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Quorum sensing signal production and inhibition by coral-associated vibrios

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Summary

Corals are inhabited by complex communities of microbes that affect their growth and survival. Several studies suggest that coral disease may be attributed to the success of vibrios in out-competing other bacteria in the mucus and tissues of corals. Vibrios utilize a variety of quorum sensing (QS) signal molecules to regulate processes that could be used to colonize corals during adverse environmental conditions. We therefore screened a range of *Vibrios* isolated from a variety of healthy and diseased corals, for the production of the QS signal molecules, *N*-acylhomoserine lactones (AHLs) and the AI-2 (autoinducer-2) small furanone signal molecule. All 29 strains examined activated the AI-2 biosensor, but only 17 activated an AHL biosensor. Using reverse phase thin-layer chromatography, we showed that the effect of temperature on AHL production varied considerably among the isolates. For the first time, the QS inhibition by *Vibrio harveyi* is reported. This only occurred at higher temperatures and does not appear to be due to degradation of AHLs. The large diversity of vibrios and the different effects of temperature on signal production may partly explain the complexity of coral-associated community changes in response to environmental factors.

Introduction

Corals are hosts to a large and diverse population of microbes associated with their mucus, tissue surface and within the coral tissue itself. These communities are distinct from the surrounding water column and, despite being highly diverse, are often specific for a particular coral species (reviewed in Rosenberg *et al*., 2007). This has led to the notion of corals as 'holobionts', consisting of the coral, symbiotic zooxanthellae and associated microbes. This complex community is expected to have an enormous effect on the physiological function and health of coral ecosystems, and their response to environmental stresses (Bourne *et al*., 2007). Indeed, shifts in bacterial populations are known to occur when corals are diseased, even when only a small part of the colony is diseased (Pantos *et al*., 2003).

Many studies have implicated vibrios in the disease of corals, and shifts to populations dominated by vibrios in diseased corals has been shown for many species of both tropical and cold water corals (Hall-Spencer *et al*., 2007). The majority of these studies relied on comparisons of bacterial isolates from diseased and healthy cultures. However, in a study of disease progression in *Acropora millepora*, Bourne and colleagues, (2007) constructed clone libraries utilizing *Vibrio*-specific PCR probes to show a clear increase in the fraction of *Vibrio*-affiliated clones during the bleaching period. Recent studies suggest that increases in vibrios occur when the holobiont is under stress due to environmental conditions that would allow vibrio growth and concomitant lost of host-resistance barriers (Gochfeld and Aeby, 2008). Although the conclusions of some studies have been criticized (Lesser *et al*., 2007), there are many reports directly implicating vibrios as the main aetiological agents of disease in corals, with some studies suggesting mechanisms by which modifications of the microbial population could facilitate infection by opportunist vibrios (Ritchie, 2006; Nissimov *et al*., 2009). For example, the seasonal bleaching of *Oculina patagonica* in the Mediterranean Sea was connected with infection by *Vibrio shiloi*. The onset of bleaching was associated with the expression at elevated temperatures of a number of potential virulence factors, including an adhesin that facilitated attachment of *V. shiloi* to *O. patagonica* (Toren *et al*., 1998), a peptide toxin (Banin *et al*., 2001), and superoxide dismutase (Banin *et al*., 2003). An increase in temperature was also linked to the bleaching and tissue lysis of the coral *Pocillopora damicomis* by *V. coralliilyticus* (Ben Haim *et al*., 2003).

Quorum sensing (QS) is an important mechanism affecting the colonization, virulence and extracellular

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enzyme production in a number of bacterial species, including the vibrios (Henke and Bassler, 2004; Croxatto *et al*., 2002). The growth capability of vibrios and their potential in causing coral disease may be linked to QS. Previously, there has been no study of QS in coral-associated bacteria. The QS pathways in vibrios are controlled by several over-lapping regulatory systems. At present, four QS circuits have been described for *Vibrios*. The first discovered regulates bioluminescence in the symbiont *V. fischeri*: a LuxI *N*-acyl homoserine lactone (AHL) synthase and a LuxR AHL-binding transcriptional regulator (reference), a system utilized by many *Proteobacteria*. In contrast, bioluminescence in *V. harveyi* is controlled by the synergistic action of three parallel systems. The first is another AHL synthase (LuxM), and its signal transducer (LuxN), the second, a small furanone signal molecule [autoinducer-2 (AI-2); LuxS] and its receptor, LuxP and the third system involves a *cqsA*-dependent signal molecule and the sensor CqsS (Henke and Bassler, 2004). In contrast, *V. cholerae* does not produce signal molecules of the AHL class, utilizing AI-2 and the product of CqsA, an α-hydroxyketone (*S*)-3-hydroxytridecan-4-one, to regulate virulence factor production (Higgins *et al*., 2007).

The aim of this study was to screen a range of vibrio strains isolated from a variety of healthy and diseased corals for the production of QS signal molecules at different temperatures.

Results and discussion

The production of AHL signal molecules by vibrios

N-acylhomoserine lactone and AI-2 production among the vibrio strains was determined using the *Escheirchia coli lux*-based AHL biosensors pSB536, pSB401 and pSB1075 (Swift *et al*., 1997; Winson *et al*., 1998) and the AI-2 biosensor *V. harveyi* BB170 (Surette and Bassler, 1998) (Table 1). While all strains were shown to activate the AI-2 biosensor, only 17 activated an AHL biosensor (Fig. 1). Of the 17 AHL positive isolates, 14 activated the short-chain biosensor pSB536, which responds to C4 acyl-chain length signal molecules.

The results detailed in this paper further stress the complexity of QS systems within vibrios. With the exception of *V. harveyi* R-21433, all other *V. harveyi* isolates produced an AHL which activated the pSB536 biosensor, and no AHL production was recorded for the *Photobacterium rosenbergii* isolates. There was considerable intra and interspecies variation in the production of signal molecules. Similar results have been shown for *V. vulnificus*: AHL-producing and non-producing representatives of this bacterium have been reported (Valiente *et al*., 2009). However, all AHL-producing *V. vulnificus* grouped with environmental isolates whereas non-producers grouped with human pathogens. In contrast, this study shows there was no correlation between the location of isolation (i.e. temperate versus tropical), the coral host, type of sample (i.e. water, tissue or mucus) or whether the coral was diseased or healthy. The QS networks of *V. anguillarum*, *V. cholerae*, *V. harveyi*, *V. fischeri* and *V. vulnificus* have been studied in detail. All have LuxSP, indicating this system may be ancestral and maintained throughout the evolution of the vibrios. However, each has different components of the four QS circuits (i.e. *luxIR*, *luxMN*, *luxSP* and *cqsAS*) with striking differences within the regulation between systems often being evident (Milton, 2006).

Attempts were made to locate the genes responsible for AHL production. It is known that *V. harveyi* produce AHLs via a *luxM* synthase, and analysis of genome sequences for strains *V. campbellii* AND4, *V. splendidus* LGP32, *V. splendidus* 12B01 and *V. shilonii* AK1 has revealed *luxM* homologues within the *V. campbellii* genome (e.g. AND4-00220, AND4-11114 and AND4-11119) and *V. splendidus* genomes (e.g. VS_II0261 and V12B01-16686) but no *luxM* homologue within the *V. shilonii* (= *V. shiloi*) *AK1* genome. However, a *luxI* homologue was located within the AK1 genome (VSAK1-16717). PCR primers specific for the *luxI* or *luxM* genes identified within the genome-sequenced strains were unsuccessful. This is not surprising given the extremely low homology between AHL synthases, even at the protein level.

Temperature influences the production of AHLs in V. harveyi *R-21446,* V. mediterranei *R-21415,* V. coralliilyticus *LMG 20984T and* V. shiloi *LMG 19703*

Increases in temperature have been linked to shifts towards vibrio-dominated populations in a number of corals, but it was not known if there is an effect of temperature on AHL production. Using reverse phase thin-layer chromatography in combination with the *Agrobacterium tumefaciens* NTL4 AHL bioreporter (pCF218; pCF372) (Fuqua and Winans, 1996), the effect of temperature on AHL production by a signal producing representative of each AHL-producing strain was examined (*V. campbellii* R-21441, *V. coralliilyticus* LMG 20984T, *V. harveyi* R-21446, *V. mediterranei* R-21415, *V. rotiferianus* R21416, *V. shiloi* LMG 19703 and *V. tasmaniensis* R-21462). Thin-layer chromatography for strains R-21415, R-21446 LMG 20984 and LMG 19703 are shown in Fig. 1. In the case of R-21416, R-21441 and LMG 20984T, AHL production remained constant at the temperatures 18°C, 25°C and 30°C. The AHL profile for strains R-21441 and LMG 20984T were identical, with three mid-chain length AHLs being produced. For *V. harveyi* R-21446 and *V. shiloi* LMG 19703, AHL production decreased with increasing temperature. This was strikingly evident in the case of R-21446. In contrast,

Table 1. Quorum sensing signal production by coral-associated vibrios.

					AHL production			
Strain	Species identification	Host species	Location[a]	Sample	pSB536	pSB401	pSB1075	AI-2
LMG 22223T	*Photobacterium rosenbergii*	*Merulina ampliata* (bleached)	MI	Water				+
LMG 22225	*Photobacterium rosenbergii*	*Pachyseris speciosa*	MI	Water				+
LMG 21426	*Photobacterium rosenbergii*	*Barabattoia amicorum*	MI	Water				+
LMG 21427	*Photobacterium rosenbergii*	*Barabattoia amicorum*	MI	Tissue				+
R-21427	*Vibrio campbellii*	*Acropora millepora*	DR	Tissue	+	+	+	+
R-21434	*Vibrio campbellii*	*Acropora millepora* (diseased)	DR	Tissue	+			+
R-21437	*Vibrio campbellii**	*Montipora catipata*	KB	Mucus				+
R-21441	*Vibrio campbellii**	*Pachyseris speciosa*	MI	Tissue				+
R-21438	*Vibrio coralliilyticus*	*Barabattoia amicorum* (bleached)	MI	Water		+	+	+
LMG 20984T	*Vibrio coralliilyticus*	*Pocillopora damicornis* (diseased)	Z	Tissue	+		+	+
LMG 21349	*Vibrio coralliilyticus*	*Pocillopora damicornis* (diseased)	RS	Tissue				+
R-21410	*Vibrio harveyi*	*Montipora capitata*	KB	Mucus	+			+
R-21435	*Vibrio harveyi*	*Pocillopora damicornis*	DR	Mucus	+			+
R-21439	*Vibrio harveyi*	*Barabattoia amicorum* (bleached)	DR	Tissue	+			+
R-21433	*Vibrio harveyi**	*Merulina ampliata*	MI	Water	+			+
R-21444	*Vibrio harveyi**	*Pachyseris speciosa*	MI	Water	+			+
R-21446	*Vibrio harveyi**	*Acropora millepora*	DR	Mucus	+			+
R-21450	*Vibrio harveyi*-like	*Acropora millepora* (diseased)	MI	Tissue	+			+
R-21455	*Vibrio harveyi*-like	*Acropora millepora*	MI	Mucus				+
R-21415	*Vibrio mediterranei*	*Merulina ampliata*	MI	Water			+	+
R-21409	*Vibrio pelagius**	*Pachyseris speciosa*	MI	Water				+
R-21431	*Vibrio pelagius**	*Pachyseris speciosa* (bleached)	MI	Mucus				+
R-21416	*Vibrio rotiferianus*	*Pachyseris speciosa*	MI	Water	+	+		+
R-21422	*Vibrio rotiferianus*	*Pachyseris speciosa* (bleached)	MI	Water	+			+
R-21433	*Vibrio rotiferianus*	*Merulina ampliata*	MI	Water				+
LMG 19703	*Vibrio shiloi*	*Oculina patagonica* (bleached)	Med	Tissue	+	+	+	+
CC096	*Vibrio splendidus***	*Eunicella verrucosa* (diseased)	PS	Tissue				+
R-21461	*Vibrio tasmaniensis*	*Eunicella verrucosa* (diseased)	LI	Tissue				+
R-21462	*Vibrio tasmaniensis*	*Eunicella verrucosa* (healthy)	LI	Tissue	+			+

The presence of AHLs (+) was detected using the *E. coli lux*-based biosensors pBS536, pSB401 and pSB1075 (Swift *et al*., 1997; Winson *et al*., 1998). These allow a range of AHL detection from C4 (pSB536), C6–C8 (pSB401) and C10–C14 (pSB1075) acyl chain lengths signal molecules, thus covering a wide range of AHL signal molecules. *N*-acylhomoserine lactones were extracted from 25 ml culture supernatants (Bainton *et al*., 1992; Cámara *et al*., 1998) and applied to white/clear bottom microtiter plate wells (Corning). Overnight cultures of biosensor diluted 1/25 and 200 μl were added to each well. The microplates were incubated at 37°C and the luminescence and absorbance (620 nm) measured using a Berthold MITHRAS microplate reader after 3 h incubation for pSB536 and 6 h for pSB401 and pSB1075.AHL standards were synthesized, purified and characterized as previously described (Chhabra *et al*., 1993; Cámara *et al*., 1998). AI-2 activity was measured from culture supernatant using the BB170 biosensor and method of Surrette and Bassler (1998).

a. MI: Magnetic Island and DR: Davies Reef, Queensland, Australia. KB: Kaneohe Bay, Hawaii. Med: Mediterranean Sea. RS: Red Sea. Z: Zanzibar. LI: Lundy Island, England.

All cultures were isolated by C. B. Munn except those obtained from the Belgian Coordinated Culture Collections; LMG 19703 (Kushmaro *et al*., 2001), LMG 20984 and LMG 21349 (Ben-Haim *et al*., 2001). Cultures were identified by MLSA as described by Thompson and colleagues (2005), except those marked *, which were identified by AFLP; **CC096 was identified by partial 16S rRNA analysis.

strain R-21415 produced two long-chain AHLs at all growth temperatures, but at 18°C, an additional two small-chain AHLs were produced. *N*-acylhomoserine lactone production has been shown to vary with temperature in other bacteria [e.g. *Yersinia pseudotuberculosis* (Atkinson *et al*., 1999); *Erwinia carotovora* (Hasegawa *et al*., 2005)], but, to our knowledge, this is the first report showing temperature effects on AHL production in vibrios.

Quorum sensing is known to control the production of enzymes in vibrios and so the relationship between enzyme activity and temperature was also investigated for *Vibrio harveyi* R-21446, *V. coralliilyticus* LMG 20984T and *V. mediterranei* R-21415. R-21446 has catalase activity and *V. coralliilyticus* LMG 20984T has haemolysin activity. *Vibrio harveyi* R-21446, *V. mediterranei* R-21415 and *V. coralliilyticus* LMG 20984T have protease activity. Generally, enzyme activity was higher at 25°C and 30°C than at 18°C. But, other than strain R-21446, where enzyme production negatively correlated with AHL production, there was no clear pattern between enzyme activity and AHL production for the other strains (Fig. 1). This highlights the complexity of QS circuits in vibrios. Often, several systems over-lap to exert control of the production of enzymes (Henke and Bassler, 2004). However, *V. harveyi*, *V. cholerae*, *V. anguillarum*, *V. fischeri* and *V. vulnificus* all converge their various signals through the transcriptional activator LuxO (Milton, 2006). If QS does regulate expression of enzymes in these coral isolates, it

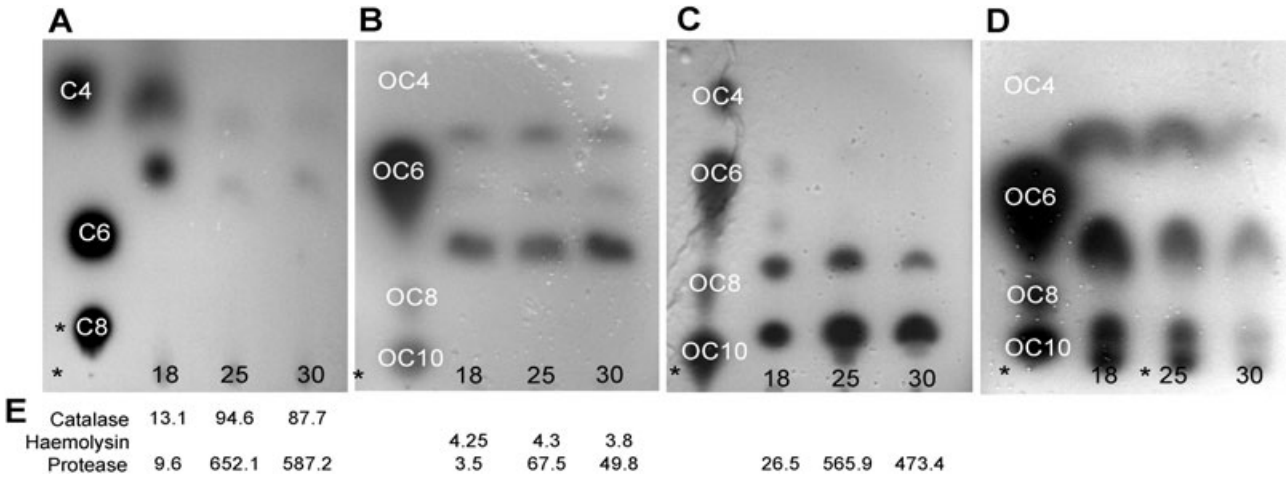

Fig. 1. Reverse phase thin-layer chromatography (TLC) profiling of AHLs produced by *Vibrio* sp. grown at 18°C, 25°C and 30°C. Cultures were grown in marine broth (Difco) until stationary phase (OD_{600} = 1.8 in all cases). Extracts were combined from the acidified (pH 2) supernatant of six 25 ml cultures for each temperature, applied to RP18 F_{254} TLC plates (20 cm × 20 cm; VWR International) and a mobile phase of 60% (v/v) methanol used to separate the extracts. The TLC plates were overlaid with the biosensors NTL4 (pCF218; pCF372) (Fuqua and Winans, 1996) or *Chromobacterium violaceum* CV026 (McClean *et al*., 1997) following the methodology of Mohamed and colleagues (2007). Following incubation at 30°C overnight, the TLC plates were examined for the presence of blue (NTL4) or purple (CV026) spots, indicative of AHL production. A is *V. harveyi* R-21446 overlaid with CV026, B is *V. coralliilyticus* LMG 20984T overlaid with NTL4, C is *V. mediterranei* R-21415 overlaid with NTL4 and D *V. shiloi* LMG 19703 overlaid with NTL4. *N*-acylhomoserine lactone synthetic standards were used as markers: 0.5 mM *N*-butanoyl-L-homoserine lactone (C4), 10 μM *N*-hexanoyl-L-homoserine lactone (C6), 1 mM *N*-octanoyl-L-homoserine lactone (C8), 2 mM *N*-(3-oxobutanoyl-L-homoserine lactone (OC4), 5 μM *N*-oxohexanoyl-L-homoserine lactone (OC6), 0.2 μM *N*-oxooctanoyl-L-homoserine lactone (OC8) and 0.5 mM *N*-oxodecanoyl-L-homoserine lactone (OC10). E displays catalase, haemolysin and protease activity for the three strains. The method of Beers and Sizer (1952) was used to measure catalase activity. One unit of catalase activity was taken as the amount to decompose 1.0 μl of H_2O_2 per min using a molar coefficient of 43.6 M s^{-1}. Haemolysin activity was measured by zones of lysis in horse blood agar. To prepare the agar, horse blood was washed in phosphate-buffered saline, diluted 1:100 with distilled water and solidified with 1.5% agar. Wells were cut into the centre of each agar plate and 45 μl culture supernatant applied. Following incubation overnight at 26°C, the plates were stained with saffranin, destained with 70% ethanol for 72 h and zones of lysis (mm) recorded. Culture supernatant was also used for protease measurements (ng trypsin degraded h^{-1}) using a Protease Fluorescent Detection Kit (Sigma). For each measurement, n = 3 and SD was ± 17.24%. Significant values (one-way analysis of variance > P = 0.05) are marked with an asterisk.

is highly likely that more than one QS pathway is involved, and the possibility that signal transduction occurs through LuxO requires further investigation.

Vibrio harveyi *R-21446 inhibits AHL production at higher temperatures*

The mechanism of AHL disappearance by strain R-21446 was investigated using *Chromobacterium violaceum*. The production of the purple pigment violacein by this strain is regulated by QS and can be exploited in a simple screen for inhibitors of QS (McLean *et al*., 2004). When R-21446 grown at different temperatures was overlaid with *C. violaceum*, a non-pigmented zone surrounding R-21446 grown at higher temperatures was evident (Fig. 2). Similar results were seen with *V. anguillarum* and *Sulfitobacter* sp. Gfp-based AHL biosensors (Tait *et al*., 2005; 2009). This suggests that R-21446 is capable of interfering with QS in other strains. Several bacteria both produce and degrade AHL signal molecules [e.g. *Pseudomonas aeruginosa* and *Shewanella* (Tait *et al*., 2009)], but this has never been shown for vibrios. To determine if the decrease in AHL production by R-21446 was due to the degradation of AHLs, stationary phase cells of R-21446 grown at 30°C were harvested and washed in phosphate-buffered saline pH 6.8. A range of long- and short-chain AHLs, both saturated and unsaturated were added to the cells, incubated for 3 h and the residual AHL concentration determined using biosensors (Tait *et al*., 2009). No AHL degradation was evident (results not shown), and it is more likely that R-21446 regulates the output of signal molecules by other means.

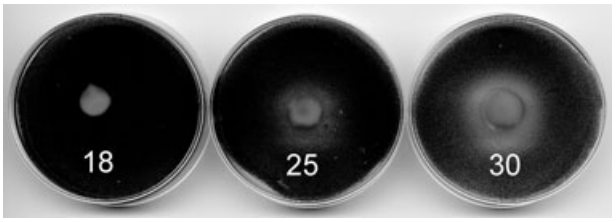

Fig. 2. The production of a quorum-sensing inhibitor by *V. harveyi* R-21446. R-21446 was spotted onto the middle of 6 cm Luria–Bertani agar and grown at 18°C, 25°C or 30°C until a healthy colony size was obtained. The plate was then overlaid with a soft agar containing the bacterium *Chromobacterium violaceum* and incubated at 30°C for a further 16 h. *Chromobacterium violaceum* produces a dark purple pigment in response to the self-produced C6 AHL (McClean *et al*., 2004). A white hallow of growth surrounding the colony is indicative of an AHL inhibitor.

This is the first attempt towards the description of QS inhibition by vibrios. Quorum sensing inhibition may have a significant impact not only on the AHL output by *V. harveyi* R-21446, but also in neighbouring bacteria of the holobiont, including other vibrios (Tait *et al*., 2009). The inhibitory activity of *V. harveyi* may be a competitive advantage that allows this species to be overrepresented among the vibrios in the holobiont during periods of high temperature (Alves *et al*., 2009). In conclusion, our study suggests that QS may play a role in the dynamics of the vibrios in the holobiont.

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