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Quorum sensing-regulated chitin metabolism provides grazing resistance to *Vibrio cholerae* biofilms

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Association of *Vibrio cholerae* with chitinous surfaces of zooplankton is important for its persistence in marine environments, as it provides accessibility to nutrients and resistance to stresses. Predation by heterotrophic protists has a major impact on the survival of *V. cholerae*. *V. cholerae* forms biofilms as its main defensive strategy, and quorum sensing (QS) additionally regulates the production of antiprotozoal factors. The role of chitin and QS regulation in *V. cholerae* grazing resistance was investigated by exposing *V. cholerae* wild-type (WT) and QS mutant biofilms grown on chitin flakes to the bacteriotrophic, surface-feeding flagellate *Rhynchomonas nasuta*. *V. cholerae* formed more biofilm biomass on chitin flakes compared with nonchitinous surfaces. The growth of *R. nasuta* was inhibited by WT biofilms grown on chitin flakes, whereas the inhibition was attenuated in QS mutant biofilms. The chitin-dependent toxicity was also observed when the *V. cholerae* biofilms were developed under continuous flow or grown on a natural chitin source, the exoskeleton of *Artemia*. In addition, the antiprotozoal activity and ammonium concentration of *V. cholerae* biofilm supernatants were quantified. The ammonium levels (3.5 mM) detected in the supernatants of *V. cholerae* WT biofilms grown on chitin flakes were estimated to reduce the number of *R. nasuta* by >80% in add-back experiments, and the supernatant of QS mutant biofilms was less toxic owing to a decrease in ammonium production. Transcriptomic analysis revealed that the majority of genes involved in chitin metabolism and chemotaxis were significantly downregulated in QS mutant biofilms when grown on chitin compared with the WT biofilms.

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Introduction

Vibrio cholerae, the causative agent of cholera, is an opportunistic pathogen that is well adapted for survival in the marine environment during interepidemic periods (Faruque *et al.*, 1998). Environmental surveillance for *V. cholerae* in Bangladesh revealed that *V. cholerae* persists in the aquatic environment predominately as viable but nonculturable cells in the planktonic phase and as culturable cells in biofilms formed on biotic and abiotic surfaces (Alam *et al.*, 2006). *V. cholerae* biofilms associated with phytoplankton and zooplankton in water columns are suggested to be important for cholera transmission, based on the reported correlation between cholera disease outbreaks and seasonal aquatic plankton blooms (Huq *et al.*, 1984, 1995). In addition, it has been shown that simple filtration of water through sari cloth removes up to 99% of *V. cholerae* cells by removing the zooplankton-associated *V. cholerae* (Huq *et al.*, 1996), and that this practice reduces cholera infections by 48% (Colwell *et al.*, 2003).

Chitin is the main component of the cell walls of fungi and the exoskeletons of crustaceans and insects. Crustaceans are the majority of zooplankton, with krill and copepods comprising the largest animal biomass in the marine environment (Murphy *et al.*, 2007). Chitin is probably the most abundant biopolymer in the marine environment, and chitin utilisation is important for C and N recycling. The chitinolytic bacteria, especially surface-associated bacteria that have higher metabolic efficiency, are essential in this process (Yu *et al.*, 1991).

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The colonisation of chitinous surfaces of zooplankton is important for the persistence of *V. cholerae* in the aquatic environment (Huq *et al.*, 1983; Pruzzo *et al.*, 2008), where *V. cholerae* has been shown to utilise chitin as a sole nutrient source, allowing for the maintenance of metabolic activity for up to 6 months (Nahar *et al.*, 2011). *V. cholerae* has several mechanisms that facilitate chitin colonisation, including the mannose-sensitive haemagglutinin (Chiavelli *et al.*, 2001; Meibom *et al.*, 2004), chitin-regulated pilus (Meibom *et al.*, 2004) and two chitin-binding proteins (36 and 53 kDa) (Tarsi and Pruzzo, 1999; Kirn *et al.*, 2005; Zampini *et al.*, 2005). The toxin coregulated pilus has also been shown to mediate biofilm differentiation on chitin surfaces (Reguera and Kolter, 2005). The chitin utilisation pathway is conserved in the Vibrionaceae and many of these genes are upregulated upon attachment of *V. cholerae* to chitin (Meibom *et al.*, 2004; Hunt *et al.*, 2008).

In addition to nutrient accessibility, colonisation of chitinous zooplankton has also been shown to increase the resistance of *V. cholerae* to low pH (Nalin *et al.*, 1979) and alum/chlorine treatment (Chowdhury *et al.*, 1997). Furthermore, association with chitin surfaces promotes horizontal gene transfer by inducing natural competence (Meibom *et al.*, 2005), allowing *V. cholerae* to acquire the genes useful for adaption to various stresses both *in vivo* and *in vitro* (Bartlett and Azam, 2005).

Protozoan grazing has been identified as one of the major mortality factors faced by bacteria in the environment, and predation has a strong top–down control on *V. cholerae* populations (Worden *et al.*, 2006). In response to protozoan grazing, *V. cholerae* has been shown to exhibit an increase in biofilm formation, where *V. cholerae* biofilms are protective against grazing pressure, whereas the planktonic counterparts are more readily consumed (Matz *et al.*, 2005). The high cell density within biofilms also allows for the quorum sensing (QS) regulated production and accumulation of antiprotozoal factors (Sun *et al.*, 2013). QS has also been reported to control the expression of an extracellular protease PrtV (Vaitkevicius *et al.*, 2006) and an inhibitory factor, VasX, secreted by type VI secretion system (Ishikawa *et al.*, 2009; Miyata *et al.*, 2011), which are important for resistance to predation.

In this study, we investigated the role of chitin in *V. cholerae* grazing resistance by exposing *V. cholerae* biofilms grown on chitin flakes and crustacean surfaces to the bacteriotrophic surface-feeding flagellate, *Rhynchomonas nasuta*. Results show that biofilms on chitinous surfaces were more resistant to predation than those on abiotic surfaces. Furthermore, we demonstrated that chitin metabolism was QS-regulated and that the ammonium produced as a by-product of chitin metabolism is toxic to *R. nasuta.*

Materials and methods

Strains and culture conditions

V. cholerae A1552 wild type (WT) and its isogenic mutant Δ*hapR*, a master response regulator of QS (Yildiz *et al.*, 2004), were routinely maintained in Luria–Bertani broth and on agar plates. *R. nasuta*, a surface-feeding flagellate, was isolated from the Sydney Institute of Marine Science (Erken *et al.*, 2011) and routinely grown on heat-killed *Pseudomonas aeruginosa* PAO1 (final concentration 10^7 cells ml^{-1}) in 50% nine-salt solution (NSS) medium (NSS is an artificial seawater medium that contains 17.6 g NaCl, 1.47 g Na_2SO_4, 0.08 g $NaHCO_3$, 0.25 g KCl, 0.04 g KBr, 1.87 g $MgCl_2 \bullet 6H_2O$, 0.45 g $CaCl_2 \bullet 2H_2O$, 0.01 g $SrCl_2 \bullet 6H_2O$ and 0.01 g H_3BO_3 in 1 l of distilled water; 50% NSS contains half of the salts of NSS medium) (Vaatanen, 1976) grown statically at room temperature (RT). Ampicillin (200 μg ml^{-1}) and gentamycin (200 μg ml^{-1}) were added to eradicate indigenous bacteria. *R. nasuta* was subcultured in antibiotic-free medium 3 days prior to grazing experiments.

Artemia cysts from the Great Salt Lake, UT, USA (INVE Aquaculture, Dendermonde, Belgium) were decapsulated and hatched as previously described with modification (Sung *et al.*, 2008). Briefly, decapsulated *Artemia* cysts were incubated in 50% NSS at 26 °C for 18 h for hatching. The axenic *Artemia* were killed at the Instar I stage by freezing at − 20 °C overnight. To rule out bacterial contamination in grazing experiments, aliquots from cultures of *R. nasuta* and *Artemia* were plated on Luria–Bertani agar.

Microtitre plate grazing assay

To compare the grazing resistance of *V. cholerae* biofilms formed on abiotic and chitinous surfaces, batch experiments were performed in 24-well tissue culture plates as previously reported with modification (Weitere *et al.*, 2005). *V. cholerae* overnight cultures were co-incubated at a final concentration of 10^6 cells ml^{-1} with 10^4 cells ml^{-1} of *R. nasuta* in 1 ml of 50% NSS medium. Chitin flakes (2% w/v, C9213, Sigma-Aldrich, St Louis, MO, USA) or glucose (0.01% w/v) or both were supplemented as carbon sources. The microtitre plates were incubated at RT with shaking at 60 r.p.m. for 3 days. After incubation, *R. nasuta* was enumerated by direct inspection with an inverted light microscope ($\times 200$ magnification, CKX41, Olympus, Tokyo, Japan). *V. cholerae* biofilm biomass was determined by MTT staining as per manufacturer's recommendation (Cell Proliferation Kit I, Roche, Basel, Switzerland). The experiments were run in replicates of four and repeated twice.

Flow cell grazing assay

V. cholerae A1552 and *R. nasuta* were incubated in continuous flow cells ($1 \times 4 \times 40$ mm), assembled

and prepared as previously described (Moller *et al.*, 1998). One millilitre of 50% NSS containing 10^6 cells ml^{-1} of *V. cholerae* A1552 and 10^4 cells ml^{-1} of *R. nasuta* was injected into the cell and allowed to settle for 2 h before the flow was restored by pumping 50% NSS into the cell at a rate of 3 ml h^{-1}. Chitin flakes were glued to the bottom of flow cells using glass silicon, and glucose (0.01% w/v) was supplemented in the medium flow. The flow cells were incubated at RT for 3 days. *R. nasuta* was enumerated daily by inverted microscopy, and the effluents were collected and plated to determine the *V. cholerae* colony-forming units at the end of the experiment. The experiments were run in replicates of three and repeated twice.

V. cholerae biofilms formed on chitinous surfaces of zooplankton and exposed to predation

To compare biofilms on chitin flakes with those formed on natural chitinous surfaces, for example, exoskeletons of *Artemia*, the microtitre plate assay was modified to include 150 ml^{-1} killed *Artemia* instead of chitin flakes. Owing to the difficulties encountered in quantifying the *V. cholerae* biofilm biomass formed on *Artemia* surfaces, only *R. nasuta* was enumerated microscopically with a haemocytometer. The experiments were run in replicates of four and repeated twice.

Supernatant toxicity assay

The cell-free supernatants of *V. cholerae* biofilms from the above assays were collected, filtered through 0.22 μm filters (Millex-GP, Millipore, Billerica, MA, USA) and stored at −20 °C. The antiprotozoal activity of collected supernatants was tested as previously described (Matz *et al.*, 2005). *R. nasuta* (at a final concentration 10^4 cells ml^{-1}) was added to biofilm supernatants supplemented with heat-killed *P. aeruginosa* PAO1 in 24-well tissue culture plates and incubated at RT with shaking at 60 r.p.m. for 3 days. *R. nasuta* was enumerated microscopically. The experiments were run in replicates of four and repeated twice. The ammonium concentration of supernatants was determined by using the Ammonia Kit (TNT832, Hach, Lakewood, CO, USA) as per manufacturer's recommendation.

Transcriptomic analysis

V. cholerae A1552 WT and Δ*hapR* were grown in 50% NSS supplemented with 2% w/v chitin flakes at RT with shaking at 60 r.p.m. for 3 days. After removing the planktonic phase, the total RNA was extracted from biofilms formed on chitin flakes using the RNeasy Protect Bacteria Mini Kit (Qiagen, Hilden, Germany) as per the manufacturer's recommendation, and paired-end sequencing was performed on the Illumina Hi-Seq 2000 platform (San Diego, CA, USA) with 237 million reads of 100 bp length.

Reads from Illumina were processed with Cutadapt (version 1.3) (Martin, 2011) with a quality trimming threshold of 20 and a minimum read length of 50 bp. The trimmed reads were subsequently depleted of ribosomal RNA with SortMeRNA version 1.8 (Koylova and Touzete, 2012), and the nonribosomal RNA reads mapped to the genome of *V. cholerae* (RefSeq accession numbers NC 002505 and NC 002506) using Bowtie version 2.1.0 (Langmead *et al.*, 2009). The transcript abundance was estimated with Cufflinks version 2.1.0 (Langmead *et al.*, 2009) using the default geometric method. Calculations of the log2 fold changes and false discovery rates were performed with Cuffdiff (Langmead *et al.*, 2009). The differentially expressed transcripts were determined using the selection criteria of more than twofold change and q-value <0.05.

Results

Grazing resistance of V. cholerae biofilms grown on chitin flakes in batch culture

Owing to the importance of chitin utilisation and resistance to protozoan grazing for the survival of *V. cholerae* in the environment, the grazing resistance of chitin-associated *V. cholerae* and the role of QS in this process were assessed. Hence, biofilms of *V. cholerae* WT and Δ*hapR* strains were grown on chitin flakes and abiotic control surfaces, and exposed to predation by *R. nasuta*.

In the absence of a carbon source, the biofilms of *V. cholerae* formed on the surface of microtitre plates were not extensive. In the presence of glucose, there was a slight, but not significant, increase in biofilm biomass (228%, $P>0.05$), whereas the biomass of biofilms formed on chitin flakes was significantly greater (3053%, $P<0.001$) (Figure 1). There was no significant removal of biofilm biomass by *R. nasuta* under any of the above batch biofilms and no significant difference in biofilm biomass of *V. cholerae* WT and Δ*hapR* strains (Figure 1).

The antiprotozoal activity of *V. cholerae* biofilms was determined by enumeration of *R. nasuta*. In *V. cholerae*-free controls, the number of *R. nasuta* increased with the supplementation of glucose and/or chitin flakes. The presence of *V. cholerae* WT biofilms did not significantly affect the number of *R. nasuta* on abiotic surfaces, but *R. nasuta* was killed by WT biofilms grown on chitin flakes ($P<0.001$). In contrast, the Δ*hapR* biofilms supported significantly more *R. nasuta* individuals on abiotic surfaces compared with the controls and the WT biofilms ($P<0.001$). There was no significant difference in the number of *R. nasuta* co-incubated with Δ*hapR* biofilms grown on chitin flakes compared with the controls. Compared with *V. cholerae* WT strain, Δ*hapR* biofilms were less toxic in all nutrient conditions ($P<0.001$) (Figure 2).

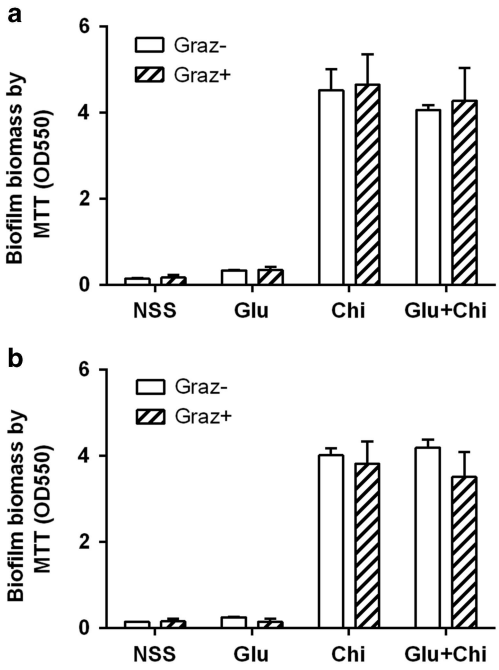

Figure 1 Grazing resistance of biofilms of *V. cholerae* grown on abiotic and chitinous surfaces. Biofilm biomass of *V. cholerae* WT (**a**) and Δ*hapR* (**b**) strains with no carbon source (NSS), with glucose (Glu) and/or chitin flakes (Chi) in the presence (Graz+) and absence (Graz−) of *R. nasuta.*

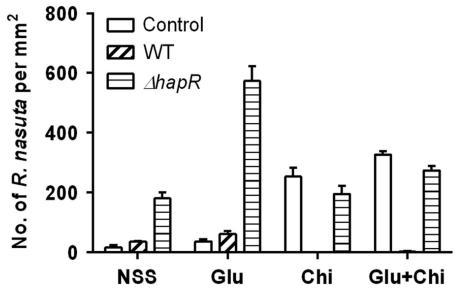

Figure 2 Effect of *V. cholerae* biofilms on survival of *R. nasuta.* Number of *R. nasuta* feeding on *V. cholerae* WT and Δ*hapR* biofilms with no carbon source (NSS), with glucose (Glu) and/or chitin flakes (Chi). *R. nasuta* concentrations in media without *V. cholerae* were used as controls.

Grazing resistance of V. cholerae biofilms grown on chitin flakes in flow cells

To determine if grazing resistance was similar under continuous flow conditions where there is dilution of extracellular components to that observed in batch cultures, *V. cholerae* WT stain and *R. nasuta* were co-incubated in flow cells with chitin or glucose. The grazing effects were determined by quantification of *V. cholerae* colony-forming units in the biofilm effluent and the numbers of *R. nasuta.*

The effluents of *V. cholerae* biofilms grown on chitin flakes contained more *V. cholerae* cells (295%, $P<0.001$) than the effluents from biofilms developed in flow cells supplemented with glucose, indicating that the biofilm biomass was higher when grown on chitinous surfaces. Similar to the above grazing assays

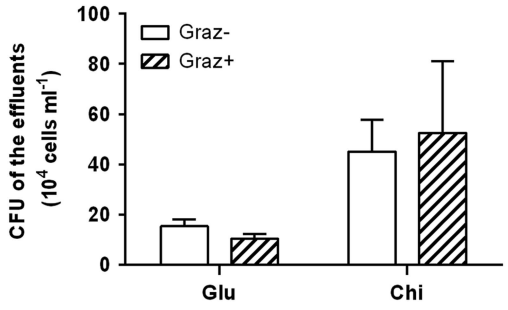

Figure 3 Biofilms formed on chitin surfaces in a flow-through system. Colony-forming units of *V. cholerae* WT biofilm effluents grown on glucose (Glu) and chitin flakes (Chi). Experiments were performed in flow cells in the presence (Graz+) and absence (Graz−) of *R. nasuta.*

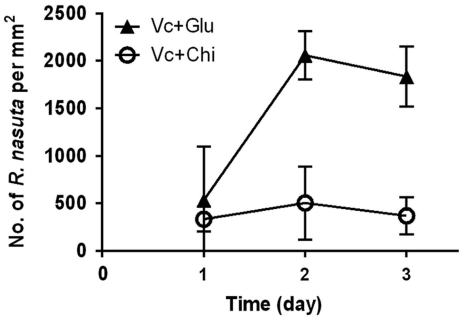

Figure 4 Effect of biofilms on survival of *R. nasuta* in a flow-through system. Number of *R. nasuta* feeding on *V. cholerae* WT biofilms grown on glucose or chitin flakes. Experiments were performed in flow cells for 3 days and *R. nasuta* was quantified by microscopy daily.

performed in microtitre plates, predation by *R. nasuta* did not significantly reduce the biofilm biomass as the colony-forming units of effluents were similar to nongrazed controls (Figure 3).

Direct microscopic enumeration revealed that the number of *R. nasuta* increased when feeding on *V. cholerae* biofilms grown in glucose, whereas the growth of *R. nasuta* was inhibited by *V. cholerae* biofilms grown on chitin flakes, by 80% on day 3 ($P<0.001$). In control flow cells lacking *V. cholerae*, the number of *R. nasuta* was below the detection limits, which was most likely due to nutrient deprivation (Figure 4).

Grazing resistance of V. cholerae biofilms grown on Artemia

In order to determine if biofilms formed on the chitinous surfaces of zooplankton were also resistant to predation, *V. cholerae* biofilms were grown first on *Artemia* in microtitre plates. In preliminary experiments, live *Artemia* fed on and depleted the *R. nasuta* population (data not shown). Thus, in the following experiments, *Artemia* was killed by freezing prior to addition of *V. cholerae* WT in batch cultures and the number of *R. nasuta* grazing on *V. cholerae* biofilms was compared with media controls.

In the absence of *V. cholerae*, the number of *R. nasuta* increased more in wells containing *Artemia* (1710%) than in those containing glucose (224%), indicating that *R. nasuta* benefited from the nutrients released by freeze-killed *Artemia* ($P<0.001$). The *V. cholerae* biofilms grown on abiotic surfaces did not affect the number of *R. nasuta* significantly, whereas *V. cholerae* biofilms formed on *Artemia* inhibited *R. nasuta* ($P<0.001$) (Figure 5).

Ammonium produced by V. cholerae during chitin metabolism is toxic to R. nasuta

Supernatants of *V. cholerae* biofilms were collected from all the above assays and tested for their inhibitory effects on *R. nasuta* by comparing with the corresponding media controls. The supernatants of *V. cholerae* WT biofilms grown in 50% NSS medium were nontoxic to *R. nasuta*, whereas supernatants from WT biofilms grown in glucose inhibited *R. nasuta* by 50% ($P<0.001$) (Figure 6).

The supernatants of WT biofilms grown on chitin flakes were significantly more toxic to *R. nasuta* than those grown in glucose and media controls, resulting in a 98% decrease in *R. nasuta* numbers ($P<0.001$). In comparison, the supernatants of Δ*hapR* biofilms were less toxic than the supernatants of WT biofilms when grown on chitin flakes ($P<0.001$). Interestingly, all of the supernatants collected from *V. cholerae* biofilms grown in flow cells and on the surfaces of *Artemia* did not show significant inhibition (data not shown), which was probably owing to high dilution in the flow cells and/or low chitin abundance in both conditions as discussed below.

Chitin is a modified derivative of glucose containing nitrogen, and the metabolism of chitin produces ammonia as a by-product. Therefore, the ammonium concentration of supernatants collected from *V. cholerae* biofilms grown in batch cultures was measured (Figure 7). Ammonium was not detected in media controls and was low in nonchitin supernatants. In contrast, chitin utilisation by *V. cholerae* generated a significant amount of ammonium (3.5 mM) in the supernatants ($P<0.001$), whereas the Δ*hapR* biofilms produced 55% less than the WT ($P<0.001$). The ammonium concentration of supernatants collected from *V. cholerae* biofilms grown in flow cells and on the surface of *Artemia* was below the detection limits (data not shown). To confirm that ammonium is toxic to *R. nasuta*, a dose–response curve was plotted using NH_4Cl in 50% NSS medium. The number of *R. nasuta* decreased with an increase in ammonium (Figure 8).

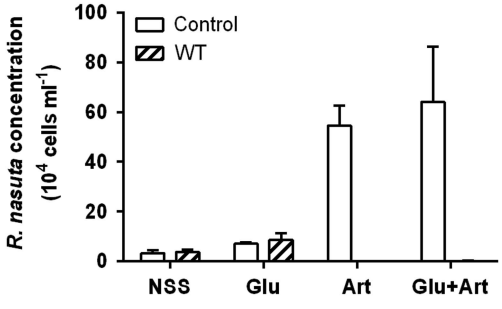

Figure 5 Grazing resistance of biofilms of *V. cholerae* formed on *Artemia.* Numbers of *R. nasuta* in media controls and feeding on *V. cholerae* WT biofilms with no carbon source (NSS) or with glucose (Glu) and/or *Artemia* (Art).

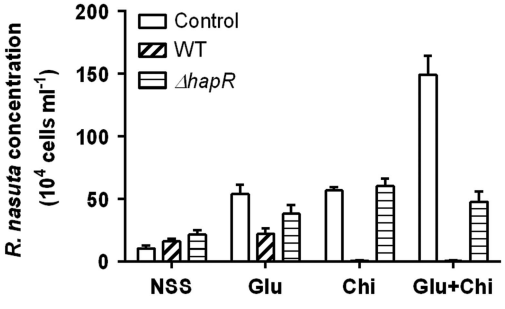

Figure 6 Toxicity of supernatants from *V. cholerae* biofilms to *R. nasuta.* Toxicity of supernatants collected from *V. cholerae* WT and Δ*hapR* biofilms grown in artificial seawater (NSS), glucose (Glu) and/or on chitin flakes (Chi). *R. nasuta* concentrations in media without *V. cholerae* were used as controls.

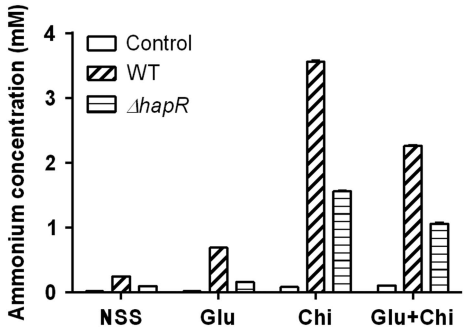

Figure 7 Ammonium concentration of *V. cholerae* biofilm supernatants. Ammonium concentration of supernatants collected from *V. cholerae* WT and Δ*hapR* biofilms grown in artificial seawater (NSS), glucose (Glu) and/or on chitin flakes (Chi). Ammonium concentrations of *V. cholerae*-free media was measured as controls.

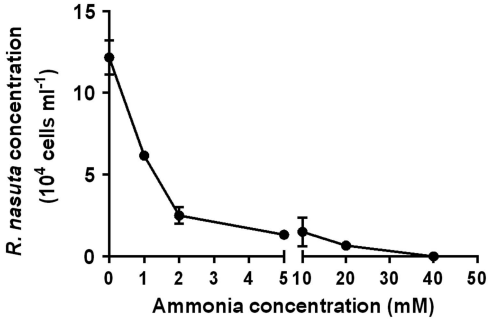

Figure 8 Effect of ammonium on survival of *R. nasuta.* Effect of increasing concentration of ammonium on *R. nasuta* survival. *R. nasuta* was quantified by microscopy after 3 days incubation in NSS medium supplemented with NH_4Cl.

QS regulation of V. cholerae chitin utilisation

As the *V. cholerae* Δ*hapR* strain was less toxic to *R. nasuta* than the WT strain and produced less ammonium when grown on chitin flakes, the role of QS regulation of chitin metabolism was investigated by transcriptomic analysis of *V. cholerae* WT and Δ*hapR* biofilms grown on chitin flakes in batch cultures. Results demonstrated that 367 transcripts were differentially expressed with more than a twofold change (Supplementary Table S1).

The chitinolytic pathway of *V. cholerae* (Hunt *et al.*, 2008) begins with the degradation of chitin by chitinase and the transport of *N*-acetylglucosamine (GlcNAc) monomers or dimers into the periplasm. The transcripts of genes that are involved in this process were largely unchanged in the Δ*hapR* biofilms compared with the WT biofilms grown on chitin, with the exception of two chitinases (VC0769 and VCA0027), which were expressed at a higher level in Δ*hapR* biofilms. Following the transport of GlcNAc monomers and dimers, the products of degradation are delivered into central metabolism as fructose-6-phosphate, acetate and ammonium. Among the 22 genes involved in GlcNAc catabolism, transcripts of 19 genes, including the entire chitin catabolic operon (VC0611-VC0620), were expressed at a significantly lower level in the QS-negative Δ*hapR* biofilms, whereas the transcripts of the remaining 3 genes were not significantly different (Table 1). In addition, transcripts of the genes coding for chitin-regulated pilus (VC2423, VC2424 and VC1612) and the majority of the methyl-accepting chemotaxis genes (29 of total 45 MCPs) were repressed in the Δ*hapR* biofilms, whereas only 2 MCP genes were upregulated. The RNA-Seq data also confirmed QS regulation of previously reported genes, including those involved in *Vibrio* polysaccharide production, flagella synthesis, virulence, natural competence, type VI secretion system and c-di-GMP synthesis/degradation (Supplementary Table S1).

Discussion

V. cholerae biofilms formed on chitin surfaces are grazing resistant

Results show that *V. cholerae* forms significantly more biofilm biomass on chitin flakes than on abiotic surfaces in both microtitre plates and continuous flow cells. It has been reported that chitin colonisation and utilisation are ubiquitous among vibrios, with all completed genomes of vibrios having multiple chitinases (Grimes *et al.*, 2009). The association with chitin is important for the long-term persistence of *V. cholerae* in the environment. *V. cholerae* can use chitin as a sole carbon source (Nahar *et al.*, 2011), and colonisation of chitinous zooplankton has been shown to increase the resistance of *V. cholerae* to low pH (Nalin *et al.*, 1979) and alum/chlorine treatment (Chowdhury *et al.*, 1997). Various chitin attachment mechanisms have been identified (Meibom *et al.*, 2004), although the role of mannose-sensitive haemagglutinin in chitin association is still under debate (Watnick *et al.*, 1999). When the *V. cholerae* biofilms grown on chitin flakes were exposed to predation by *R. nasuta*, there was little effect of grazing on biofilm biomass and this was in line with our previous observation that biofilm formation protects *V. cholerae* from protozoan grazing (Matz *et al.*, 2005).

Ammonium produced by V. cholerae chitin metabolism is toxic to R. nasuta

In both microtitre plates and flow cells, *V. cholerae* biofilms formed on chitin flakes were more toxic to *R. nasuta* compared with biofilms formed on abiotic

Table 1 Differentially regulated genes between *V. cholerae* WT and Δ*hapR* biofilms in GlcNAc catabolism

Gene	*Description*	ΔhapR/*WT log2 fold*
VC0611	Phosphoglucomutase/phosphomannomutase	−1.74
VC0612	Cellobiose/cellodextrin-phosphorylase	−1.92
VC0613	Beta-*N*-acetylhexosaminidase	−1.54
VC0614	Hypothetical protein	−1.55
VC0615	Endoglucanase-like protein	−1.01
VC0616	Peptide ABC transporter ATP-binding protein	−1.63
VC0617	Peptide ABC transporter ATP-binding protein	−1.78
VC0618	Peptide ABC transporter permease	−1.42
VC0619	Peptide ABC transporter permease	−1.96
VC0620	Peptide ABC transporter substrate-binding protein	−1.10
VC0994	*N*-acetylglucosamine-6-phosphate deacetylase	−1.22
VC0995	PTS system *N*-acetylglucosamine-specific transporter subunit IIABC	−1.48
VC1280	Hypothetical protein	−1.47
VC1281	PTS system cellobiose-specific transporter subunit IIB	−2.29
VC1282	PTS system cellobiose-specific transporter subunit IIC	−2.17
VC1284	6-Phospho-beta-glucosidase	−1.77
VC1285	Hypothetical protein	−1.06
VC2217	Beta-*N*-acetylhexosaminidase	−1.93
VCA1025	Glucosamine-6-phosphate deaminase	−1.48

Abbreviations: ABC, ATP-binding cassette; PTS, phosphotransferase system.

surfaces, indicating the existence of antiprotozoal factor(s) produced by *V. cholerae* biofilms in association with chitin. Furthermore, the chitin-dependent antiprotozoal activity was also observed when biofilms were grown on the surface of *Artemia*. Supernatants collected from *V. cholerae* biofilms grown on chitin flakes in batch cultures were inhibitory to *R. nasuta* and, subsequently, ammonium was identified to be responsible for the antiprotozoal activity in the supernatant. This metabolite-based grazing resistance may be a general feature of environmental bacteria. For example, biofilms of *Pseudomonas fluorescens* CHA0 produce the metabolites hydrogen cyanide and 2,4-diacetylphloroglucinol, which have antiprotozoal activities against bacteriotrophic protists (Jousset *et al.*, 2006).

Ammonia/ammonium at high concentrations is generally toxic to eukaryotic cells. It has been reported that the amphipod *Gammarus pulex* is inhibited by <0.1 mM ammonium (Berenzen *et al.*, 2001; Prenter *et al.*, 2004), and bacterial-derived ammonia in the intestine is largely accepted as the main source for hyperammonaemia in patients with liver disease (Sherlock, 1987). Interestingly, ammonia is also an important environmental signal in the regulation of the life cycle of *Dictyostelium discoideum*, where high ammonia concentrations inhibit fruiting body formation (Mahadeo and Parent, 2006). The dose–response curve of *R. nasuta* to ammonium demonstrated that 2 mM ammonium was lethal for 80% of *R. nasuta* individuals. As the supernatants of *V. cholerae* WT biofilms grown on chitin flakes contained 3.5 mM ammonium, these biofilms are even more toxic than *R. nasuta*.

R. nasuta was also inhibited when co-incubated with *V. cholerae* biofilms formed on chitin flakes in flow cells or on *Artemia*. In the marine environment, seasonal plankton blooms may provide enough chitin for *V. cholerae* metabolism to produce inhibitory amounts of ammonium (Huq *et al.*, 1984). It has also been proposed that the inhibitory factors may accumulate to higher concentrations within biofilms than in supernatants owing to high cell density and retention of cell-derived compounds by exopolymeric substances (Matz *et al.*, 2005). In addition, the antiprotozoal activity of *V. cholerae* biofilms in association with chitinous surfaces may also benefit from other inhibitory factors, for example, PrtV (Vaitkevicius *et al.*, 2006), VasX (Ishikawa *et al.*, 2009; Miyata *et al.*, 2011) and the unknown factor secreted by *V. cholerae* biofilms (Matz *et al.*, 2005).

QS regulation of chitin metabolism and antiprotozoal activity in V. cholerae biofilms

The reduced production of ammonium and decreased toxicity against *R. nasuta* of *V. cholerae* Δ*hapR* biofilms grown on chitin flakes indicates that QS controls *V. cholerae* chitin utilisation. Transcriptomic analysis revealed that 19 of the 22 genes involved in GlcNAc catabolism, as well as functionally related chitin-regulated pilus synthesis and chemotaxis, were positively regulated by QS in *V. cholerae* biofilms.

Interestingly, two putative chitinases, VC0769 and VCA0027, were downregulated by QS in *V. cholerae* biofilms, indicating that the five chitinases in *V. cholerae* might be redundant or functionally differentiated. The negative regulation of chitinase by QS has been reported previously, where VCA0811 was found to be repressed by QS in a microarray study of *V. cholerae* rugosity (Yildiz *et al.*, 2004), and chitinase A of *Vibrio harveyi* was expressed at higher level in a QS-negative mutant strain (Defoirdt *et al.*, 2010). One possible explanation is that the expression of chitinases may be a nutrient-exploring strategy used by planktonic *V. cholerae* cells rather than in high-density biofilms. When chitin is present in the surrounding environment, the secreted chitinases degrade chitin and generate soluble oligomers of GlcNAc, which act as signals for *V. cholerae* chemotaxis, chitin attachment and utilisation (Meibom *et al.*, 2004). To date, only VC1952 and VCA0027 have been confirmed to have catabolic activity for chitin and the double-deletion mutant could not grow on chitin as the sole carbon source (Meibom *et al.*, 2004). Thus, it is possible that the remaining three chitinases may not have catabolic activity.

It has been proposed that many mechanisms involved in *V. cholerae* environmental persistence are important in pathogenesis as well (Vezzulli *et al.*, 2008). Indeed, it has been reported that six genes (VC0613, VC0614, VC0616, VC0619, VC1284 and VC2424) involved in chitin utilisation are also induced during colonisation of the infant mice small intestine (Mandlik *et al.*, 2011). Therefore, the QS-regulated chitin utilisation genes of *V. cholerae* biofilms may play a role in infection by *V. cholerae.*

Chitin association has been reported to provide *V. cholerae* nutrient accessibility and to induce natural competence of *V. cholerae*. The data presented here show that biofilm formation of *V. cholerae* on chitin provides increased grazing resistance and is toxic to the heterotrophic protist, *R. nasuta*, owing to the production of ammonia as a by-product of chitin metabolism. Furthermore, we show that chitin metabolism is QS-regulated. These results provide an explanation for the association of *V. cholerae* with chitinous exoskeletons of zooplankton as well as a role for QS-regulated phenotypes in persistence and survival in the marine environment.

Conflict of Interest

The authors declare no conflict of interest.

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