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***Vibrio cholerae* in an Historically Cholera-Free Country**

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Summary

We report the autochthonous existence of *Vibrio cholerae* in coastal waters of Iceland, a geothermally active country where cholera is absent and has never been reported. Seawater, mussel, and macroalgae samples were collected close to and distant from sites where geothermal activity causes a significant increase in water temperature during low tides. *V. cholerae* was detected only at geothermal-influenced sites during low-tides. None of the *V. cholerae* isolates encoded cholera toxin (*ctxAB*) and all were non-O1/non-O139 serogroups. However, all isolates encoded other virulence factors that are associated with cholera as well as extra-intestinal *V. cholerae* infections. The virulence factors were functional at temperatures of coastal waters of Iceland, suggesting an ecological role. It is noteworthy that *V. cholerae* was isolated from samples collected at sites distant from anthropogenic influence, supporting the conclusion that *V. cholerae* is autochthonous to the aquatic environment of Iceland.

Introduction

Vibrio cholerae, a Gram-negative bacterium and the causative agent of cholera, has caused seven pandemics since 1816, as well as sporadic inter-epidemic outbreaks. Studies of *V. cholerae* in the environment have often focused on geographic regions of cholera endemicity, in order to elucidate links between its aquatic reservoir and clinical cases of cholera. This has led to the assumption that disease-causing strains of *V. cholerae* are confined to geographical regions where cholera occurs annually or sporadically, that the human gastrointestinal tract is an essential environment for *V. cholerae* presence and dissemination, and that the primary role of virulence factors is infection of the human body. Here we report the presence of *V. cholerae* in Iceland, a geothermally active island in the subarctic north Atlantic with active marine and terrestrial hot-springs, where cholera has never been recorded, and *V. cholerae* has never been isolated from humans (Islam et al., 1993; Gunnardsdóttir, 2008; Haraldur Briem, Chief Epidemiologist, Infectious Disease Control, personal communication). Results demonstrated the presence along the coast of this cholera-free country of genetically diverse *V. cholerae* populations encoding virulence

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factors known to be integral in human disease, namely cholera. This study demonstrates that *V. cholerae* is autochthonous in a region of the world where cholera never occurs and that the human body is not an obligate environment for the presence and dispersal of this organism. Moreover, virulence factors shown to be conserved in these strains are concluded to be related to the ecology of this bacterium in its native aquatic habitat.

Results and Discussion

Sampling

One liter water samples were filtered through 0.22 μm nitrocellulose membranes. Each membrane was placed in 200 ml of 1% alkaline peptone water (APW) and incubated overnight at 37°C with shaking at 100 rpm. The meat of each mussel was removed aseptically and 10 g were weighed and diluted in 100 ml of PBS + 2% NaCl, and homogenized in a stomacher (Seward, West Sussex, UK). The homogenate (10 ml) was transferred to a flask with 200 ml of 1% APW and incubated overnight at 37°C with shaking at 100 rpm. Kelp samples were also processed following this procedure. After incubation, 20 μl of the top-most layers of the overnight APW cultures were streaked onto TCBS agar and incubated overnight at 37°C. Yellow sucrose-fermenting colonies were presumptively identified as *V. cholerae*, aseptically removed from the TCBS agar, and stored at -80°C in 2-ml cryotubes with 1.5-ml Luria-Bertani broth amended with 20% sterile glycerol.

Sampling locations chosen near geothermal activity were labeled geothermal-influenced, while locations far from geothermal activity were labeled non-geothermal-influenced (Figure 1). At sites positive for *V. cholerae*, all surface water, macroalgae, and mussel samples collected were positive for *V. cholerae* (Tables 1 and 2). *V. cholerae* were recovered at all geothermal-influenced sites from the samples collected at low-tide, but not at any of the non-geothermal-influenced sites or geothermal-influenced sites at high tide, when surface water temperatures are low because of cold seawater inflow (Table 1). Friedman's test demonstrated that median surface water temperatures at times of sampling when *V. cholerae* was isolated (median = 27°C, mean = 25.5°C) came from a different distribution than surface water temperatures when *V. cholerae* was not isolated (median = 5.5°C, mean = 6.5°C) ($P < 0.05$). Three of the sites positive for *V. cholerae* are located significantly distant from cities or towns, centers of tourism, or major international shipping routes (Berserkseyri and Stykkishólmur in Breiðafjörður, and Vatnsnes in northern Iceland). Reykjavík harbor (the old port of Reykjavík) and Álftanes and Hlíðnes near the shipping port of Hafnarfjörður near Reykjavík, are not geothermal-influenced and those samples were negative for *V. cholerae*.

Isolate Characterization and Diversity

In total, 380 presumptive *V. cholerae* isolates were recovered primarily from geothermal-influenced sites. One non-geothermal-influenced site, Hvalfjörður, yielded 22 yellow colonies on TCBS that were determined by PCR not to be *V. cholerae*. Other non-geothermal-influenced sites yielded no growth on TCBS after incubation of water samples overnight at 37°C and incubation of inoculated TCBS plates at 37°C for 24 to 48 hours. Mesophilic bacteria that grow on TCBS at 37°C are either absent from or in low abundance in these areas or, most likely, may be present in the viable but nonculturable (VBNC) state and not detectable by the methods employed in this study. The remaining 358 isolates were isolated from samples collected at the geothermal-influenced sites and all, except 22 isolates from water samples collected at Stykkishólmur, were presumptively identified as *V. cholerae* by biochemical identification following a previously published protocol (Choo-pun et al., 2002). A subset (127) of the isolates were subjected to PCR and were confirmed *V.*

cholerae. From these results it is concluded that *V. cholerae* was recovered from five of the coastal sampling sites in nine of 19 sample collections that were carried out (Table 1).

Confirmation of *V. cholerae* by PCR was achieved by targeting *V. cholerae* intergenic spacer and *toxR* gene (Chun et al., 1999; Vora et al., 2005), and further analysis was done to detect virulence factors and mobile genetic elements. All *V. cholerae* isolates were non-O1/non-O139 serogroups, and *ctxAB* negative (Table 2), and did not encode the *Vibrio* seventh pandemic island I (VSP-I) on either chromosome or the *Vibrio* pathogenicity island 1 (VPI-1) also known as the TCP island. All strains were evaluated for presence of the *Vibrio* seventh pandemic island II (VSP-II) by employing the PCR typing scheme for presence of the island and its variants (Taviani et al., 2010). Amplification was not observed for any of the VSP-II variants. However, 50 strains (39%) demonstrated amplification of the 451 bp region that has been reported in all described *V. cholerae* VSP-II variants, except the *V. cholerae* CIRS101 variant. It is concluded that the Iceland strains encode a VSP-II variant similar to that described in *V. cholerae* RC385, an environmental isolate from the Chesapeake Bay and present in other *V. cholerae* non-O1/non-O139 strains isolated from the mid-Atlantic coast of the United States and from Bangladesh (Taviani et al., 2010). PCR targeting VSP-II flanking regions of the 77 VSP-II-negative isolates did not result in amplification, demonstrating that another genomic island not yet described is inserted at this locus in these isolates.

All but three strains encoded sialidase (NanH) of the *Vibrio* pathogenicity island 2 (VPI-2) that acts as a sialic acid scavenger by cleaving two sialic acid groups from the triasialogangliosides of the intestinal mucus, thereby releasing sialic acid and making the epithelial cell gangliosides in the human gut more accessible to cholera toxin (Moustafa et al., 2004; Almagro-Moreno and Boyd, 2009). We used *nanH* as a marker of VPI-2 which often encodes a suite of sialic acid transport and catabolism genes along with *nanH*, all shown to be expressed in models of *V. cholerae* infections (Almagro-Moreno and Boyd, 2009).

All strains encoded *hlyA*, *rtxA*, HA/P, which are involved in *V. cholerae* virulence in humans (Finkelstein et al., 1992; Olivier et al., 2007). Fifty eight strains (45%) encoded cholix toxin, a novel ADP-ribosylating toxin and 114 (89%) also encoded the integrase of an integrative and conjugative element (ICE). DNA of all strains encoded the hemagglutinin/protease (HA/P), known to be involved in mucin penetration, detachment, spread of the infection through the gastrointestinal tract, and full expression of enterotoxicity (Silva et al., 2006; Shinoda, 2011). This protease has also been shown to be involved in the degradation of chironomid egg masses (Halpern et al., 2003) which inhabit the aquatic environment of the Iceland coast (Ingólfsson, 1995; Sæther, 2009; Kaiser et al., 2010). Chironomids can inhabit the sandy intertidal zone, where we were able to isolate *V. cholerae* in Berserkseyri.

Diversity of a subset of strains isolated from different locations in Iceland was analyzed by pulsed field gel electrophoresis (PFGE), revealing genomic variability within Icelandic *V. cholerae* populations (Figure 4). The genomic patterns did not match those of other environmental non-O1/non-O139 and clinical isolates reported in other regions of the world. Interestingly, two sets of strains from different sampling sites: (1) strain 226 isolated from a sand pore water sample collected at Berserkseyri on 12/08/2006 and strain 334 isolated from mussels in Ægisíða isolated on 2/09/2007; (2) strain 310 isolated from macroalgae in Seltjarnarnes on 1/09/2007 and strain 295 from macroalgae in Ægisíða, on 1/09/2007 yielded identical banding patterns. These two patterns suggest genomic clonality of a subset of strains circulating around Iceland between regions of geothermal activity. However, no other conserved set of PFGE patterns was observed among the other Iceland strains, indicating significant diversity of the *V. cholerae* strains.

Phenotypic diversity among seven strains randomly selected for analysis was observed at 25 and 34°C, showing different patterns of carbon substrate utilization on Biolog PM2A plates (Figure 3 and Supplementary Figure 1). Results of other phenotypic tests showed only one strain to be bioluminescent, a phenotype encoded in GI-64 of the global *V. cholerae* mobilome reported by (Chun et al., 2009). Antibiotic disk diffusion assays of a subset of 44 strains showed all were susceptible to chloramphenicol (30 µg), ciprofloxacin (5 µg), vibriostatic agent O/129 (150 µg), streptomycin (10 µg), sulfamethoxazole/trimethoprim (23.75/1.25 µg), and tetracycline (30 µg), while 25% were resistant to ampicillin (10 µg). These results demonstrate genetic and phenotypic diversity of the *V. cholerae* populations of the Iceland coast, further evidence that *V. cholerae* in Iceland is neither imported nor derived from a single source. Moreover, similarity in some of the features suggests that strains of *V. cholerae* can circulate spatially along the coast of Iceland.

Expression of Virulence Factors

The role of virulence factors encoded in the *V. cholerae* strains was evaluated by expression *in vitro* at the temperatures of the geothermal-influenced environmental sampling sites. The phenotypes of 44 strains at were tested at 4, 14, 25, and 34, and 50°C. Motility, previously reported to be associated with virulence of *V. cholerae* virulence (Guentzel and Berry, 1975; Watnick et al., 2001; Krukonis and DiRita, 2003) was tested by inoculating motility media incubated for 24 hours. Those incubated at 14°C were evaluated after 96 hours. Motility was most active at 34°C (mean = 12.5 mm), followed by 25°C (mean = 6.25 mm), with no motility observed at temperatures 50°C or 14°C (Figure 4). Hemolysis was detected on blood agar plates incubated at 34°C (mean = 3 mm) and weak hemolysis at 25°C (mean = 1 mm). Growth without hemolysis was observed for the blood agar plates incubated at 14°C. Neither hemolysis nor growth was observed for plates incubated at 50°C or 4°C.

The sialic acid utilization cluster of VPI-2 was studied using seven randomly selected strains. These were inoculated in a medium containing sialic acid as sole carbon source and all utilized sialic acid when incubated at 34, 25, and 14°C. Sialic acid utilization was estimated as follows: area under the curve $\text{Strain} / \text{area under the curve Background}$ in a Biolog assay. Utilization was higher at 34°C (4.12) than at 25°C (3.57) (Table 4). Quantitative evaluation at of sialic acid utilization at 14°C could not be measured since this temperature falls below the Biolog temperature range. Plates with sialic acid as a carbon source were inoculated and incubated at 14°C, with end point color change recorded at 72 hours compared with a negative control. All strains tested utilized sialic acid at 14°C. Since marine bivalves contain free sialic acid in their hemolymph, this pathogenicity island may be instead ecologically significant in utilization of sialic acid of the mussels with which the bacterium is associated.

The *V. cholerae* strains were actively proteolytic at 14, 25 and 34°C, with the largest zones of proteolysis on the milk agar plates incubated at 34°C (6.1 mm). Milk agar plates inoculated with the *V. cholerae* isolates and incubated at 4°C for 2 weeks were also positive for proteolysis. To confirm proteolysis aliquots of overnight cultures were centrifuged at 20,000 × g for 20 minutes and the supernatant was removed and placed on ice for 1 hour, after which 10 µl was transferred to the top layer of chilled milk agar and incubated at 4°C for two weeks. Proteolysis was observed, with proteolysis as a virulence factor being functional at environmental temperatures relative to Iceland, a cholera-free region.

Conclusions

It is concluded that *V. cholerae* is naturally occurring and readily isolated from environmental samples collected along the coast of Iceland, a country where cholera has never been reported. *V. cholerae* was readily culturable in areas of geothermal activity

where water temperature was elevated, but *in vitro* growth experiments did not demonstrate growth of these isolates at 50°C, i.e., the bacteria are not hyperthermophilic. We hypothesize that *V. cholerae* is present in the VBNC state in areas that are not geothermal-influenced or when tides are high (and the water temperature is low) near sites of geothermal activity. *V. cholerae* in the mesophilic layer resulting from mixing of hot and cold water remained culturable. A mesophilic layer is present at all times at sites of geothermal-influence but during high tide it is overlaid with cold sea water. The similarity of the PFGE patterns of *V. cholerae* isolated from different locations in Iceland indicates circulation of *V. cholerae* strains around coastal Iceland that become culturable with temperature upshift of geothermal heated water. Such temperature upshifts have been shown to resuscitate VBNC cells of *V. vulnificus* to the culturable state (Oliver, 2005) and very likely *V. cholerae* (Chaiyanan et al., 2007).

DNA of *V. cholerae* isolated during this study were found to encode many of the virulence factors associated with intestinal and extraintestinal infections caused by *V. cholerae*. Icelanders have very little exposure to the usual pathways of cholera transmission, since municipal water is supplied by groundwater recharged by precipitation or glacial melt and uncooked molluscan shellfish, a common vehicle of vibrioses in developed countries, is not typically consumed in Iceland (Petursson, 1968; Guðfinnsson, 2007).

Historically, Iceland has experienced both sporadic cases and epidemics of other infections (plague, smallpox, and influenza) evidence that it is not isolated from pathogens that are global in their epidemiology (Hjaltelin, 1871; Karlsson, 1996; Dowell and Bresee, 2008, Cliff et al., 2009; Sigurdsson et al., 2009). It is one of the very few countries never to have recorded even a single case of cholera or related *Vibrio* infection (Islam et al., 1993). The absence of any record of cholera, a reportable disease in Iceland (Gunnarsdóttir, 2008; Icelandic Directorate of Health), combined with the extensive health record keeping for all citizens, with the presence of potentially virulent *V. cholerae* strains in the most remote locations in Iceland, suggest *V. cholerae* is autochthonous to Iceland. The ecology of *V. cholerae*, as has been documented elsewhere by other investigators does not require human transmission for persistence (Kenyon et al., 1984; Louis et al., 2003; Schuster et al., 2011). However, sporadic cases of cholera or *V. cholerae* infections in or near areas of cholera outbreaks or ballast water exchange in shipping ports where cholera has occurred has been suggested to be a source of *V. cholerae*. To our knowledge this is the first report of *V. cholerae* with functional virulence factors isolated from a region where a case of cholera has never occurred. It is concluded that *V. cholerae* was not introduced to Iceland, i.e. from ballast water or infected persons, but rather is a component of its natural ecological and microbiological environment. The results further suggest global distribution of *V. cholerae* in the aquatic environment without the necessity of anthropogenic activity as a source. The presumed absence of *V. cholerae* in regions where cholera has never occurred or has not occurred for many years earlier, is most likely because the presence of *V. cholerae* has not been recognized as naturally occurring in the aquatic environment and, therefore, not monitored.

Both presence of the microorganism and the high degree of conservation of virulence factors where cholera has not been documented strongly indicate these factors have an ecological function other than pathogenicity for humans. The results of this study fully support the autochthonous nature of this bacterium in aquatic systems on a global scale and not its confinement to those regions where cholera or *V. cholerae* infections are endemic or sporadic or only to warmer tropical and subtropical regions of the world. Clearly, human intestinal amplification and shedding is not required for the presence of *V. cholerae* in the natural aquatic environment and conservation of virulence factors does not serve solely to

maintain cell viability between human infections, but rather they play a role in the natural ecology of *V. cholerae*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Sampling sites showing presence and absence of geothermal activity. Ægissíða and Nauthólsvík are both located within the city limits of Reykjavík. Symbols that correspond to Reykjavík are bordered in black. Seltjarnarnes is a suburb of Reykjavík. Water, mussel, and macroalgae samples were collected at stations along the coast of Iceland (Fig. 1). Photo Credit: National Aeronautics and Space Administration (NASA). Use of this image is licensed under the Creative Commons Attribution-Share Alike 3.0 Unported license (http://commons.wikimedia.org/wiki/File:Iceland_sat_cleaned.png).

Figure 2. Pulse-Field Gel Electrophoresis images of strains collected along the coast of Iceland as well as globally distributed clinical and environmental strains. PFGE was performed using methods developed for *V. cholerae* (Cooper et al., 2006).

Figure 3. Carbon utilization patterns of 7 randomly selected *V. cholerae* strains using Biolog PM2A plate, Biolog Phenotype MicroArrays™ (Biolog Inc., Hayward, California). Strain ID is listed in far left column. Red = area under curve divided by background < 1. Yellow = area under curve divided by background 2 < 1. Green = area under curve divided by background > 2. Top row is carbon utilization profile at 25°C, bottom row is carbon utilization at 34°C.

Figure 4.

Expression of virulence factors on agar plates. Columns show the average expression; zones of hemolysis, proteolysis, and motility on blood, milk, and motility agar plates, at different temperatures, of strains isolated from different locations along the coast of Iceland (n = 44). Assays were conducted following standard methods (Son and Taylor, 2011).

Table 1

Sample sites, water temperature, salinity, presence of geothermal activity, tidal height, type of sample collected (water, kelp, mussels), and presence of *V. cholerae* are presented. All samples were collected within ca. 5 meters of a geothermal outlet or source (geothermal-influenced) or from a distance of ca. 1 km or greater from a geothermal source (non-geothermal-influenced). Sampling was not paired at some of the sampling locations because of inclement weather conditions at the sampling sites. Iceland experiences extreme weather in some locations where sampling sites are located with access difficult. Paired samples were obtained when conditions permitted. Ægissíða and Nauthólsvík are both located within the city limits of Reykjavík.

Site	Temp (°C)	Salinity	Month	Year	Geothermally Influenced	Tide	Sample	<i>V. cholerae</i> Detected
Hvalfjörður	6	NR	Oct	2006	-	Low	Ma, W, M	-
Ægissíða	5	NR	Oct	2006	+	High	W	-
Hlíðnes	7	NR	Oct	2006	-	Low	W	-
Álfanes	7	NR	Oct	2006	-	Low	W	-
Nauthólsvík	7	NR	Oct	2006	+	High	W	-
Seltjarnarnes	NR	NR	Nov	2006	+	High	Ma, W, M	-
Reykjanestá	2	35	Dec	2006	-	Low	Ma, W	-
Hafnir	6	32	Dec	2006	-	Low	Ma, W	-
Reykjavík Harbor	8	30	Apr	2007	-	High	W, M	-
Nauthólsvík	11	NR	Sept	2008	+	High	W	-
Stykkishólmur	21	20	Dec	2006	+	Low	Ma, W	+
Berserkseyri	17	6	Dec	2006	+	Low	W	+
Ægissíða	26	7	Jan	2007	+	Low	Ma, W, M	+
Seltjarnarnes	18	20	Jan	2007	+	Low	Ma, W	+
Ægissíða	31	2	Feb	2007	+	Low	Ma, W, M	+
Seltjarnarnes	32	15	Feb	2007	+	Low	W	+
Ægissíða	34	NR	Sept	2008	+	Low	Ma, W, M	+
Seltjarnarnes	28	NR	Sept	2008	+	Low	Ma, W	+
Skarðshver	34	NR	Nov	2008	+	Low	Ma, W	+
Skarðshver	14	NR	Nov	2008	+	Low	Ma, W	+

NR = not recorded

Ma = macroalgae

W = water

M = mussel

+ = yes

- = no

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Table 2

Results of PCR analysis of *V. cholerae* isolates collected during this study.

Date	Site	Source	Strains	O1/O139	ctxA	ctxB	ace	zot	ctxA (cholix)	toxR	ompU	hlyA	rtxA	HAP	luxO	tcpA	nag-ST	nanH	ICE	VSP-II	VSP-I
8 Dec 2006	Stykkishólmur	Water	10	0	0	0	0	0	10 (100)	10 (100)	10 (100)	10 (100)	10 (100)	10 (100)	10 (100)	0	0	8 (80)	10 (100)	7(70)	0
8 Dec 2006	Stykkishólmur	Macroalgae	10	0	0	0	0	0	10 (100)	10 (100)	10 (100)	10 (100)	10 (100)	10 (100)	10 (100)	0	0	10 (100)	10 (100)	6 (60)	0
8 Dec 2006	Berserkseyri	Water	10	0	0	0	0	0	0	10 (100)	10 (100)	10 (100)	10 (100)	10 (100)	10 (100)	0	0	10 (100)	10 (100)	7(70)	0
9 Jan 2007	Ægisiða	Water	11	0	0	0	0	0	0	11 (100)	11 (100)	11 (100)	11 (100)	11 (100)	11 (100)	0	0	11 (100)	11 (100)	0	0
9 Jan 2007	Ægisiða	Mussel	8	0	0	0	0	0	0	8 (100)	8 (100)	8 (100)	8 (100)	8 (100)	8 (100)	0	0	8 (100)	8 (100)	2(25)	0
9 Jan 2007	Ægisiða	Macroalgae	8	0	0	0	0	0	0	8 (100)	8 (100)	8 (100)	8 (100)	8 (100)	8 (100)	0	0	8 (100)	8 (100)	1 (12)	0
9 Jan 2007	Seltjarnarnes	Macroalgae	9	0	0	0	0	0	1 (11)	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	0	0	9 (100)	9 (100)	4 (44)	0
9 Jan 2007	Seltjarnarnes	Water	8	0	0	0	0	0	3 (37)	8 (100)	8 (100)	8 (100)	8 (100)	8 (100)	8 (100)	0	0	8 (100)	8 (100)	3 (37)	0
9 Feb 2007	Ægisiða	Mussel	11	0	0	0	0	0	5 (45)	11 (100)	11 (100)	11 (100)	11 (100)	11 (100)	11 (100)	0	0	11 (100)	11 (100)	4 (36)	0
9 Feb 2007	Ægisiða	Water	6	0	0	0	0	0	3 (50)	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)	0	0	6 (100)	6 (100)	2 (33)	0
9 Feb 2007	Ægisiða	Macroalgae	4	0	0	0	0	0	2 (50)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	0	0	4 (100)	4 (100)	1 (25)	0
9 Feb 2007	Seltjarnarnes	Water	9	0	0	0	0	0	7 (77)	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	0	0	9 (100)	7 (77)	2 (22)	0
4 Sep 2008	Ægisiða	Water	5	0	0	0	0	0	1 (20)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	0	0	5 (100)	5 (100)	0	0
4 Sep 2008	Ægisiða	Mussel	2	0	0	0	0	0	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	0	0	2 (100)	0	1 (50)	0
4 Sep 2008	Ægisiða	Macroalgae	2	0	0	0	0	0	1 (50)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	0	0	2 (100)	0	0	0
24 Sep 2008	Seltjarnarnes	Water	2	0	0	0	0	0	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	0	0	2 (100)	0	1 (50)	0
24 Sep 2008	Seltjarnarnes	Macroalgae	5	0	0	0	0	0	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	0	0	4 (80)	3 (60)	4 (80)	0
27 Oct 2008	Skarðshver	Water	4	0	0	0	0	0	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	0	0	4 (100)	3 (75)	3 (75)	0
27 Oct 2008	Skarðshver	Macroalgae	2	0	0	0	0	0	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	0	0	2 (100)	1 (50)	2 (100)	0
27 Oct 2008	Skarðshver	Water	1	0	0	0	0	0	0	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0	0	1 (100)	0	0	0
			127	0	0	0	0	0	58 (45)	127 (100)	127 (100)	127 (100)	127 (100)	127 (100)	127 (100)	0	0	124 (97)	114 (89)	50 (39)	0

Table 3

PCR primers used in the study.

	Target	Forward Primer	Reverse Primer	Reference
VSP-I		GCCGAGAACTCTAAAGCGCTTCTC	CCAAGTACAGATGATACACAGCA	
VSP-I insertion site (Chr I)	<i>Vibrio</i> Seventh Pandemic Island I	AAACTGGCGACCTTTGAGCAAGC	GATGGTAGCCTGACGCTGCATCTG	(Grim et al., 2010)
VSP-I insertion site (Chr II)		ATAGCGGGAGTTGGCTCTGCA	GGTGACTTGGTGCCCATCGTA	
pVSP2-I		CACCTGTCAATGTTATGAGGTGCA	AACAGGTCTCTTATCGGCTTTGC	
pVSP2-II	<i>Vibrio</i> Seventh Pandemic Island II	GCACAACTTGTAAAGATAGCCTTGC	ACGCAAGACAAAACACTACAGCTTGC	(Taviani et al., 2010)
pVSP2-III		CCAGCAAACGGTCATTCGCT	TGGTTGGAAGGTGGGTGTGT	
VSP-II insertion site		AGATCAACTACGATCAAGCC	CGCAGTCACAGCTTAAAC	(O'Shea et al., 2004)
O1	O1 antigen	GTTTCACTGAACAGATGGG	GGTCATCTCTAAAGTACAAC	
O139	O139 antigen	AGCCTCTTTATTACGGGTGG	GTCAAAACCCGATCGTAAAGG	(Hoshino et al., 1998)
<i>ctxA</i>	A subunit of cholera toxin	ACAGAGTGAATACCTTTGACC	ATACCATCCATATATTTGGGAG	
<i>ctxAB</i>	CTX _φ	AGTCAGGTGGTCTTTATGCC	TTGCCATACTAAATTGCGG	(Zhu et al., 2007)
HA/protease	Hemagglutinin/Protease	ACGTTAGTGCCCATGAGGTC	ACGGCAACACATTCAAAACC	Our Laboratory
nag-ST	non-agglutinating heat stable toxin	CAATCGCATTTAGCCAAAACA	GCAAGCTGGATTGCAACATA	Our Laboratory
<i>ctxA</i>	cholix toxin	TGGTGAAGATTCTCCTGCAA	CTTGGAGAAATGGATGCGCTG	(Purdy et al. 2010)
<i>tcpA</i>	A subunit of toxin co-regulated pilus	CACGATAAGAAAACCGGTCAAGAG	CGAAAGCACCTTCTTTCACGTTG	(Rivera et al., 2001)
<i>ace</i>	accessory cholera enterotoxin	TGATGGCTTTACGTGGCTTGTGATC	GCCTGTTGGATAAGCGGATAGATGG	
<i>zot</i>	zona occludens toxin	ATCTGCCCTAACCAACGCTAACATTG	ACCGCTTGCTCCGACAG	
<i>toxR</i>	global regulator of virulence	ACCGCAGCCAGCCAATGTG	TGGCAATGACTTCTATCGGCTTGAG	
<i>ompU</i>	outer membrane protein U	TACGCTGGTATCGGTGGCACTTAC	TCCATGCGGTAAAGAACGCGCTAG	(Vora et al., 2006)
<i>rtxA</i>	repeat in toxin	CTGAATATGAGTGGGTGACTTACG	GTGTATTGTTGATATCCGCTACG	
<i>luxO</i>	global regulatory gene	CGCTGTATCGTTCTTACCTCACACC	GCTCGCCGACAGAGTCAATGG	
<i>nanH</i>	sialidase	CTTCCTCCAATACGGTCTGTCTCTTATGC	TTCGGCTACCATCGGCAACTTGTATC	
<i>hlyA</i>	hemolysin A	GGCAAAACAGCGAAACAAATACC	CTCAGCGGGCTAATACGGTTTA	(Rivera et al., 2001)
ICE	integrative and conjugative element	GAGCCGGCATTCATCTGAAT	CTCTATGGGCACTGTCCACATTG	(Hochhut et al., 2001)

Table 4

Results of virulence assays. Numbers in columns show average results in millimeters (n=44 for hemolysis, proteolysis, and motility assays).

Assay Temperature (°C)	Hemolysis (mm)	Proteolysis (mm)	Motility (mm)	Sialic Acid Metabolism
50	0	0	0	ND
34	3	6.11	12.52	4.12 A
25	1	3.32	6.25	3.57
14	0	0.95	0	+
4	0	weak	0	ND

A = area under the curve $\text{Strain} / \text{area under the curve Background}$

ND = not done