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ORIGINAL ARTICLE

Temperature regulation of virulence factors in the pathogen *Vibrio coralliilyticus*

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Sea surface temperatures (SST) are rising because of global climate change. As a result, pathogenic *Vibrio* species that infect humans and marine organisms during warmer summer months are of growing concern. Coral reefs, in particular, are already experiencing unprecedented degradation worldwide due in part to infectious disease outbreaks and bleaching episodes that are exacerbated by increasing SST. For example, *Vibrio coralliilyticus*, a globally distributed bacterium associated with multiple coral diseases, infects corals at temperatures above 27 °C. The mechanisms underlying this temperature-dependent pathogenicity, however, are unknown. In this study, we identify potential virulence mechanisms using whole genome sequencing of *V. coralliilyticus* ATCC (American Type Culture Collection) BAA-450. Furthermore, we demonstrate direct temperature regulation of numerous virulence factors using proteomic analysis and bioassays. Virulence factors involved in motility, host degradation, secretion, antimicrobial resistance and transcriptional regulation are upregulated at the higher virulent temperature of 27 °C, concurrent with phenotypic changes in motility, antibiotic resistance, hemolysis, cytotoxicity and bioluminescence. These results provide evidence that temperature regulates multiple virulence mechanisms in *V. coralliilyticus,* independent of abundance. The ecological and biological significance of this temperature-dependent virulence response is reinforced by climate change models that predict tropical SST to consistently exceed 27 °C during the spring, summer and fall seasons. We propose *V. coralliilyticus* as a model Gram-negative bacterium to study temperature-dependent pathogenicity in *Vibrio*-related diseases.
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Introduction

The correlation between temperature and disease is of an escalating concern because of observed and predicted changes attributed to global climate change (Hoegh-Guldberg and Bruno, 2010). Record breaking temperatures are occurring more frequently with the ten warmest years in recorded history experienced over the last 13 years (NOAA, 2011), and the average global temperature is predicted to increase from 1.8 to 4.0 °C in the 21st Century (IPCC, 2007). Concurrently, increased incidence and/or severity of diseases have been observed in human (Patz *et al.*, 2005) and marine (Harvell *et al.*, 2009) ecosystems. In coral reefs, an estimated one third of coral species are at a risk of extinction largely because of global warming and disease (Carpenter *et al.*, 2008). Mass mortality of Caribbean coral ecosystems occurred in 1998 (Aronson *et al.*, 2000) and 2005 (Eakin *et al.*, 2010), two of the hottest years recorded, with record breaking sea surface temperatures (SST) (NOAA, 2011). Localized temperature-related bleaching episodes have also increased in frequency (Whiteman, 2010) and are predicted to occur biannually within 20 years (Donner *et al.*, 2007).

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Pathogens of the genus *Vibrio* are associated with temperature-related diseases exhibiting peak infection rates in humans (Igbinosa and Okoh, 2008) and corals (Vezzulli *et al.*, 2010) following the warmer summer months. High summer temperatures correlate with increased *V. cholerae* outbreaks (Fernandez *et al.*, 2009; Hashizume *et al.*, 2011), as well as infections caused by *V. parahaemolyticus* and *V. vulnificus* (Iwamoto *et al.*, 2010). Increased SST undoubtedly causes an increase in abundance of vibrios (Vezzulli *et al.*, 2010); however, temperature also has a more direct role in *Vibrio* pathogenicity (Oh *et al.*, 2009), although little is known regarding specific mechanisms involved in temperature-related infections.

V. coralliilyticus is of interest because of its global distribution, broad host range and temperature-dependent pathogenicity in corals. *V. coralliilyticus* has been isolated from marine organisms in the Atlantic (Ben-Haim *et al.*, 2003a; Alves *et al.*, 2010; Vizcaino *et al.*, 2010), Indian (Ben-Haim *et al.*, 2003a) and Pacific Oceans (Sussman *et al.*, 2008; Kesarcodi-Watson *et al.*, 2009), as well as the Mediterranean (Vezzulli *et al.*, 2010) and Red Seas (Ben-Haim *et al.*, 2003a) (Figure 1). It causes fatal infections in a wide range of organisms, including unicellular algae (Ben-Haim *et al.*, 2003b; de Oliveira Santos *et al.*, 2011), corals (Ben-Haim *et al.*, 2003b), oysters (Jeffries, 1982), shrimp (Austin *et al.*, 2005; de Oliveira Santos *et al.*, 2011), rainbow trout (Austin *et al.*, 2005) and flies (Alves *et al.*, 2010; de Oliveira Santos *et al.*, 2011) during experimental infection assays. Although it is uncertain whether *V. coralliilyticus* is a primary or opportunistic coral pathogen, evidence strongly suggests that this endemic member of global coral holobionts (Pollock *et al.*, 2010) has a role in coral disease (Rosenberg and Kushmaro, 2011). Infection experiments establish the ability of *V. coralliilyticus* to cause bacterial bleaching (Ben-Haim *et al.*, 2003b), white syndrome (Sussman *et al.*, 2008) and mortality in corals (Alves *et al.*, 2010; Vezzulli *et al.*, 2010), in addition to being associated with the microbial consortium of black band disease (Arotsker *et al.*, 2009). *V. coralliilyticus* type strain ATCC (American Type Culture Collection, Manassas, VA, USA) BAA-450 (*Vc*450), isolated from bleached corals near Zanzibar, displays a tightly regulated temperature-dependent virulence; it is capable of invading and lysing coral tissue of *Pocillopora damicornis* at temperatures $>27\,^{\circ}C$, it attacks the symbiotic algae of this coral at temperatures between 24 °C and 26.5 °C and is avirulent at temperatures $\leqslant 24\,^{\circ}C$ (Ben-Haim *et al.*, 2003b). Further, *Vc*450 was recently shown to provoke a physiological response in *P. damicornis* during a temperature-induced infection experiment (Vidal-Dupiol *et al.*, 2011). *V. coralliilyticus* P1 (*Vc*P1), isolated from diseased corals in the Great Barrier Reef, infects corals at 28–31 °C (Sussman *et al.*, 2008). It has been speculated that a zinc-metalloprotease may be driving these infections (Ben-Haim *et al.*, 2003a; Sussman *et al.*, 2009); however, recent infection experiments using a zinc-metalloprotease mutant of *Vc*P1 (*vcpA*) revealed no significant differences in pathogenicity (de Oliveira Santos *et al.*, 2011).

In this study, we identify potential virulence factors in *Vc*450 using whole genome sequencing and compare our results with that of the recently published *Vc*P1 draft genome (de Oliveira Santos *et al.*, 2011). In addition, we use two-dimensional liquid chromatography coupled with tandem mass spectrometry and bioassays to investigate the influence of nonpathogenic (24 °C) and pathogenic (27 °C) temperatures on the expression of virulence

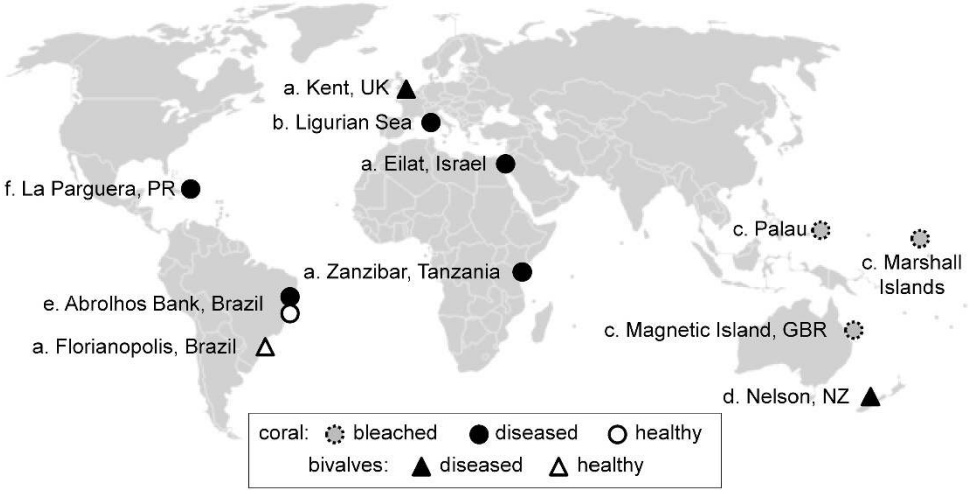

Figure 1 Global distribution of *V. coralliilyticus* strains. The *V. coralliilyticus* strains represented here are (**a**) type strains (Ben-Haim *et al.*, 2003a), as well as strains identified using (**b**) DnaJ PCR (Vezzulli *et al.*, 2010), (**c**, **d**) 16S rRNA sequencing (Sussman *et al.*, 2008; Kesarcodi-Watson *et al.*, 2009), (**e**) multi-locus sequencing (Alves *et al.*, 2010) and (**f**) multiple molecular analyzes, that is, 16S rRNA sequencing, recA PCR and repetitive extragenic palindromic - polymerase chain reaction (REP-PCR) (Vizcaino *et al.*, 2010).

factors in *Vc*450. We demonstrate that *Vc*450 maintains a broad array of virulence mechanisms, similar yet distinct from *Vc*P1, and provide evidence that increased temperature results in a significant increase in the number and expression level of numerous virulence factors, including flagellar-mediated motility, secretion systems, host degradation and antimicrobial resistance, as well as transcriptional regulators including quorum sensing (QS).

Materials and methods

Vc450 genome sequencing

The *Vc*450 genome was sequenced, assembled and finished at the Joint Genome Institute (Los Alamos, NM, USA). Draft sequences were obtained from paired-end Sanger sequencing on 8 kb plasmid libraries (5 times coverage) and 454 sequences (20 times coverage), providing 6.5 times total coverage. Details regarding sequencing and library construction can be found at http://www.jgi.doe.gov/. Gene finding and annotation were achieved using the RAST server (Aziz *et al.*, 2008), and all genome comparisons were performed using SEED Viewer 2.0 (Overbeek *et al.*, 2005). The *Vc*450 whole genome sequence data have been submitted to the GenBank database under the accession no. ACZN00000000. See Supplementary Materials and methods for further details.

Growth curves and protein quantification

*Vc*450 was grown on glycerol artificial sea water (GASW) agar (Smith and Hayasaka, 1982), with inocula taken from frozen glycerol stocks and tested for purity prior to use. Individual colonies were grown at either 24 °C or 27 °C overnight, and 4 ml of each *Vc*450 inoculum was transferred to 96 ml of GASW media. After 24 h, the optical density (OD_{610}) was measured using a spectrophotometer (Beckman Coulter DU 800 Spectrophotometer, Fullerton, CA, USA), and 1 ml of 2.4 OD_{610} *Vc*450 inoculum was added to 99 ml GASW media. The OD_{610} and the number of colony forming units were determined for *Vc*450 cultures grown at 24 °C and 27 °C at 0, 2, 4, 6, 8, 12 and 24 h after inoculation. Total protein was quantified from aliquots collected concurrently with OD and CFU samples. Samples were centrifuged and cell pellets washed with 0.09% NaCl. Pellets were resuspended in 200 µl of 0.045% NaCl, 1 M NaOH and boiled for 10 min. Extracted protein (100 µl) was added to 1 ml Coomassie Blue (Sigma, St Louis, MO, USA) and measured by a spectrophotometer at a 595-nm wavelength. The protein quantification was calculated using the regression equation of a bovine serum albumin standard curve.

Protein extraction

*Vc*450 cultures were grown as described above for 12 h to early stationary phase. The liquid culture was centrifuged and the resulting *Vc*450 pellets were resuspended in lysis buffer (40 mM Tris pH 8.0, 10 mM sodium fluoride and 1 × Complete Protease Inhibitor Cocktail Tablet stock (Roche Diagnostics, Pleasanton, CA, USA)). The resuspended pellet was vortexed with 0.1 mm silica beads (90 s) in a Mini-Bead Beater (BioSpec Products Inc., Bartlesville, OK, USA) three times, and the lysate was recovered and centrifuged to remove remaining beads. The protein concentration was determined with Commassie Plus—The Better Bradford Assay (Thermo Scientific, Rockford IL, USA). Isolated proteins were reduced using 10 mM dithiothreitol and 1.6 mg l^{-1} RapiGest (Waters, Milford, MA, USA), and alkylated by adding 50 mM iodoacetamide. An additional incubation was performed at room temperature for 30 min after adding 50 mM dithiothreitol. The proteins were desalted and washed three times with Tris buffer (25 mM Tris with 1 mg l^{-1} RapiGest) and concentrated using Ultrafree centrifuge tubes (membrane cutoff at >10 kDa; Millipore, Billerica, MA, USA). The concentrated proteins were resuspended in Tris buffer and digested overnight at 37 °C with a 1:50 ratio of trypsin and 1 mg l^{-1} RapiGest. To stop digestion, 3 M HCl was added to each sample and incubated at 37 °C for 60 min. The supernatant was transferred to a fresh vial after centrifugation, and the protein was dried under vacuum and stored at −20 °C.

Two-dimensional liquid chromatography coupled with tandem mass spectrometry

In two independent experiments, *Vc*450 peptides were fractionated by strong cation exchange chromatography in a 2.1-mm Polysulfoethyl A ion-exchange column (PolyLC, Columbia, MD, USA). The peptides were separated at a flow rate of 200 µl min^{-1} using a 100-min gradient. Each fraction was further analyzed by LC-MS/MS using a reverse-phase C18 1 mm column (Waters) or a C18 75 µm column (Microtech Scientific, Anaheim, CA, USA) on an LTQ (linear trap quadruple) linear ion trap mass spectrometer (Thermo Fischer Scientific, San Jose, CA, USA). Mass spectra from both experiments were matched to predicted tryptic peptides from the *Vc*450 genome using Turbo SEQUEST (Eng *et al.*, 1994). SEQUEST search result files (.srf) of the combined dataset (Supplementary Figure 1) were loaded into Scaffold (Proteome Software Inc., Portland, OR, USA; version Scaffold_2_05_01) for validation of peptide and protein identifications. Only proteins identified by Turbo SEQUEST and validated by Scaffold (Supplementary Figure 1) were included in the spectral counting analysis performed in Scaffold. We applied the *G*-test of independence, a likelihood ratio test for discreet data, to quantify the relative expression of proteins (that is, the number of spectral counts per protein) between *Vc*450 grown at 24 °C and 27 °C. Spectral counts were normalized according to the

total number of spectral counts for both data sets, as suggested previously (Old *et al.*, 2005; Hendrickson *et al.*, 2006). See Supplementary Materials and methods for further details.

Electron microscopy
A *Vc*450 cell suspension ($\sim 10^{10}$ cells ml^{-1}) was prepared in 1 ml of fixative (3% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2). After 24 h of incubation at 4 °C, the cells were washed twice in 0.9% saline and added to 200 µl of 1% phosphotungstic acid (pH 6.8). Fifteen µl of cells were applied to the surface of a 300-mesh, carbon-coated, formvar-coated copper grid. Excess stain was removed, and the grids were air-dried. A JEOL 1011 transmission electron microscope (JEOL USA Inc., Peabody, MA, USA) operating at an accelerating voltage of 80 kV was used to examine the *Vc*450 cells.

Motility assay
A single colony each of *Vc*450 grown at 24 °C and 27 °C was inoculated into 3 ml GASW media and incubated at the corresponding temperature overnight at 180 r.p.m. Cell densities were adjusted to OD_{595} 1.0, and 1 µl of the adjusted culture was stabbed into the center of a 0.35% GASW agar plates. The plates were then incubated at the respective temperatures for 24 h before the diameter of the growth zone was measured. Three independent cultures were performed in triplicate ($N = 9$) for each temperature.

Chinese hamster ovary (CHO) cell assay
*Vc*450 was grown in casamino-yeast extract, Proteose peptone no. 3 and brain heart infusion medium with 2% salt at 24 °C, 27 °C and 30 °C, with agitation. Culture aliquots were taken after 6–8 h and after 24 h of growth. Cell supernatants were obtained by centrifugation of cells and cell lysates were prepared by incubating the cell pellet in tris-buffered saline containing 2 mg ml^{-1} polymixin B. CHO cells were grown in Eagle's minimum essential medium supplemented with 10% heat-inactivated fetal calf serum, 10% tryptose phosphate broth, penicillin (100 IU ml^{-1}), 0.01% streptomycin and 0.14% sodium bicarbonate. The ability of the culture supernatant fluids and cell lysates to alter the morphology of CHO cells or to lyse them was determined using the same medium without the tryptose phosphate broth, but supplemented with 1% heat-inactivated fetal calf serum.

Hemolysis assay
*Vc*450 was grown on Trypticase Soy Agar amended with 5% Sheep Blood (Becton Dickinson, Sparks, MD, USA) at 24 °C and 27 °C in three independent cultures at each temperature. *V. vulnificus* and *Escherichia coli* were used as positive and negative controls, respectively.

Assays for autoinducer (AI)-1 and AI-2 signaling molecules
*Vc*450 was cultured as described above and grown in triplicate at 21 °C, 24 °C, 27 °C, 30 °C, 33 °C or 37 °C, and 1.5 ml was collected from each sample after 3, 12 and 24 h. The cell cultures were centrifuged, and the supernatant was filtered (0.2 µm) and stored at 4 °C. The *V. harveyi* reporter strains, BB886 (ATCC BAA-1118, luxPQ::tn5kAN) and BB170 (ATCC BAA-1117, luxN::tn5 kAN) were used to determine the presence of AI-1 and AI-2 signaling molecules, respectively. The bioluminescence assays were performed as described previously (Bassler *et al.*, 1994). Luminescence measurements were taken using a luminometer (BMG Novostar, Ortenberg, Germany), normalized to background controls (that is, reporter strains with sterile media added), and presented as the fold change compared with endogenous levels of luminescence expressed by the reporter strains. See Supplementary Materials and methods for further details.

Results and Discussion

V. coralliilyticus *genome*
To investigate the presence of potential virulence factors, we performed whole genome sequencing of *Vc*450 and identified an asymmetrical, two-chromosome structure consistent among all *Vibrio* genomes examined (Okada *et al.*, 2005; Chun *et al.*, 2009). The larger (C1—3 416 103 bp) and smaller (C2—1 865 911 bp) chromosomes follow a gene distribution pattern typical for vibrios with C1 predominantly carrying genes for viability and growth, and C2 mostly bearing genes for adaptation to environmental change (Makino *et al.*, 2003). A total of 5078 protein-coding sequences were identified (GenBank ACZN00000000): 3047 from C1 and 1656 from C2, with 81.1% and 74.7% showing sequence homology to proteins with known or putative functions, respectively. In addition, *Vc*450 contains a megaplasmid of ~398 614 bp, encoding 369 coding sequences with 55% annotated as hypothetical proteins, indicating that the majority of the genes code for potentially novel proteins. The *Vc*450 genome lacks the IntI4 integrase associated with the superintegron cassette present in most vibrios; however, elements of the superintegron (for example, the RelEB toxin-antitoxin replicon stability system) are located on the megaplasmid, indicating acquisition and/or relocation of cassettes of the superintegron to a mobile conjugative replicon.

A gross comparison of the *Vc*450 and *Vc*P1 genomes (Table 1) yields a conserved genome structure, consisting of two chromosomes, consistent for *Vibrio* species, a large plasmid and a conserved gene content (4478 shared genes). However, closer scrutiny reveals notable differences between the

Table 1 Characteristics of the *Vc*450 and *Vc*P1 genome

Characteristics	Vc*450*				Vc*P1*
	C1	*C2*	p*BAA-450*	*Total*	*Total*
Size (bp)	3 416 103	1 865 911	398 614	5 680 628	5 513 256
Contigs	17	2	1	20	230
G+C content	45.60%	45.30%	48.50%	46.50%	46%
CDS	3053	1656	369	5078	5107
Hypothetical	607	419	173	1199	1245
Identified	2440	1237	196	3873	3862
Total RNAs	117	5	0	122	58
tRNAs	87	5	0	92	53
rRNAs	30	0	0	30	5

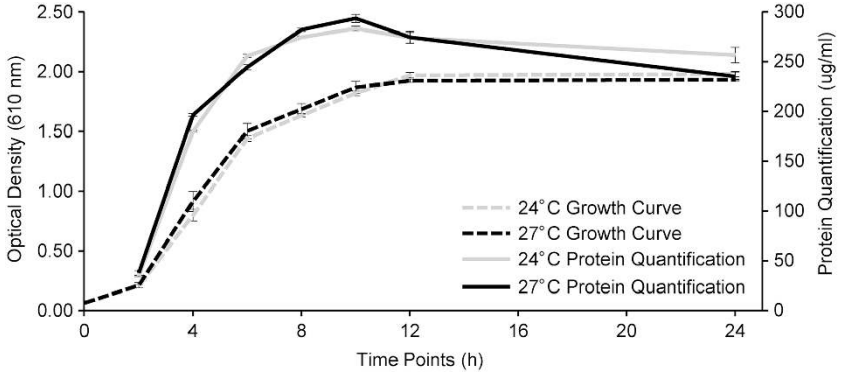

Figure 2 The growth rate and total protein production of *Vc*450 is similar whether grown at 24 °C or 27 °C. *Vc*450 was grown in GASW media at 24 °C and 27 °C. The growth curves were prepared using optical density at time points 0, 2, 4, 6, 8, 10, 12 and 24 h for both 24 °C (—) and 27 °C (- - -). Protein production was measured using the Bradford assay for both 24 °C (···) and 27 °C (—) from time points 2–24 h. Error bars represent the standard deviation of three replicate samples.

two strains. Although both genomes contain large plasmids, the *Vc*450 plasmid is considerably larger (~399 kbp) than the one observed in *Vc*P1, estimated at 252 kbp by comparative genomics (Supplementary Figure 2) and pulsed field gel electrophoresis (Supplementary Figure 3). Additionally, 12% of each genome is unique, representing 600 and 629 coding sequences in *Vc*450 and *Vc*P1, respectively. This includes important virulence factors, such as a repeats-in-toxin (RTX) toxin, type 3 secretion genes and pilus proteins that are unique to *Vc*450. *Vc*P1, in contrast, contains 12 unique prophage, transposon and integron regions, as well as a unique flagellar operon. This is consistent with a recent report that closely related *Vibrio* strains harbor unique integrons as a result of lateral gene transfer (Koenig *et al.*, 2011). Further, genomic comparison at the nucleotide level shows a substantial level of divergence, ANIb (average nucleotide identity via BLAST) = 96.6, between the two strains (Goris *et al.* 2007).

V. corallilyticus *proteome*

Vibrio pathogenicity is multifactorial, requiring the expression of numerous virulence factors and other essential genes for infectivity. Differential expression of these proteins regulates the multiple stages of microbial disease, including transmission, adhesion, penetration, survival and host injury. To assess gene expression in *Vc*450, we compared proteins expressed at its avirulent (24 °C) and virulent (27 °C) temperatures, using two-dimensional liquid chromatography coupled to tandem mass spectrometry. Growth curve analyses and protein assays did not reveal a significant difference in *Vc*450 growth at the temperatures utilized in this study (Figure 2), demonstrating that the differential regulation of virulence factors was not caused by changes in growth or abundance. Spectral counting provided quantification of the relative abundance of individual proteins between the two temperatures, revealing significant changes in gene expression (Supplementary Table 1). Our results revealed significant upregulation of 136 virulence-associated genes encoded in the genome of *Vc*450 grown at 27 °C, including factors involved in motility, host degradation, QS, antimicrobial resistance, secretion and transcriptional regulation (Table 2). In contrast, ribosomal proteins and general stress proteins (that is, heat shock and cold shock proteins) were downregulated at the higher temperature (Supplementary

Table 2 Number of *Vc*450 genes and proteins identified within a given virulence category

Virulence categories	*Genome*	*24 °C proteome*[a]	*27 °C proteome*[a]
Chemotaxis/motility			
Chemotaxis proteins[b]	57	20 (2)	38 (31)
Flagellar proteins[b]	82	13	23 (16)
Host degradation			
Toxins[b]	4	1	3 (3)
Hemolysin/cytolysin[b]	14	3	4 (2)
Proteases	45	18 (7)	21 (7)
Resistance			
Multidrug efflux pumps	32	3 (2)	4 (2)
Multidrug resistance	11	4 (2)	3
Specific antibiotic	14	1	2
Secretion			
T1[b]	203	50 (11)	68 (35)
T2	15	7 (1)	9 (6)
Tfp[b]	50	10	13 (10)
T3	21	1	1
T4	14	0	0
T6[b]	42	10 (1)	16 (12)
Regulation			
Sigma factors[b]	28	7 (1)	10(5)
H-NS[b]	1	1	1 (1)
Quorum sensing signaling/receptors	8	6 (1)	5 (2)
Quorum sensing response regulators[b]	12	4 (1)	7 (4)

[a]The number of proteins significantly upregulated at the temperature indicated is given in parenthesis.
[b]Virulence categories significantly upregulated as a whole at 27 °C using a *G*-test statistic ($P > 0.05$).

Table 1), providing additional evidence that the upregulation of these putative virulence factors is not a growth-dependent response.

In the initial stage of a *Vibrio* infection, chemotaxis and motility are essential for *Vibrio* species to locate and initiate infection in their host. *V. vulnificus* (Lee *et al.*, 2004), *V. anguillarum* (Ormonde *et al.*, 2000) and *V. fischeri* (Millikan and Ruby, 2004), all display attenuated infection in motility-deficient mutants. Similarly, nonmotile *Vc*450 mutants are unable to infect the coral, *Pocillopora damicornis* (Meron *et al.*, 2009). *Vc*450 and *Vc*P1 contain two adjacent regions involved in lateral flagella gene system (VIC_004722–VIC_004762); however, no gene products were present in the proteome and no lateral flagella were observed by electron microscopy. In contrast, the upregulation of polar flagellar proteins (Table 2) and increased motility observed at 27 °C (Supplementary Figure 4) demonstrate that temperature influences *Vc*450 motility via its single polar flagellum. Furthermore, there is an increase in methyl-accepting chemotaxis proteins that relay environmental signaling cues to flagellar motor controls, with 80% of these significantly upregulated (Table 2). Increased diversity of the methyl-accepting chemotaxis proteins allows a more sensitive response to changing conditions in the environment (Tran *et al.*, 2008), whereas increased expression provides the ability to amplify a response signal (Parkinson, 2004). These results support the hypothesis that a temperature-dependent increase in chemotaxis and motility of *Vc*450 contributes to its increased virulence at higher temperatures.

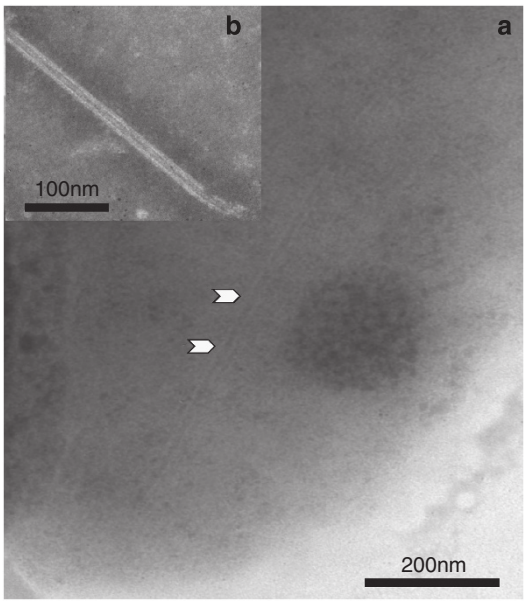

Figure 3 Transmission electron photomicrograph of *Vc*450 T6SS tubular structure. *Vc*450 cell stained with 0.5% sodium phosphotungstic acid, pH 6.8. (**a**) A VipA/VipB-like T6SS tubular structure, similar to that described for *V. cholerae*, is evident in the cytoplasm (black arrowheads). (**b**) A *Vc*450 VipA/VipB-like T6SS tubular structure found outside of a cell.

Following transmission, secretion systems are utilized by vibrios to transport macromolecules necessary for the remaining stages of infection. The *Vc*450 genome encodes five of the six described bacterial secretion systems (T1-T6SS): T1-4SS and T6SS (two clusters). In addition, we identified type IV pilus (Tfp) subsystems, including three tight adherence (Tad) locus colonization islands, mannose-sensitive hemagglutinin (MSHA) genes and Pil components. *Vc*P1 contains the same five secretion systems and three Tfp subsystems; however, there are notable differences. Whereas T2SS (that is, general secretion genes) and T6SS genes display 98–100% homology between *Vc*450 and *Vc*P1, the T3SS genes (VIC_001039–VIC_001055) and one of the Tfp Tad clusters (VIC_001023–VIC_001030) located on the *Vc*450 megaplasmid display <50% sequence similarity with the corresponding *Vc*P1 genes. Similarly, *Vc*450 and *Vc*P1 T4SS conjugation genes of the megaplasmid exhibit only 67% sequence similarity.

Expression of T1SS, T2SS, Tfp (MSHA and Tad) and T6SS genes are upregulated by *Vc*450 at 27 °C (Supplementary Table 1). Both pathogenic and nonpathogenic vibrios secrete factors necessary for host colonization, including RTX toxins, proteases and hemolysins via T1SS and T2SS, whereas the

Tfp subsystems are important for biofilm formation, colonization and phage transductions by pathogens, including *V. cholerae* (Wooldridge, 2009). Of the two clusters of T6SS genes, 12 out of 19 genes from one cluster (VIC_003912–VIC_003930) are upregulated at 27 °C, whereas only 3 of the 23 genes from the second cluster (VIC_003136–VIC_003158), including the *hcp* effector gene (ZP_05886652.1), are upregulated at the higher temperature. Interestingly, VipA (annotated as ImpB) is expressed from both clusters (ZP_05886644.1 and ZP_05887414.1). It is unclear whether both copies need to be expressed to produce the T6SS tubules observed in *Vc*450 (Figure 3), which resemble those observed in other vibrios (Bonemann *et al.*, 2009). The upregulation observed in such a broad array of secretion systems indicates an increased capacity for a variety of functions that facilitate establishment of *Vc*450 at higher temperatures.

Once associated with a host, pathogenic *Vibrio* species employ various mechanisms, including antibiotic resistance, to maintain competitiveness against other microorganisms and to ward off host defenses. *Vc*450 exhibits a temperature-dependent increase in resistance to the antimicrobial activity of coral-associated bacteria, as well as to therapeutic antibiotics (Vizcaino *et al.*, 2010). We observed a larger number of multidrug-resistance efflux pump proteins expressed at 27 °C, with two of these (ZP_05885878.1 and ZP_05883974.1) displaying significant upregulation (Supplementary Table 1). Multidrug efflux pumps have a significant role in virulence of *V. cholerae,* and are required for resistance to the host innate immune system (Bina *et al.*, 2008). This may be relevant to *Vc*450, as the innate immune system of cnidarians, including corals, shares some conserved defenses with that of higher vertebrates, including humans (Dunn, 2009). Evidence also suggests that multidrug-resistance efflux pumps provide an alternative function in bacterial pathogenicity, including transport of virulence factors (Piddock, 2006). The convergence of temperature-dependent virulence and increased antibiotic resistance in *Vc*450 highlights its unique attributes as a model organism in a warming environment compounded by multiple stressors.

Host degradation factors, such as proteases and toxins, also contribute to *Vibrio* pathogenicity (Thompson *et al.*, 2004). In the *Vc*450 genome, there are 45 annotated proteases, 2 of which (VIC_003472 thermolysin/zinc-metalloprotease and VIC_002633 neutral protease precursor) have homologous regions with metalloproteases previously identified in *V. coralliilyticus* infection studies (Ben-Haim *et al.*, 2003b; Sussman *et al.*, 2009). Out of the 21 proteases identified in the *Vc*450 proteome, neither VIC_003472 nor VIC_002633 were detected. This could be the result of strict parameters employed in identifying proteins in the *Vc*450 proteome, or it could indicate that there is redundancy in the metalloprotease functionality as suggested previously (de Oliveira Santos *et al.*, 2011).

Toxins, including hemolysins, have a significant role in *Vibrio* pathogenicity (Thompson *et al.*, 2004; Igbinosa and Okoh, 2008), and their activity has been shown to be directly affected by temperature (for example, enterohaemorrhagic *E. coli*) (Li *et al.*, 2008). *Vc*450 exhibits upregulation of two hemolysins (ZP_05886322.1 and ZP_05888459.1) at 27 °C, with corresponding enhanced hemolytic activity (Supplementary Figure 5). *Vc*450 also displays significant upregulation of the RTX toxin (ZP_05887531.1), which is not present in *Vc*P1. Further screening of *Vc*450 supernatants and cell lysates with CHO cells revealed the production of multiple active proteins. *Vc*450 produced a cell elongation factor at all temperatures assayed early in its growth curve and into stationary phase. At 24 h of growth, *Vc*450 secreted a nonhemolytic cytotoxic substance at higher temperatures (27 °C and 30 °C) and a hemolysin, which had low cytotoxic activity against CHO cells, present at all temperatures tested. Although we could not predict the cell elongation factor of *Vc*450 from the proteome, we hypothesize that the RTX toxin is the most likely candidate as the cytotoxic substance, as it is a pore-forming toxin and the CHO cells were not completely lysed during the assay. In *V. cholerae*, RTX toxin acts as a virulence cofactor disrupting the cell wall integrity of the host cells (Olivier *et al.*, 2007), whereas the *V. vulnificus* RTX toxin causes cell lysis through pore formation, resulting in the degradation of phagocytic host cells (Lo *et al.*, 2011). An increased expression of RTX toxin in *Vc*450 at the virulent temperature of 27 °C may allow for increased survival of *Vc*450 owing to degradation of the host's innate immune system.

V. coralliilyticus *pathogenicity islands*

Pathogenic vibrios, like many bacteria, commonly acquire virulence factors via horizontal transfer of bacteriophages and pathogenicity islands (Chun *et al.*, 2009). We identified two novel pathogenicity islands in the genome of *Vc*450. Coralliilyticus pathogenicity island-1 (CPI-1) is located on C1 at an integration site consistent with the *dif*-like region of *V. cholerae* and *V. parahaemolyticus,* the insertion site for CTX and f237 phages in these species, respectively. CPI-1 contains homologs of the VvhA cytolysin (VIC_004014) and the associated secretory protein VvhB (VIC_004013), which produce and secrete the primary toxin of *V. vulnificus*, respectively. CPI-1 additionally carries a putative bacteriocin (VIC_004006) and the RTX toxin mentioned above (VIC_004043). This integration locus also possesses two T3SS clusters, presumably for secretion of the different effector proteins within the island. The *Vc*P1 genome carries a similar pathogenicity island to the CPI-1 (Figure 4), with most corresponding genes sharing >90% homology. However, the RTX toxin (ZP_05887531.1), one of 12 CPI-1 proteins upregulated at 27 °C in the *Vc*450 proteome, is unique to the *Vc*450 CPI-1. The

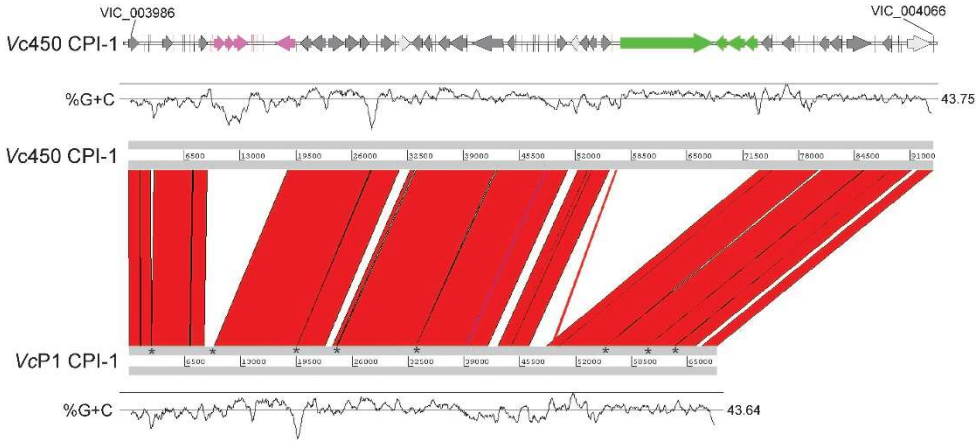

Figure 4 Schematic representation of *Vc*450 CPI-1 and comparison with *Vc*P1 using Artemis Comparison Tool (ACT, Wellcome Trust Sanger Institute, Hinxton, UK). Coding sequences of CPI-1 from *Vc*450 are shown on the top, with G + C content directly below. Two large regions are present in *Vc*450 and not in *Vc*P1, one with mostly hypothetical proteins (fuschia) and the other containing an RTX homolog, transporter and associated genes (green). Additionally, there were three regions of gene-level divergence (light gray) at VIC_4016, VIC_4037 and within VIC_4065. CPI-1 of *Vc*P1 is composed of nine contigs: AEQS01000075, −105, −183, −138, −135, −025, −161, −224 and −071, with contig gaps (asterisks) indicated on the bottom ACT scale.

upregulation of numerous proteins from the CPI-1 at 27 °C indicates that CPI-1 contributes to *Vc*450 pathogenicity at a higher temperature, whereas the low homology between the *Vc*450 and *Vc*P1 RTX toxins indicates a potential difference in virulence between the two strains.

The *Vc*450 genome contains a second novel pathogenicity island, coralliilyticus pathogenicity island-2, which is not found in *Vc*P1. Coralliilyticus pathogenicity island-2 is similar to vibrio seventh pandemic island-II of *V. cholerae* and located on C1, inserted between a tRNA (Uracil54-C5-)-methyltransferase (VIC_000153) and an adenosine triphosphatases (VIC_000179) of the AAA + class (Supplementary Figure 6). The tandem arrangement of these two genes is highly conserved among *Vibrio*, *Allivibrio* and *Photobacterium* species, with only four species (*Vc*450, *V. furnissii* CIP102972, *V. alginolyticus* 12G01 *and V. cholerae* RC385) known to contain a genomic island at this site.

Temperature-dependent regulation

The altered regulation of virulence factors and phenotypic changes documented in this study indicate that temperature affects *Vc*450 virulence mechanisms independent of growth or abundance. We hypothesize that global transcriptional regulators, which by definition are capable of affecting the expression of numerous genes from multiple pathways, are driving the temperature modulation observed. The *Vc*450 genome contains bacterial thermosensors and regulators known to influence downstream virulence signaling in other vibrios, and these transcriptional regulators are differentially expressed between 24 °C and 27 °C (Table 2). For example, nucleoid-associated protein (H-NS) binds DNA at lower temperatures blocking the transcription of multiple genes, whereas higher temperatures cause loosening in the DNA structure allowing transcription to occur. H-NS suppresses virulence-associated genes, such as RTX and CTX, in *V. vulnificus* (Liu *et al.*, 2009) and *V. cholerae* (Stonehouse *et al.*, 2011), respectively. The concurrent upregulation of H-NS (ZP_05887985.1) and RTX toxin (ZP_05887531.1) on CPI-1 in the *Vc*450 proteome at 27 °C could be the result of a conserved *Vc*450 H-NS unable to regulate a more recently acquired RTX toxin. Alternatively, decoupling of the H-NS protein from the RTX toxin DNA could allow greater detection of H-NS protein concurrent with the resulting upregulation of RTX toxin.

QS is another global mechanism by which temperature can directly regulate virulence in *Vc*450. In *V.cholerae*, *V. harveyi* and *V. parahaemolyticus* QS is achieved through AI stimulation of histidine kinase receptor pathways (Ng and Bassler, 2009). Activation of these pathways result in the transcription of small RNAs, which subsequently degrade the messenger RNA of virulence factors (Ng and Bassler, 2009). Temperature has recently been shown to affect QS mechanisms (Tait *et al.*, 2010). For example, *V. mediterranei* produces four *N*-Acyl homoserine lactone at 18 °C compared with only two at 25 °C and 30 °C (Tait *et al.*, 2010), and there is evidence that temperature can affect the level of AHL production positively (Hasegawa *et al.*, 2005; Latour *et al.*, 2007) and negatively (Tait *et al.*, 2010). The *Vc*450 genome possesses three (AI-1/LuxMN, AI-2/LuxSPQ and CAI-1/CqsAS) two-component histidine kinase QS pathways (Figure 5) and three small RNAs (Supplementary Figure 7), characteristic of those identified in the regulation of other *Vibrio* species QS (Lenz *et al.*, 2004). In addition, the

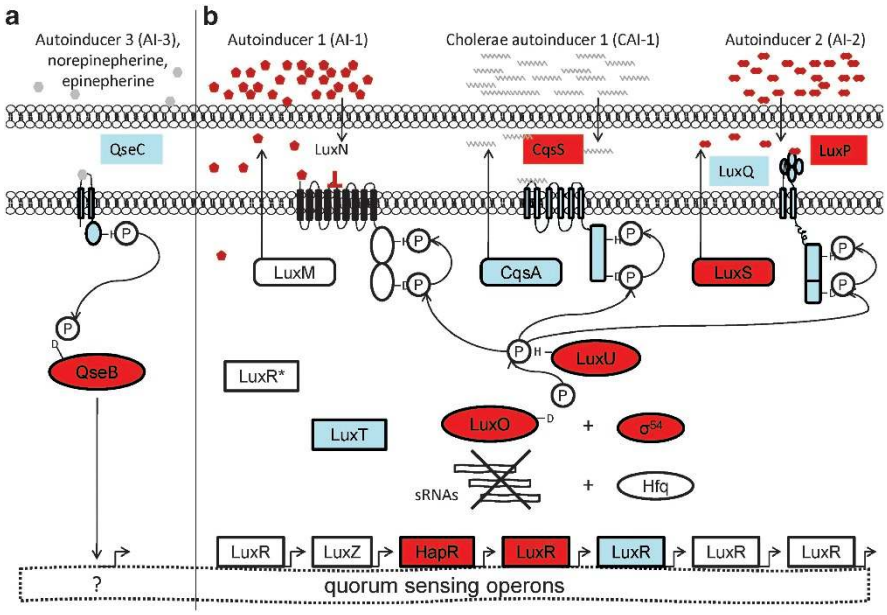

Figure 5 Proposed *Vc*450 QS systems. This figure illustrates the potential QS mechanisms utilized by *V. coralliilyticus* at high density. (**a**) The QseBC system is a two-component system, in which the QseC histidine kinase receptor becomes phosphorylated when bound to cognate ligands and subsequently activates QseB through phosphorelay. The activated QseB molecule binds DNA, acting as a direct transcriptional regulator. (**b**) The three two-component histidine kinase receptor systems previously described in *Vibrio* species, each produce and detect a specified class of AI. Ligand binding, at levels above the density threshold, blocks the kinase activity of the membrane-bound receptors, reversing the phosphorelay. This results in the dephosphorylation of the response regulator, LuxO, via LuxU. In its unactivated state, LuxO is unable to transcriptionally activate the sRNAs that degrade the messenger RNA (mRNA) of LuxR-type genes. Thus, LuxR-type mRNA is stabilized and proteins are produced. The LuxR-type proteins in turn act as transcriptional regulators of virulence-associated genes. All of the proteins shown are present in the *Vc*450 genome. The shaded (blue and red) proteins are present in the *Vc*450 proteome, with (red) proteins representing those significantly affected by temperature.

AI-3/QseBC QS pathway, originally described in enterohemorrhagic *E. coli* is present (Figure 5), providing a potential mechanism for direct interactions with a host (Hughes and Sperandio, 2008). Although this system is not well characterized in vibrios, genomic comparisons reveal the presence of *qseBC* genes in numerous *Vibrio* species (Supplementary Figure 8). The four QS systems present in *Vc*450 are also present in *Vc*P1 and share >98% homology, indicating that these systems are common between two geographically distinct strains of *V. coralliilyticus*.

The AI2/LuxSPQ, CAI-1/CqsAS and the AI-3/QseBC QS systems are detected in the *Vc*450 proteome (Figure 5), whereas AI signaling from AI-1/LuxMN and AI-2/LuxSPQ QS systems were established using bioluminescence reporter assays (Supplementary Figure 9). Collectively, these results indicate that all four QS pathways found in the *Vc*450 genome are active (Figure 5). Furthermore, the *Vc*450 proteome exhibits upregulation at 27 °C of numerous QS proteins (Supplementary Table 1), including the following: histidine kinase receptors (ZP_05879449.1, ZP_05886587.1), response regulators (ZP_05888198.1, ZP_05888199.1) and transcriptional regulators (ZP_05887548.1, ZP_05884374.1). Bioluminescence reporter assays also reveal significant temperature effects on AI-1 and AI-2 (Supplementary Figure 9) signaling, indicating that temperature has a direct effect on *Vc*450 QS.

Conclusions

V. coralliilyticus is considered an endemic member of coral reef ecosystems, consisting of geographically distinct strains that exhibit genetic variations (Pollock *et al.*, 2010). *Vc*450, isolated from the Indian Ocean, and *Vc*P1, isolated from the GBR, represent two geographically distinct strains that cause different coral infections (that is, bleaching and white syndrome, respectively), indicating that each of them harbor unique virulence mechanisms in addition to shared virulence characteristics. The complexity of virulence-associated factors expressed by *Vc*450, similar to the complexity described for *Vc*P1 (de Oliveira Santos *et al.*, 2011), suggests that *V. coralliilyticus* infections depend on the coordinated expression of multiple factors. Although the two strains share conserved virulence-associated genetic components, such as flagellar, secretion and QS systems, we describe here a number of genomic differences between *Vc*450 and *Vc*P1, namely RTX toxin, proteases and pathogenicity islands, that most likely account for their unique physiological characteristics. Further, the ANIb of 96.6 between the two genomes argues that although *Vc*450 and *Vc*P1 are strains of the same species, they have diverged significantly in a vertical fashion, in addition to the lateral differences described above, and may represent distinct ecotypes or subspecies. Much remains unknown,

however, regarding the ecological and physiological differences among strains of *V. coralliilyticus*.

In a warming ocean, the confluence of genetic mobility, temperature-dependent virulence and increased antimicrobial resistance makes *V. coralliilyticus* a formidable global pathogen with broad host specificity. *Vc*450 exhibits resistance to many common antibiotics (that is, tetracycline, erythromycin and quinolones (Vizcaino *et al.*, 2010)) and to date, phage therapy is the only proposed strategy for mitigation of *V. coralliilyticus* infections (Efrony *et al.*, 2009). Elucidating temperature-dependent virulence mechanisms of *V. coralliilyticus* may assist in the design of antivirulence therapies (Cegelski *et al.*, 2008) for this organism, as well as for other vibrios, which exhibit temperature-related disease outbreaks, including *V. cholerae*. With the world's oceans changing rapidly (Hoegh-Guldberg and Bruno, 2010), we hypothesize that *V. coralliilyticus* will become a sustained threat to coral reefs and propose that *V. coralliilyticus* establishes a model to further elucidate temperature-dependent virulence mechanisms.

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