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ORIGINAL ARTICLE

Virulence of luminescent and non-luminescent isogenic vibrios towards gnotobiotic *Artemia franciscana* larvae and specific pathogen-free *Litopenaeus vannamei* shrimp

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luminescent, non-luminescent, SPF shrimp, *V. campbellii, V. harveyi.*

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Abstract

Aims: This study was conducted to test the virulence of luminescent (L) and non-luminescent (NL) isogenic strains of *Vibrio campbellii* LMG21363, *Vibrio harveyi* BB120 (wild type) and quorum-sensing mutant strains derived from the wild type such as *Vibrio harveyi* BB152, BB170, MM30 and BB886.
Methods and Results: The NL strains could be obtained by culturing rifampicin-resistant luminescent strains in the dark under static condition. The virulence of the L and NL strains was tested in gnotobiotic *Artemia franciscana* larvae challenged with 10^4 CFU ml^{-1} of bacteria. All luminescent isogenic tested strains showed higher virulence compared to the NL strains. The virulence of L and NL *V. campbellii* and *V. harveyi* BB120 was also tested in specific pathogen-free juvenile shrimp upon intramuscular injection with 10^6 CFU of bacteria. In contrast with *Artemia*, there was no significant difference in mortality between the groups challenged with L and NL strains ($P > 0{\cdot}05$). The non-luminescent strains were not able to revert back to the luminescent state and quorum sensing did not influence this phenotypic shift.
Conclusions: Luminescent *Vibrio* strains can switch to a non-luminescent state by culturing them in static conditions. The NL strains become less virulent as verified in *Artemia*.
Significance and Impact of the Study: The luminescent state of *Vibrio* cells in a culture needs to be verified in order to assure maintenance of virulence.

Introduction

Vibriosis is considered as one of the most important infectious diseases in aquaculture hatcheries as well as in grow-out shrimp ponds. Vibrios are commonly present in various marine and brackish habitats. The rapid increase in shrimp culture area is facilitating disease outbreak. *Vibrio harveyi* is a luminescent bacterium most notably associated with disease in cultured shrimp worldwide (Liu and Lee 1999). Mass mortalities of *Penaeus monodon* larvae or juveniles associated with luminous vibrios have been observed in hatcheries or farms in Indonesia (Sunaryanto and Mariam 1986), Philippines (Lavilla-Pitogo *et al.* 1990), India (Karunasagar *et al.* 1994), Taiwan (Song and Lee 1993; Liu *et al.* 1996), Thailand (Jiravanichpaisal *et al.* 1994), Australia (Pizzutto and Hirst 1995) and China (Vandenberghe *et al.* 1998).

It is known that bioluminescence is regulated by quorum sensing. The light intensity of luminous bacteria depends on bacterial density both *in situ* and *in vitro*. Quorum sensing is a process that allows bacteria to communicate using secreted chemical signaling molecules called autoinducers (Miller and Bassler 2001). Indirect evidence suggests that toxin production is regulated by quorum-sensing signal molecules (Manefield *et al.* 2000). Quorum sensing in *V. harveyi* utilizes three cell signalling systems that function in parallel to positively regulate bioluminescence (Bassler *et al.* 1993, 1994; Henke and Bassler 2004a), metalloprotease

(Mok *et al.* 2003), siderophore and exopolysaccharide production (Lilly and Bassler 2000) and to negatively regulate type III secretion (Henke and Bassler 2004b) in a cell population density-dependent manner. The LuxM/N system relies on *N*-(3-hydroxybutanoyl)-L-homoserine lactone (3-hydroxy-C4-HSL), which is synthesized by LuxM (Cao and Meighen 1989; Bassler *et al.* 1993). The LuxS/PQ system utilizes the signal molecule 3A-methyl-5,6-dihydro-furo(2,3-D)(1,3,2) dioxaborole-2,2,6,6A-tetraol (termed AI-2). The unborated AI-2 precursor is synthesized by LuxS (Surette *et al.* 1999; Schauder *et al.* 2001; Chen *et al.* 2002). The CqsA/S system utilizes the signal molecule CAI-1, which is dependent on CqsA for its synthesis. CAI-1 is (S)-3-hydroxytridecan-4-one, a new type of bacterial autoinducer (Higgins *et al.* 2007). These three signal molecules are distinct from each other and work synergistically in gene regulation (Milton 2006).

It was noticed that a luminescent strain of *Vibrio campbellii*, shown to be virulent to *Artemia* and shrimp (Halet *et al.* 2007; Yik Sung *et al.* 2007; Phuoc *et al.* 2008), produced non-luminescent colonies on Marine Agar (MA) at low frequency. The aim of this study is to document this phenomenon and to compare the virulence of luminescent and non-luminescent isogenic *Vibrio* towards gnotobiotic *Artemia* and SPF juvenile shrimp *Litopenaeus vannamei*.

Materials and methods

Preparation of bacterial stocks

The bacterial strains (Table 1), previously stored in 20% glycerol at −80°C, were aseptically inoculated in MA. The plates were incubated for 24 h at 28°C. Single colonies were subsequently transferred and grown in Marine Broth (MB) 2216 (Difco Laboratories, MI, USA) by incubation overnight in the shaker (28°C, 150 rev min^{-1}). The culture was then transferred to centrifugation tubes and centrifuged at 2200 ***g*** for 15 min. The supernatant was discarded and pellets were washed twice and finally re-suspended in filtered autoclaved seawater (FASW). The bacterial densities were determined spectrophotometrically at an optical density (OD) of 550 nm assuming that an OD of 1·0 at 550 nm corresponds to $1{\cdot}2 \times 10^9$ cells ml^{-1}, the McFarland standard (BioMerieux, France).

Qualitative detection of autoinducers and measurement of luminescence

Cell-free culture fluids of the strains collected in stationary phase were tested for the presence of *V. harveyi* BB120 and *V. campbellii* autoinducers by an assay from Defoirdt *et al.* (2008) using the double mutants JAF375 (sensor HAI-1^-, sensor AI-2^-, sensor CAI-1^+), JMH597

Table 1 Bacterial strains used in this study

Strain	Relevant features	References
LVS3	Isolate that enhances growth and survival of *Artemia*	Verschuere *et al.* (1999)
Vibrio campbellii LMG21363	Pathogen for shrimp and *Artemia,* luminescent	Soto-Rodríguez *et al.* (2003)
V. campbellii LMG21363 (RR-L)	Rifampicin-resistant, luminescent	This study
V. campbellii LMG21363 (RR-NL)	Rifampicin-resistant, non-luminescent	This study
Vibrio harveyi BB120	Wild type, luminescent	Bassler *et al.* (1997)
V. harveyi BB120 (RR-L)	Rifampicin-resistant, luminescent	This study
V. harveyi BB120 (RR-NL)	Rifampicin-resistant, non-luminescent	This study
V. harveyi BB152	Mutation in *LuxM* (AI-1 synthase)	Bassler *et al.* (1994)
V. harveyi BB152 (RR-L)	Rifampicin-resistant, luminescent	This study
V. harveyi BB152 (RR-NL)	Rifampicin-resistant, non-luminescent	This study
V. harveyi BB170	Mutation in *LuxN* (AI-1 receptor)	Bassler *et al.* (1994)
V. harveyi BB170 (RR-L)	Rifampicin-resistant, luminescent	This study
V. harveyi BB170 (RR-NL)	Rifampicin-resistant, non-luminescent	This study
V. harveyi MM30	Mutation in *LuxS* (AI-2 synthase)	Surette *et al.* (1999)
V. harveyi MM30 (RR-L)	Rifampicin-resistant, luminescent	This study
V. harveyi MM30 (RR-NL)	Rifampicin-resistant, non-luminescent	This study
V. harveyi BB886	Mutation in *LuxP* (AI-2 receptor)	Bassler *et al.* (1994)
V. harveyi BB886 (RR-L)	Rifampicin-resistant, luminescent	This study
V. harveyi BB886 (RR-NL)	Rifampicin-resistant, non-luminescent	This study
V. harveyi JAF375	Mutation in *luxN*: Cm^R*luxQ* :Kan^R, luminescent	Freeman and Bassler (1999)
V. harveyi JMH597	Mutation in *luxN*: Tn5 *cqsS*: Cm^R, luminescent	Henke and Bassler (2004b)
V. harveyi JMH612	Mutation in *luxPQ*: Tn5 *cqsS*: Cm^R, luminescent	Henke and Bassler (2004b)

RR, rifampicin-resistant; L, luminescent; NL, non-luminescent.

(sensor HAI-1$^-$, sensor AI-2$^+$, sensor CAI-1$^-$) and JMH612 (sensor HAI-1$^+$, sensor AI-2$^-$, sensor CAI-1$^-$) as reporters for CAI-1, AI-2 and HAI-1, respectively. The reporter strains were grown at 28°C with shaking (100 rev min^{-1}) in MB to an OD of approximately 1 at 550 nm and diluted 1 : 5000 in fresh medium. Of the diluted reporter cultures, 50 μl was mixed with 50 μl of cell-free culture fluids in 3-ml test tubes. Cell-free culture fluids of *V. harveyi* BB120 were used as a positive control; fresh medium was used as a negative control. The test tubes were incubated at 28°C and luminescence was measured every hour with a Lumac Biocounter M2500 luminometer (Lumac b.v., Landgraaf, Netherlands) until the luminescence with fresh medium was minimal (after 3–4 h).

Rifampicin-resistant strains

Rifampicin-resistant (RR) *V. campbellii* and *V. harveyi* BB120 were produced by an assay from Phuoc *et al.* (2008). A colony picked from MA plates was cultured for 24 h in 25 ml MB in a 100 ml erlenmeyer. After incubation at 28°C, 50 μl of the culture was taken by micropipette and transferred to 25 ml of fresh MB containing 0·5 mg l^{-1} rifampicin (R3501, Sigma-Aldrich). The culture was incubated for 24 h at 28°C. The growth of bacteria was monitored by the turbidity of the culture. The following days, further sub-cultures were made in MB, increasing the rifampicin concentration gradually from 1, 2, 4, 8, 16, 32, 64 to finally 100 mg l^{-1}. When bacteria cultures were growing well in the final concentration of rifampicin (100 mg l^{-1}), they were inoculated on MA plates containing 100 mg l^{-1} rifampicin (MAR) for obtaining single colonies. For long-term storage, the stock was preserved in 20% glycerol at −80°C.

Production of non-luminescent bacteria and staining of luminescent and non-luminescent colonies by Congo red

The non-luminescent (NL) bacteria were produced by culturing RR luminescent strains in the dark under static conditions in a 250 ml erlenmeyer. One single freshly grown luminescent colony was picked and inoculated in a 500 ml erlenmeyer containing 50 ml MB. The culture was kept in the dark under static conditions for 3 days or longer depending on the bacterial strain. Subsequently, the broth was discarded and the cells attached to the bottom of the erlenmeyer were collected by adding 5 ml of FASW and shaking the erlenmeyer. These cells were plated on MA and incubated for 24 h at 28°C to check for the presence of NL colonies. Totally dark colonies were picked and inoculated on MAR to verify for rifampicin resistance, ensuring that dark colonies were not accidental contaminants. In case no NL colonies were detected, fresh MB was added to the erlenmeyer and the above described procedure was repeated until a NL strain was found. For staining of luminescent (L) and NL colonies, the strains were inoculated in Marine Agar with Congo red (MACR). The medium was prepared with 37·4 g MB, 50 g sucrose, 15 g agar and 0·8 g Congo red l^{-1}. Congo red was prepared as a stock solution, autoclaved at 121°C for 15 min and then added to the culture medium when it had cooled to 55°C. Plates inoculated with L or NL strains were incubated for 24 h at 28°C. Strains that produce extracellular slime develop black colonies (Freeman *et al.* 1989).

Restoration of luminescence

Culturing of NL-RR strains in the supernatant of rifampicin-sensitive V. harveyi BB120

In an attempt to restore the luminescence, NL-RR strains were cultured in filter-sterilized supernatant from the culture of rifampicin-sensitive *V. harveyi* BB120 (wild type). The samples were collected at 30 min, 1, 2, 3, 6, 12, 24 h and plated on MAR. The plates were incubated for 24 h at 28°C for checking of L-RR colonies.

Co-culture of L-RS and NL-RR strains

The NL-RR strains were co-cultured with the original isogenic L-RS strains in a 250 ml erlenmeyer containing 50 ml MB. The samples were collected at 3, 6, 9, 12, 24, 48 h after inoculation and plated on MAR for detecting of L-RR colonies.

Passing of NL-RR strains through the hosts

In an attempt to restore the luminescence, NL-RR strains were passed through *Artemia* and juvenile *L. vannamei* shrimp. *Artemia* were challenged by immersion with 10^4 CFU ml^{-1} of NL-RR strains. Twenty-four hours after the challenge, infected *Artemia* were collected and homogenized by the stomacher to re-isolate strains on MAR. The NL strains were re-isolated and passed through the host for three times. Every passage cycle, the strains were plated on MAR for detecting L colonies. The same procedure was applied in *L. vannamei* shrimp but shrimp were challenged with NL-RR strains by injection. The challenge test is described in item 2·8 in the Materials and methods section.

Restoration of luminescence by chemicals

Two chemical products, H_2O_2 and *S*-nitroso-*N*-acetylpenicillamine (SNAP), were used in this study. The NL strains were prepared in FASW; the initial bacterial concentration was 5×10^6 CFU ml^{-1} and different concentrations of chemical products were added: 20, 30, 40, 50, 60,

70, 80, 90 and 100 mg l^{-1} of H_2O_2 or 0·01, 0·1, 0·5, 1, 2, 3, 4, 5, 10, 20, 30, 40 and 50 mg l^{-1} of SNAP. The samples were collected at 10, 20, 30 min, 1, 2, 3, 4, 5, 6, 12, 24 h and after plating on MAR were checked for L colonies.

Decapsulation and challenge test in *Artemia*

All experiments were performed with high quality hatching cysts of *Artemia franciscana* (EG® Type, batch 6940; INVE Aquaculture, Baasrode, Belgium). The modified decapsulation method of Sorgeloos *et al.* (1986) was chosen in order to obtain sterile *Artemia* nauplii. The protocol used was previously optimized by Marques *et al.* (2004). After hatching, groups of 30 nauplii (instar II) were transferred to sterile 50 ml falcon tubes containing 30 ml of FASW. They were challenged with vibrios at 10^4 CFU ml^{-1}. *Artemia* were fed once with autoclaved LVS3 (equivalent to 10^7 CFU ml^{-1}) at the beginning of the experiment. The challenge protocol was adapted from the protocol described by Defoirdt *et al.* (2005). Each treatment was carried out in quadruplicate. All manipulations were performed under a laminar flow hood in order to maintain sterility of the cysts and nauplii. Finally, the tubes were put back on the rotor and kept at 28°C. The mortality was scored at 36 and 48 h after exposure to bacteria. In each test, the sterility of the feed and the control treatments (to which no live bacteria were added) were checked at the end of the challenge by taking 100 μl of the feed suspension or *Artemia* culture water and spreading it on fresh MA. The plates were incubated for 48 h at 28°C. If a control tube was found to be contaminated, the data from the corresponding experiment were not considered and the experiment was repeated.

SPF shrimp

SPF *L. vannamei* of the Kona-Hawaii (USA) strain (Wyban *et al.* 1992) were used in this study. Shrimp were imported from Molokai Sea Farms, Hawaii, USA. Animals were certified to be free of WSSV, IHHNV, MBV, HPV, Taura syndrome virus (TSV), Yellow head virus (YHV) and Gill-associated virus (GAV), as well as other pathogens (fungi, protozoa) as verified by PCR and histopathology. Batches of shrimp arrived at the Laboratory of Aquaculture and Artemia Reference Center (ARC), Ghent University, as post-larvae (PL_{8-12}). They were kept in a recirculation system at a water temperature of 28–29°C, 34 g l^{-1} salinity, and pH of 7·8–8·1. During the first week, the animals were fed twice daily with *Artemia* nauplii. After 1 week their diet was shifted to A2 monodon high performance shrimp feed (2·2 mm fraction; INVE Aquaculture NV, Dendermonde, Belgium). The feeding ratio was 2·5% of the mean body weight (MBW) per day. Before starting the experiments, shrimp were transferred to the challenge room where they were acclimated for 2 days in PVC tanks.

Injection challenge in SPF shrimp

Challenge tests were conducted in quadruplicate with ten shrimps per replicate. After acclimation, shrimp were distributed to PVC tanks in which air stones and air tubes were installed. Those experimental materials were disinfected with sodium hypochlorite. The tanks were covered to prevent the shrimp from jumping out and to prevent cross-contamination by aerosols. Salinity was maintained at 35 g l^{-1}, pH = 7·9 to 8·2 and temperature at 28 ± 1°C during the experimental periods. To increase susceptibility to vibrios, shrimp were shocked with ammonium chloride at the concentration of 50 mg l^{-1} NH_4^+ (L.H. Phnoc *et al.*, unpublished data). The water was refreshed after 12 h of ammonium stress and replaced with new seawater containing 20 mg l^{-1} NH_4^+. Subsequently the shrimp were injected with 10^6 CFU of *V. campbellii* or 5×10^6 CFU of *V. harveyi* BB120. The bacteria were inoculated into shrimps by intramuscular injection between the second and third abdominal segment. Control shrimps were injected with an equal volume of FASW. After injection, each group was held in a separate 20 l plastic tank containing 5 l seawater (35 g l^{-1} salinity) at 28°C with aeration. Shrimp were fed with formulated shrimp feed twice a day up to 5 days. Forty-eight hours post-injection, the water was refreshed and replaced with new NH_4Cl-free seawater.

The relative percentage mortality

The relative percentage mortality (RPM) was calculated using the following formula:

$$\text{RPM}\ (\%) = \frac{\text{Percent mortality caused by non-luminescent isogenic strain}}{\text{Percent mortality caused by luminescent isogenic strain}} \times 100$$

Statistical analysis

Values of shrimp mortality (%) were Arcsin transformed to satisfy the requirement for a normal distribution.

Table 2 Luminescence and quorum-sensing molecules of luminescent and non-luminescent *V. campbellii* and *V. harveyi* BB120 strains

	V. harveyi BB120		*V. campbellii*		
Parameters	RR-L	RR-NL	RR-L	RR-NL	Control
Lu (RLU)	$5{\cdot}6 \times 10^7$	18	$2{\cdot}5 \times 10^7$	10·3	8·7
AI1	+	+	+	+	–
AI2	+	+	+	+	–
AI3	+	+	+	+	–

RR, rifampicin resistant; L, luminescent; NL, non-luminescent; Lu, luminescence; RLU, relative unit of luminescence reported by the Lumac Biocounter M2500 luminometer; AI, autoinducer.

Differences between treatments were evaluated by performing analysis of variance (ANOVA) using statistical analysis software SPSS (version 13·0 for Windows).

Results

The luminescence and quorum-sensing molecules of luminescent and non-luminescent strains

The luminescence of NL strains was very low compared to L strains (Table 2). Actually, the NL strains derived from *V. campbellii* and *V. harveyi* BB120 were completely dark since the obtained values were not significantly different from the control. The quorum-sensing molecules (AI1, AI2, AI3) were detected not only in L but also in NL strain of *V. harveyi* BB120 as well as in the NL strain of *V. campbellii*. The NL *V. campbellii* colonies seem to be more mucoid and less round shaped on MA compared to the L strains. The two types of colonies stained differently *in situ* with Congo red. The NL colonies were black while L colonies were red on MACR. The L and NL strains of *V. harveyi* BB120 and quorum-sensing mutants did not show any difference in colour on MACR.

Relative percentage mortality of *Artemia* after 36 h challenge with luminescent and non-luminescent *V. campbellii*

RPM caused by *V. campbellii* was 66 ± 7 and 70 ± 7% in experiment 1 and 2 respectively, meaning that luminescent *V. campbellii* caused higher mortality compared to the non-luminescent isogenic strain.

Relative percentage mortality of *Artemia* after 48 h challenge with luminescent and non-luminescent *Vibrio harveyi* BB120 and quorum-sensing mutant strains

In two experiments, all luminescent isogenic strains showed higher virulence compared to NL ones (Table 3). The RPM in the *Artemia* challenge with the wild type *V. harveyi* BB120 and quorum-sensing mutant strains ranged from 48% to 88% in experiment 1 and 53% to 81% in experiment 2. The mortality in the control treatment without *Vibrio* challenge was 18% and 13% in experiments 1 and 2, respectively.

Mortality of SPF *L. vannamei* after challenge with luminescent and non-luminescent *V. campbellii*

There was no significant difference in mortality between the groups challenged with luminescent and non-luminescent *V. campbellii*. Most shrimp died at around 24 h post-injection (Table 4). Cumulative mortality of luminescent and non-luminescent injection groups was more or less the same at 24, 48, 72, 96 and 120 hpi. At the end of the experiment, cumulative mortality in the *V. campbellii* injection groups ranged from 53% to 59% in experiment 1 and 38% to 40% in experiment 2.

Similar to *V. campbellii*, no significant difference in mortality was observed between the groups challenged with L and NL *V. harveyi* BB120. At the end of the experiment, the cumulative mortality in shrimp challenged with NL strains ranged from 33% to 40% and approximately 40% mortality was observed in shrimp challenged with L strains (Table 5).

Table 3 Relative percentage mortality of *Artemia* (mean ± SD) after 48 h challenge with NL strains of *V. harveyi* BB120 and isogenic mutants (L strains were used as control and set at 100%, not included in the table)

Treatments	Experiment 1	Experiment 2
V. harveyi BB120 (NL)	48 ± 7	53 ± 10
V. harveyi BB152 (NL)	65 ± 7	71 ± 6
V. harveyi BB170 (NL)	57 ± 7	57 ± 7
V. harveyi MM30 (NL)	61 ± 7	70 ± 18
V. harveyi BB886 (NL)	88 ± 16	81 ± 12

NL, non-luminescent; L, luminescent; values are means of four replicates.

Table 4 Cumulative shrimp mortality (%) after challenge with luminescent and non-luminescent *Vibrio campbellii*

Treatments	6 h	12 h	24 h	36 h	48 h	72 h	96 h	120 h
Experiment 1								
Control	0 ± 0	0 ± 0^{a}	0 ± 0^{a}	3 ± 6^{a}	3 ± 6^{a}	9 ± 6^{a}	9 ± 6^{a}	13 ± 10^{a}
VC (RS)	3 ± 6	41 ± 12^{b}	53 ± 19^{b}	56 ± 16^{b}	56 ± 16^{b}	56 ± 16^{b}	56 ± 16^{b}	59 ± 16^{b}
VC (NL)	0 ± 0	38 ± 10^{b}	50 ± 18^{b}	50 ± 18^{b}	50 ± 18^{b}	50 ± 18^{b}	53 ± 21^{b}	53 ± 21^{b}
VC (L)	0 ± 0	34 ± 6^{b}	53 ± 12^{b}	56 ± 16^{b}	56 ± 16^{b}	56 ± 16^{b}	56 ± 16^{b}	56 ± 15^{b}
Experiment 2								
Control	0 ± 0	0 ± 0^{a}	0 ± 0^{a}	3 ± 6^{a}	3 ± 6^{a}	6 ± 7^{a}	6 ± 7^{a}	6 ± 7^{a}
VC (RS)	3 ± 6	16 ± 12^{a}	22 ± 16^{a}	28 ± 12^{a}	34 ± 16^{b}	38 ± 18^{a}	38 ± 18^{b}	41 ± 21^{ab}
VC (NL)	0 ± 0	16 ± 12^{a}	9 ± 12^{a}	22 ± 19^{a}	25 ± 14^{ab}	31 ± 16^{a}	31 ± 16^{ab}	34 ± 12^{ab}
VC (L)	3 ± 6	13 ± 10^{a}	19 ± 16^{a}	25 ± 14^{a}	31 ± 16^{b}	34 ± 19^{a}	38 ± 14^{b}	38 ± 14^{b}

VC, *V. campbellii*; L, luminescent; NL, non-luminescent; RS, rifampicin-sensitive; values within a column with different superscripts are significantly different ($P < 0{\cdot}05$).

Table 5 Cumulative shrimp mortality (%) after challenge with luminescent and non-luminescent *Vibrio harveyi* BB120

Treatments	12 h	24 h	36 h	48 h	72 h	96 h	120 h
Experiment 1							
Control	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
BB120 (RS)	17 ± 6^{b}	23 ± 6^{b}	37 ± 6^{b}	37 ± 6^{b}	40 ± 10^{b}	40 ± 10^{b}	40 ± 10^{b}
BB120 (NL)	10 ± 0^{b}	23 ± 6^{b}	37 ± 6^{b}	37 ± 6^{b}	40 ± 0^{b}	40 ± 0^{b}	40 ± 0^{b}
BB120 (L)	13 ± 6^{b}	33 ± 6^{b}	37 ± 6^{b}	37 ± 6^{b}	37 ± 6^{b}	37 ± 6^{b}	37 ± 6^{b}
Experiment 2							
Control	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	3 ± 5^{a}	3 ± 5^{a}	3 ± 5^{a}
BB120 (RS)	13 ± 6^{a}	30 ± 0^{b}	37 ± 6^{b}	37 ± 6^{b}	37 ± 6^{b}	37 ± 6^{b}	37 ± 6^{b}
BB120 (NL)	10 ± 0^{a}	23 ± 6^{b}	30 ± 10^{b}	30 ± 10^{b}	33 ± 15^{b}	33 ± 15^{b}	33 ± 15^{b}
BB120 (L)	13 ± 6^{a}	30 ± 0^{b}	37 ± 6^{b}	37 ± 6^{b}	37 ± 6^{b}	37 ± 6^{b}	37 ± 6^{b}

No mortality was observed at 6 hpi; values within a column with different superscripts are significantly different ($P < 0{\cdot}05$).

Switching from non-luminescent to luminescent

As mentioned in Materials and methods section, many different ways were tried to make the NL strains switch back to the luminescent state, but none of the described procedures was successful in generating L strains from NL strains.

Discussion

In this study, the non-luminescent (NL) strains were produced by sub-culturing luminescent (L) strains in dark under static conditions. Probably the contact with the hydrophilic glass surface under static conditions helps L strains to switch to NL strains. *V. campbellii* was switching faster than *V. harveyi*. After 3 days of culturing luminescent *V. campbellii* in static condition, NL cells were detected. On the one hand, more than 2 months were needed before NL *V. harveyi* BB120 and quorum-sensing mutant strains started to appear (results not shown). A difference in morphology of L and NL *V. campbellii* colonies was noticed as the NL colonies were less round shaped on MA compared to the L ones. The difference in colony appearance was substantiated by the different staining on MACR. On the other hand, staining of *V. harveyi* BB120 and quorum-sensing mutant strains with Congo red did not reveal a difference between L and NL strains as seen in *V. campbellii*. In this study, three simultaneous phenotypic changes are described in *V. campbellii*, namely a change in staining on MACR, a switch from L to NL, and a switch from virulent to less virulent state. The latter two phenotypic changes were also obtained in *V. harveyi* BB120. This strongly argues against the idea that NL were obtained by a spontaneous mutation in the luciferase gene. Rather a phenotypic shift is likely to be responsible. A phenotypic shift has been described in other species under identical culture conditions (Bossier and Verstraete 1996; Drenkard and Ausubel 2002; Kussell *et al.* 2005). In *Comamonas testosteroni*, Bossier and Verstraete (1996) found two types of colonies on Luria-Bertani (LB) agar plates, namely colonies with mucoid appearance and colonies with a non-mucoid appearance. Also in that study, in absence of agitation and in contact with a glass surface, a culture with

predominantly non-mucoid-colony forming (NMCF) cells very rapidly shifted to a culture dominated by mucoid-colony forming (MCF) cells. A difference in morphology was also mentioned.

In this study, it was verified whether quorum sensing would be involved in the phenotype switch. Two types of experiments were performed to substantiate this hypothesis. It was found that *V. harveyi* strains with mutation in the AI1 and AI2 quorum-sensing pathway were still able to switch to NL strains. In addition, the production of quorum-sensing molecules in the NL strains was measured. It was verified that the quorum-sensing molecules (AI1, AI2, AI3) were still present in cultures of NL *V. harveyi* BB120 and *V. campbellii* (Table 2). Hence, the results did not reveal any support for quorum-sensing molecule production being drastically reduced in NL strains. Neither does quorum-sensing abolishment prevents L strains from becoming dark under static culture conditions. Hence, it is more likely that another mechanism is regulating this phenomenon.

The difference in virulence between the L and the NL strains of *V. campbellii* was demonstrable in *Artemia* but not in shrimp. In *Artemia*, the relative percentage mortality was less than 100% in all NL tested strains, indicating a reduced virulence of the non-luminescent isogenic strains. In the shrimp experiments the *Vibrio* was injected intramuscularly. Hence under these experimental circumstances the *Vibrio* does not need to colonize the gut and overcome barriers such as the mucosal layer, to finally infect otherwise sterile tissue. In *Artemia*, we assume that infection can only happen through the gut (Verschuere *et al.* 2000). Hence the reduced virulence of NL *V. campbellii* in *Artemia* might be caused by its reduced capability to infect the gut.

Bossier and Verstraete (1996) reported that the forward and backward shift between NMCF cells and MCF cells could be the result of a genetic switch mechanism. According to Van den Broek *et al.* (2005), colony phase variation is a regulatory mechanism at the DNA level which usually results in high frequency, reversible switches between different phenotype of colonies. Mutational events can be involved in the mechanism of phenotypic variation (Massey and Buckling 2002). The environment can regulate the rates of mutation at specific sites. Other mechanisms include elevated genome-wide mutation rates, environmentally regulated genome-wide mutation rates and elevated site-specific mutations.

In laboratory conditions, the switching between two phenotypes can be controlled by chemicals. For instance, the mucoid colonies can be switched to non-mucoid colonies by H_2O_2 (Bossier and Verstraete 1996). It was hypothesized that under stress of reactive oxygen, NL strains would switch to L. In this study, luminescence could not be restored by treatment with H_2O_2 or SNAP (NO generator). Co-culture of L and NL and passing the NL strains through the host were also not successful in generating NL strains. Hence, the loss of the described phenotypes in luminescent *Vibrio* strains does not seem to be caused by phase variation, instead rather a unidirectional phenotypic shift seems to occur.

In conclusion, luminescent *Vibrio* strains can switch to a non-luminescent state by culturing them in static conditions. Concomitant with the loss of luminescence, the NL strain stains less on MACR and becomes less virulent.

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