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Genetic diversity of culturable *Vibrio* in an Australian blue mussel *Mytilus galloprovincialis* hatchery

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ABSTRACT: Bacillary necrosis associated with *Vibrio* species is the common cause of larval and spat mortality during commercial production of Australian blue mussel *Mytilus galloprovincialis*. A total of 87 randomly selected *Vibrio* isolates from various stages of rearing in a commercial mussel hatchery were characterised using partial sequences of the ATP synthase alpha subunit gene (*atp*A). The sequenced isolates represented 40 unique *atp*A genotypes, overwhelmingly dominated (98%) by *V. splendidus* group genotypes, with 1 *V. harveyi* group genotype also detected. The *V. splendidus* group sequences formed 5 moderately supported clusters allied with *V. splendidus*/*V. lentus*, *V. atlanticus*, *V. tasmaniensis*, *V. cyclitrophicus* and *V. toranzoniae*. All water sources showed considerable *atp*A gene diversity among *Vibrio* isolates, with 30 to 60% of unique isolates recovered from each source. Over half (53%) of *Vibrio atp*A genotypes were detected only once, and only 7 genotypes were recovered from multiple sources. Comparisons of phylogenetic diversity using UniFrac analysis showed that the culturable *Vibrio* community from intake, header, broodstock and larval tanks were phylogenetically similar, while spat tank communities were different. Culturable *Vibrio* associated with spat tank seawater differed in being dominated by *V. toranzoniae*-affiliated genotypes. The high diversity of *V. splendidus* group genotypes detected in this study reinforces the dynamic nature of microbial communities associated with hatchery culture and complicates our efforts to elucidate the role of *V. splendidus* group bacteria in vibriosis.

KEY WORDS: Bacillary necrosis · Vibriosis · *Vibrio splendidus* · *atp*A · Shellfish · Aquaculture

INTRODUCTION

Gammaproteobacteria of the genus *Vibrio* and allied genera are significant disease-causing agents in shellfish hatcheries worldwide and are a common cause of bacillary necrosis, a disease that results in severe hatchery stock losses (DiSalvo et al. 1978, Sugumar et al. 1998). In the vast majority of cases, *Vibrio* spp. have been implicated as the causative agents, such that bacillary necrosis has been alternatively termed vibriosis.

V. splendidus-related bacteria are a diverse and abundant component of the temperate coastal bacterial community (Thompson et al. 2004) that have previously been considered of little significance as pathogens (Gay et al. 2004). However, bacteria within this group have increasingly been associated with mortality of hatchery-reared shellfish larvae, post-settlement juveniles (spat) and juvenile stock (Nicolas et al. 1996, Lacoste et al. 2001, Le Roux et al. 2002, Gay et al. 2004, Gómez-León et al. 2005, Kesarcodi-Watson et al. 2009, Beaz-Hidalgo et al. 2010, Saulnier et al. 2010).

Since the first description of *V. splendidus* (Reichelt et al. 1976), the group has been divided into 15 related species (Sawabe et al. 2007, Beaz-Hidalgo et al. 2010), making the *V. splendidus* group the most diverse among the *Vibrionales* (Sawabe et al. 2007).

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A number of related species are considered pathogenic to shellfish, including *V. splendidus* (Saulnier et al. 2010), *V. crassostreae* (Faury et al. 2004), *V. gigantis* (Le Roux et al. 2005), *V. celticus* (Beaz-Hidalgo et al. 2010) and *V. chagasii* (Teng et al. 2012). The shellfish species affected include oysters (*Crassostrea* spp.; Sugumar et al. 1998, Lacoste et al. 2001, Le Roux et al. 2002, Gay et al. 2004), clams (*Ruditapes* spp. and *Solen* spp.; Gómez-León et al. 2005), scallops (*Pecten* spp.; Nicolas et al. 1996) and mussels (*Perna* spp.; Kesarcodi-Watson et al. 2009). Bacteria affiliated with the *V. splendidus* group have often been isolated from diseased shellfish, and some were pathogenic to shellfish (Sugumar et al. 1998, Saulnier et al. 2010). While a loose correlation exists between shellfish larval mortality and increased concentration of *V. splendidus* (Sugumar et al. 1998, Lacoste et al. 2001, Le Roux et al. 2002, Gómez-León et al. 2005), understanding of the disease is limited partly due to the genetic diversity and highly variable pathogenicity among isolates (Le Roux & Austin 2006). While molecular approaches can distinguish a range of *Vibrio* (Le Roux et al. 2002, 2004), the 16S rRNA gene contains insufficient sequence variation to resolve distinct genotypes within the *V. splendidus* group (Le Roux et al. 2004). However, the ATP synthase alpha subunit gene (*atp*A) appears to show higher sequence variation among *V. splendidus* strains (Thompson et al. 2007) and thus may be a more useful tool to examine diversity in this group.

Our preliminary studies in an Australian blue mussel *Mytilus galloprovincialis* hatchery indicated that mass mortality events were associated with bacterial communities dominated by the *V. splendidus* group; however, the potential sources and diversity of *V. splendidus*-associated mortalities were unknown. Here we used partial *atp*A gene sequences to examine and compare the diversity of culturable *Vibrio* from hatchery rearing systems and seawater sources in hatchery culture of juvenile Australian blue mussels.

MATERIALS AND METHODS

Bacterial sampling

Culturable bacteria were isolated on the same day in May 2009 (autumn) from seawater and hatchery rearing systems used for production of Australian blue mussel at a Tasmanian marine hatchery (42.5° S, 147.9° E). Seawater samples were collected in triplicate from 2 points in the hatchery seawater system: (1) untreated hatchery intake bay seawater (Ba) pumped from 30 m depth in Spring Bay; and (2) 20 µm filtered seawater stored in a header tank (Hd). Triplicate seawater samples were also collected from 3 different culture stages of the hatchery production: (1) broodstock tank (Br), (2) larvae culture tank (Lr) and (3) spat culture tank (Sp). Bay water entering the intake pipe and header tank was at seasonal ambient temperature (<12°C), and broodstock tank seawater was held at 18°C using a heat exchanger. Both the larval and spat tank systems received 20 µm filtered header tank seawater heated to 22°C and ultraviolet (UV) irradiated which reduced culturable bacteria (and *Vibrio*) to below the routine detection limit of 10 CFU ml^{-1}. Header tanks were operated with an approximately 30 min residence time. Spat tanks were flow-through systems at flow rates achieving complete water replacement in approximately 6 h. Broodstock tank seawater contained expelled gametes at the time of sampling. Larval tanks were static 3000 l tanks with cleaning and water replacement every 48 h. At the time of sampling, larval cultures were 48 h post-fertilisation and had not received water changes or live microalgae as food. The spat cultures sampled were at 4 wk post-settlement and received microalgae feed 4 times a day, maintaining a concentration of 200 algal cells ml^{-1}. At the time of sampling, the hatchery was neither experiencing elevated larval nor spat mortality.

Samples of seawater (10 ml) were collected using sterile 20 ml syringes, transferred into sterile 30 ml McCartney tubes and vigorously shaken for 1 min prior to serial 10-fold dilution (to 10^{-5}). A volume of 100 µl from each dilution was spread plated onto thiosulphate citrate bile sucrose (TCBS) agar (Oxoid), and plates were incubated at 21 ± 3°C for 48 h. The concentration of culturable *Vibrio* was determined for each source by triplicate plate counts from plates containing 30 to 300 colonies. A library of presumptive *Vibrio* strains was established by random selection of a minimum of 20 colonies from each source. Colonies were streak-plated onto fresh TCBS agar to ensure purity and then maintained at 8°C over the course of study. All confirmed *Vibrio* isolates were archived at −80°C in cryovials containing 10% glycerol and Protect Beads (Oxoid).

Colony PCR and DNA sequencing of the *atp*A gene

Genomic DNA was prepared using the colony stab technique. Briefly, isolates were incubated for 24 to 72 h at 25°C, and a single bacterial colony was

stabbed with a sterile wooden toothpick and resuspended in 200 µl of milliQ water in a sterile 1.5 ml centrifuge tube. The tube was vortexed until the colony was dispersed, and the cell suspension was then stored frozen at −20°C until use. Aliquots of suspension were defrosted for use directly in PCR. Degenerate PCR primers for the *Vibrio atp*A gene were adopted from Thompson et al. (2007) and were designed to anneal to positions 37 (*atp*A37F, 5′-CTD AAT TCH ACN GAA ATY AGY G-′3) and 1554 (*atp*A1554R, 5′-TTA CCA RGW YTG GGT TGC-′3) of the ATP synthase A subunit gene. The PCR reaction mixture and thermal cycling parameters were modified from Thompson et al. (2007) and optimised for colony PCR. Reactions were 50 µl in volume, and all reagents were supplied by Bioline. The components at final concentration were: 1× ammonium buffer (160 mM $[NH_4]_2SO_4$, 670 mM Tris-HCl, pH 8.8 at 25°C, 0.1% Tween-20), 0.25 mM of each dNTP, 1.5 mM $MgCl_2$, 0.3 µM of *atp*A37F and *atp*A1554R primers, 2 U of BioTaq DNA polymerase and 5.0 µl of *Vibrio* colony suspension. Cycling was carried out using an Eppendorf Master Cycler Gradient with a thermal cycling program consisting of (1) 5 min at 95°C, (2) 3 cycles of 1 min at 95°C, 2 min at 58°C and 1 min at 72°C, (3) 25 cycles of 35 s at 95°C, 1 min at 58°C and 1 min at 72°C and (4) a final extension of 10 min at 72°C. PCR products were visualised by electrophoresis through 1% (w/v) agarose Tris-Borate-EDTA (TBE) gels stained with ethidium bromide. Amplicons of the expected size (around 1500 bp) were confirmed by examination under UV illumination and imaging with DigiDoc-It version 1.1.27 (SelectScience) and size estimated by comparison to a set of DNA size standards (Hyperladder II; 100–2000 bp; Bioline). All isolates producing no amplified product were repeated using fresh colony PCRs; colonies resulting in 3 negative PCRs were considered to be non-*Vibrio.*

Successful PCR products were purified using Montage™ PCR ultra-filtration spin columns (Millipore) according to the manufacturer's manual. DNA concentration (ng μl^{-1}) was then estimated using a Turner Designs TBS-380 DNA fluorometer and 60 ng of PCR product used for DNA sequencing. The distal portion of the *atp*A gene was sequenced using the reverse amplification primer (*atp*A1554R) because it consistently returned higher-quality chromatograms than sequences using the forward primer. Sequence reactions and electrophoresis were prepared and carried out by the Australian Genome Research Facility (AGRF, Brisbane) using an ABI 3730 DNA sequencer (Applied Biosystems) and ABI Big-dye terminator chemistry. Resulting chromatograms were checked manually for base-calling accuracy using Geneious 5.6.4. Preliminary comparative multiple alignments of chromatograms were carried out using the Geneious alignment algorithm, and all base variations detected were verified by comparison of chromatogram traces.

Phylogenetic and diversity analyses

All strains with identical *atp*A sequences were determined by direct pairwise comparison of aligned electropherograms. Diversity measures and indices (Shannon-Wiener Index, *H*′) were determined from the frequency of each distinct genotype recovered from each seawater source (see Table 1). The resulting 40 distinct partial *atp*A gene sequences obtained from hatchery isolates were subjected to BLAST searches to verify nearest neighbour taxa, and aligned using ClustalW (in Geneious 5.6.4). Preliminary guide trees were constructed with more than 200 *V. splendidus*-related *atp*A sequences to define major clusters and relationships to type species/strains, and to guide selection of taxa for inclusion in the final analyses (see the Appendix). Phylogenetic trees of the 39 *V. splendidus* group *atp*A sequence types were constructed from Tamura-Nei distances with the neighbour-joining (NJ) algorithm (Saitou & Nei 1987) using PAUP* version 4.0b10 (Swofford 1993) implemented via the Geneious Pro software interface. Analyses were rooted using 3 outgroup taxa (*V. tapetis* LMG19704, *V. tapetis* LMG19705 and *V. penaeicida* LMG19663^T) identified as the nearest relatives to the *V. splendidus* group in the broader *atp*A gene analysis of Thompson et al. (2007). Consistency of major clusters (A to E) was determined by comparison with NJ trees constructed using Tamura-Nei, Kimura-2-parameter and maximum likelihood distances; only the Tamura-Nei NJ tree is shown. Support for major clusters was assessed by bootstrap resampling (500 replicates), and the identity of clusters was determined by reference to *atp*A sequences of *Vibrio* type strains where possible (see Table 2). The proportion of genotype clusters among water sources was compared by chi-squared analysis of proportion using SPSS (IBM SPSS Statistics for Windows, Version 20.0). The phylogenetic similarity of culturable *Vibrio* recovered from each source was also determined by weighted UniFrac analysis (Lozupone et al. 2006) using the Tamura-Nei NJ tree used as the input tree. UniFrac distances were then subjected to principal coordinate analysis (PCoA).

RESULTS

Culturable *Vibrio* were detected in all samples with concentrations ranging from approximately 2×10^4 CFU ml^{-1} in the spat tank water up to 1.6×10^7 CFU ml^{-1} in broodstock tank water (Table 1). The targeted 1500 bp *atp*A amplicon was successfully amplified from 91 of 112 isolates. Three products failed to produce a readable sequence, and 1 isolate was found to be affiliated with *Shewanella* and excluded from further analysis. The 21 isolates that did not yield an amplified product in repeated PCRs were considered to be not *Vibrio* and excluded from further study. After sequence correction, trimming of poor-quality data and comparative alignment, the high-quality, unambiguous sequence recovered from each isolate ranged from 609 to 931 bp in length in the distal half (base 750–1554, approximately) of the *atp*A gene.

In total, 40 unique *atp*A genotypes could be resolved among the 87 hatchery *Vibrio* isolates sequenced. Alignment with published sequences from *V. splendidus* group type strains and outgroup taxa resulted in an alignment dataset comprising 1458 nucleotide positions. The phylogenetic affiliations based on *atp*A sequence comparisons are shown in Table 2. The vast majority (98%) were allied with the *V. splendidus* group (Thompson et al. 2007), with the hatchery isolates forming 5 consistent major clusters (Groups A to E) with moderate to strong bootstrap support (60–100%). Clusters were allied with *V. cyclitrophicus* (Group A), *V. celticus* (Group B), *V. atlanticus* and *V. tasmaniensis* (Group C), *V. splendidus* and *V. lentus* (Group D) and *V. toranzoniae* (Group E) (Fig. 1). Two isolates with identical *atp*A genotypes were affiliated with the *V. harveyi* group (Group F, not shown). The majority (>60%) of isolates clustered within group D, which contained 2 weakly supported clusters (Subgroup D1 and D) allied with type strains of *V. lentus* and *V. splendidus*, respectively. Isolates of groups B, C and E formed 9 to 13% of the isolates recovered during the study. The 2 *V. harveyi*-related isolates (Group F) were recovered only from spat tank samples (Table 2).

All hatchery water sources showed a high level of *Vibrio atp*A gene diversity (Table 2). Over half of *Vibrio atpA* genotypes (53%) were detected only once. Each source examined contained between 30 and 60% of isolates recovered only once from that source; only 7 genotypes were recovered from more than 1 water source. The most common genotype

Table 1. Abundance (CFU ml^{-1}) and diversity (*H'*) of culturable *Vibrio atp*A genotypes in seawater sources associated with hatchery culture of Australian blue mussel *Mytilus galloprovincialis*. Poor-quality and non-*Vibrio atp*A sequences were removed. Unique types (%) = *atp*A sequence types (N)/Isolates sequenced (N) × 100

Source (code)	Culturable presumptive *Vibrio* (CFU ml^{-1}) Mean	SE	Isolates (N)	Unique *atp*A types (%)	Dominant *Vibrio atp*A type (%)	Diversity (*H'*)
Hatchery intake from Spring Bay (Ba)	2.04×10^5	5.60×10^4	18	61.1	*V. lentus* 39, (22)	2.27
Broodstock tank (Br)	1.58×10^7	1.25×10^6	20	60.0	*V. lentus* 17, (20)	2.35
Larval tank (Lr)	2.00×10^5	8.00×10^4	22	72.7	*V. celticus* 14, *V. lentus* 21, (14)[a]	2.67
Spat tank (Sp)	2.12×10^4	1.88×10^4	19	31.6	*V.* cf. *toranzoniae* 15, (42)	1.38
Header tank (Hd)	8.10×10^5	4.70×10^5	8	62.5	*V.* cf. *atlanticus* 4, (50)	1.39

[a]Co-dominance at same proportions

Table 2. Genetic affiliation and frequency of 6 *Vibrio atp*A genotypic clusters detected in seawater sources associated with hatchery culture of Australian blue mussel *Mytilus galloprovincialis*. Frequency (%) = count (N) / total 87 isolates × 100

*atp*A genotypic clusters	Type species affiliation	*atp*A sequence type (N)	Count (N)	Frequency (%)
Group A	*V. cyclitrophicus*	3	5	5.7
Group B	*V. celticus*	3	8	9.2
Group C	*V. atlanticus*, *V. tasmaniensis*	8	11	12.6
Group D	*V. lentus*, *V. splendidus*	24	53	60.9
Subgroup D1	*V. lentus*	14	35	40.2
Subgroup D2	*V. splendidus*	8	16	18.4
Group E	*V. toranzoniae*	1	8	9.2
Group F	*V. harveyi*	1	2	2.3

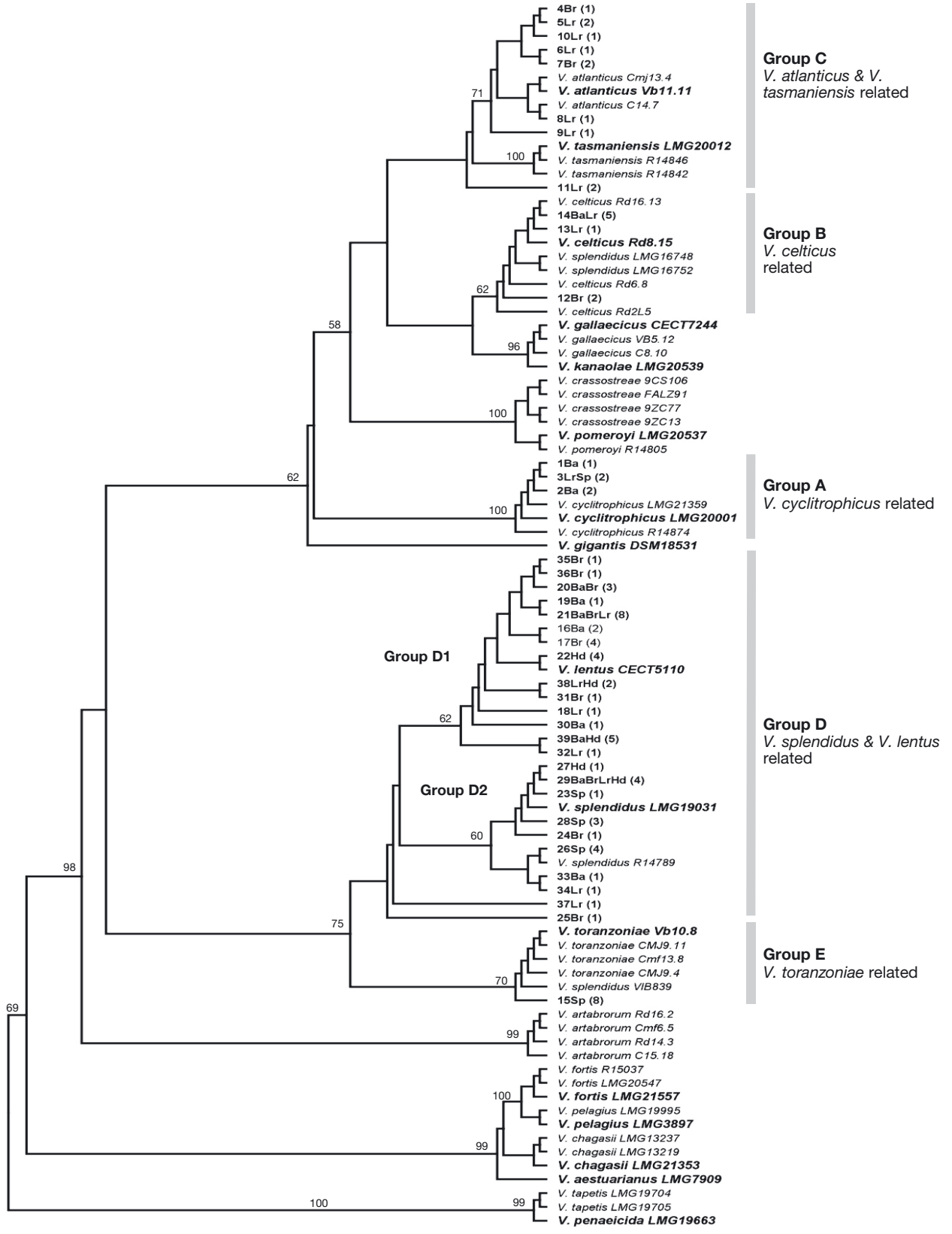

Fig. 1. Phylogenetic relationships of 39 partial *Vibrio splendidus* group *atp*A genotypes associated with hatchery culture of Australian blue mussel *Mytilus galloprovincialis*. Genotype clusters of the hatchery isolates are indicated by Groups A–E; *Vibrio* type strains are indicated by larger **bold** text. Each hatchery isolate genotype is identified by genotype number (1–40), the water source(s) in which it was detected (codes as in Table 1), and frequency (n) at which the genotype was detected in the study. Tree was constructed by neighbour-joining from Tamura-Nei genetic distances. Clusters with >50% bootstrap support (500 replicates) are shown at branch points. Genotype 40 allied with *V. harveyi* (Group F) was not included in the tree

recovered differed in each water source, with the spat and header tank being dominated (42–50% of isolates) by a single genotype. Genotypes recovered from spat tank isolates were not detected in any other source with the exception of *V.* cf. *cyclitrophicus* (genotype 3), which was also recovered once from larval tank samples.

When considered as genotypic clusters (Groups A–F), *V. lentus* and *V. splendidus*-related types (Group D) dominated all seawater sources examined (41–100%), particularly intake seawater, the hatchery header tank and broodstock tanks, although different mixtures of individual genotypes dominated in each source (Fig. 2, Table 2). Due to the high *Vibrio* genotype diversity, frequency of the *V. splendidus* Groups A–E did not differ among water sources. However, after phylogenetic pooling into higher-order clusters (Groups D/E and Groups A/B/C), the proportion of Group D/E genotypes was lower in the larval tank samples compared to other water samples (χ^2 = 17.78, df = 4, p = 0.0014). UniFrac-PCoA analysis showed that culturable *Vibrio* communities from intake, header, broodstock and larval tanks were phylogenetically similar while spat tank communities were different (UniFrac significance test, p = 0.02; Fig. 3).

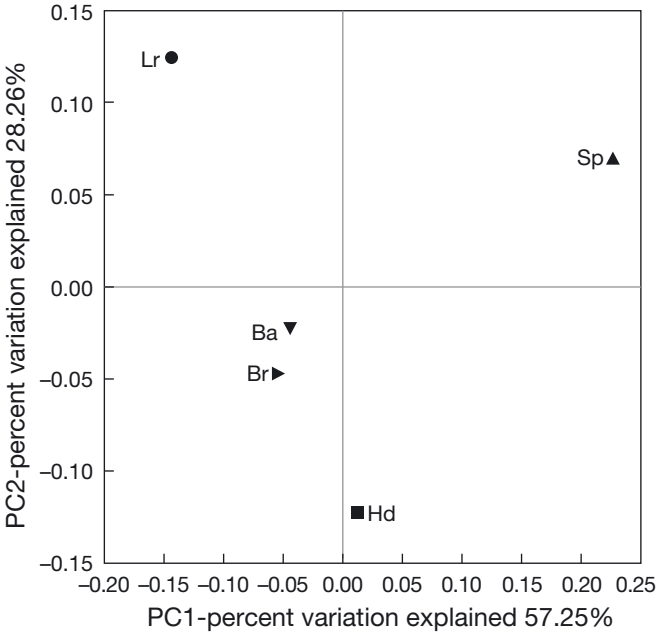

Fig. 3. Phylogenetic similarity of culturable *Vibrio* communities associated with different water sources (codes as in Table 1) in an Australian blue mussel *Mytilus galloprovincialis* hatchery based on 87 partial *atp*A gene sequences. The first 2 principal coordinates of UniFrac principal coordinate analysis explain 85.51% of variation. Spat tank community significantly differed from all 4 other sources (UniFrac significance test, p = 0.02)

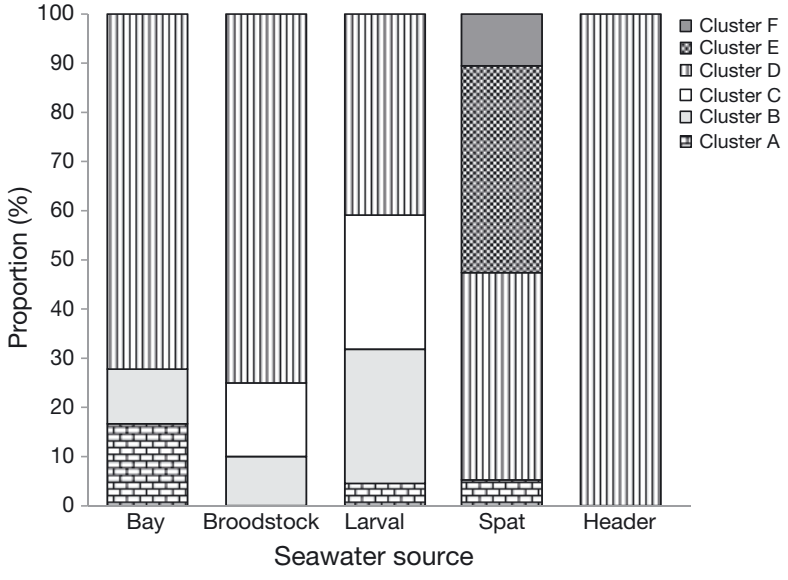

Fig. 2. Proportion of *Vibrio atp*A genotype clusters (Groups A–F; see Fig. 1) associated with hatchery culture systems for Australian blue mussel *Mytilus galloprovincialis*. Frequencies of the 5 clusters (Groups A–E) were not significantly different. After pooling higher-order phylogenetic clusters (Groups D/E and A/B/C), the proportion of Groups A/B/C was significantly higher in larval samples than other samples (χ^2 = 17.78, df = 4, p = 0.0014)

DISCUSSION

The samples examined in this study were not associated with vibriosis or significantly elevated larval mortality and therefore represent culturable *Vibrio* present during typical commercial mussel hatchery operation. While limited published data are available from mussel hatcheries, the order of magnitude of abundance of culturable *Vibrio* observed in this study is similar to previous reports from the same source types (e.g. larval rearing seawater, broodstock tanks, settlement tanks) reported from marine hatcheries of other molluscs such as oysters (*Crassostrea* spp.; DiSalvo et al. 1978), scallops (*Pecten* spp.; Nicolas et al. 1996) and clams (*Ruditapes* spp.; Mechri et al. 2012), suggesting a common underlying factor controlling *Vibrio* abundance. The design and operation of most invertebrate hatcheries, including water treatment, stocking densities, flow manage-

ment, algal food and husbandry practices, are typically very similar. This contributes to environments of enriched organic carbon and higher water temperatures, both of which potentially are the major control of *Vibrio* and perhaps total bacterial abundance in hatchery systems (Thompson et al. 2004, Le Roux & Austin 2006).

The culturable *Vibrio* community associated with this Australian blue mussel hatchery was overwhelmingly dominated by a diverse assemblage of *V. splendidus* group genotypes. There are few other studies of comparable depth from a single hatchery; however, a high diversity of *V. splendidus*-related genotypes is commonly recovered from hatchery-rearing of other molluscs (see Supplementary Table S1 of Thompson et al. 2007). Our findings are similar to those from *Ruditapes decussatus* clam hatcheries where 56% of culturable *Vibrio* were found to belong to the *V. splendidus* group (Beaz Hidalgo et al. 2008). Using amplification fragment length polymorphism, the same study demonstrated very low clonality among 29 different cultured *Vibrio* phenotypes. In this study, more than 50% of *atp*A genotypes were unique, i.e. detected only once, indicating that culturable *Vibrio* diversity was considerably high, and that high *Vibrio* diversity is a general feature of hatchery-rearing systems. The high diversity of *V. splendidus* detected in the hatchery resembles that of coastal seawater in a study by Thompson et al. (2005). Using pulsed-field gel electrophoresis, those authors characterised 87% unique genomic patterns in over 200 *V. splendidus* isolates. The higher level of diversity is probably due to the use of the whole genome as a marker and the fact that more isolates were examined. It nevertheless suggests that the remarkable diversity of *V. splendidus* in marine environments is more common than anticipated.

Analysis of our intake water samples indicated that *V. splendidus* group genotypes dominated the culturable *Vibrio* community of the coastal bay water, which may act as a major reservoir of *Vibrio* entering the hatchery. However, water entering the hatchery larval and spat culture systems is subjected to bacteriocidal UV irradiation, and *Vibrio* were never detected in treated water (<10 CFU ml^{-1}). Therefore, bay water appears unlikely to be the major source of the culturable *Vibrio* entering the hatchery rearing systems, although we cannot rule out the selective recovery (up to 15%) from UV treatment as a significant source of *V. splendidus* (Abraham & Palaniappan 2000). The substantial differences in presence/absence, dominance and proportion of genotypes recovered from each water source indicate that communities are more likely to be derived from the broodstock, larval stock and/or water reticulation at each stage of rearing. The rearing system environment (static versus flow-through operation), developmental stage and husbandry differences at each stage (e.g. water exchange rates, stocking and feeding rates) are also thought to further influence growth dynamics, diversity and dominance of *Vibrio* in hatchery-reared species including oysters (*Crassostrea virginica*; Murchelano & Bishop 1969) and rock lobster *Panulirus ornatus* (Bourne et al. 2004).

Both the frequency of major clusters (Groups A–F) and UniFrac-PCoA analysis indicated that spat tank water harboured a culturable *Vibrio* community that was phylogenetically distinct from the bay water and other hatchery tanks. Despite relatively short water residence times (30 min), header water *Vibrio* displayed a distinct reduction in diversity compared to the bay water, most likely associated with 20 µm filtration before the header tank. This treatment would remove a high proportion of the particle-associated bacterial community including those associated with molluscan larvae.

The phylogenetic similarity of culturable *Vibrio* communities of bay water, header, larvae and broodstock tank is surprising considering the differing water filtration and treatment applied to the hatchery water, stocking density, water flow management, and the much higher temperature of larval and broodstock tank water (22°C and 18°C, respectively) and bay water (<12°C), all of which might be expected to change the nature and concentration of organic carbon in the systems and the selective forces operating on the microbial community. The similarity suggests a common *Vibrio* inoculum source and/or that the environments exert similar selective pressure on the culturable *Vibrio* community. The only clear common factor is the presence of mussel gametes and larvae in the systems. Tasmanian wild and farmed Australian blue mussels spawn from April to September, peaking during May when this study was carried out (Dix & Ferguson 1984, Fearman & Moltschaniwskyj 2010), and developing larvae would also have been abundant in bay water at the time of sampling. Overall, our findings suggest that the major influence on the culturable hatchery *Vibrio* community is the presence of mussel larvae rather than inefficient water treatment or diffuse/cryptic sources such as water pipes, pumps and surfaces.

The spat tank culturable *Vibrio* community was the most phylogenetically divergent, and the only sam-

ples in which *V. toranzoniae*-related and *V. harveyi*-group genotypes were detected. The spat tank environment differs from other hatchery static and flow-through tanks in many ways, such as increased size and age of stock (4 wk after settlement in our study), higher stocking densities and also the presence of complex settlement matrix (500 m of poly-rope) with a high effective surface area. Settlement rope is pre-conditioned for 7 d prior to addition of pediveligers to establish a microbial community. Preliminary studies of rope conditioning showed that the settlement rope harboured a substantial community of culturable *Vibrio* ($>3 \times 10^6$ CFU cm^{-1}) equivalent to 1.5×10^5 CFU ml^{-1} inoculum to the conditioning tank (A. Azizi & C. J. S. Bolch unpubl. results). The nature and diversity of the community during pre-conditioning and at pediveliger settlement is not known, but our data indicate that by 4 wk after settlement, the water-associated *Vibrio* community is very different from other hatchery systems.

Previous studies have shown that *V. splendidus* group bacteria are capable of causing bacillary necrosis when present at sufficiently high abundance (Guisande et al. 2008, Kesarcodi-Watson et al. 2009). However, our study shows that all stages of hatchery mussel production harbour a substantial and diverse community of culturable *Vibrio*, even when stock mortalities are within normal commercial production limits. *Vibrio splendidus* group bacteria are also regularly isolated from healthy molluscan larvae (e.g. Macián et al. 2000, Beaz Hidalgo et al. 2008), indicating that *V. splendidus* group members are part of the normal microflora of marine molluscan larvae and provide a substantial reservoir of potentially virulent *V. splendidus* genotypes. While we did not examine virulence of our *V. splendidus* isolates, the diversity that we detected in a single hatchery may suggest that vibriosis is associated with changes in *Vibrio* community dominance and diversity to more virulent *Vibrio* genotypes.

It is relevant to note that culture-independent studies have shown that *Vibrio* are rarely the dominant taxa in hatchery culture environments even during mortality events (Bourne et al. 2004). Recent pyro-sequencing studies have also shown that *Vibrio* typically represent <1% of the total bacterial community associated with oyster larval production (Powell et al. 2013). While low relative abundance suggests that *Vibrio* may not be the primary cause of larval mortality, distinct 'spikes' in relative abundance of cultivable *Vibrio* (up to 25–100% of total viable count) sometimes coincide with onset of disease and/or mortality of commercial hatchery-reared larvae (Chapman 2012). The cultivable *Vibrio* (dominated by the *V. splendidus* group) may instead have an indirect role in larval mortality, exacerbated by environmental conditions including behavioural factors such as increased larval aggregation (DiSalvo et al. 1978, Chapman 2012) and interactions within the total bacterial community associated with larval molluscan cultures.

In conclusion, our data show that a substantial and genetically diverse community of *V. splendidus* group bacteria is associated with all stages of commercial hatchery production even when stock mortalities are within normal production limits. This high diversity may allow rapid shifts in genotype dominance and community pathogenicity/virulence. What is not yet clear is how changes in seawater quality and husbandry practices select for more virulent *V. splendidus* genotypes.

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Appendix. Reference strains of *Vibrio* spp. used in the final phylogenetic analysis of *atp*A genotypic clusters (as shown in Fig. 1)

Strain	GenBank acc. no.	Strain	GenBank acc. no.
Vibrio aestuarianus LMG7909^T	EF601228	*Vibrio gallaecicus* CECT7244^T	EU541559
Vibrio artabrorum Cmf6.5	FN668901	*Vibrio gallaecicus* VB5.12	EU931119
Vibrio artabrorum Rd16.2	FN668905	*Vibrio gallaecicus* C8.10	EU541560
Vibrio artabrorum Rd14.3	FN668903	*Vibrio gigantis* DSM18531^T	EU541556
Vibrio artabrorum C15.18	FN668899	*Vibrio kanaolae* LMG20539^T	EF601307
Vibrio atlanticus Vb11.11^T	FN582252	*Vibrio lentus* CECT5110^T	EU541558
Vibrio atlanticus Cmj13.4	FN582248	*Vibrio pelagius* LMG3897^T	EF601269
Vibrio atlanticus C14.7	FN582250	*Vibrio pelagius* LMG19995	EF601332
Vibrio celticus Rd8.15^T	FN582232	*Vibrio penaeicida* LMG19663^T	EF601263
Vibrio celticus Rd16.13	FN582230	*Vibrio pomeroyi* R14805	EF601290
Vibrio celticus Rd6.8	FN582231	*Vibrio pomeroyi* LMG20537^T	EF601318
Vibrio celticus Rd2L5	FN582233	*Vibrio splendidus* LMG16752	EF601258
Vibrio chagasii LMG13237	EF601256	*Vibrio splendidus* LMG16748	EF601257
Vibrio chagasii LMG13219	EF601255	*Vibrio splendidus* LMG19031^T	EF601244
Vibrio chagasii LMG21353^T	EF601280	*Vibrio splendidus* R14789	EF601289
Vibrio crassostreae FALZ91	GU378426	*Vibrio splendidus* VIB839	EF601323
Vibrio crassostreae 9CS106	GU378407	*Vibrio tapetis* LMG19704	EF601366
Vibrio crassostreae 9ZC77	GU378410	*Vibrio tapetis* LMG19705	EF601367
Vibrio crassostreae 9ZC13	GU378409	*Vibrio tasmaniensis* LMG20012^T	EF601325
Vibrio cyclitrophicus LMG20001	EF601372	*Vibrio tasmaniensis* R14846	EF601292
Vibrio cyclitrophicus LMG21359^T	EF601304	*Vibrio tasmaniensis* R14842	EF601291
Vibrio cyclitrophicus R14847	EF601293	*Vibrio toronzoniae* CMJ9.11	HE805625
Vibrio fortis LMG21557^T	EF601322	*Vibrio toronzoniae* Vb10.8^T	HE820043
Vibrio fortis R15037	EF601369	*Vibrio toronzoniae* Cmf13.8	HE805626
Vibrio fortis LMG20547	EF601336	*Vibrio toronzoniae* CMJ9.4	HE805624

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