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In vitro and *in vivo* expression of virulence genes in *Vibrio* isolates belonging to the Harveyi clade in relation to their virulence towards gnotobiotic brine shrimp (*Artemia franciscana*)

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Summary

Vibrios belonging to the Harveyi clade are pathogenic marine bacteria affecting both vertebrates and invertebrates, thereby causing a severe threat to the aquaculture industry. In this study, the expression of haemolysin, metalloprotease, serine protease, the quorum sensing master regulator LuxR and the virulence regulator ToxR in different Harveyi clade isolates was measured with reverse transcriptase real-time PCR with specific primers. There was relatively low variation in the *in vitro* expression levels of the quorum sensing master regulator *luxR* (sevenfold), whereas for the other genes, the difference in expression between the isolates showing lowest and highest expression levels was over 25-fold. Furthermore, there was a significant correlation between expression levels of *toxR* and *luxR* and between the expression levels of these regulators and the protease genes. The expression levels of *luxR*, *toxR* and haemolysin were negatively correlated with the survival of brine shrimp larvae challenged with the isolates. Finally, a non-virulent, a moderately virulent and a strongly virulent isolate were selected to study *in vivo* expression of the virulence genes during infection of gnotobiotic brine shrimp larvae. The *in vivo* gene expression study showed a clear difference in virulence gene expression between both virulent isolates and the non-virulent isolate.

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Introduction

World aquaculture production has grown tremendously during the last 50 years and this development shifted the farming methods from extensive to intensive to achieve maximum profits. Due to these changes in the culture systems, aquaculture industry is threatened by the occurrence of diseases caused by bacteria and viruses. According to the Food and Agriculture Organization (FAO), disease outbreaks are a significant constraint to the development of the aquaculture sector, causing severe economical losses (FAO, 2004). *Vibrio harveyi* has been recognized as one of the most significant pathogens in aquaculture of marine fish, crustaceans and mollusks (Zhang and Austin, 2000; Bourne *et al*., 2006). *Vibrio campbellii* is very closely related to *V. harveyi* (showing 61–74% DNA–DNA similarity and over 97% 16S rDNA similarity) and has also been identified as an important cause of shrimp disease (de la Pena *et al*., 2001; Gomez-Gil *et al*., 2004; Defoirdt *et al*., 2008). Both species, together with the closely related species *Vibrio natriegens*, *V. alginolyticus* and *V. parahaemolyticus*, were grouped in a clade, called the Harveyi clade (Cano-Gomez *et al*., 2009).

Many virulence factors have been identified in Harveyi clade vibrios, including proteases, phospholipases, haemolysins, cysteine protease, metalloprotease, serine protease and chitinase (Zhang and Austin, 2000; Defoirdt *et al*., 2010). Virulence of vibrios is controlled by different regulatory systems, including quorum sensing and the transmembrane transcriptional regulator ToxR (Miller *et al*., 1987; Milton, 2006). Quorum sensing, bacterial cell-to-cell communication by means of small signal molecules, is known to be linked to virulence of several pathogenic bacteria (De Kievit and Iglewski, 2000). In *V. harveyi*, quorum sensing controls the production of a siderophore, type III secretion system components and extracellular toxin (Higgins *et al*., 2007). In a gnotobiotic

environment, using brine shrimp (*Artemia franciscana*) as model organism, the AI-2 (autoinducer 2) channel of the *V. harveyi* quorum sensing system has been shown to regulate virulence of the bacterium (Defoirdt *et al*., 2005). The *toxR* gene was first discovered as a positive transcriptional regulator in *Vibrio cholerae* regulating the *ctxA* gene (encoding the cholera toxin; Miller and Mekalanos, 1984). ToxR can directly affect gene expression by binding to promoters, including those for the *ctx* operon, the genes encoding two outer membrane proteins OmpU and OmpT (Miller *et al*., 1987). Later on, *toxR* was found to be one of the main regulators for more than 20 genes (Merrell *et al*., 2001). The *toxR* gene is present in many vibrios other than *V. cholerae*, including *V. campbellii*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus* and *V. anguillarum* (Lee *et al*., 2000; Wang *et al*., 2002; Pang *et al*., 2006; Ruwandeepika *et al*., 2010). The ToxR regulon is not well studied in other vibrios except *V. cholerae*. However, it seems to be involved in the expression of outer membrane proteins and bile resistance in *V. anguillarum, V. mimicus*, *V. fluvialis* and *V. parahaemolyticus* (Provenzano *et al*., 2000; Wang *et al*., 2002; 2003).

At this moment, it is not yet clear which virulence factors are most important in the progression of the disease caused by vibrios belonging to the Harveyi clade. Some studies revealed that the presence of virulence genes within the genome of these bacteria is not directly linked to virulence, with differences in virulence not being correlated to presence or absence of a specific virulence factor (Zhang and Austin, 2000; Ruwandeepika *et al*., 2010). The quantity of the virulence products produced by these bacteria might be a critical factor and the virulence of the bacteria might be more closely linked to the expression level of the virulence genes rather than by their presence.

Studies on virulence factors and virulence mechanisms of pathogenic vibrios reported in literature thus far are usually performed in nutrient-rich synthetic growth media. However, the environmental conditions the bacteria experience in such experiments are largely different from those they experience in the real world and mechanisms that occur *in vivo* might be distinct from those that occur in simplified model systems (Fux *et al*., 2005). Therefore, it is of utmost importance to study virulence factor expression under conditions that are as close as possible to those the bacteria experience during infection, using host cell cultures or animal models, in order to understand the real mechanisms behind virulence gene expression.

Hence, in this study, we quantified the expression level of the virulence regulators *luxR* and *toxR* and the virulence factors metalloprotease (*vhp*), serine protease (*srp*) and haemolysin (*vhh*) in a group of isolates belonging to the Harveyi clade. The expression levels of the genes were first studied under *in vitro* conditions, and the relation between virulence gene expression and survival of challenged brine shrimp (*A. franciscana*) was determined. Finally, virulence gene expression in three selected isolates (one non-virulent, one moderately virulent and one strongly virulent) was monitored during infection of gnotobiotic brine shrimp larvae.

Results

Virulence gene expression in the isolates

In this study, we used 10 isolates belonging to the Harveyi clade of vibrios to determine the expression levels of genes encoding the quorum sensing master regulator LuxR (*luxR*), the virulence regulator ToxR (*toxR*), a metalloprotease (*vhp*), a haemolysin (*vhh*) and a serine protease (*srp*). The presence of the genes was first confirmed by PCR. Expected amplicons were generated from DNA extracts of the isolates, indicating that all isolates were positive for all the target genes (data not shown). In further experiments, in order to obtain an indication of the production capacity of the virulence factors and regulators, the isolates were grown to late exponential phase and relative expression of the target genes was measured by reverse transcriptase real-time PCR, using the RNA polymerase A subunit (*rpoA*) as a reference gene (Defoirdt *et al*., 2007).

luxR, the main regulatory gene in the quorum sensing cascade, was highly expressed in isolates BB120 and LMG21363 followed by VIB645, LMG22891, LMG22893 and LMG22894 (Table 1). LMG11226 showed the least expression of this gene. The *luxR* gene was expressed in all isolates at different levels, with relatively low variation (Table 2).

ToxR is a transmembrane transcription regulator, and known to be an important virulence regulator in many vibrios. The highest expression was found in LMG21363

Table 1. Expression of the quorum sensing master regulator gene *luxR*, the virulence regulator gene *toxR*, the *srp* serine protease gene, the *vhp* metalloprotease gene and the *vhh* haemolysin gene relative to *rpoA* mRNA in the pathogenic Harveyi clade isolates.

Isolate	*luxR*	*toxR*	*vhh*	*srp*	*vhp*
LMG11226	1.0^{a}	1.0^{A}	1.0^{x}	1.0^{X}	1.0^{i}
BB120	4.3^{d}	30.1^{D}	$21.7^{z,u}$	$12.2^{Y,Z}$	127.4^{iii}
LMG21363	6.8^{d}	31.2^{D}	$21.8^{z,u}$	$12.6^{Y,Z}$	$47.1^{ii,iii}$
VIB571	$1.8^{b,c}$	$5.8^{B,C}$	32.8^{u}	$16.4^{Y,Z}$	$2.3^{i,ii}$
VIB645	2.0^{c}	4.9^{C}	69.2^{u}	$15.4^{Y,Z}$	$3.8^{i,ii}$
LMG22891	2.2^{c}	8.1^{C}	$2.7^{x,y}$	$5.4^{X,Y,Z}$	$4.1^{i,ii}$
LMG22893	$1.8^{b,c}$	6.9^{C}	1.6^{x}	$3.2^{X,Y,Z}$	$3.6^{i,ii}$
LMG22894	2.0^{c}	6.1^{C}	2.4^{x}	$4.3^{X,Y,\ Z}$	$3.5^{i,ii}$
ACMM642	$1.3^{a,b,c}$	2.8^{B}	$3.8^{x,y,z}$	26.7^{Z}	$19.4^{i,ii,iii}$
ACMM20	$1.1^{a,b}$	1.2^{A}	1.2^{x}	$2.5^{X,Y}$	$5.0^{i,ii}$

For each gene, the relative expression in the isolate LMG11226 was set at 1 and the expression in all other strains was normalized accordingly, using the $2^{-\Delta\Delta CT}$ method. Values in the same column with a different superscript are significantly different ($P < 0.05$).

Table 2. Variation (standard deviation of the $2^{-\Delta\Delta CT}$ values) between the different strains in the expression levels of the quorum sensing master regulator *luxR*, the virulence regulator *toxR*, the *srp* serine protease, the *vhp* metalloprotease and the *vhh* haemolysin.

Gene	Variation (fold difference)
luxR	1.8
toxR	11.2
vhh	22.0
srp	8.1
vhp	39.7

and *toxR* expression showed a wide variation (Table 2), with 31-fold difference between the highest and the lowest value (Table 1) indicating that a high variability exists in the expression of this regulatory gene in closely related bacteria.

Haemolysin is one of the vital virulence products in many vibrios having cytotoxic and haemolytic activity in the host tissues subsequently leading to haemorrhagic septicaemia and diarrhoea. Isolates VIB645 and VIB571 showed the highest expression of the *vhh* haemolysin gene, whereas isolates LMG11226 and ACMM20 had the lowest expression (Table 1). This gene also showed a high variation in expression levels (Table 2), and there was 69-fold difference in expression levels between the isolate showing the highest expression and the one showing the lowest (Table 1).

In this study, we quantified the expression level of two genes coding for proteases, i.e. the *srp* serine protease gene and the *vhp* metalloprotease gene, both of which are known to be virulence-mediated genes in vibrios. The *srp* gene showed the highest expression in the isolate ACMM642 and the lowest expression was again noted for isolates LMG11226 and ACMM20 (Table 1) and a relatively low variability (Table 2). The *vhp* gene was expressed at the highest level in BB120 and the lowest in LMG11226 (Table 1). The variation in expression was extremely large for this gene (Table 2), with a 127-fold difference between BB120 and the strain showing the lowest expression level (Table 1).

Correlation analysis revealed that the expression of the quorum sensing master regulator gene *luxR* is positively correlated with the expression of the virulence regulator gene *toxR*, the *vhp* metalloprotease and the *srp* serine protease (Table 3), but it is not significantly correlated with the expression of the *vhh* haemolysin gene ($P > 0.05$). The expression of the transcriptional regulatory gene *toxR* showed a statistically significant positive correlation with the expression of *luxR*, the *vhp* metalloprotease gene and the *srp* serine protease gene. Finally, *vhh* haemolysin gene expression showed a significant positive correlation with the expression of the *srp* serine protease gene (Table 3).

Virulence of the isolates towards gnotobiotic brine shrimp larvae and relation with in vitro *virulence gene expression*

In order to investigate the virulence of the isolates used in this study, challenge experiments were carried out using gnotobiotically grown brine shrimp larvae as host. Among all the isolates tested, we found the lowest relative percent survival when the shrimp were challenged with isolate LMG21363 (Table 4). In this highly virulent isolate, we could find a high expression level of the quorum sensing master regulator gene *luxR*, the virulence regulator gene *toxR*, the *vhh* haemolysin gene, the *vhp* metalloprotease gene and the *srp* serine protease gene. BB120, VIB645 and VIB571 also showed relatively low survival of larvae in both experiments. These isolates had relatively high *vhh* haemolysin and *toxR* gene expression. The highest survival was observed for nauplii challenged with LMG11226 and ACMM20 and was not significantly different from the survival of uninfected nauplii. LMG11226 and ACMM20 showed the lowest expression for all genes, except for the *vhp* metalloprotease gene. Isolate ACMM642, which caused relatively low mortality, showed a high expression of *toxR*, *srp* serine protease and *vhp* metalloprotease.

Table 3. Significant correlations[a] in expression levels of the quorum sensing master regulator *luxR*, the virulence regulator *toxR*, the *srp* serine protease, the *vhp* metalloprotease and the *vhh* haemolysin.

Genes	Pearson correlation	*P*-value
luxR–toxR	0.95	< 0.0001
luxR–vhp	0.87	0.002
luxR–srp	0.68	0.044
toxR–vhp	0.81	0.008
toxR–srp	0.73	0.027
vhh–srp	0.93	< 0.0001

a. ΔC_T values were used to calculate the correlation coefficients.

Table 4. Relative percentage of survival[a] of brine shrimp larvae (mean ± standard error of three replicates) after 48 h challenge with the Harveyi clade isolates.

Isolate	Challenge 1	Challenge 2
Control	100 ± 0^{a}	100 ± 0^{A}
LMG11226	$98 \pm 3^{a,b}$	98 ± 4^{A}
BB120	53 ± 7^{d}	$49 \pm 5^{D,E}$
LMG21363	25 ± 8^{e}	23 ± 5^{E}
VIB571	$44 \pm 4^{d,e}$	$41 \pm 7^{D,E}$
VIB645	53 ± 8^{d}	$51 \pm 10^{C,D,E}$
LMG22891	$62 \pm 4^{c,d}$	$60 \pm 6^{B,C,D}$
LMG22893	$60 \pm 7^{c,d}$	$59 \pm 7^{B,C,D}$
LMG22894	49 ± 6^{d}	$51 \pm 28^{C,D,E}$
ACMM642	$77 \pm 12^{b,c}$	$78 \pm 13^{A,B,C}$
ACMM20	$86 \pm 16^{a,b}$	$85 \pm 10^{A,B}$

a. Survival in the uninfected control was set at 100% and the other treatments were normalized accordingly. Values in the same column with a different superscript are significantly different ($P < 0.05$).

Table 5. Spearman's rank correlation[a] between the relative expression levels of the quorum sensing master regulator *luxR*, the virulence regulator *toxR*, the *srp* serine protease, the *vhp* metalloprotease and the *vhh* haemolysin and brine shrimp survival.

	Challenge 1		Challenge 2	
Gene	Correlation coefficient	*P*-value	Correlation coefficient	*P*-value
luxR	0.74	0.015	0.77	0.010
toxR	0.68	0.030	0.73	0.017
vhh	0.71	0.021	0.73	0.018
srp	0.51	0.137	0.51	0.137
vhp	0.12	0.738	0.19	0.590

a. ΔC_T values were used to calculate the correlation coefficients; ΔC_T is inversely related to the expression level.

We hypothesized that the level of virulence gene expression in the vibrios rather than presence of these genes was the reason for their difference in virulence. Consequently, we calculated the correlation between survival of challenged brine shrimp and gene expression (using ΔC_T values). We found significant positive correlations between brine shrimp survival and ΔC_T values for the quorum sensing master regulator *luxR*, the virulence regulator *toxR* and the *vhh* haemolysin gene (Table 5). ΔC_T is inversely related to the expression level and therefore, a positive correlation between ΔC_T and survival indicates that higher expression levels of the virulence genes are correlated with lower survival, thus confirming that higher virulence gene expression could be found in the more virulent isolates.

In vivo *virulence gene expression during infection of brine shrimp larvae*

In order to obtain more relevant data on virulence gene expression, we studied the expression of the virulence genes during infection of gnotobiotic brine shrimp larvae. The larvae were challenged by adding three wild-type isolates (BB120, LMG11226 and LMG21363) to the culture water (according to our standardized challenge protocol; Defoirdt *et al.*, 2005). Because the larvae are relatively small, 500 larvae were collected per sample. Bacterial mRNA was extracted and virulence gene expression was measured with reverse transcriptase real-time PCR. In a first experiment, using strain BB120, we investigated whether preparation of the *Vibrio* inoculum has any impact on the *in vivo* expression pattern. The pathogen was prepared in three different ways prior to addition to the brine shrimp culture water: grown in nutrient-rich broth (marine broth), grown in marine broth and subsequently starved in sea water for 24 h and grown in sterile brine shrimp hatching water. The three different methods of inoculum preparation resulted in the same pattern of expression of the quorum sensing master regulator *luxR* in wild-type *V. harveyi* during infection of brine shrimp: the expression level increased up to the 24 h time point with approximately threefold, after which it decreased again to the initial expression level (Fig. 1). We also tested the effect of inoculum preparation on the expression of the *srp* serine protease gene. The expression of this gene did not change over time for any of the treatments (data not shown). Based on these observations, we could conclude that the preparation method of the inoculum has no effect on the subsequent expression

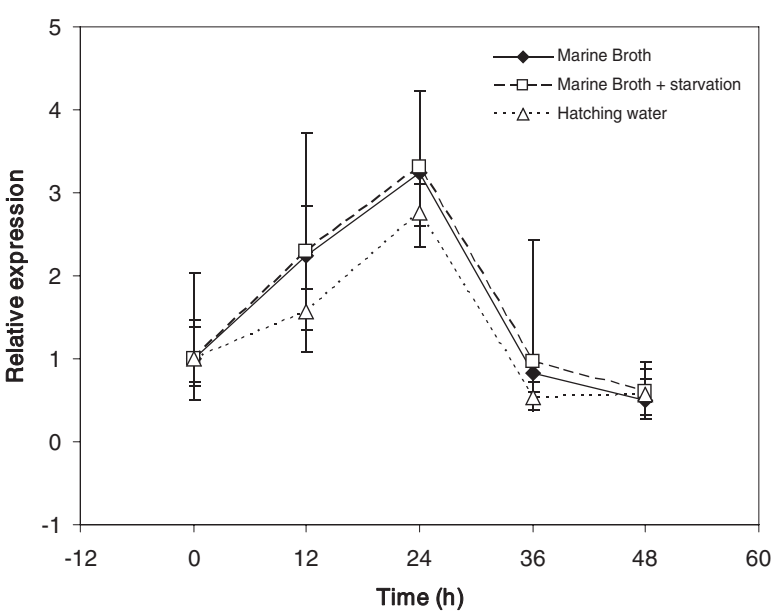

Fig. 1. *In vivo* expression of *luxR* in wild-type *V. harveyi* BB120 during infection of brine shrimp nauplii. The expression level at the 0.5 h time point was set at 1 and the expression at the other time points was normalised accordingly using the $2^{-\Delta\Delta CT}$ method. The RNA polymerase A subunit (*rpoA*) mRNA was used as an endogenous control. The error bars represent the standard deviation of three independent measurements of $\Delta\Delta C_T$ based on bacterial mRNA extracted from 500 larvae.

pattern during infection of the brine shrimp larvae and we used non-starved marine broth-grown inocula in all further experiments.

Based on the brine shrimp challenge results, we selected an avirulent, a moderately virulent and a strongly virulent isolate (LMG11226, BB120 and LMG21363 respectively) to study the expression of the virulence genes during the infection of brine shrimp larvae. The expression of virulence genes showed quite a remarkably similar pattern of *in vivo* expression, with a peak in expression at the 24 h time point for the moderately virulent and the strongly virulent isolate and a relatively constant and low expression level in the avirulent isolate (Fig. 2). The pattern of gene expression was similar to the one observed in the preliminary *in vivo* experiment (Fig. 1). When compared with the avirulent isolate, the virulent isolates showed on average 18-, 122-, 56-, 24- and 160-fold higher *in vivo* expression levels of the quorum sensing master regulator gene *luxR,* the virulence regulator gene *toxR,* the *vhh* haemolysin gene, the *srp* serine protease gene and the *vhp* metalloprotease gene, respectively, at the 24 h time point. These differences in expression were two to four times more pronounced than those obtained under *in vitro* conditions (Table 1).

We used the gene encoding the RNA polymerase A subunit (*rpoA*) as an endogenous control. When expressed relative to this endogenous control, the expression levels of the moderately virulent isolate BB120 and the highly virulent isolate LMG21363 were very similar. However, *in vivo rpoA* mRNA levels were higher in LMG21363 than in BB120, as manifested by lower C_T values throughout the experiment (Fig. 2F). Therefore, total *in vivo* virulence gene expression levels were higher for the highly virulent strain LMG21363 than for the moderately virulent strain BB120. The lower *in vivo rpoA* mRNA levels observed for isolate BB120 might reflect lower metabolic activity and/or lower cell density *in vivo*. Importantly, the *in vivo rpoA* mRNA levels of the avirulent isolate were similar to those of the highly virulent isolate (Fig. 2F), indicating that the low levels of virulence gene expression were not due to absence or inactivity of the avirulent isolate.

Discussion

Vibrios belonging to the Harveyi clade (such as *V. harveyi* and *V. campbellii*) are among the most important pathogens in aquaculture (Zhang and Austin, 2000). These bacteria regulate the expression of some virulence-associated genes by quorum sensing – bacterial cell-to-cell communication with small signal molecules (Henke and Bassler, 2004). Three quorum sensing signals have been identified in *V. harveyi*: HAI-1 (harveyi autoinducer 1), AI-2 (autoinducer 2) and CAI-1 (cholera autoinducer 1) (Waters and Bassler, 2006). These extracellular signal molecules are detected by membrane-bound two component receptors that feed a common phosphorylation/dephosphorylation signal transduction cascade and at the end is the quorum sensing master regulator LuxR (Taga and Bassler, 2003). In the current study, a high expression of the *luxR* gene was found in the more virulent isolates such as LMG21363 and BB120, both *in vitro* and *in vivo* (Tables 1 and 4 and Fig. 2A). The lowest expression was found in the avirulent isolates LMG11226 and in ACMM20 (Tables 1 and 4). Moreover, there was a significant positive correlation between *in vitro luxR* expression and virulence to brine shrimp larvae (Table 5) providing evidence that isolates with a higher LuxR production capacity tend to be more virulent. This was confirmed in the *in vivo* expression study, where virulent isolates BB120 and LMG21363 showed on average 18-fold higher expression than the avirulent isolate LMG11226. LuxR is the quorum sensing master regulator and therefore, these results corroborate our previous reports in which we found that quorum sensing regulates the virulence of luminescent vibrios towards brine shrimp (Defoirdt *et al*., 2005) and rotifers (Tinh *et al*., 2007). An increased virulence of isolates producing higher levels of *luxR* could be explained by a high expression level of *luxR*-regulated virulence genes. Quorum sensing has been reported earlier to regulate the expression of a type III secretion system (Henke and Bassler, 2004), extracellular toxin (Manefield *et al*., 2000), metalloprotease (Mok *et al*., 2003), a siderophore (Lilley and Bassler, 2000) and chitinase A (Defoirdt *et al*., 2010). In this study, we found that *luxR* expression levels were positively correlated with expression levels of the *vhp* metalloprotease and the *srp* serine protease and the regulatory gene *toxR* (which might possibly regulate the expression of other, yet unidentified virulence genes). Previous studies have shown that *luxR* homologues regulate the expression of virulence genes in other vibrios as well. Rui and colleagues (2009) found that serine protease, extracellular polysaccharide and motility in *V. alginolyticus* are regulated by a *V. harveyi* type LuxR protein. In *V. cholerae,* some virulence-related genes such as haemolysin are negatively regulated by the quorum sensing mechanism, whereas others such as *hap* protease are positively regulated (Miller *et al*., 2002; Zhu *et al*., 2002; Tsou and Zhu, 2010). The LuxR homologue SmcR in *V. vulnificus* was demonstrated to positively regulate metalloprotease and negatively regulate cytolysin expression respectively (Shao and Hor, 2001). In *V. anguillarum,* the LuxR homologue VanT has been reported to regulate production of serine protease, metalloprotease and pigment, and formation of biofilms (Croxatto *et al*., 2002).

The *toxR* gene, one of the main regulators for more than 20 genes in *V. cholerae* (Merrell *et al*., 2001), is

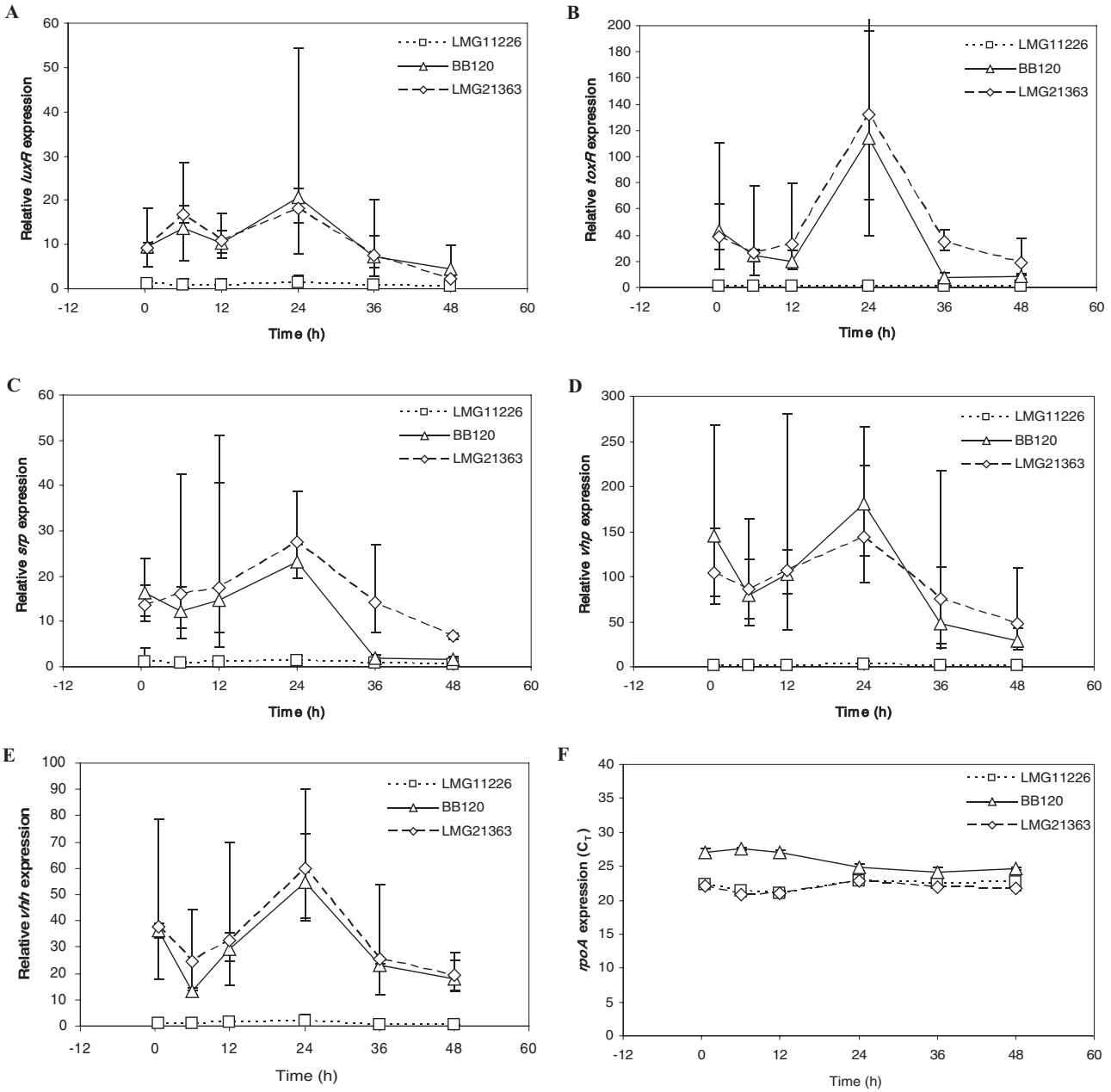

Fig. 2. A–E. *In vivo* expression of the quorum sensing master regulator *luxR* (A), the virulence regulator *toxR* (B), the *srp* serine protease (C), the *vhp* metalloprotease (D) and the *vhh* haemolysin (E) in wild-type strains BB120, LMG11226 and LMG21363 during infection of brine shrimp larvae. The expression was calculated relative to the endogenous control RNA polymerase A subunit (*rpoA*) and expression in LMG11226 at the 0.5 h time point was set at 1 and the expression at the other time points was normalised accordingly using the $2^{-\Delta\Delta CT}$ method. The error bars represent the standard deviation of three independent measurements of $\Delta\Delta C_T$ based on bacterial mRNAs extracted from 500 larvae.
F. The expression levels of the endogenous control *rpoA* for all sampling points.

known to be present in many vibrios including *V. harveyi* (Pang *et al*., 2006), and has been reported to regulate haemolysin and outer membrane protein production in different vibrios (Lin *et al*., 1993; Lee *et al*., 2000; Provenzano *et al*., 2000; Wang *et al*., 2002). The complete *toxR* gene of *V. harveyi* shares 87%, 84% and 83% sequence similarity with *toxR* of *V. parahaemolyticus*, *V. fluvialis* and *V. vulnificus* respectively (Franco and Hedreyda, 2006). Moreover comparison of *toxR* amino acid sequences from several *Vibrio* spp. revealed relatively conserved transcription activation and transmembrane domains (Conejero and Hedreyda, 2003). Therefore, we hypothesised that the ToxR protein might regulate the production of virulence factors in vibrios belonging to the Harveyi clade

as well. In this study, we observed a relatively large variability in the expression of *toxR* within the group of tested isolates, with 31-fold difference in expression between the isolate showing the highest expression and the one showing the lowest (Table 1). This is especially remarkable since ToxR is a regulatory protein and consequently, small differences in the expression level of this regulator can be expected to result in larger differences in the expression of the genes it regulates. Correlation analysis showed that *toxR* expression was significantly correlated with survival of challenged brine shrimp, which indicates that strains that have a higher ToxR production capacity tend to be more virulent. The *in vivo* expression study showed that on average 122-fold higher *toxR* expression occurred in the virulent isolates BB120 and LMG21363 in comparison with the avirulent isolate LMG11226 (Fig. 2B), further suggesting that this gene is playing a role in the virulence of these organisms. As far as we know, this is the first report demonstrating involvement of ToxR in virulence of vibrios belonging to the Harveyi clade. At this moment, the virulence genes that are included in the ToxR regulon in these bacteria are not known. However, our data suggest that metalloprotease and serine protease might be good candidates since we observed a significant positive correlation between expression levels of these genes and *toxR* (Table 3).

Haemolysins have been identified as an important virulence determinant in many vibrios. In the current study, we found a high variation in the expression levels of the *vhh* haemolysin gene (Table 2), with a low expression in the avirulent isolates and higher expression in the more virulent isolates *in vitro*. Correlation analysis showed a strong correlation between the survival of challenged brine shrimp and expression of the *vhh* haemolysin gene (Table 5), indicating that differences in the capacity of the isolates to produce haemolysin could explain a good part of the differences in their virulence towards the shrimp. The *in vivo* expression study showed that over 50-fold higher *in vivo* expression of the *vhh* haemolysin gene occurs in the virulent isolates BB120 and LMG21363 than in the avirulent isolate LMG11226. This confirms previous literature reports which stated that haemolysin is one of the major virulence determinants in *V. harveyi* (Zhang and Austin, 2000; Zhang *et al*., 2001; Zhu *et al*., 2006). Zhang and colleagues (2001) reported that haemolysin activity in extracellular products of *V. harveyi* was responsible for the pathogenesis towards salmonids and found that strain VIB645, which was very pathogenic towards salmonids, contains a duplication of the haemolysin gene (Zhang *et al*., 2001). This is consistent with our findings that this isolate showed the highest expression level of the *vhh* haemolysin gene (Table 1) *in vitro*. Liu and colleagues (1996) reported that *V. harveyi* isolates exhibiting higher haemolytic activity were more virulent towards giant tiger shrimp. More recently, Sun and colleagues (2007) found that disrupting the active site of phospholipase activity of *V. harveyi* haemolysin resulted in loss of haemolytic activity and pathogenicity to fish.

Extracellular proteases have been identified to play an important role in virulence and pathogenicity of many bacteria, including the Harveyi clade vibrios *V. alginolyticus* (Cai *et al*., 2007), *V. parahaemolyticus* (Sudheesh and Xu, 2001) and *V. harveyi* (Zhang and Austin, 2000; Teo *et al*., 2003; Won and Park, 2008). For both the *srp* serine protease and the *vhp* metalloprotease gene, there was considerable variation in expression levels between the different isolates, with an exceptionally high variability for the *vhp* metalloprotease gene, which showed over 100-fold difference in *in vitro* expression levels between the isolate that had the highest expression level and the one having the lowest (Table 1). Expression of the *srp* serine protease and the *vhp* metalloprotease genes was positively correlated with virulence towards challenged brine shrimp, but the correlations were not significant (Table 5). Isolate ACMM642 caused low mortality in brine shrimp whereas it showed the highest expression of the *srp* serine protease gene and it was also among the highest metalloprotease-producing strains. This isolate has been shown to harbour a *vhml* (*V. harveyi* Myovirus-like) phage (Ruwandeepika *et al*., 2010), which could explain the high protease expression because *vhml* phages are known to transfer proteases to *V. harveyi* (Munro *et al*., 2003). The *in vivo* expression levels of the *vhp* metalloprotease and the *srp* serine protease genes confirmed the relation with the virulence of the isolate since they were on average 160- and 24-fold higher in the virulent isolates BB120 and LMG21363 than in the avirulent isolate LMG11226. Finally, we found that *in vitro* expression of the *srp* serine protease gene had a strong positive correlation with expression of the *vhh* haemolysin gene (Table 3), suggesting the possibility of both genes being physically linked or under the control of a common regulatory mechanism.

In conclusion, the data presented in this study demonstrate that there is variation in the expression level of virulence regulators and virulence factors in *Vibrio* isolates belonging to the Harveyi clade. We studied the expression of virulence genes and regulators under optimal conditions (i.e. during growth in a nutrient-rich medium) in order to obtain an indication of the production capacity of the isolates. The production capacity of the regulators *luxR* and *toxR* and the *vhh* haemolysin gene were correlated with the survival of challenged brine shrimp larvae indicating that variation in the expression of these genes might be the cause of the variation in the virulence of the isolates. Monitoring of the *in vivo* virulence gene expression in three selected isolates during infection of brine shrimp larvae revealed a remarkably

Table 6. Isolates used in this study.

Isolate	Source and relevant feature	Reference
BB120	= ATCC BAA-1116; marine isolate (1993)	Bassler *et al*. (1997)
VIB571	Isolated from seabass (*Dicentrarchus labrax*), Spain (1990)	Zhang *et al*. (2001)
VIB645	Isolated from seabass (*Dicentrarchus labrax*), Tunisia (1990)	Zhang *et al*. (2001)
LMG22891	= CAIM88; isolated from the haemolymph of shrimp (*Litopenaeus* spp.), Seprofin, Mexico (1998)	Defoirdt *et al*. (2008)
LMG22893	= CAIM148; isolated from diseased shrimp (*Penaeus* spp.) haemolyph, Técnica Aquacultural del Matatipac, Mexico (1995)	Defoirdt *et al*. (2008)
LMG22894	= CAIM151; isolated from the haemolymph of diseased shrimp (*Penaeus* spp.), Costa Azul Sinaloa, Mexico (1995)	Defoirdt *et al*. (2008)
ACMM20	Sea water isolate	Payne *et al*. (2004)
ACMM642	Isolate from *Penaeus monodon* larvae	Payne *et al*. (2004)
LMG11226	Sea water isolate, Hawaii	Thompson *et al*. (2002)
LMG21363	= CAIM372 = PN9801; isolated from the lymphoid organ of diseased shrimp (*Penaeus* spp.) *juveniles*, Philippines	Soto-Rodriguez *et al*. (2003)

ACMM, Australian Collection of Marine Microorganisms; LMG, Laboratory of Microbiology Collection (Ghent University, Ghent, Belgium); CAIM, Collection of Aquacultural Important Micro-organisms (CIAD/Mazatlan Unit for Aquaculture, Mazatlan, Mexico).

similar expression pattern for all virulence genes, with a peak in expression after 24 h in the virulent isolates and a constantly low expression in the avirulent isolate.

Experimental procedures

Bacterial strains and growth conditions

The isolates used in the study were obtained from the *Vibrio* collection in the Laboratory of Aquaculture and Artemia Reference Center, Ghent University, Belgium (Table 6). Ten microlitres of stored cultures (in 40% glycerol at –80°C) were plated onto fresh Marine agar (Himedia, India) and incubated for 24 h at 28°C. Single colonies were picked from the plates and cultured in marine broth (Himedia, India) at 28°C under constant agitation (150 min^{-1}).

Sampling and RNA extraction

The isolates were grown to late exponential phase in three independent cultures. The cell density was measured spectrophotometrically (Shimadzu UV-1601, Kyoto, Japan) at 600 nm. The bacteria were harvested and suspended in bacterial RNA protective reagent (Qiagen) according to the manufacturer's instructions in order to increase the RNA stability. The pellet was stored at –80°C.

For the *in vivo* gene expression study, challenged brine shrimp larvae (obtained as described below) were sampled at 0.5, 6, 12, 24, 36 and 48 h after addition of *Vibrio* isolates to the culture water (500 larvae per sample from three independent brine shrimp cultures), washed with autoclaved sea water and stored in –80°C for RNA extraction. Before the RNA extraction, the larvae were grinded using an eppendorf grinder (Kontes Pellet Pestle® Micro Grinders, Daigger and company, Llinois, USA) under septic conditions. Tissue debris of the larvae was removed using the Qiashredder (Qiagen, Hilden, Germany) apparatus to avoid clogging of the RNA extraction columns during the subsequent RNA extraction.

RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Extracts were subsequently treated with DNase I (Fermentas, Germany), according to producer's guidelines to remove the remaining DNA. The RNA quantity was checked spectrophotometrically (ND-1000, V3.3.0, Thermo Fisher Scientific, USA) and adjusted to 200 ng μl^{-1} in all samples. The complete DNA degradation within the RNA samples was confirmed by subjecting DNase-treated RNA to PCR. The RNA quality was confirmed by electrophoresis. The RNA samples were stored in –80°C for subsequent use.

Primers used in this study

Specific primers for *luxR*, *toxR*, haemolysins, serine protease and metalloprotease genes were designed using the Primer 3.0 software (http://frodo.wi.mit.edu/primer3). The *rpoA* gene, which is considered to be a housekeeping gene, was used as a control in the real-time PCR (Defoirdt *et al*., 2007). Specific primers were designed based on the consensus of sequences that are deposited in GenBank. Primers used in this study are listed in Table 7.

Reverse transcription

Reverse transcription was performed using the reverse transcriptase (Fermentas International, Canada) in accordance to the manufacturer's instructions. Briefly, the mixture of 2 μg of RNA and 2 μl of reverse primer was incubated at 70°C for 5 min and then chilled on ice. Subsequently, 8 μl of reaction mixture containing 4 μl of 5× reaction buffer (0.25 mol l^{-1} Tris-HCl pH 8.3, 0.25 mol l^{-1} KCl, 0.02 mol l^{-1} $MgCl_2$, 0.05 mol l^{-1} DTT), 2 μl of 0.01 mol l^{-1} dNTP mix, 20 units of ribonuclease inhibitor (Fermentas life sciences), 200 units of RevertAid™ H minus M MuLV reverse transcriptase (Fermentas Life Sciences) was added, the reaction mixture was incubated at 42°C for 60 min followed by heating at 70°C for 10 min and then cooled to 4°C. cDNA samples were checked by PCR and stored at –20°C for further use.

Real-time PCR

Real-time PCR was used to quantify the level of expression of *luxR*, *toxR*, *vhpA* (metalloprotease), *vhh* (haemolysin) and

Table 7. Primers used for PCR and real-time PCR amplification.

Gene	Primer sequence	Product size (bp)	Reference
luxR	F: TCAATTGCAAAGAGACCTCG R: AGCAAACACTTCAAGAGCGA	84	Defoirdt *et al.* (2007)
toxR	F: CGACAACCAAAATACGGAA R: AGAGCAATTTGCTGAAGCTA	131	NCBI database (AAWP01000030)
vhh[a]	F: GCGCTTGGTATCTTCTCTGC R: CAGACAGCTCATCACGCATT	226	NCBI database (NC_009783)
vhh	F: TTCACGCTTGATGGCTACTG R: GTCACCCAATGCTACGACCT	234	NCBI database (AF293430)
srp	F: TGCACGACCAGTTGCTTTAG R: AAGTGGTCGTCAGCAAATCC	232	NCBI database (NC_009783)
vhp	F: CTGAACGACGCCCATTATTT R: CGCTGACACATCAAGGCTAA	201	NCBI database (AY630354)
rpoA	F: CGTAGCTGAAGGCAAAGATGA R: AAGCTGGAACATAACCACGA	197	Defoirdt *et al.* (2007)

a. This primer pair was used to amplify the *vhh* haemolysin gene in strains BB120 and LMG21363; the other pair was used for all other strains.

serine protease. The appropriate primer concentration (200 nm) was determined for subsequent use in the real-time PCR. Dissociation curve analysis in the real-time PCR was performed for each gene to check for the amplification of untargeted fragments. Real-time PCR was performed in an ABI PRISM 7300 Fast Real Time System thermal cycler (Applied Biosystems) in a total volume of 25 μl, consisting 12.5 μl of 2× SYBR green master mix, appropriate volumes of forward and reverse primers and 5 μl of template cDNA. The volume of each reaction mixture was adjusted to 25 μl by adding sterile RNase-free water. The thermal cycle parameters used for the real-time amplification were: initial activation at 50°C for 2 min, initial denaturation at 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 15 s, primer annealing at 55°C for 20 s and elongation at 72°C for 30 s. Data acquisition was performed with the 7300 SDS software (v 1.3.1) at the end of each elongation step.

Real-time PCR data analysis

The real-time PCR was validated by amplifying of serial dilutions of cDNA synthesized from 1 μg of RNA isolated from bacterial samples (Livak and Schmittgen, 2001). Serial dilutions of cDNA were amplified by real-time PCR using gene-specific primers. ΔC_T (average C_T value of target – average C_T value of *rpoA*) was calculated for the different dilutions and plotted against the cDNA concentration. The slope of the graph was almost equal to 0 for all of the target genes (*luxR*, *toxR*, *vhh*, *vhp* and *srp*). Therefore, the amplification efficiency of reference and the target genes is equal.

The expression of the target genes was normalized to the endogenous control *rpoA* (RNA polymerase A subunit) by calculating ΔC_T:

$$\Delta C_T = C_{T,\text{target}} - C_{T,rpoA}$$

and expressed relative to a calibrator strain by calculating $\Delta\Delta C_T$:

$$\Delta\Delta C_T = \Delta C_T - \Delta C_{T,\text{calibrator}}.$$

Isolate LMG11226 was used as calibrator for the *in vitro* expression experiment and for the *in vivo* expression experiment, the calibrator was the 0.5 h time point of LMG11226. The relative expression was then calculated as

$$\text{Relative expression} = 2^{-\Delta\Delta CT}.$$

Axenic brine shrimp hatching

High-quality hatching cysts of *A. franciscana* (INVE Aquaculture, Baasrode, Belgium) were used in the experiments. The hatching was performed according to the method developed by Marques and colleagues (2004), briefly, 200 mg of cysts were hydrated in 18 ml of tap water for 1 h and sterile cysts and nauplii were obtained via decapsulation, by adding 660 μl of NaOH (32%) and 10 ml of NaOCl (50%) to the hydrated cyst suspension. The decapsulation was stopped after 2 min by adding 14 ml of $Na_2S_2O_3$ (10 g l^{-1}). Filtered 0.22 μm aeration was provided during the reaction. The decapsulated cysts were washed with filtered (0.22 μm) and autoclaved natural sea water. The cysts were resuspended in a 50 ml tube containing 30 ml of filtered and autoclaved natural sea water and hatched for 24 h on a rotor (4 min^{-1}) at 28°C with constant illumination (approximately 2000 lux). After 24 h of hatching, groups of 20 nauplii were counted and transferred to new sterile 50 ml tubes that contained 20 ml of filtered and autoclaved sea water. For the *in vivo* gene expression study, approximately 600 animals were transferred into sterilized 11 flasks. The tubes were put back on the rotor and flasks were kept on a shaker at 28°C. All manipulations were performed under a laminar flow in order to maintain sterility of the cysts and nauplii.

Brine shrimp challenge test

Challenge tests were performed according to the method developed by Defoirdt and colleagues (2005). Bacterial isolates used for the challenge were washed twice in filtered and autoclaved sea water. The bacterial dose was 10^5 cfu per ml of shrimp culture water. At the start of the challenge test, an autoclaved suspension of autoclaved LVS3 bacteria (Verschuere *et al.*, 1999) in filtered and autoclaved sea water was added as feed, equivalent to approximately 10^7 cfu ml^{-1}

culture water. The falcon tubes, to which only autoclaved LVS3 bacteria were added, were used as controls. The survival of the larvae was counted 48 h after the addition of the pathogens. Each treatment was performed in triplicate and each experiment was repeated twice to see the reproducibility. The sterility of the control treatments were checked at the end of the challenge by inoculating 1 ml of brine shrimp culture water to Marine Broth in a test tube and incubating the mixture for 2 days at 28°C as well as 100 µl of brine shrimp culture water was spread on LB (Luria–Bertani) and MA plates and incubated at 37°C and 28°C respectively. If the control was contaminated, the results were not considered and the experiment was repeated.

The relative percentage of survival

The relative percentage of survival (RPS) was calculated using the following formula:

$$\text{RPS} = (\%\ \text{survival in challenge with the tested strain} / \%\ \text{survival in uninfected control}) \times 100.$$

Statistical analysis

The analysis of data was carried out using the SPSS statistical software (version 15). Gene expression (ΔC_T) and survival data were compared with one-way ANOVA, followed by a Tukey's *post hoc* test. Spearman's rank correlations were calculated between brine shrimp survival and ΔC_T data. Pearson correlations were calculated between expression levels of the different genes using ΔC_T data. For all statistical analyses, a 5% significance level was used.

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