Journal of
Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Expression of virulence genes in luminescent and nonluminescent isogenic vibrios and virulence towards gnotobiotic brine shrimp (*Artemia franciscana*)

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Keywords
gene expression, quorum sensing, virulence.

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2010/1700: received 24 September 2010, revised 18 October 2010 and accepted 19 October 2010

doi:10.1111/j.1365-2672.2010.04892.x

Abstract

Aims: This study aimed to evaluate the expression levels of virulence gene regulators (*luxR* and *toxR*) and virulence factors (serine protease, metalloprotease and haemolysin) in luminescent and nonluminescent isogenic *Vibrio harveyi* and *Vibrio campbellii*.
Method and Results: Nonluminescent variants have been reported before to become dominant in cultures of luminescent vibrios when grown under static conditions in the dark. Wild-type *V. harveyi* BB120, *V. campbellii* LMG 21363, quorum sensing mutants of *V. harveyi* BB120 and their previously reported nonluminescent isogenic counterparts were used in this study. The expression level of the virulence genes *srp* serine protease, *vhp* metalloprotease and *vhh* haemolysin, the quorum sensing master regulator gene *luxR* and the virulence regulator gene *toxR* in isogenic luminescent and nonluminescent strains were quantified using reverse transcriptase real-time PCR. These experiments revealed that the nonluminescent strains produced lower levels of the quorum sensing master regulator gene *luxR* and the *vhp* metalloprotease gene (which is known to be regulated by quorum sensing). Finally, challenge tests with gnotobiotic brine shrimp (*Artemia franciscana*) larvae revealed that the nonluminescent strains are less virulent than their luminescent isogenic counterparts.
Conclusion: Nonluminescent variants of *V. harveyi* and *V. campbellii* strains produce lower levels of the quorum sensing master regulator gene *luxR* and the *vhp* metalloprotease gene and are less virulent to brine shrimp than their isogenic luminescent counterparts.
Significance and Impact of the study: These results indicate that adaptation of luminescent vibrios to specific growth conditions that result in a dominant nonluminescent phenotype is accompanied by a decreased adaptation to a host environment because of altered virulence gene regulation.

Introduction

Vibrio harveyi is a ubiquitous, bioluminescent bacterium causing life-threatening vibriosis in marine vertebrates and invertebrates, leading to severe losses in the aquaculture industry (Austin and Zhang 2006). The species *Vibrio campbellii* is closely related to *V. harveyi*, showing 61–74% DNA–DNA similarity and over 97% 16S rDNA similarity (Gomez-Gil *et al.* 2004). The pathogenicity mechanisms of these bacteria are imprecisely understood and it is suggested that the ability to attach and to form biofilms, the secretion of various extracellular products including proteases and haemolysin, lipopolysaccharide and bacteriocin like substances and the presence bacteriophages are playing a major role (Austin and Zhang 2006). In a previous study, we have shown the presence of

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virulence genes (including the regulatory genes *toxR* and *luxR* and the genes for serine protease, metalloprotease and haemolysin) in vibrios belonging to the Harveyi clade (Ruwandeepika *et al.* 2010b). In a further study, we quantified the expression levels of these virulence genes and found that the expression levels were correlated with the virulence towards gnotobiotic brine shrimp (*Artemia franciscana*) (Ruwandeepika *et al.* 2010a).

Quorum sensing, a process of bacterial cell-to-cell communication, has been studied in many bacteria as it is thought to be one of the virulence regulatory mechanisms (Henke and Bassler 2004b; Waters and Bassler 2005). Quorum sensing is studied in depth, in *V. harveyi*, which uses three channels that are mediated by the signal molecules Harveyi Autoinducer 1 (HAI-1), autoinducer 2 (AI-2) and Cholerae Autoinducer 1 (CAI-1), respectively (Waters and Bassler 2006). The autoinducers are recognized by cell membrane-bound two component receptor proteins and feed a common phosphorylation/dephosphorylation signal transduction cascade influencing the quorum sensing master regulator LuxR (Taga and Bassler 2003). Binding of LuxR to the promoter regions activates or inactivates the expression of the target genes in the quorum sensing regulon. In *V. harveyi*, quorum sensing controls bioluminescence (Bassler *et al.* 1993), biofilm formation, type III secretion (TTS) (Henke and Bassler 2004a), extracellular toxin (Manefield *et al.* 2000), siderophore (Lilley and Bassler 2000) and metalloprotease production (Mok *et al.* 2003). It has been shown that the quorum sensing process regulates the virulence of luminescent vibrios towards different hosts *in vivo* (Defoirdt *et al.* 2008).

Luminescence is one of the phenotypes that are regulated by quorum sensing in vibrios (Bassler *et al.* 1993). Although the biochemistry and genetics of bioluminescence have been investigated in detail, its biological role in free-living and pathogenic bacteria remains mysterious (Wegrzyn and Czyz 2002). Bioluminescence consumes 20% of the cellular energy (Makemson 1986), and consequently, it must have a positive selective value because otherwise it would have been lost during evolution. One of the hypotheses is that *V. harveyi* emits light to stimulate DNA repair (Wêgrzyn and Czyz 2002). Interestingly, *V. harveyi* bioluminescence was also shown to be involved in the detoxification of H_2O_2, thus playing a role in the protection against oxidative stress (Czyz and Wêgrzyn 2001). Because H_2O_2 is an important part of the defence of eukaryotic hosts against infections (Murray and Cohn 1980), bioluminescence might be considered as a virulence factor *per se*. Recently, Phuoc *et al.* (2009) obtained nonluminescent variants of luminescent vibrios by culturing them under static conditions in the dark and found that these nonluminescent variants were indeed less virulent towards brine shrimp (*A. franciscana*) than their luminescent counterparts.

This study was designed to quantitate the expression of the virulence factors serine protease, metalloprotease and haemolysin and the quorum sensing master regulator *luxR* and the virulence regulator *toxR* in isogenic luminescent and nonluminescent strains described previously (Table 1; Phuoc *et al.* 2009).

Materials and methods

Bacterial strains used

Wild-type *V. harveyi* BB120, *V. campbellii* and quorum sensing mutants of *V. harveyi* and nonluminescent strains of isogenic strains were used in the study (Table 1). Ten microlitres of stored cultures (in 40% glycerol at −80°C) was plated onto Marine agar (Himedia, Mumbai, India) and incubated for 24 h at 28°C. Single colonies were picked from the plates and cultured in marine broth (Himedia) at 28°C under constant agitation (150 min^{-1}).

RNA extraction

The strains used in the study 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, Hilden, Germany) according to the manufacturer's instructions to increase the RNA stability. The pellet was stored at −80°C.

RNA was extracted using the Qiagen RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Extracts were subsequently treated with DNase I (Fermentas, St Leon-Rot, Germany), according to producer's guidelines to remove the remaining DNA. The RNA quantity was checked spectrophotometrically (nanodrop) 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 EXPRESS 3.0 software (Applied Bioscience, Life Technologies Corp., Carlsbad, CA). The *rpoA* gene, which is considered to be a house-keeping gene, was used as a control in the real-time PCR (Defoirdt *et al.* 2007). Specific primers were designed based on the consensus of

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Table 1 Phenotype and source of *Vibrio harveyi* and *Vibrio campbellii* isolates used in this study

Strain	Source and relevant feature of the strain	References
Wild types		
BB120	Wild type from which strains BB152, BB170, MM30, and BB886 are derived	Bassler *et al.* (1997)
LMG 21363	*V. campbellii,* CAIM372 = PN9801; isolated from the lymphoid organ of diseased shrimp (*Penaeus* spp.) juveniles, Philippines	Soto-Rodriguez *et al.* (2003)
Quorum sensing mutants derived from BB120		
BB152	Mutation in *luxM* (AHL synthase that produces *N*-(3-hydroxybutanoyl) -L-homoserine lactone)	Bassler *et al.* (1994)
BB170	Mutation in *luxN* (AHL receptor that detects the AHL produced by LuxM)	Bassler *et al.* (1993)
MM30	Mutation in *luxS* (AI-2 synthase)	Surette *et al.* (1999)
BB886	Mutation in *luxP* (AI-2 receptor)	Bassler *et al.* (1994)
Nonluminescent strains (NL)		
LMG 21363 NL	*V. campbellii* Nonluminescent. From Laboratory of Aquaculture and Artemia Reference Center, Gent Univerisity, Belgium	Phuoc *et al.* (2009)
BB170 NL	BB170 Nonluminescent. From Laboratory of Aquaculture and Artemia Reference Center, Gent Univerisity, Belgium	Phuoc *et al.* (2009)
BB152 NL	BB152 Nonluminescent. From Laboratory of Aquaculture and Artemia Reference Center, Gent Univerisity, Belgium	Phuoc *et al.* (2009)
BB120 NL	BB120 Nonluminescent. From Laboratory of Aquaculture and Artemia Reference Center, Gent Univerisity, Belgium	Phuoc *et al.* (2009)
BB886 NL	BB886 Nonluminescent. From Laboratory of Aquaculture and Artemia Reference Center, Gent Univerisity, Belgium	Phuoc *et al.* (2009)
MM30 NL	MM30 Nonluminescent. From Laboratory of Aquaculture and Artemia Reference Center, Gent Univerisity, Belgium	Phuoc *et al.* (2009)

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

sequences that are deposited in GenBank and the primer sequences were blasted against GenBank. Primers used in this study are listed in Table 2.

Reverse transcription

Reverse transcription was performed using the reverse transcriptase (Fermentas International Inc., Harrington Court, Burlington, 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 [250 mmol l^{-1} Tris–HCL (pH 8·3)-250 mmol l^{-1} KCl-20 mmol l^{-1} $MgCl_2$-50 mmol l^{-1} DTT], 2 μl of 10 mmol l^{-1} dNTP mix, 20 units of ribonuclease inhibitor (Fermentas Life Sciences, Germany), 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.

Table 2 Primers used for PCR and real-time PCR amplification

Gene	Primer	Product size	References
vhh	F: GCGCTTGGTATCTTCTCTGC R: CAGACAGCTCATCACGCATT	226	NC_009783, NCBI data base
Serine protease	F: TGCACGACCAGTTGCTTTAG R: AAGTGGTCGTCAGCAAATCC	232	NC_009783, NCBI data base
Metalloprotease	F: CTGAACGACGCCCATTATTT R: CGCTGACACATCAAGGCTAA	201	AY630354, NCBI data base
luxR	F: TCAAGATTGCAAAGAGACCTCG R: AGCAAACACTTCAAGAGCGA	84	Defoirdt *et al.* (2007)
toxR	F: CGACAACCAAAATACGGAA R: AGAGCAATTTGCTGAAGCTA	131	AAWP01000030, NCBI data base
rpoA	F: CGTAGCTGAAGGCAAAGATGA R: AGCTGGAACATAACCACGA	197	Defoirdt *et al.* (2007)

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Real-time PCR

Real-time PCR was used to quantitate the level of expression of *luxR*, *toxR*, *vhpA* (metalloprotease), *vhh* (haemolysin) and serine protease. The appropriate primer concentration ($200\ \text{nmol l}^{-1}$) 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 as follows: 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, Applied Bioscience) at the end of each elongation step.

Real-time PCR data analysis

Analysis of relative gene expression was performed using the $2^{-\Delta\Delta C_t}$ method according to Livak and Schmittgen (2001) after validation of the method. Briefly, the validation was carried out by amplifying of serial dilutions of cDNA synthesized from 1 μg of RNA isolated from bacterial samples. 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 reference) was calculated and plotted against the cDNA concentration. The slope of the graph derived was almost equal to 0. Thus, it was proven that the amplification efficiency of reference and the target is almost equal, fulfilling the requirements for applying the $2^{-\Delta\Delta C_t}$ method. The expressions of target genes were normalized to the endogenous control *rpoA* gene by calculating ΔC_t:

$$\Delta C_t = C_{t\,\text{target}} - C_{t\,\text{rpoA}}$$

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

$$\Delta\Delta C_t = \Delta C_t - \Delta C_{t\,\text{calibratior}}$$

Luminescent strain of each nonluminescent strains was used as calibrator. Fold change in expression was then calculated as:

$$\text{Fold change} = 2^{-\Delta\Delta C_t}$$

Axenic *Artemia* 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 *et al.* (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 $Na_2S_2O_3$ ($10\ \text{g l}^{-1}$). Filtered 0·22 μm aeration was provided during the reaction. The decapsulated cysts were washed with filtered (0·22 μm) and autoclaved natural seawater (121°C for 15 min). The cysts were resuspended in a 50-ml tube containing 30 ml of filtered and autoclaved natural seawater and hatched for 24 h on a rotor ($4\ \text{min}^{-1}$) at 28°C with constant illumination (*c.* 2000 lux). The axenity of cysts were checked by inoculating 1 ml of culture water in to 9 ml of Marine broth and incubating at 28°C for 24 h. 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 seawater. Falcon tubes with decapsulated cysts were kept in the rotor at 28°C. All manipulations were performed under a laminar flow to maintain sterility of the cysts and nauplii.

Artemia challenge test

Challenge tests were performed according to the method developed by Defoirdt *et al.* (2005). Bacterial isolates used for the challenge were washed twice in filtered and autoclaved seawater. The animals were challenged with 10^5CFU of the vibrios per ml *Artemia* culture water. At the start of the challenge test, an autoclaved suspension of autoclaved LVS3 bacteria (Verschuere *et al.* 1999) in filtered and autoclaved seawater was added as feed, equivalent to *c.* $10^7\ \text{CFU ml}^{-1}$ culture water. The falcon tubes, to which only autoclaved LVS3 bacteria were added, were used as controls. The survival of *Artemia* 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 was checked at the end of the challenge by inoculating 1 ml of *Artemia* 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 *Artemia* culture water was spread on LB (Luria–Bertani) and MA plates and incubated at 37 and 28°C, respectively. If the control was contaminated, the results were not considered and the experiment was repeated. At the end of the challenge tests, the relative percentage of survival (RPS) was calculated according to Amend (1981).

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Statistical analyses

The analysis of data was carried out using the SAS® statistical software (v. 9.1, SAS Institute Inc., Cary, NC). Survival data of *Artemia* was analysed by an independent sample *t*-test test. The gene expression levels were analysed by an independent sample *t*-test using ΔC_t data. Significant level was chosen to be 5 and 1% for survival and gene expression, respectively.

Results

Expression of virulence gene regulators and virulence factors

In this study, we used 12 strains of *V. harveyi* and *V. campbellii* to determine the expression levels of the quorum sensing master regulator *luxR*, the virulence regulator *toxR*, metalloprotease, haemolysin and serine protease. The presence of the genes was first confirmed by PCR in the culture collection used in the study. Expected amplicons were generated from DNA extracts of the isolates, which indicate that all isolates were positive for all the target genes (data not shown). To quantify the gene expression, the strains 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 is the main regulatory gene in the quorum sensing cascade as shown by previous studies and in this study, we compare the level of *luxR* expression between luminescent and nonluminescent strains. The reverse transcriptase real-time PCR results indicate that the expression of the quorum sensing master regulator gene *luxR* was significantly affected in the nonluminescent wild-type strains BB120 and LMG 21363 (Table 3). Also for the AI-2 mutants, *luxR* mRNA levels were lower in the nonluminescent variants, although only significant in the AI-2 receptor mutant BB886. In contrast, there were no differences in *luxR* mRNA levels in the HAI-1 mutants. Apparently, the nonluminescent variation has a more significant effect on *luxR* mRNA levels in wild-type and AI-2 mutants than it has on *luxR* mRNA in the HAI-1 mutants (Table 4).

Proteases are known to be putative virulent factors in most of the vibrios including the *Harveyi* clade vibrios. In this study, we quantified the expression level of two virulence-mediated genes in vibrios coding for proteases, i.e. serine protease and metalloprotease. In this study, the metalloprotease gene expression was showing approximately twofold lower expression levels in the nonluminescent variants of all tested strains, although the difference was only significant for the wild-type BB120 and the AI-2 synthase mutant MM30 (Table 3). Serine protease gene was not shown any significant difference in expression between the luminescent and nonluminescent strains.

The ToxR that is a transmembrane transcription regulator known to be an important virulence regulator in many vibrios also (the gene *toxR*) did not show difference in expression level between luminescent and nonluminescent strains (Table 3). Apart from the regulators such as LuxR and ToxR, the virulence factor, haemolysin is also one of the vital virulence products in many vibrios having cytotoxic and haemolytic activity in the host tissues causing severe damages to the host. But in the current study, we could not find significant difference in expression level of this putative gene in nonluminescent strains when compare to the luminescent strains (Table 3).

In vivo virulence towards gnotobiotic brine shrimp (*Artemia franciscana*) larvae

Virulence of strains used in the study was examined according to the method described previously, using challenge experiments with gnotobiotically grown *Artemia*

Table 3 Expression of the quorum sensing master regulator *luxR*, the virulence regulator *toxR*, serine protease, metalloprotease and haemolysin in nonluminescent *Vibrio harveyi* strains grown to log phase relative to their isogenic luminescent counterparts

	QS master regulator *luxR*		Virulence regulator *toxR*		Metalloprotease		Serine protease		Haemolysin	
Strain	Fold ≠	*P* value*	Fold ≠	*P* value	Fold ≠	*P* value*	Fold ≠	*P* value	Fold ≠	*P* value
BB120 NL	*–1·7*	*0·017*	–1·3	0·272	***–3·1***	***0·002***	–1·2	0·671	–1·8	0·028
BB152 NL	–1·3	0·192	–1·3	0·164	–2·3	0·051	–1·5	0·357	–1·1	0·875
BB170 NL	–1·1	0·565	–1·3	0·209	–1·7	0·055	–1·2	0·326	–1·1	0·861
BB886 NL	*–1·7*	*0·049*	–1·4	0·107	–2·2	0·159	–1·2	0·771	–1·1	0·606
MM30 NL	–1·6	0·064	–1·3	0·180	***–2·2***	***0·001***	–1·1	0·841	–1·1	0·741
LMG21363 NL	***–2·0***	***0·005***	–1·3	0·179	–2·0	0·151	–1·1	0·730	–1·5	0·403

Expression levels are reported as fold change relative to the luminescent variant.

*Significant differences with $P < 0·05$ and $P < 0·01$ are in italics and in bold and italics, respectively.

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Table 4 Expression levels of *luxR* relative to *rpoA* mRNA in *Vibrio harveyi* BB120 and its quorum sensing mutants

Strain	Inactivated component	Relative *luxR* expression (%)	
		Luminescent	Nonluminescent
BB120	–	100	61
BB152	HAI-1 receptor	81	62
BB170	HAI-1 synthase	77	68
BB886	AI-2 receptor	78	46
MM30	AI-2 synthase	68	42
JAF548	QS completely inactive	28	–

Relative expression in the luminescent wild type was set at 100% and the expression in all other strains was normalized accordingly.
HAI-1, Harveyi Autoinducer 1.

as the host animals. The survival of larvae challenged to nonluminescent variants of the wild-type strains BB120 and LMG 21363, and the HAI-1 mutants BB152 and BB170 was significantly higher than survival of larvae challenged to the corresponding luminescent strains ($P < 0{\cdot}05$) (Table 5). Survival of larvae challenged to the nonluminescent variants was also higher than that of larvae challenged to their luminescent counterparts for the AI-2 mutants, but the difference was not significant (Table 5).

Discussion

At this moment, the cause of the nonluminescent phenotype still is unclear. Phuoc *et al.* (2009) reported the presence of quorum sensing molecules in the nonluminescent strains and thus the nonluminescent phenotype was not caused by inability of the strains to produce signals. Our findings indicate that it might be a mutation or rearrangement in the quorum sensing system or in another regulatory protein that is integrated in the quorum sensing response. Importantly, although similar effects were observed for the different strains, we cannot exclude the possibility that the nonluminescent phenotype is caused by different mutations in the different strains. Interestingly, Nelson *et al.* (2007) recently reported that all of the bioluminescence structural genes (*luxABCDE*) were present in cryptically bioluminescent *Vibrio salmonicida* (recently reclassified as *Alliivibrio salmonicida*) strains, whereas the strains showed a novel arrangement and copy number of the homologues of the *Vibrio fischeri* quorum sensing regulatory genes $luxI_{Vf}$ and $luxR_{Vf}$ (which is not a homolog of the *V. harveyi* quorum sensing master regulator *luxR*). The authors argued that the novel arrangement of these quorum sensing regulatory genes generates antisense transcripts that might be responsible for the reduced luminescence.

Extracellular proteases are playing an important role in the virulence of many vibrios (Hase and finkelstein 1993). Serine protease and metalloprotease seem to be the major proteases in *V. harveyi* (Won and Park 2008; Zhang *et al.* 2008). Interestingly, metalloprotease levels were found to be lower in the nonluminescent variants when compared to the respective luminescent strains. According to the previous findings of Mok *et al.* (2003), the expression of metalloprotease is positively regulated by quorum sensing. Hence, the nonluminescent variants producing lower levels of metalloprotease mRNA are consistent with our observation that *luxR* mRNA levels were lower in nonluminescent variants when compared to their luminescent counterparts. On the other hand, serine protease expression in the nonluminescent strains was not different from the luminescent strains. The difference in metalloprotease expression might explain the lower virulence of the nonluminescent variants in the challenge tests. Moreover, the level of the quorum sensing master regulator *luxR* was also lower in the nonlu-

Table 5 Relative percentage of survival* of *Artemia* nauplii (mean ± standard error of three replicates) after 48-h challenge with isogenic luminescent and nonluminescent *Vibrio campbellii* and *Vibrio harveyi* strains

Strain	Experiment 1			Experiment 2		
	Nonluminescent	Luminescent	*P* value†	Nonluminescent	Luminescent	*P* value†
Control	100 ± 0	100 ± 0		100 ± 0	100 ± 0	
BB120	***78 ± 1***	***53 ± 7***	***0·008***	***80 ± 12***	***49 ± 5***	***0·007***
BB152	***65 ± 4***	***43 ± 10***	***0·036***	***66 ± 3***	***45 ± 2***	***0·025***
BB170	***69 ± 8***	***49 ± 7***	***0·025***	***68 ± 1***	***49 ± 7***	***0·019***
BB886	96 ± 8	91 ± 7	0·468	96 ± 9	88 ± 11	0·609
MM30	96 ± 8	85 ± 8	0·184	94 ± 1	85 ± 10	0·398
LMG21363	***49 ± 11***	***25 ± 8***	***0·031***	***45 ± 9***	***23 ± 5***	***0·036***

*For each experiment, the survival in the uninfected control was set at 100% and all other survival data were normalizing accordingly.
†Survival in challenge with luminescent and corresponding nonluminescent strain was compared using an independent samples *t*-test. Significant differences ($P < 0{\cdot}05$) are in bold and italics.

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minescent strains. Quorum sensing has been shown before to regulate virulence towards brine shrimp larvae (Defoirdt *et al.* 2005) and in addition to metalloprotease, quorum sensing has been shown to regulate the expression of a TTS system (Henke and Bassler 2004a), extracellular toxin (Manefield *et al.* 2000) and a siderophore (Lilley and Bassler 2000) *in vitro*. Hence, a lower activity of this regulatory mechanism might also be an explanation for the reduced virulence of the nonluminescent variants, not only by decreasing metalloprotease levels but also the expression levels of other virulence factors that were not investigated in this study. Survival of larvae challenged to the nonluminescent variants was higher than that of larvae challenged to their luminescent counterparts for the AI-2 mutants, but the difference was not significant (which is because of the fact that survival of larvae challenged to luminescent AI-2 mutants was already high). This is consistent with the findings of Phuoc *et al.* (2009), who also found lower mortality of *Artemia* when challenged with nonluminescent strains.

Conclusion

In this study, we found that nonluminescent variants of *V. harveyi* and *V. campbellii* described before by Phuoc *et al.* (2009) produced lower levels of the quorum sensing master regulator *luxR* and metalloprotease when compared to their isogenic luminescent counterparts under *in vitro* conditions. Moreover, challenge tests with gnotobiotic brine shrimp revealed that the nonluminescent variants were less virulent under *in vivo* conditions.

Acknowledgments

This study was financially supported by the Flemish Interuniversity Council–University Development Cooperation (VLIR), and by the Research Foundation of Flanders (FWO) project 'Probiont-induced functional responses in aquatic organisms?' (project no. G.0491.08) Belgium. T.D. is a postdoctoral fellow of FWO-Vlaanderen. The guidance and assistance of the staff of the Artemia Reference Center (Ghent University, Belgium) and UNESCO MIRCEN for Marine Biotechnology (College of Fisheries, Mangalore, India) is highly appreciated.

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