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The impact of the multichannel quorum sensing systems of *Vibrio tasmaniensis* and *Vibrio crassostreae* on virulence towards blue mussel (*Mytilus edulis*) larvae

Islam Shikder Saiful ^{1,2}, Zhang Shanshan ³, Eggermont Mieke ³, Bruto Maxime ⁴, Le Roux Frederique ⁴, Defoirdt Tom ^{3,*}

¹ Fisheries and Marine Resource Technology Discipline, Khulna University, Khulna, Bangladesh

² Institute for Marine and Antarctic Studies, University of Tasmania, Launceston, Australia

³ Center for Microbial Ecology and Technology (CMET), Ghent University, Ghent, Belgium

⁴ Station Biologique de Roscoff, Roscoff, France

* Corresponding author : Tome Defoirdt, email address : Tom.Defoirdt@UGent.be

Shanshan.Zhang@UGent.be ; Mieke.Eggermont@UGent.be ; frederique.le-roux@sb-roscoff.fr

Abstract :

Mussel culture in Europe is currently suffering from mass mortality events with mortality rates up to 100%. Vibrios belonging to the Splendidus clade have recently been implicated in these events. In this study, we demonstrate that *V. tasmaniensis* LGP32 and *V. crassostreae* J2-9, two model pathogens of oysters, are also pathogenic to blue mussel larvae in an immersion challenge test. After five days, LGP32 and J2-9 had killed 93 and 73% of challenged mussel larvae, respectively. Because quorum sensing, bacterial cell-to-cell communication with small signal molecules, has been demonstrated to control the virulence of various vibrios, we further investigated whether it had an impact on the virulence of *V. tasmaniensis* and *V. crassostreae*. We identified the components of a multichannel quorum sensing system (as also found in many other vibrios) in the genomes of both species. Knock out of selected components of this pathway revealed that in general quorum sensing in *V. tasmaniensis* LGP32 and *V. crassostreae* J2-9 has no impact on motility, protease activity, biofilm formation and virulence towards mussel larvae. Finally, the quorum sensing inhibitor cinnamaldehyde did not protect mussel larvae from these pathogens. Together, these data indicate that the multichannel quorum sensing systems of *V. tasmaniensis* LGP32 and *V. crassostreae* J2-9 have no impact on virulence of the bacteria towards blue mussel larvae. Hence, quorum sensing controlling virulence is not a general feature in vibrios as it has different outcomes on virulence in different species.

Highlights

► Splendidus clade vibrios are major pathogens of bivalves. ► *V. tasmaniensis* and *V. crassostreae* contain multichannel quorum sensing systems. ► These systems have no impact on virulence to blue mussel larvae. ► The link between quorum sensing and virulence is different for different vibrios.

Keywords : Bivalve, Splendidus clade, Larviculture, Immersion challenge, Quorum sensing

64 **1. Introduction**

65 Mussels are the most important aquaculture species in Europe based on live weight. The two main
66 cultivated species are the blue mussel (*Mytilus edulis*) and the Mediterranean mussel (*Mytilus*
67 *galloprovincialis*) (FAO, 2017). Recently, mussel culture in Europe has suffered from mass mortality events
68 with mortality rates up to 100%. In addition to environmental and genetic factors (Benabdelmouna and
69 Ledu, 2016; Polsenaere et al., 2017), bacterial pathogens have been linked to mussel mortality (Eggermont
70 et al., 2014; Ben Cheikh et al., 2016). Vibrios belonging to the *Splendidus* clade (including *V. crassostreae*
71 and *V. tasmaniensis*) are amongst the major bacterial pathogens of marine bivalves (Beaz-Hidalgo et al.,
72 2010; Travers et al., 2015; Le Roux et al., 2016; Dubert et al., 2017) and *V. splendidus* strains have also
73 been implied in mussel mortality events (Ben Cheikh et al., 2016; Oden et al., 2016; Eggermont et al.,
74 2017). The implication of vibrios in mortality events in mussels is a very recent observation and the
75 pathogenicity mechanisms are currently unknown.

76 Quorum sensing, bacterial cell-to-cell communication with small signal molecules, is known to control
77 the virulence of many bacteria, including vibrios, and inactivation of these systems often decreases the
78 virulence of these pathogens (Milton, 2006; Defoirdt, 2014). Vibrios usually contain multichannel quorum
79 sensing systems, in which multiple signal molecules determine the output of the system (Figure 1). Three
80 types of signal molecules can be produced by vibrios (although not all vibrios produce all types): acylated
81 homoserine lactones (AHLs), autoinducer-2 (AI-2), and cholerae autoinducer-1 (CAI-1) (Milton, 2006). The
82 concentrations of the signal molecules determine the cellular level of the quorum sensing master regulator
83 (a homolog of *V. harveyi* LuxR), which in turn controls the expression of many genes (van Kessel et al.,
84 2013). Virulence-related phenotypes that are controlled by this kind of systems include motility, biofilm
85 formation, and the production of lytic enzymes such as proteases (Sultan et al., 2006; Tian et al., 2008;
86 Natrah et al., 2011; Defoirdt, 2014; Yang and Defoirdt, 2015). Moreover, multichannel quorum sensing
87 systems have been documented to be required for full virulence of vibrios towards various host organisms
88 (Ye et al., 2008; Bjelland et al., 2012; Defoirdt and Sorgeloos, 2012), and inhibitors of these systems (such
89 as cinnamaldehyde, brominated furanones and brominated thiophenones) protect aquatic animals from
90 vibriosis (Defoirdt et al., 2006; Brackman et al., 2008; Yang et al., 2015).

91 In this study, we aimed at identifying multichannel quorum sensing systems in these pathogens, and
92 at determining the impact of these systems on the virulence of *V. tasmaniensis* and *V. crassostreae*
93 towards blue mussel larvae.

94

95

96 2. Materials and methods

97 2.1. Bacterial strains and growth conditions

98 *V. tasmaniensis* and *V. crassostreae* strains used in this study are described in **Table 1**. The bacteria were
99 grown in marine LB broth (m-LB; tryptone 10 g/l, yeast extract 5 g/l, Instant Ocean synthetic sea salt 35
100 g/l) at 18°C with shaking (100 rpm). Cell densities were determined spectrophotometrically at 600 nm.

102 2.2. Identification of multichannel quorum sensing system genes in *V. tasmaniensis* and *V.* 103 *crassostreae* and confirmation of the activity of the quorum sensing system

104 GenBank was screened for homologs of components of the *V. campbellii* multichannel quorum sensing
105 system in *V. tasmaniensis* and *V. crassostreae* by performing BLAST searches at the website of the National
106 Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

107 The activity of the quorum sensing system of the vibrios was assessed by introducing the cosmid pBB1
108 (Bassler et al., 1993) into natural rifampicin resistant mutants through conjugation using the donor strain
109 *E. coli* SM10 (λ pir). The pBB1 cosmid contains a tetracycline resistance gene and the *V. campbellii lux*
110 operon under control of its native promoter and is activated by homologs of the *V. campbellii* quorum
111 sensing master regulator LuxR. Hence, vibrios with an active quorum sensing system will produce quorum
112 sensing-dependent bioluminescence. Transconjugants containing the pBB1 cosmid were selected on agar
113 containing rifampicin (100 mg/l) and tetracycline (20 mg/l).

115 2.3. Construction of *V. tasmaniensis* and *V. crassostreae* quorum sensing mutants

116 Deletion of selected genes was performed by allelic exchange using pSW7848T (Val et al., 2012), a R6K γ -
117 *ori*-based suicide vector. This vector encodes the *ccdB* toxin gene under the control of an arabinose-
118 inducible and glucose-repressible promoter, P_{BAD} (Le Roux et al., 2007). Briefly, two 500 bp fragments
119 flanking the gene to delete were amplified and cloned into pSW7848T using the Gibson assembly method
120 according to the manufacturer's instructions (New England Biolabs, NEB). The construct was transferred
121 from an *E. coli* donor strain to the *Vibrio* LGP32 or J2-9 by conjugation as described previously (Le Roux et
122 al., 2007). Subsequently, the first and second recombination leading to pSW7848T integration and
123 elimination was selected on chloramphenicol + glucose and arabinose media, respectively. After re-
124 isolations, the gene deletion was confirmed by PCR using external primers.

125

126

127 2.4. Motility, protease and biofilm formation assays

128 Swimming motility was assessed as described previously by [Yang and Defoirdt \(2015\)](#). Briefly, 5 µl aliquots
129 of the isolates ($OD_{600} = 1$) were inoculated in the centre of LB₃₅ soft agar (0.2 % agar) plates. Plates were
130 incubated upright at 18°C, and motility halos were measured after 24 h.

131 Caseinase and gelatinase activities were determined according to [Natrah et al. \(2011\)](#). For each assay,
132 overnight grown cultures were diluted to an OD_{600} of 0.5, and 5 µl aliquots of the diluted cultures were
133 inoculated to the centre of the test plates. Caseinase and gelatinase activities were assessed on agar plates
134 containing 2% skimmed milk powder and 0.5% gelatin, respectively. Colony and clearing zone diameters
135 were measured after 4 and 7 days incubation at 18°C for caseinase and gelatinase, respectively. In order
136 to visualise the clearing zones in the gelatinase assay, saturated ammonium sulphate (80% solution in
137 distilled water) was poured over the plates.

138 Biofilm formation was measured by crystal violet staining as described by [Brackman et al. \(2008\)](#).
139 Briefly, overnight grown cultures were diluted to OD_{600} of 0.5 and inoculated into the wells of a 96-well
140 polystyrene plate. The plates were incubated for 24h. After incubation, the OD_{600} of the wells were
141 measured. After this, the wells were rinsed 3 times with tap water, and stained for 20 min with a 0.1%
142 crystal violet solution. The stain was removed, wells were rinsed 3 times with tap water, and air dried. The
143 dye bound to adherent cells was redissolved in 95% ethanol, and absorbance was measured at 570 nm
144 and expressed relative to the OD_{600} .

145

146 2.5. Challenge tests with blue mussel D-larvae

147 Challenge tests were performed as described previously ([Eggermont et al., 2017](#)). Wild-caught mature blue
148 mussels were stimulated to spawn by thermal shocks in sterile sea water at 5°C and 20°C until gametes
149 were released. Spawning males and females were transferred to sterile plastic cups containing 50 ml sterile
150 sea water and allowed to spawn for 15 minutes. Sperm and eggs were collected and gently mixed at a 10:1
151 ratio in a beaker containing 1 l of sterile sea water. After the appearance of polar bodies, the eggs were
152 gently rinsed with sterile sea water using a sterile 30 µm sieve to remove excess sperm. Fertilized eggs
153 were incubated in 2 l of sterile sea water (max 100 eggs/ml) containing chloramphenicol, nitrofurazone
154 and enrofloxacin (each at 10 mg/l). After two days of incubation, D-larvae were harvested on a sterile 60
155 µm sieve. The larvae were washed gently with sterile sea water to remove the antibiotics. Rinsed D-larvae
156 were transferred to a beaker containing 1 l of sterile sea water and distributed uniformly using a plunger.
157 Subsamples were taken to calculate the larval density, and the density was corrected in order to obtain a
158 final concentration of 250 larvae/ml. All manipulations were performed under a laminar flow hood.

159 One ml aliquots of the larval suspension in synthetic sea water supplemented with 10 mg/l tryptone
160 and 5 mg/l yeast extract were subsequently transferred to 24-well plates. The final larval density was 200
161 larvae/ml. Vibrios were inoculated into the rearing water at 10^5 cells/ml. In the experiment with
162 cinnamaldehyde, 10 μ M of cinnamaldehyde (Sigma) was added to the wells (Pande et al., 2013). Larvae to
163 which no bacteria were added and that were otherwise treated in the same way as challenged larvae,
164 were used as controls. Each treatment was performed in 24 replicates. The plates were incubated at 18°C.
165 Each day, four replicates per treatment were stained with lugol (5% (v/v)), and stained larvae were counted
166 under a binocular microscope (*Nikon Eclipse E 200, Nikon Instruments Europe*). Larvae were considered
167 alive when stained black by lugol, death if only parts of the larvae were stained or if shells were empty.
168

169 2.6. Statistics

170 All statistical analyses were performed using the SPSS software, version 24. A significance level of 1% was
171 used in all analyses.
172

173

174 3. Results and discussion

175 3.1. Virulence of *V. crassostreae* J2-9 to blue mussel larvae

176 *V. tasmaniensis* LGP32 and *V. crassostreae* J2-9 are well-known pathogens of oysters, that have been used
177 as model strains to explore virulence mechanisms and immune responses in oysters (Duperthuy et al.,
178 2010; Duperthuy et al., 2011; Toffiano-Nioche et al., 2012; Lemire et al., 2015; Vanhove et al., 2015; Le
179 Roux et al., 2016; Bruto et al., 2017). Presence of genetically related strains has also been documented in
180 mussels (Vezzulli et al., 2015). We previously reported that *V. tasmaniensis* LGP32 is also pathogenic to
181 blue mussel larvae (Eggermont et al., 2017). In order to determine pathogenicity of *V. crassostreae* J2-9 to
182 blue mussel larvae, we used the same immersion challenge test with sterile D-larvae (Eggermont et al.,
183 2017). *V. crassostreae* J2-9 showed to be pathogenic to the larvae, leading to significant mortality from
184 day 2 onwards when compared to the unchallenged control (independent samples t-tests, $P < 0.01$)
185 (Figure 2).
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190 3.2. Identification of components of a multichannel quorum sensing system in the genomes of
191 *V. tasmaniensis* and *V. crassostreae* and confirmation of the activity of the system

192 Similar to many other *Vibrio* spp., vibrios belonging to the *Splendidus* clade have recently been suggested
193 to contain multichannel quorum sensing systems (Zhang and Lee, 2021). Based on the genome sequences
194 that are available in GenBank (Le Roux et al., 2009; Wang et al., 2013; Lemire et al., 2015), we identified
195 homologs of the components of the *V. campbellii* multichannel quorum sensing system in the genomes of
196 *V. tasmaniensis* and *V. crassostreae* (Table 2). This suggests that these two species also contain a
197 multichannel quorum sensing system, with AHL, AI-2 and CAI-1 as signal molecules and a signal
198 transduction cascade with LuxU, LuxO and LuxR, and is consistent with detection of AHLs, AI-2 and CAI-1
199 in *Splendidus* clade vibrios (including *V. splendidus*, *V. chagasii* and *V. pomeroyi*) by using signal molecule
200 reporter strains (Yang et al., 2011). Recently, Girard et al. (2017) reported that *V. tasmaniensis* LGP32
201 produces various AHLs, which were identified by mass spectrometry.

202 The activity of the quorum sensing system was investigated by conjugating the cosmid pBB1 into the
203 vibrios. This cosmid contains the *V. campbellii* *lux* operon under control of its native promoter and is
204 activated by homologs of the *V. campbellii* quorum sensing master regulator LuxR. We could confirm
205 activity of the *V. tasmaniensis* quorum sensing system as *V. tasmaniensis* LGP32 pBB1 produced the typical
206 V-shaped bioluminescence pattern that is the hallmark of quorum sensing-regulated bioluminescence in
207 vibrios (Figure 3). Unfortunately, it was not possible to introduce cosmid pBB1 into *V. crassostreae* J2-9 as
208 this strain is naturally resistant to tetracycline, the antibiotic required to select pBB1 carrying bacteria.

210 3.3. Impact of quorum sensing on motility, protease activity and biofilm formation of *V.*
211 *tasmaniensis* and *V. crassostreae*

212 To explore their role in QS-related phenotypes, the *luxM*, *luxS* and *luxR* genes were successfully deleted in
213 *V. tasmaniensis* LGP32, and the *luxM*, *luxS* and *cqsA* genes were deleted in *V. crassostreae* J2-9. We
214 determined the impact of these knock outs on phenotypes that have been previously associated with
215 quorum sensing-regulated virulence of other vibrios, i.e. motility, protease activity and biofilm formation
216 (Defoirdt, 2014). Both *V. tasmaniensis* LGP32 and *V. crassostreae* J2-9 were highly motile (Table 3). There
217 were no significant differences between wild types and mutants, except for the *luxM* deletion mutant of
218 *V. tasmaniensis* LGP32 (which was more motile than the wild type) and the *cqsA* deletion mutant of *V.*
219 *crassostreae* J2-9 (which showed lower motility than the wild type). The higher and lower motility of the
220 *luxM* and *cqsA* deletion mutant of *V. tasmaniensis* and *V. crassostreae*, respectively, might be explained
221 by a (yet unknown) signal transduction pathway that does not involve the known shared signal

222 transduction pathway (using LuxU, LuxO and LuxR). A similar effect has been previously observed for the
223 *vhh* hemolysin gene in *V. harveyi*, which was differentially expressed in a *luxS* deletion mutant, whereas
224 inactivation of other components of the multichannel quorum sensing system had no effect
225 (Ruwandepika et al., 2011). Another explanation for the higher motility of the *luxM* mutant of
226 *V. tasmaniensis* is that both the production of AHLs and motility are energy-consuming processes
227 (McCarter, 2001; Keller and Surette, 2006) and consequently, inactivation of the AHL synthase LuxM might
228 result in increased cellular energy levels which in turn might lead to higher motility. Further, the strains
229 tested positive for caseinase and gelatinase activities, with no significant differences in these activities
230 between wild types and quorum sensing mutants. Finally, all strains were capable to form biofilms on
231 polystyrene (Table 3). The quorum sensing mutants of *V. tasmaniensis* showed lower biofilm formation
232 than the wild type. However, the difference was only significant for the *luxS* deletion mutant. There were
233 no significant differences between wild type and mutants of *V. crassostreae*.

234 235 3.4. Impact of the multichannel quorum sensing system of *V. tasmaniensis* and *V. crassostreae* 236 on virulence towards blue mussel larvae

237 We further compared mortality in blue mussel larvae caused by wild types and quorum sensing mutants
238 of *V. tasmaniensis* and *V. crassostreae*. For *V. tasmaniensis*, there were no significant differences in
239 virulence between wild type and quorum sensing mutants (Figure 4A). For *V. crassostreae* a slightly (but
240 significantly) increased mortality was observed for larvae challenged with the *luxS* deletion mutant when
241 compared to larvae challenged with the wild type from day 2 onwards (independent samples t-test, $P <$
242 0.01) (Figure 4B). Survival of larvae challenged with the *luxM* and *cqsA* deletion mutants was similar to
243 that of larvae challenged with the wild type.

244 In a last experiment, we used a chemical biological approach to block the multichannel quorum sensing
245 systems of *V. tasmaniensis* and *V. crassostreae* by adding cinnamaldehyde to the rearing water.
246 Cinnamaldehyde has been shown before to block the multichannel quorum sensing in vibrios by inhibiting
247 the DNA-binding activity of the quorum sensing master regulator LuxR, and the compound blocked the
248 virulence of *V. harveyi* towards brine shrimp and giant river prawn larvae (Brackman et al., 2008; Pande et
249 al., 2013). However, no significant differences in survival of mussel larvae challenged with *V. tasmaniensis*
250 or *V. crassostreae* were observed after treatment with cinnamaldehyde (Figure 5). Together, our data
251 indicate that inactivation of the quorum sensing system of *V. tasmaniensis* and *V. crassostreae* does not
252 attenuate their virulence. A multichannel quorum sensing system has been reported to be required for full
253 virulence in several *Vibrio* species, such as *V. alginolyticus*, *V. campbellii* and *V. salmonicida* (Ye et al., 2008;

254 Bjelland et al., 2012; Defoirdt and Sorgeloos, 2012), whereas it has no effect on the virulence for *V.*
255 *tasmaniensis* and *V. crassostreae*, which is similar to what has been reported for *V. anguillarum* (Li et al.,
256 2018).

257
258

259 4. Conclusions

260 In this study, we identified the components of a multichannel quorum sensing system in the genomes of
261 the bivalve model pathogens *V. tasmaniensis* LGP32 and *V. crassostreae* J2-9. We found that motility is
262 controlled by HAI-1 in *V. tasmaniensis* and by CAI-1 in *V. crassostreae* and that biofilm formation is
263 controlled by AI-2 quorum sensing in *V. tasmaniensis*, whereas the multichannel quorum sensing systems
264 of *V. tasmaniensis* and *V. crassostreae* have no effect on protease activity, and on virulence of the
265 pathogens towards blue mussel larvae. Hence, since quorum sensing controlling virulence is not a general
266 feature in vibrios, it is not possible to extrapolate the impact of quorum sensing on virulence of vibrios
267 from one species to another.

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270 5. References

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435 **Tables**

436 **Table 1.** Strains used in this study.

| Strain | Genotype | Reference |
|------------------------------|---|---------------------------------------|
| <i>V. tasmaniensis</i> LGP32 | | |
| LGP32 WT | Wild type from which the <i>luxM</i> , <i>luxS</i> and <i>luxR</i> deletion mutants were derived. | Le Roux et al. (2009) |
| LGP32 <i>luxM</i> | Deletion of <i>luxM</i> (AHL synthase gene) | This study |
| LGP32 <i>luxS</i> | Deletion of <i>luxS</i> (AI-2 synthase gene) | This study |
| LGP32 <i>luxR</i> | Deletion of <i>luxR</i> (quorum sensing master regulator) | This study |
| <i>V. crassostreae</i> J2-9 | | |
| J2-9 WT | Wild type from which the <i>luxM</i> , <i>luxS</i> and <i>cqsA</i> deletion mutants were derived. | Lemire et al. (2015) |
| J2-9 <i>luxM</i> | Deletion of <i>luxM</i> (AHL synthase gene) | This study |
| J2-9 <i>luxS</i> | Deletion of <i>luxS</i> (AI-2 synthase gene) | This study |
| J2-9 <i>cqsA</i> | Deletion of <i>cqsA</i> (CAI-1 synthase gene) | This study |

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444 **Table 2.** Homologs of components of the *V. campbellii* ATCC BAA-1116 (Vca) multichannel quorum sensing system identified within the genomes
 445 of *V. tasmaniensis* LGP32 (Vta) and *V. crassostreae* J2-9 (Vcr), and nucleotide identities between the homologs.

| Gene | Function | Locus tag ¹ Vca (chromosome) | Locus tag ¹ Vta (chromosome) | Locus tag ¹ Vcr | % identity Vca-Vta (% coverage) | % identity Vca-Vcr (% coverage) | % identity Vta-Vcr (% coverage) |
|-------------|---------------------|--|--|----------------------------|------------------------------------|------------------------------------|------------------------------------|
| <i>luxM</i> | AHL synthesis | M892_17055 (chr I) | VS_RS16040 (chr II) | VCR9J2v1_1350180 | 70 (95) | 72 (96) | 77 (94) |
| <i>luxN</i> | AHL detection | M892_17050 (chr I) | VS_RS16035 (chr II) | VCR9J2v1_1350181 | 73 (95) | 76 (96) | 80 (95) |
| <i>luxS</i> | AI-2 synthesis | M892_13670 (chr I) | VS_RS12075 (chr I) | VCR9J2v1_700111 | 81 (100) | 81 (100) | 89 (100) |
| <i>luxP</i> | AI-2 detection | M892_24610 (chr II) | VS_RS16480 (chr II) | VCR9J2v1_110050 | 68 (80) | 68 (81) | 84 (100) |
| <i>luxQ</i> | AI-2 detection | M892_24605 (chr II) | VS_RS16485 (chr II) | VCR9J2v1_110051 | 64 (57) | 64 (63) | 81 (99) |
| <i>cqsA</i> | CAI-1 synthesis | M892_21495 (chr II) | VS_RS08055 (chr I) | VCR9J2v1_720342 | 76 (90) | 75 (90) | 83 (99) |
| <i>cqsS</i> | CAI-1 detection | M892_21490 (chr II) | VS_RS08060 (chr I) | VCR9J2v1_720343 | 72 (98) | 73 (98) | 83 (99) |
| <i>luxU</i> | Signal transduction | M892_16185 (chr I) | VS_RS04560 (chr I) | VCR9J2v1_50013 | 69 (56) | 71 (26) ² | 83 (100) |
| <i>luxO</i> | Signal transduction | M892_16180 (chr I) | VS_RS04555 (chr I) | VCR9J2v1_50012 | 76 (95) | 77 (95) | 88 (100) |
| <i>luxR</i> | Master regulator | M892_13795 (chr I) | VS_RS11960 (chr I) | VCR9J2v1_700132 | 77 (86) | 79 (86) | 89 (100) |

¹locus tags refer to the genome sequences in Genbank: Vca chr. I: CP006605; Vca chr. II: CP006606; Vta chr. I: NC_011753; Vta chr. II: NC_011744; Vcr: CCJY0000000.1

²60% identity at amino acid level (70% coverage)

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450 **Table 3.** Motility, caseinase and gelatinase activities and biofilm formation of *V. tasmaniensis* LGP32 and
 451 *V. crassostreae* J2-9 wild types and quorum sensing mutants (average \pm standard deviation of three
 452 independent experiments).

| Strain | Motility halo ¹ | Caseinase activity ² | Gelatinase activity ² | Biofilm formation ³ |
|------------------------------|----------------------------|---------------------------------|----------------------------------|--------------------------------|
| <i>V. tasmaniensis</i> LGP32 | | | | |
| LGP32 WT | 62 \pm 4 | 1.4 \pm 0.1 | 2.6 \pm 0.2 | 0.22 \pm 0.03 |
| LGP32 <i>luxM</i> | 78 \pm 2* | 1.4 \pm 0.0 | 2.7 \pm 0.1 | 0.14 \pm 0.03 |
| LGP32 <i>luxS</i> | 72 \pm 5 | 1.6 \pm 0.1 | 2.6 \pm 0.2 | 0.11 \pm 0.02* |
| LGP32 <i>luxR</i> | 63 \pm 3 | 1.4 \pm 0.1 | 2.7 \pm 0.2 | 0.18 \pm 0.04 |
| <i>V. crassostreae</i> J2-9 | | | | |
| J2-9 WT | 72 \pm 5 | 1.4 \pm 0.0 | 2.5 \pm 0.2 | 0.17 \pm 0.04 |
| J2-9 <i>luxM</i> | 72 \pm 4 | 1.4 \pm 0.0 | 2.6 \pm 0.2 | 0.13 \pm 0.02 |
| J2-9 <i>luxS</i> | 75 \pm 4 | 1.4 \pm 0.0 | 2.7 \pm 0.2 | 0.19 \pm 0.01 |
| J2-9 <i>cqsA</i> | 56 \pm 3* | 1.3 \pm 0.1 | 2.6 \pm 0.2 | 0.22 \pm 0.02 |

¹ Diameter of the motility zone (mm)

² Ratio between the activity zone and the colony diameter

³ OD₅₇₀ of crystal violet-stained biofilms on polystyrene multiwell plates/OD₆₀₀

* Significantly different from the wild type (independent samples t-test; P < 0.01)

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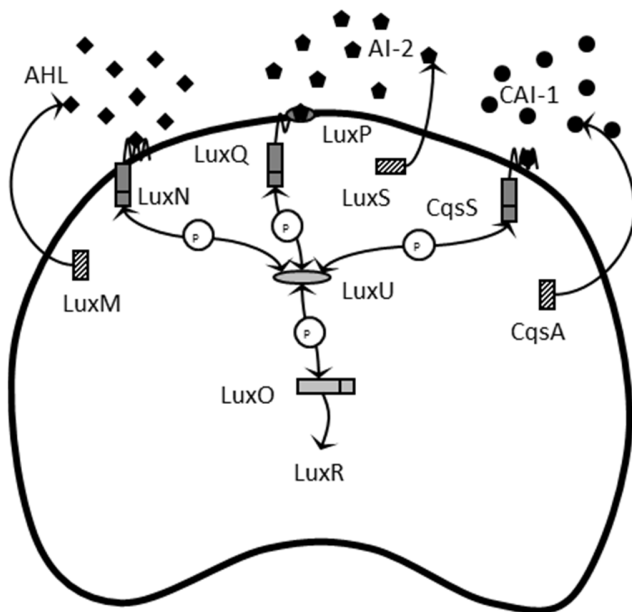
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469 **Figures**



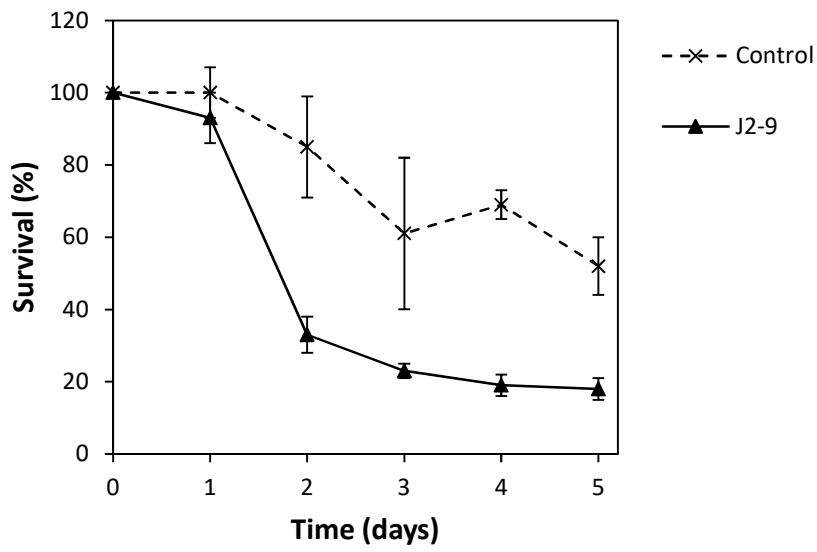
470 **Figure 1.** Generic scheme of multichannel quorum sensing systems of vibrios. The LuxM, LuxS and CqsA
471 enzymes synthesise acylated homoserine lactone (AHL), autoinducer-2 (AI-2) and cholerae autoinducer-1
472 (CAI-1), respectively. These signal molecules are detected at the cell surface by the LuxN, LuxPQ and CqsS
473 receptors, respectively. The receptors feed a shared phosphorylation/ dephosphorylation signal
474 transduction cascade involving the LuxU and LuxO proteins. This cascade controls the production of the
475 master regulator LuxR. The level of LuxR is proportional to the concentration of the signal molecules, and
476 LuxR determines the transcription of the quorum sensing target genes. "P" denotes phosphotransfer.
477 Based on Defoirdt et al. (2008).
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483 **Figure 2.** Survival of blue mussel D-larvae challenged with *V. crassostreae* J2-9. Error bars represent the
 484 standard deviation of four mussel cultures. "Control" refers to unchallenged larvae that were otherwise
 485 treated the same as the other larvae.
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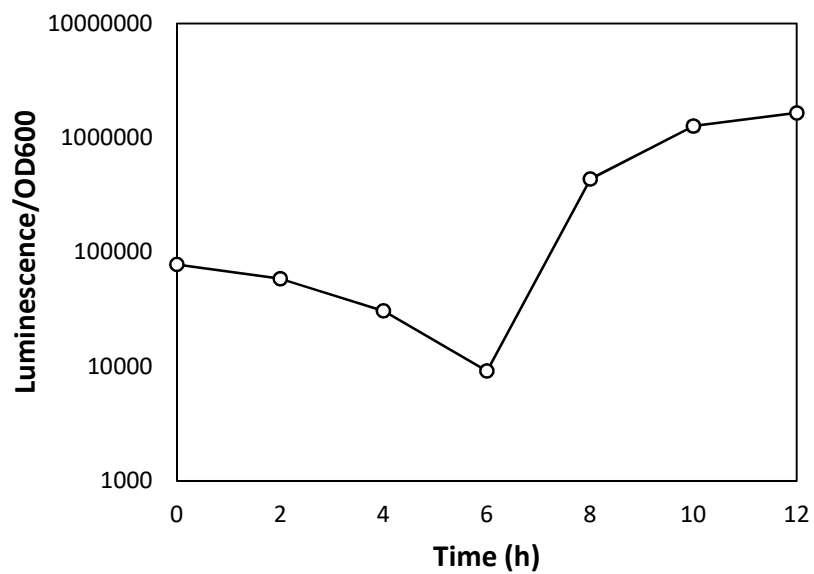
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502 **Figure 3.** Quorum sensing-dependent bioluminescence of *V. tasmaniensis* LGP32 pBB1. The data points
503 are averages of three replicates. Error bars are too small to be visible on the graph.

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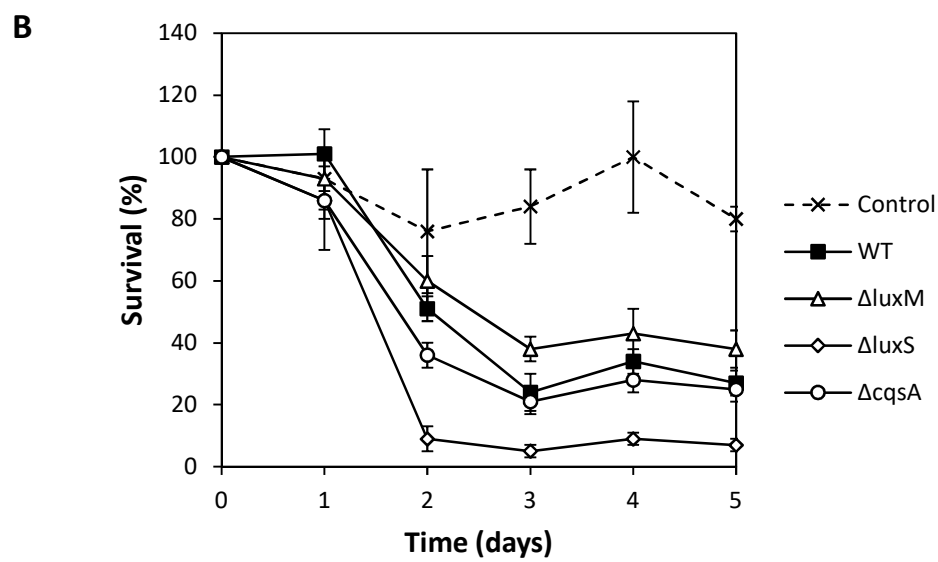
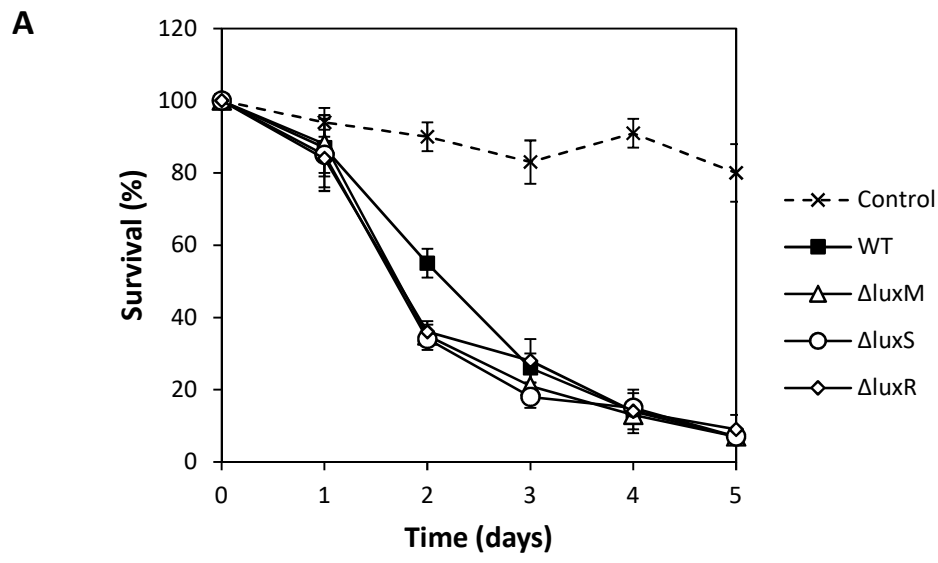
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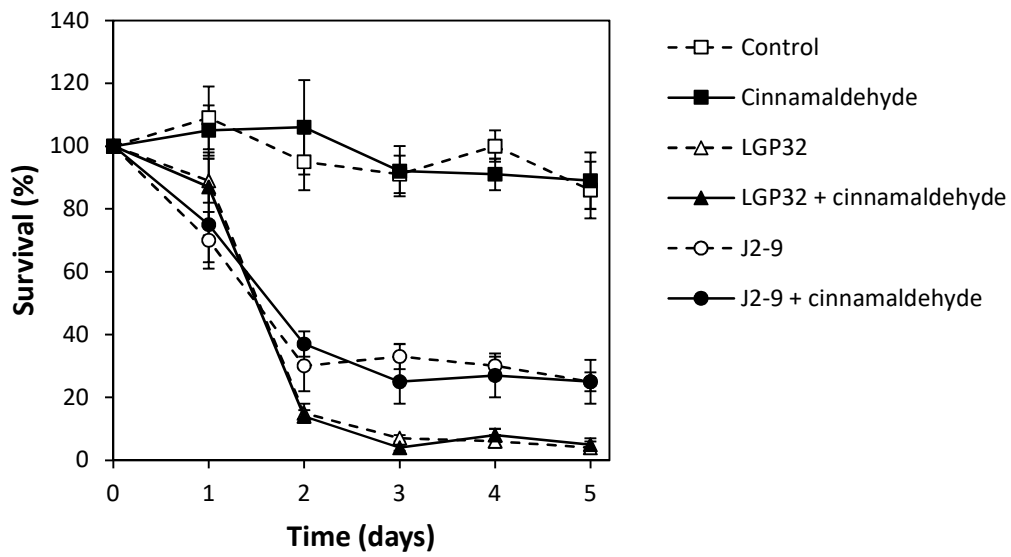
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519 **Figure 4.** Survival of blue mussel D-larvae challenged with *V. tasmaniensis* LGP32 (panel A) and *V.*
 520 *crassostreae* J2-9 (panel B) wild type (WT) and quorum sensing mutants. Error bars represent the standard
 521 deviation of four mussel cultures. “Control” refers to unchallenged larvae that were otherwise treated the
 522 same as the other larvae.

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 529 **Figure 5.** Survival of blue mussel D-larvae challenged with *V. tasmaniensis* LGP32 or *V. crassostreae* J2-9,
 530 with and without the quorum sensing inhibitor cinnamaldehyde (added at 10 μ M to the rearing water at
 531 the start of the experiment). Error bars represent the standard deviation of four mussel cultures. "Control"
 532 refers to unchallenged larvae that were otherwise treated the same as the other larvae.
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