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# Quorum sensing negatively regulates chitinase in *Vibrio harveyi*

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## Summary

**Quorum sensing, bacterial cell-to-cell communication, regulates the virulence of *Vibrio harveyi* towards different hosts. Chitinase can be considered as a virulence factor because it helps pathogenic bacteria to attach to the host and to penetrate its tissues (e.g. in case of shrimp). Here, we show that quorum sensing negatively regulates chitinase in *V. harveyi*. Chitinolytic activity towards natural chitin from crab shells, the synthetic chitin derivative chitin azure, and fluorogenic chitin oligomers was significantly higher in a mutant in which the quorum-sensing system is completely inactivated when compared with a mutant in which the system is maximally active. Furthermore, the addition of signal molecule containing cell-free culture fluids decreased chitinase activity in a Harveyi Autoinducer 1 and Autoinducer 2-deficient double mutant. Finally, chitinase A mRNA levels were fivefold lower in the mutant in which the quorum-sensing system is maximally active when compared with the mutant in which the system is completely inactivated. [Correction added on 25 September 2009, after first online publication: the preceding sentence was corrected from 'Finally, chitinase A mRNA levels were fivefold lower in the mutant in which the quorum-sensing system is completely inactivated when compared with the mutant in which the system is maximally active.'] We argue that this regulation might help the vibrios to switch between host-associated and free-living life styles.**

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## Introduction

Chitin is a polymer of β-1,4-linked N-acetyl-D-glucosamine and is the second most abundant biopolymer on earth (Keyhani and Roseman, 1999). Vibrios and other marine bacteria can utilize chitin as the sole carbon and nitrogen source by producing chitinases and chitin-binding proteins (Colwell, 1996; Svitil *et al.*, 1997). The chitinolytic proteins are located extracellularly, in the cell envelope, the periplasmic space, the inner membrane and the cytoplasm (Keyhani and Roseman, 1999). Suginta and co-workers reported constitutive chitinase activity in *Vibrio harveyi* and closely related species (Suginta *et al.*, 2000). However, the chitinase activity increased approximately twofold in the presence of chitin. Svitil and co-workers reported that *V. harveyi* excretes a total of 10 different chitin-degrading proteins (Svitil *et al.*, 1997). Some of the enzymes were produced in the presence of different forms of chitin, whereas others were unique to a particular chitin. In addition to the regulation by chitin and N-acetyl-D-glucosamine mono- and oligomers, chitinase activity appears to be highly regulated by other factors as well. Quorum sensing, bacterial cell-to-cell communication with small signal molecules, has been reported to positively regulate chitinase activity in *Chromobacterium violaceum*. Indeed, a *C. violaceum* mutant defective in the production of the signal N-hexanoyl-L-homoserine lactone was completely deficient in chitinase activity, whereas the addition of the signal restored wild-type chitinase activity (Chernin *et al.*, 1998).

Quorum sensing has also been extensively studied in *V. harveyi*. *Vibrio harveyi* uses a three-channel quorum-sensing system. The three channels of this system are mediated by the Harveyi Autoinducer 1 (HAI-1), the Autoinducer 2 (AI-2) and the Cholerae Autoinducer 1 (CAI-1) (Cao and Meighen, 1989; Chen *et al.*, 2002; Higgins *et al.*, 2007). The three autoinducers are detected at the cell surface by membrane-bound, two-component receptor proteins that feed a common phosphorylation/dephosphorylation signal transduction cascade (Taga and Bassler, 2003). Central in the quorum sensing signal

**Table 1.** Bacterial strains used in this study.

| Strain | Relevant features | Reference |
|---|---|---|
| BB120 | Wild-type frm which strains JAF483, JAF548 and MM77 were derived | Bassler *et al.* (1997) |
| JAF483 | LuxO locked in high cell density conformation | Freeman and Bassler (1999) |
| JAF548 | LuxO locked in low cell density conformation | Freeman and Bassler (1999) |
| MM77 | HAI-1 and AI-2 deficient (mutation in *luxM* and *luxS*) | Mok *et al.* (2003) |

transduction cascade is the LuxO protein. Phosphorylated LuxO indirectly inhibits production of the transcriptional regulator protein LuxR (which binds to the promoter region of quorum sensing-regulated genes), whereas unphosphorylated LuxO is incapable of inhibition of LuxR production (due to a conformational change) (Tu and Bassler, 2007). Phenotypes that were found to be controlled by quorum sensing in *V. harveyi in vitro* include bioluminescence (Bassler *et al*., 1993) and the production of several virulence factors such as a type III secretion system (Henke and Bassler, 2004a), extracellular toxin (Manefield *et al*., 2000), metalloprotease (Mok *et al*., 2003) and a siderophore (Lilley and Bassler, 2000). Moreover, quorum sensing has been shown to regulate virulence of luminescent vibrios towards different hosts *in vivo* (for a review see Defoirdt *et al*., 2008).

Chitinase production by pathogenic vibrios can be considered as a virulence factor *per se* because it helps the bacteria to penetrate host tissues containing chitin (Aguirre-Guzmán *et al*., 2004). Interestingly, recent research has revealed that in *Vibrio cholerae*, chitin-binding proteins are also important for the colonization of the host by mediating attachment to the epithelium (which surface ligands contain N-acetyl-D-glucosamine) (Kirn *et al*., 2005; Bhomwick *et al*., 2008). Moreover, DebRoy and colleagues (2006) reported that chitinase A of *Legionella pneumophila* is involved in persistence in the lung in a mouse model of Legionnaires' disease, emphasizing the important role chitin-degrading proteins may have in infecting the host. However, regulation of chitinase by quorum sensing in vibrios has not yet been studied and therefore, in this study, we aimed at investigating whether quorum sensing has an impact on chitinolytic activity and chitinase expression in *V. harveyi*.

## Results and discussion

### *The effect of quorum sensing on degradation of natural chitin and the synthetic chitin derivative chitin azure*

In order to test whether quorum sensing regulates chitinase activity, we used the *V. harveyi luxO* mutants JAF483 and JAF548 (Table 1), which contain a point mutation in *luxO*, resulting in LuxO proteins locked in the high and low cell-density conformation respectively (Freeman and Bassler, 1999). The LuxO protein is central in the quorum-sensing signal transduction cascade and controls the production of the transcriptional regulator protein LuxR (which binds to the promoter region of quorum sensing-regulated genes, e.g. the bioluminescence operon) (Taga and Bassler, 2003). Hence, the quorum-sensing system is completely inactivated in mutant JAF548 (rendering it dark) and maximally activated in mutant JAF483 (making it constitutively luminescent), irrespective of cell density or signal molecule concentration. Chitinase activity was measured as described previously (Chernin *et al*., 1998). Briefly, dense cultures of the wild type and mutants were diluted to an $OD_{600}$ of 0.5 and spotted in quadruplicate onto Luria–Bertani (LB) agar containing 20 g $l^{-1}$ NaCl and 0.1% $\alpha$-chitin from crab shells (Sigma, Bornem, Belgium) and incubated for 3 days at 28°C. After the incubation, colony diameter and clearing zone diameter were determined and the ratio between both was calculated as a measure for chitinase activity. The clearing zone produced by the *luxO* mutant containing LuxO locked in low cell density conformation was significantly larger than that formed by the wild type and the mutant containing LuxO locked in high cell density conformation ($P < 0.01$; Table 2), indicating that activation of the quorum-sensing system results in a decreased chitinase activity.

In a second test, the wild type and mutants were grown for 3 days in LB medium containing 20 g $l^{-1}$ NaCl, 0.1% chitin from crab shells (Sigma) and 5 mg $ml^{-1}$ of the chitin derivative chitin azure (Sigma), in which a soluble dye

**Table 2.** Chitinolytic activity towards natural chitin from crab shells (mean ± standard deviation of four replicates) and towards chitin azure (mean ± standard deviation of three independent cultures) of *Vibrio harveyi* wild type and *luxO* mutants.

| | | Activity towards natural chitin | | | | |
|---|---|---|---|---|---|---|
| Strain | Characteristic | Colony diameter (mm) | Clearing zone (mm) | Ratio | Activity towards chitin azure ($OD_{560}$) | Luminescence |
| BB120 | Wild type | 9.8 ± 0.5 | 12.5 ± 0.6 | 1.28 ± 0.06 | 0.045 ± 0.005 | Yes |
| JAF483 | LuxO high density | 10.0 ± 0.0 | 12.8 ± 0.5 | 1.28 ± 0.05 | 0.015 ± 0.003 | Yes |
| JAF548 | LuxO low density | 9.8 ± 0.5 | 15.8 ± 0.5** | 1.62 ± 0.12** | 0.065 ± 0.009** | No |

** Significant difference between both *luxO* mutants ($P < 0.01$).

is linked covalently to chitin. Cleaving of chitin azure by chitinases results in the release of a blue coloured dye, which is quantified as absorbance at 560 nm (Ramirez *et al.*, 2004). The suspensions were incubated for 3 days at 28°C with shaking (100 $min^{-1}$). After the incubation, cultures were centrifuged in a microcentrifuge at 10 000 r.p.m. for 5 min and the absorbance of the supernatants was measured. The supernatants of the *luxO* mutant containing LuxO locked in low cell density conformation showed significantly higher chitinase activity than supernatants of the mutant containing LuxO locked in high cell density conformation ($P < 0.01$; Table 2).

*Activity towards fluorogenic chitin oligomers*

In order to quantify the difference in chitinase activity, the activities of wild-type *V. harveyi* and the two *luxO* mutants towards 4-methylumbelliferyl β-D-N,N′-diacetylchitobioside (MUF-$GlcNAc_2$) (Sigma) and 4-methylumbelliferyl β-D-N,N′,N″-triacetylchitotrioside (MUF-$GlcNAc_3$) (Sigma) were studied. In these compounds, a 4-methylumbelliferyl moiety is linked to chitobiose and chitotriose respectively. Cleaving of these compounds by chitin degrading enzymes results in the release of methylumbelliferone, which is brightly fluorescent under UV (excitation wavelength 355 nm; emission wavelength 465 nm). A standard curve with 4-methylumbelliferone allows to quantify chitinase activity (Howard *et al.*, 2003). Wild type and mutants were inoculated into LB medium supplemented with 20 g $l^{-1}$ NaCl and 0.1% chitin from crab shells (Sigma) and incubated for 2 days at 28°C with shaking. After the incubation, cultures were diluted to a concentration of approximately $5 \times 10^8$ cells per ml and 200 μl aliquots of the diluted cultures were mixed with 25 μl of 0.5 mM solutions of the chitin analogues. The mixtures were incubated at 28°C for 30 min and the reaction was stopped and fluorescence enhanced by adding 25 μl of a 50 mM glycine buffer (pH 10). Fluorescence was measured with a Tecan infinite 200 microplate reader (Tecan, Mechelen, Belgium). For these compounds, chitinolytic activity of the *luxO* mutant containing LuxO locked in low cell density conformation was four to five times higher than that of the mutant containing LuxO locked in high cell density conformation (Fig. 1). If no chitin was present in the medium, chitinolytic activity decreased twofold (data not shown), which is in accordance to what was reported by Suginta and colleagues (2000).

*The effect of signal molecules on chitinolytic activity*

In a further experiment, we investigated whether the addition of quorum-sensing signals to a signal-deficient mutant would result in a decrease of chitinolytic activity. For this experiment, we used the HAI-1 and AI-2 negative double mutant MM77 (Mok *et al.*, 2003) because those two signals are dominant in liquid growth medium, whereas the third signal, CAI-1, has only a minor effect on quorum sensing-regulated gene expression under these conditions (Henke and Bassler, 2004b; Tu and Bassler, 2007). We used cell-free culture fluids of wild-type *V. harveyi*, prepared as described previously (Defoirdt *et al.*, 2006), as a source of HAI-1 and AI-2, and supernatants of MM77 as control. Briefly, wild-type *V. harveyi* and the HAI-1 and AI-2 negative mutant MM77 were grown to late exponential phase (corresponding to an $OD_{600}$ of approximately 1) in LB medium containing 20 g $l^{-1}$ NaCl. Cell-free culture fluids were prepared by removing the cells from the growth medium by centrifugation at 10 000 r.p.m. for 5 min in a microcentrifuge. The cleared fluids were passed through 0.22 μm Millipore filters (Millipore, Bedford, USA). Subsequently, mutant

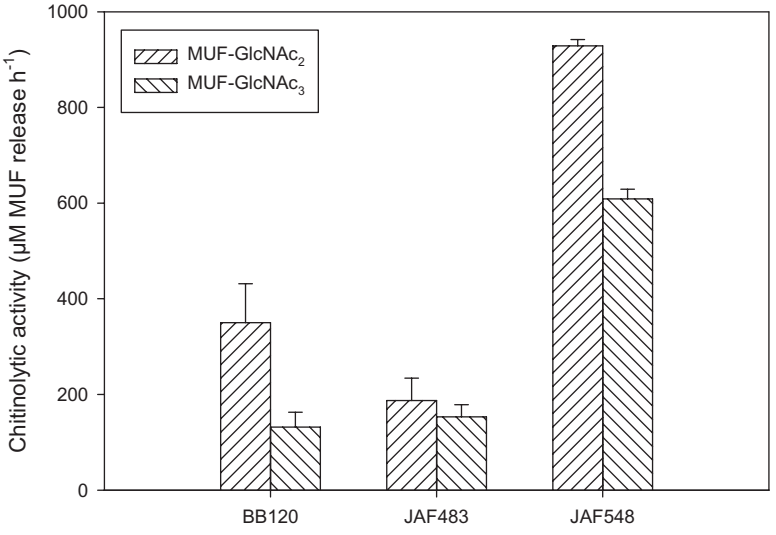


**Fig. 1.** Release of 4-methylumbelliferone (MUF) from 4-methylumbelliferyl β-D-N,N′-diacetylchitobioside (MUF-$GlcNAc_2$) and 4-methylumbelliferyl β-D-N,N′,N″-triacetylchitotrioside (MUF-$GlcNAc_3$) by *Vibrio harveyi* wild type and *luxO* mutants (mean ± standard deviation of three independent cultures) after 2 days of incubation in LB medium containing natural chitin. The mutants JAF483 and JAF548 contain LuxO locked in high and low cell density conformation respectively.

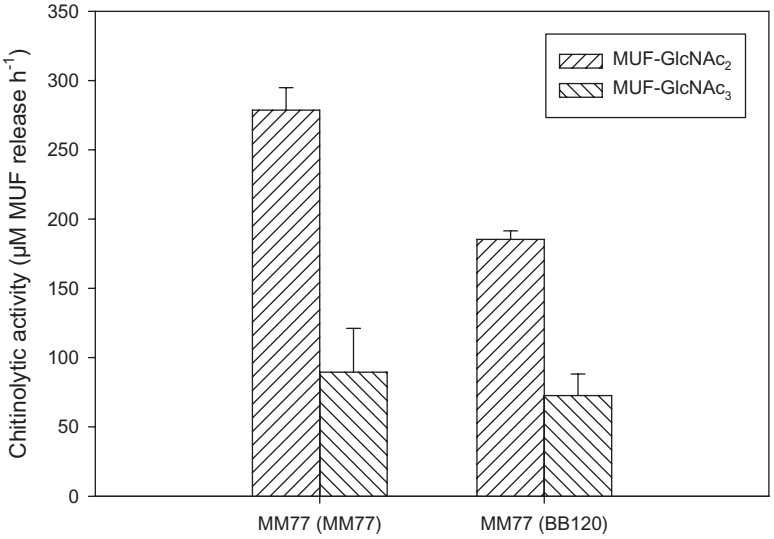


**Fig. 2.** Release of 4-methylumbelliferone (MUF) from 4-methylumbelliferyl β-D-N,N′-diacetylchitobioside (MUF-$GlcNAc_2$) and 4-methylumbelliferyl β-D-N,N′,N″-triacetylchitotrioside (MUF-$GlcNAc_3$) by the *Vibrio harveyi* signal-deficient mutant MM77 ($HAI\text{-}1^-$ $AI\text{-}2^-$) (mean ± standard deviation of three replicates) after 2 days of incubation in LB medium containing natural chitin. The mutant was incubated in the presence of its own cell-free culture fluids and in the presence of cell-free culture fluids of the wild-type BB120 (containing both HAI-1 and AI-2). The chitinase activities of the added cell-free culture fluids (treated in the same way as cell-free culture fluids inoculated with MM77) were subtracted from those of the grown MM77 cultures.

MM77 was grown for 2 days in LB medium containing 20 g $l^{-1}$ NaCl, 0.1% chitin from crab shells and 70% vol. of the cell-free culture fluids, after which chitinase activity was determined by the release of methylumbelliferone from the fluorogenic chitin oligomers MUF-$GlcNAc_2$ and MUF-$GlcNAc_3$. The chitinase activity of sterile cell-free culture fluids (treated in the same way as inoculated cell-free culture fluids) was subtracted from the activity of MM77 cultures grown in the presence of the cell-free culture fluids to correct for the chitinase activity present in the cell-free culture fluids.

The signal-deficient double mutant showed significantly lower chitinolytic activity towards MUF-$GlcNAc_2$ in the presence of the HAI-1 and AI-2 containing supernatant than in the presence of its own supernatant ($P < 0.01$; Fig. 2). The difference in chitinase activity of the double mutant between the treatments with and without signal molecules was less pronounced when compared with the difference between the two *luxO* mutants. This was as expected because the *V. harveyi* quorum-sensing system is maximally and minimally activated in the *luxO* mutants JAF483 and JAF548 respectively. On the other hand, the quorum-sensing system is not completely inactivated in double mutant MM77 (because it still produces and detects CAI-1) and addition of BB120 cell-free culture fluids most probably did not maximally activate the system throughout the incubation period due to signal decay.

### *The effect of quorum sensing on chitinase A mRNA levels*

In a final experiment, we aimed at investigating the effect of quorum sensing on chitinase at the transcriptional level. We chose the chitinase A (*chiA*) gene for this experiment because it is the main chitinase in *V. harveyi* (Svitil *et al*., 1997). *ChiA* mRNA levels were measured in late log phase cultures of the *V. harveyi luxO* mutants JAF483 and JAF548 by reverse transcriptase real-time PCR, performed as described previously, using the RNA polymerase A subunit (*rpoA*) mRNA as a control (Defoirdt *et al*., 2007). Specific primers for the amplification of *chiA* and *rpoA* mRNA were designed based on sequences that have been deposited before in GenBank. The combinations of the two primer sequences were blasted against GenBank. The primer sequences were as follows: $rpoA_{forward}$ 5′-CGTAGCTGAAGGCAAAGATGA-3′, $rpoA_{reverse}$ 5′-AAGCTGGAACATAACCACGA-3′, $chiA_{forward}$ 5′-GGAAGATGGCGTGATTGACT-3′ and $chiA_{reverse}$ 5′-GG CATCAATTTCCCAAGAGA-3′. Amplicon lengths are 232 and 197 bp for *chiA* and *rpoA* respectively.

*ChiA* mRNA levels were significantly lower in the *luxO* mutant containing LuxO locked in high cell density conformation when compared with the mutant containing LuxO locked in low cell density conformation ($P < 0.05$), whereas *rpoA* mRNA levels were not different. The log of the difference in mRNA levels between both strains was calculated as described previously (Defoirdt *et al*., 2007) to be 0.71 ± 0.37 and 0.12 ± 0.35 for *chiA* and *rpoA* respectively (mean ± standard deviation of three independent cultures). This indicates that there was approximately a fivefold difference in *chiA* mRNA levels between the two *luxO* mutants, which agrees well with the four- to five-fold difference between both mutants in activity towards the fluorogenic chitin oligomers MUF-$GlcNAc_2$ and MUF-$GlcNAc_3$ (see higher), although both compounds are also hydrolysed by *V. harveyi* chitinase B and chitinase C (Svitil *et al*., 1997).

*Conclusion and ecological interpretation*

In conclusion, our data show that quorum sensing negatively regulates chitinase activity and chitinase A expression in *V. harveyi*. Activation of the quorum-sensing system resulted in a decrease in chitinolytic activity, but not in a complete inhibition. This is in contrast to what has been found in *C. violaceum*, where chitinase was shown to be positively regulated by quorum sensing and where the regulation had the characteristics of an on/off switch because no chitin degradation occurred in a quorum sensing-deficient mutant (Chernin *et al*., 1998).

*Vibrio harveyi* is a marine species which can be found free-living as well as associated with different higher organisms. A downregulation of chitinases at high cell density might be a mechanism of *V. harveyi* to switch from the host-associated to the free-living life style. Indeed, chitinase A of *Legionella pneumophila* has been shown to be involved in persistence in the lung in the A/J mouse model of Legionnaires' disease (DebRoy *et al*., 2006). Moreover, a chitin-binding protein in *V. cholerae* has also been shown to be important for the colonization of the host by mediating attachment to the epithelium (Kirn *et al*., 2005). More recently, Bhomwick and colleagues (2008) found that this chitin-binding protein specifically bound to N-acetyl-D-glucosamine residues of mouse intestinal mucin. The authors also reported a coordinated interaction between the chitin-binding protein and mucin to upregulate each other's gene expression in a co-operative manner, emphasizing the role of N-acetyl-D-glucosamine ligands in infection. By decreasing chitinase production at high cell density, *V. harveyi* could increase the proportion of cells that are detached from the host's epithelium, allowing them to reach the external environment and finally colonize a new host. A similar mechanism has been proposed in the human pathogen *V. cholerae*, where downregulation of biofilm formation at high cell density has been explained as a mechanism of the bacteria to switch between host-associated and free-living life styles (Zhu and Mekalanos, 2003). Alternatively, down-regulation of chitinolytic activity at high cell density could be a mechanism by which *V. harveyi* avoids the release of too high levels of water-soluble chitin degradation products that might be toxic towards the bacteria. The chitin derivative chitosan, for instance, has been shown to be toxic to different Gram-negative bacteria including the closely related species *Vibrio parahaemolyticus* (Chaiyakosa *et al*., 2007).

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