T2	08-066	28/05/2008	Golfe du Morbihan (56)	spat	negative	KY923651	
08-067 2T2	08-067	05/06/2008	Esnandes-Marsilly (17)	spat	negative	KY923652	
08-067 3T3	08-067	05/06/2008	Esnandes-Marsilly (17)	spat	negative	KY923653	
08-072 4T1	08-072	19/06/2008	Baie des Veys (50)	juvenile	negative	KY923654	

08-072 4T5	08-072	19/06/2008	Baie des Veys (50)	juvenile	negative	KY923655	
08-072 4T6	08-072	19/06/2008	Baie des Veys (50)	juvenile	negative	KY923656	
08-074 4T1	08-074	01/07/2008	Meuvaine (50)	juvenile	negative	KY923657	
08-075 3T2	08-075	30/06/2008	Arcachon (33)	spat	negative	KY923659	
08-075 3T3	08-075	30/06/2008	Arcachon (33)	spat	negative	KY923658	
08-076 2T1	08-076	02/07/2008	Marseillan (34)	spat	negative	KY923261	
08-076 3T1	08-076	02/07/2008	Marseillan (34)	spat	negative	KY923660	
08-076 4T2	08-076	02/07/2008	Marseillan (34)	spat	negative	KY923262	
08-076 4T3	08-076	02/07/2008	Marseillan (34)	spat	negative	KY923661	
08-077 1T1	08-077	02/07/2008	Thau (34)	spat	negative	KY923263	
08-077 2T4	08-077	02/07/2008	Thau (34)	spat	negative	KY923264	
08-077 3T1	08-077	02/07/2008	Thau (34)	spat	negative	KY923265	
08-077 4T1	08-077	02/07/2008	Thau (34)	spat	negative	KY923266	
08-077 4T2	08-077	02/07/2008	Thau (34)	spat	negative	KY923662	
08-077 5T2	08-077	02/07/2008	Thau (34)	spat	negative	KY923663	
08-079 2T3	08-079	02/07/2008	Blainville (50)	spat	negative	KY923664	
08-080 3T2	08-080	30/06/2008	Ronce-Perquis (17)	spat	negative	KY923665	
08-080 4T1	08-080	30/06/2008	Ronce-Perquis (17)	spat	negative	KY923666	
08-081 4T1	08-081	30/06/2008	Ronce-Perquis (17)	juvenile	negative	KY923667	
08-081 5T2	08-081	30/06/2008	Ronce-Perquis (17)	juvenile	negative	KY923668	
08-081 5T3	08-081	30/06/2008	Ronce-Perquis (17)	juvenile	negative	KY923669	
08-082 1T1	08-082	02/07/2008	Baie de Bourgneuf (44)	larvae	negative	KY923267	
08-082 1T2	08-082	02/07/2008	Baie de Bourgneuf (44)	larvae	negative	KY923670	
08-082 1T5	08-082	02/07/2008	Baie de Bourgneuf (44)	larvae	negative	KY923268	
08-083 2T1	08-083	02/07/2008	Blainville (50)	juvenile	negative	KY923671	
08-084 3T2	08-084	02/07/2008	Gouville (50)	spat	negative	KY923672	
08-084 4T1	08-084	02/07/2008	Gouville (50)	spat	negative	KY923673	
08-085 5T2	08-085	03/07/2008	Penthièvre (56)	spat	negative	KY923674	

08-087 5T2	08-087	03/07/2008	Etel (56)	spat	negative	KY923675	
08-088 5T2	08-088	03/07/2008	Etel (56)	spat	negative	KY923676	
08-090 3T4	08-090	04/07/2008	Chatelaillon (17)	juvenile	negative	KY923677	
08-091 3T1	08-091	07/07/2008	Cancale (35)	juvenile	negative	KY923678	
08-091 5T2	08-091	07/07/2008	Cancale (35)	juvenile	negative	KY923679	
08-097 1T1	08-097	08/07/2008	Baie de Bourgneuf (44)	juvenile	negative	KY923680	
08-097 1T2	08-097	08/07/2008	Baie de Bourgneuf (44)	juvenile	negative	KY923269	
08/103 3T2	08/103	17/07/2008	Rivière dAuray (56)	adult	negative	KY923681	
08/103 5T4	08/103	17/07/2008	Rivière dAuray (56)	adult	negative	KY923682	
08/104 1T4	08/104	17/07/2008	Ronce les bains (17)	larvae	negative	KY923683	
08/105 3T1	08/105	17/07/2008	Ronce les bains (17)	adult	negative	KY923270	
08/106 3T2	08/106	17/07/2008	Bonne anse (17)	spat	negative	KY923684	
08/108 4T1	08/108	21/07/2008	Baie des Veys (50)	spat	negative	KY923685	
08/110 1T2	08/110	20/07/2008	Ronce les bains (17)	adult	negative	KY923686	
08/110 1T3	08/110	20/07/2008	Ronce les bains (17)	adult	negative	KY923687	
08/110 3T1	08/110	20/07/2008	Ronce les bains (17)	adult	negative	KY923688	
08/110 4T1	08/110	20/07/2008	Ronce les bains (17)	adult	negative	KY923271	
08/111 1T1	08/111	21/07/2008	Ile d'aix (17)	adult	negative	KY923689	
08/111 2T1	08/111	21/07/2008	Ile d'aix (17)	adult	negative	KY923690	
08/111 3T1	08/111	21/07/2008	Ile d'aix (17)	adult	negative	KY923691	
08/114 1t1	08/114	21/07/2008	Saint Germain sur Ay (50)	juvenile	negative	KY923692	
08/114 4T2	08/114	21/07/2008	Saint Germain sur Ay (50)	juvenile	negative	KY923693	
08/114 5T1	08/114	21/07/2008	Saint Germain sur Ay (50)	juvenile	negative	KY923694	
08/122 3T2	08/122	07/08/2008	Arcachon (33)	spat	negative	KY923695	
08/122 4T1	08/122	07/08/2008	Arcachon (33)	spat	negative	KY923696	

Table S1 (supplementary data): Genbank accession numbers and origin of the bacterial strains isolated from *Crassostrea gigas* in 2008. Their sources (sampling period, age class of the oysters and origin of the mortality batches), as well as DTA results and Genbank accession numbers (for 16s rDNA and *gyrB* genes) are indicated.