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Rise and fall of pandemic *Vibrio parahaemolyticus* serotype O3:K6 in southern Chile

Katherine García, Roberto Bastías, Gastón Higuera, Rafael Torres, Alex Mellado, Paulina Uribe and Romilio T. Espejo*

Biotechnology Laboratory, Nutrition and Food Technology, INTA, University of Chile, Santiago, Chile.

Summary

Seafood consumption-related diarrhoea increased drastically in Chile when the pandemic strain of *Vibrio parahaemolyticus* serotype O3:K6 reached Region de Los Lagos, where most of Chile's seafood is produced. Outbreaks peaked in 2005 with 3725 clinical cases in this region and gradually decreased to fewer than 10 cases in 2010 and 2011. We show here that the pandemic strain concurrently vanished from mussels; we also report further environmental data. Integration of the 2010/2011 data with those obtained since 2004 suggests that after its arrival in southern Chile, the pandemic strain grew in mussels, likely facilitated by a minor rise in surface seawater temperature and by warming of the mussels in the intertidal region due to frequent sunny days. However, since these environmental parameters probably equally affected the pandemic strain and more than 30 *V. parahaemolyticus* DNA restriction clusters that inhabit local shellfish, a selective effect of bacteriophages is proposed. Lytic bacteriophage VP93 may have favoured the growth of the pandemic strain versus similar phage-sensitive strains, as shown here in a particular case. However, the pandemic strain's decline may have been promoted by temperate phage VP58.5, which kills the pandemic strain and increases the UV sensitivity of lysogenized phage-resistant cells.

Introduction

The bacterial species *Vibrio parahaemolyticus* includes a large number of strains that inhabit the sea. As with all bacteria, each of these strains reproduces via binary fission without systematic exchange of genes with other individuals of the same species, leading to essentially clonal reproduction. Different clones that evolve independently would thus form the population of *V. parahaemolyticus*, except for occasional lateral transfers that occurred between clones (Feil, 2004; Gevers *et al*., 2005). Only some of these clones of *V. parahaemolyticus* cause diarrhoea in humans when they are ingested in uncooked seafood. Although diarrhoea from ingestion of shellfish was rare in Chile before 1998, the rate increased greatly with the arrival of the pandemic strain O3:K6, which was originally observed in South-East Asia (Gonzalez-Escalona *et al*., 2008) and corresponds to a clonal complex.

The clonal nature of the *V. parahaemolyticus* pandemic isolates obtained worldwide has been ascertained by the high degree of similarity among their genomes. This comparison includes the presence of specific genetic markers and similarity of the restriction patterns of their genomes, demonstrated by genome restriction fragment length polymorphism–pulsed-field gel electrophoresis (Wong *et al*., 2000), direct genome restriction enzyme analysis (DGREA; Fuenzalida *et al*., 2006), arbitrarily primed PCR (Okuda *et al*., 1997; Matsumoto *et al*., 2000) and multilocus sequence typing (Chowdhury *et al*., 2004; Gonzalez-Escalona *et al*., 2008). Characteristics of isolates of the O3:K6 pandemic clone include the O3:K6 antigens, a distinctive *toxRS* sequence (*toxRS*new; Matsumoto *et al*., 2000), the presence of the *orf8* (Nasu *et al*., 2000) and *tdh* genes, and the absence of the *trh* gene in some pathogenic strains.

The pandemic strain was first observed in Chile in Antofagasta in 1998, when it produced a large outbreak that generated more than 300 clinical cases (Córdova *et al*., 2002). Large diarrhoea outbreaks related to seafood consumption started in Puerto Montt, Region de Los Lagos in 2004. In 2005, cases reported by the Ministry of Health reached peaks of 3600 and 10 984 in Region de Los Lagos and the entire country respectively. It is generally accepted that the seafood from this region caused most of the clinical cases of *V. parahaemolyticus*-associated diarrhoea observed in Chile because Region de Los Lagos produces approximately 80% of the seafood consumed in Chile [Anuario 2008 Sernapesca (http://www.sernapesca.cl)]. Since 2005, reported cases

Received 15 August, 2012; revised 16 August, 2012; accepted 23 August, 2012. *For correspondence. E-mail respejo@inta.cl; Tel. (+56) 2 9781426; Fax (+56) 2 2214030.

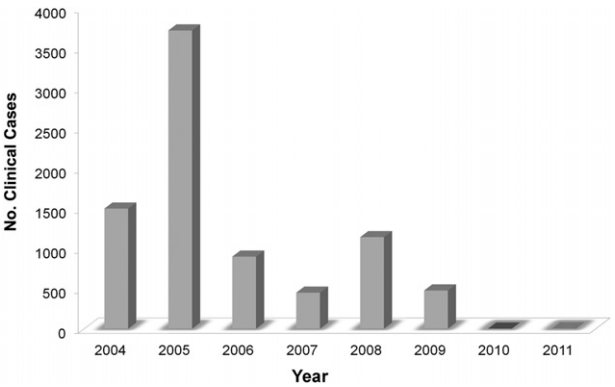

Fig. 1. Number of clinical cases of seafood-related diarrhoea in Region de Los Lagos.

have gradually decreased to a total of 441 in 2009, and practically no cases were reported in 2010 and 2011 (Fig. 1). Our observations previously suggested 'the pandemic strain has become a relatively stable bacterial subpopulation of the diverse *V. parahaemolyticus* population present in shellfish in Chile (García *et al*., 2009). However, the data from 2010/2011 showed that the pandemic strain has practically disappeared in the region, completing a cycle already observed in other regions.

The local rise and fall of the *V. parahaemolyticus* pandemic strain has been observed in several locations including Antofagasta, Chile, where it lasted a single summer, the Khanh Hoa province of Vietnam, where infection by the pandemic clone that was causing 49% of diarrhoea cases from 1996 onward abruptly stopped in November 1999 (Tuyet *et al*., 2002; Chowdhury *et al*., 2004), and Galveston Bay, TX, USA, where a particular clone has not been detected since it caused the largest *V. parahaemolyticus* outbreak ever reported in the USA in 1998 (DePaola *et al*., 2000).

In an effort to understand this cycle of proliferation and disappearance of pathogenic strains in the environment, we have assessed the influence of physical and biological environmental variables on *V. parahaemolyticus* growth in shellfish sampled at Quillaipe, a small harbour south of Puerto Montt, Chile, from 2004 to 2011. The *V. parahaemolyticus* population isolated from shellfish in Region de Los Lagos consists of a large set of strains belonging to different DGREA groups whose composition changes considerably each summer (Fuenzalida *et al*., 2006; 2007; Harth *et al*., 2009; García *et al*., 2009). Isolates belonging to 28 DGREA groups were isolated between 2004 and 2009 (García *et al*., 2009). Certain environmental variables seem relevant to *V. parahaemolyticus*' cycle in Region de Los Lagos, such as seawater and shellfish temperature, salinity, and biological variables including the presence of predators, bacteriophages and competing *Vibrio* species. Paradoxically, surface seawater temperatures seldom reach 16°C in this region. Our analysis indicates that both seawater temperature and warming of the abundant intertidal shellfish may have affected the life cycle of the pandemic strain. We also assess the possible effect of two pandemic *V. parahaemolyticus* bacteriophages found in this region (Zabala *et al*., 2009; Bastías *et al*., 2010). Here we report the practical disappearance of the pandemic strain of *V. parahaemolyticus* in Region de Los Lagos, and we evaluate likely physical and biological factors that may have caused its rise in 2004 and its fall in 2010 and 2011.

Results and discussion

Retreat of seafood-related diarrhoea in humans and pandemic V. parahaemolyticus in shellfish during 2010 and 2011

The large summer outbreaks occurring in Chile since 2005 that resulted in thousands of clinical cases in Region de Los Lagos fewer than 1 and 11 cases per year in 2010 and 2011 respectively (http://epi.minsal.cl/, search 'parahaemolyticus informe'). Not a single case among the 10 symptomatic persons seeking attention in the General Hospital of the Region these two summers was positive for *V. parahaemolyticus*. Coincidentally, the pandemic strain was not detected in mussels collected during these two summers (Table 1).

Twenty-eight and 32 shellfish samples (from 2010 and 2011 respectively) were analysed by *V. parahaemolyticus* enrichment via parallel serial dilutions of the soft meat in alkaline peptone water. After enrichment, the presence of *tlh*, which is specific for this species, and *tdh* and *trh*, which are associated with pathogenic strains, was tested by multiplex PCR (Bej *et al*., 1999) to asses the load of *V. parahaemolyticus*. Isolates obtained from the positive enrichment were then analysed and grouped according to their DNA restriction patterns using DGREA (Fuenzalida *et al*., 2006). Neither *tdh*- nor *trh*-positive isolates were obtained in 2010 and 2011 and most exhibited DGREA patterns observed in previous years; only one new pattern was observed for each of these years (Fig. 2; Table 1).

In addition to the absence of pandemic *V. parahaemolyticus* in the summers of 2010 and 2011, total loads in shellfish assessed by the most probable number method (Kaysner and DePaola, 2004) were considerably lower than those observed in previous years (García *et al*., 2009). Total *V. parahaemolyticus* (calculated according to the number of tubes positive for *tlh*) in shellfish sampled in 2010 ranged from 0.7 g^{-1} to 110 g^{-1}, with a MPN geometric mean of 2.1 g^{-1}. In samples from 2011, it ranged from < 0.3 MPN g^{-1} to 110 MPN g^{-1} with a geometric mean of 1.4 MPN g^{-1}, substantially lower than the values of 25.5 and 22.6 observed during the summer of 2006 and 2007,

Table 1. Properties of *V. parahaemolyticus* isolates obtained from shellfish during the summers of 2010 and 2011 in Region de Los Lagos, Chile.

	tlh	*tdh*	*trh*	DGREA group
V. parahaemolyticus isolate 2010				
PMA2.10	+	–	–	PMA34.6
PMA1.10, 3.10	+	–	–	PMA1.10
PMA4.10, 5.10, 6.10, 7.10, 8.10, 9.10	+	–	–	PMA118
PMA10.10, 11.10, 12.10, 13.10, 14.10, 15.10, 16.10, 17.10	+	–	–	PMA18.9
PMA18.10	+	–	–	PMA11.7
V. parahaemolyticus isolate 2011				
PMA1.11, 6.11, 11.11, 13.11, 19.11, 24.11	+	–	–	PMA1.11
PMA2.11, 3.11, 7.11, 8.11, 9.11, 14.11, 15.11, 17.11, 18.11, 20.11, 21.11, 22.11, 23.11, 25.11, 26.11, 30.11	+	–	–	PMA18.9
PMA4.11, 5.11, 10.11	+	–	–	PMA1.10
PMA27.11, 28.11	+	–	–	PMA118

+: present; –: absent. Strains in bold correspond to the prototype strain of each DGREA group.

respectively, the worst outbreak summers (García *et al*., 2009). However, the percentage of shellfish samples containing *V. parahaemolyticus* was similar to previous years (García *et al*., 2009). The genetic marker *tlh* was detected in 89% and in 88% of the sample enrichments from 2010 and 2011 respectively. Neither *tdh* nor *trh*, recognized markers of pathogenicity, were detected in these enrichments during these two summers.

V. parahaemolyticus *population diversity in shellfish*

Characterization by DGREA of single colonies from the enrichment cultures (Fuenzalida *et al*., 2006) indicated the appearance of only two new groups (Table 1). The 18 characterized isolates from 2010 were differentiated into five DGREA groups; four of these groups had been observed in previous years. The 27 isolates obtained in 2011 were differentiated into four DGREA groups, only one of which had not been observed previously. The two new groups appearing in 2010 and 2011 increased to 30 the number of DGREA groups observed since 2005 (Fuenzalida *et al*., 2006; 2007; Harth *et al*., 2009; García *et al*., 2009). Given this increase in the number of groups observed each year, the richness of the DGREA groups in seafood is estimated at 62 by Chao1 (Thompson *et al*., 2004). The number of strains of *V. parahaemolyticus* in seafood is probably much higher, considering that the shellfish included in this study were mostly taken from an area of less than 1 km^2.

Figure 3 depicts the number of samples containing isolates from the various DGREA groups since 2004, using data reported here and previously (Fuenzalida *et al*., 2006; 2007; Harth *et al*., 2009; García *et al*., 2009). The prevailing DGREA groups are rapidly changing; most of the strains appear and disappear each year. While 60% of the DGREA groups were detected in only a single year, other groups such as 118, 34.6 and VpKX (the pandemic strain) were more abundant and persistent. The pandemic strain persisted at detectable levels in shellfish from 2004 to 2008. Some DGREA groups, such as 187 and 339, were detected after 3 or more years of absence. These data probably reflect the abundance rather the presence or absence of each group, although in some cases absence may indicate actual disappearance from the environment. The significance of these observations is therefore important for predicting the possibility of the

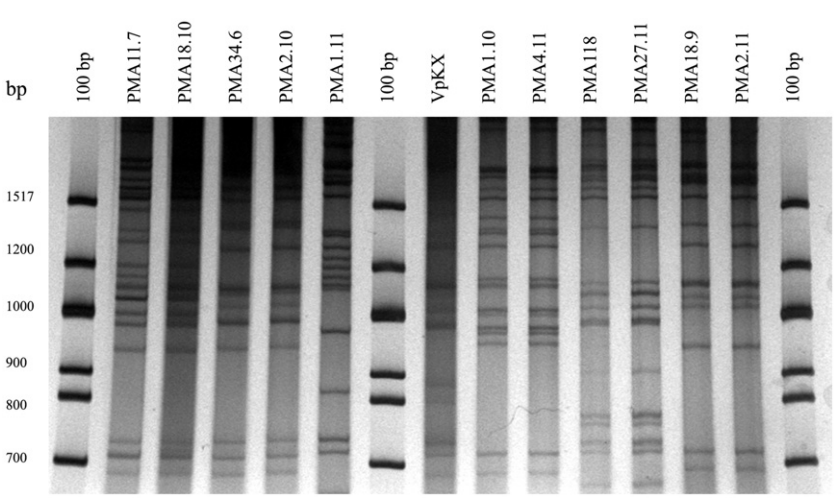

Fig. 2. DGREA with *Nae*I of *V. parahaemolyticus* isolates from shellfish collected in Puerto Montt, Chile, in the summers of 2010 and 2011. The gel shows representative strains for the new DGREA groups observed in 2010 or/and 2011; PMA1.10 and PMA1.11. It also shows those patterns for previously observed groups next to the type isolate of that group. Lanes marked '100 bp' contain a 100 bp size ladder; lane VpKX shows the DGREA pattern of RIMD 2210633.

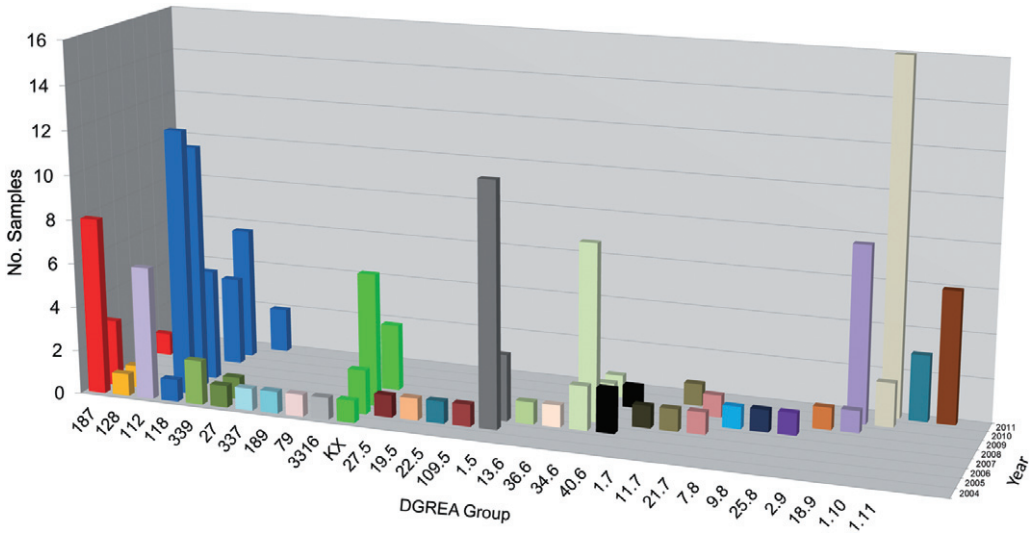

Fig. 3. Histogram of the number of shellfish samples containing *V. parahaemolyticus* corresponding to the DGREA groups observed each summer since 2004. Data of years preceding 2010 were obtained from García and colleagues (2009).

return of large outbreaks in Region de Los Lagos. It is a matter of speculation as to when the pandemic strain arrived in Region de Los Lagos before reaching detectable levels in shellfish and producing large disease outbreaks.

Environmental variables and the load of the pandemic strain in shellfish

The pandemic strain probably competed in shellfish with a large and diverse *V. parahaemolyticus* population. The balance between the rates of growth plus recruitment – increase by incorporation of bacteria from the external environment – and death plus migration – decrease by movement of the bacteria to the external environment – determines the increase or decrease of the load of the pandemic strain in shellfish according to the following equation:

$$dN/dt = N(\mu_g + \mu_r) - N(\mu_d + \mu_m)$$

where N is the number of organisms and μ_g, μ_r, μ_d and μ_m are the specific rates of growth, recruitment, death and migration respectively. After its arrival in Region de Los Lagos, the pandemic strain colonized successfully, with a $\mu_g + \mu_r$ higher than $\mu_d + \mu_m$ to reach a detectable level in shellfish that was maintained for 5 years. This load was high enough to achieve infective doses when raw seafood was consumed or when it contaminated nearby processed cooked food. Differences in environmental conditions probably determined the initial success of the strain, as well as its later decline. We further consider three variables that may have impacted the cycle of the pathogen: seawater surface temperature, solar radiation, and the presence and activity of bacteriophages.

Effect of seawater surface temperature and solar exposure on intertidal shellfish temperature and the V. parahaemolyticus *load*

One of the most important physical variables in the growth of *V. parahaemolyticus* is temperature. The surface seawater temperature in the Puerto Montt coastal area exhibited a small increase during the worst outbreaks to an average of 17.3°C in 2005, and a small drop during the summers of 2010 and 2011 to 14.0°C and 15.2°C respectively. The temperatures from 2000 to 2003 – years preceding the outbreaks – were 15.3°C, 15.0°C, 16.9°C and 14.9°C, with an average temperature of 15.5°C. This 1–2°C difference in seawater surface temperature may have strongly influenced the load of *V. parahaemolyticus* in shellfish and the number of clinical cases.

Solar radiation may also have had some effect. Natural banks of mussels that are very abundant in the large intertidal zone of Region de Los Lagos are exposed to solar radiation during low tide. Mussels exposed to sunlight during low tide on a sunny day can reach temperatures of 30°C, becoming practical incubators for bacterial growth (Fig. 4). We assessed the apparent relationships between clinical cases and *V. parahaemolyticus* load in shellfish with surface seawater temperature (Fig. 5A) and solar radiation (Fig. 5B), estimated as the number of sunny days at the collection site. Monthly number of sunny days from 2004 to 2011 were 16.7, 17.3, 16.0, 16.2, 16.9, 16.4 14.0 and 15.2. The rise of pandemic *V. parahaemolyticus* and its subsequent fall seems to be related to seawater temperature and solar radiation. The Pearson Coefficient and their *P*-values supported this correlation. Values for the correlation between the logs of both clinical cases and pandemic *V. parahaemolyticus* load

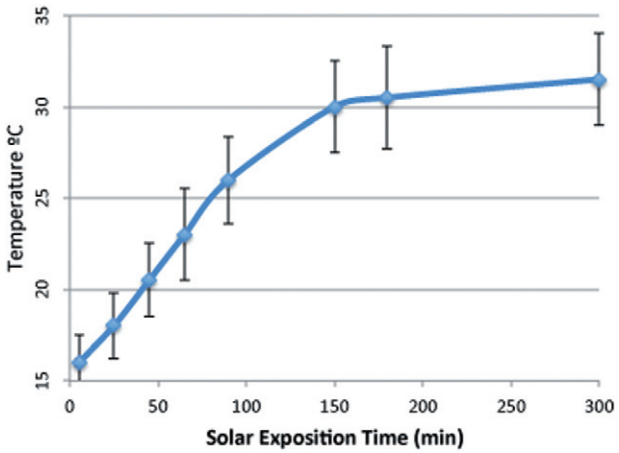

Fig. 4. Internal temperatures and their standard deviation of mussels exposed to solar radiation during low tide.

with seawater temperature were 0.96 ($P < 0.01$) and 0.83 ($P = 0.03$) respectively. Those for the correlation of the same variables with sunny days were 0.64 ($P = 0.08$) and 0.42 ($P = 0.04$). Altogether these results indicate that the correlation is higher with seawater temperature suggesting that this variable had greater effect. The relative concentration of the pandemic strain in the total *V. parahaemolyticus* population of shellfish seems to have been higher during summers with larger numbers of clinical cases (2006 and 2008) (Fig. 5; García *et al*., 2009), indicating the possible presence of a selective advantage for the growth of the pandemic strain.

Effect of bacteriophages

It is generally accepted that bacteriophages play an important role in the regulation of the amount of bacteria in the sea (Bergh *et al*., 1989; Fuhrman, 1999; Wilhelm and Suttle, 1999). This regulatory effect can be highly selective given the exquisite specificity of phage for bacteria, even when they are of the same species. Since 2005 phages able to infect pandemic *V. parahaemolyticus* strain PMC57.5 were systematically searched but only two phage groups able to infect this strain were found in the coast of Region de Los Lagos: Vp93; a lytic bacteriophage group (Bastías *et al*., 2010), and Vp58.5, a temperate phage (Zabala *et al*., 2009). Although lytic, VP93 can cohabitate with its host, and for this reason it was thought that it exerts little (if any) control on the propagation of the pandemic strain. However, the presence of VP93 may provide a selective advantage for the pandemic strain when growing in competition with other *V. parahaemolyticus* strains that are sensitive to the phage.

We explored the effect of the presence of VP93 when the pandemic strain (PMC57.5) was co-cultured with another non-pandemic *V. parahaemolyticus* strain (PMA112) isolated from shellfish of the same site. Among 28 isolates from different DGREA groups 5 (18%) showed larger sensitivity to VP93. These isolates showed almost clear lysates after infection of bacterial lawns on solid medium, in contrast with the turbid lysates observed with pandemic host PMC 57.5. On the other hand, 15 isolates (54%) showed to be resistant to VP93 (data not shown).

The non-pandemic strain, which overgrows the pandemic strain in the absence of the phage (Fig. 6A), is overgrown by the pandemic strain when VP93 is present (Fig. 6B). On the other hand, bacteriophage VP93 did not affect the relative growth of PMC57.5 and PMA337, non-pandemic *V. parahaemolyticus* strains that are resistant to VP93 (data not shown). Taken together, our observations suggest that the presence of bacteriophage VP93, which can propagate in the pandemic strain without affecting its growth, may become advantageous for this strain when VP93-sensitive *V. parahaemolyticus* strains are present in the same habitat. However, the presence of this phage

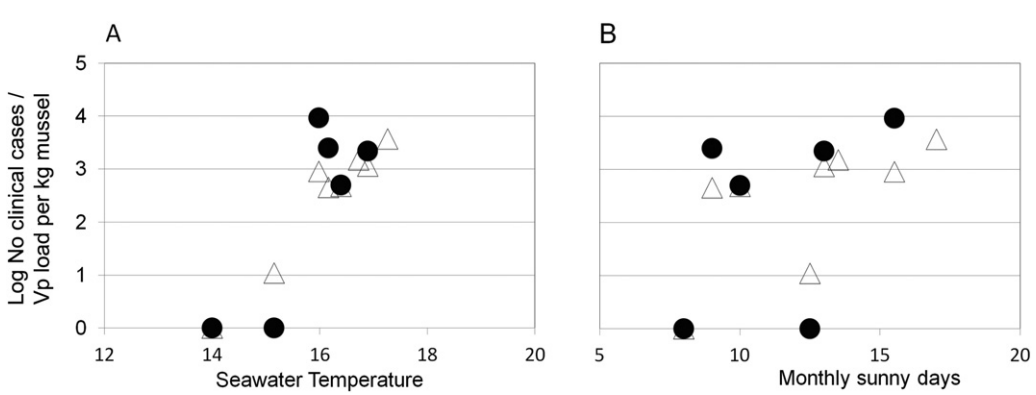

Fig. 5. Log of the number of clinical diarrhoeal cases (triangles) and log of pandemic *V. parahaemolyticus* (Vp) load in shellfish (circles) versus mean surface seawater temperature (A) and mean number of sunny days (B) during January and February 2004–2011, Puerto Montt, Region de Los Lagos, Chile.

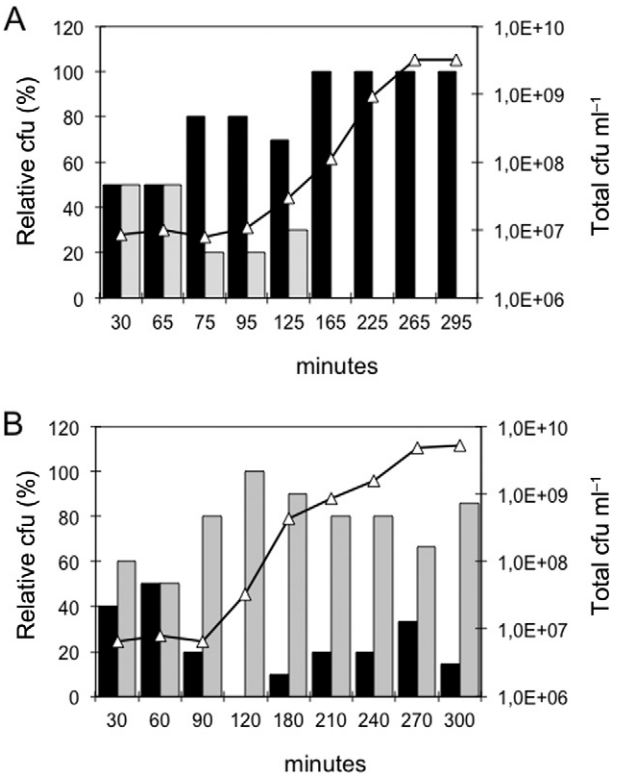

Fig. 6. Growth of pandemic *V. parahaemolyticus* strains PMC57.5 (black) and non-pandemic strain PMA112 (grey) co-cultured in the absence (A) and presence (B) of bacteriophage VP93. Curves indicate total bacterial growth.
[Correction added on 11 December 2012, after first online publication: In the figure legend, 'bacteriophage VP58.5' has been amended to read 'bacteriophage VP93'.]

could be of greater advantage for VP93-resistant strains that add up to 54% of the tested *V. parahaemolyticus* strains.

On the other hand, the presence of the temperate phage Vp58.5 may be a disadvantage. VP58.5 killed most of the pandemic strain after infection and 28% of the few resistant cells became lysogenic for the phage. Lysogenic cells were detected by PCR of VP58.5 genes as reported (Zabala *et al*., 2009). These lysogenic cells could grow in presence of the phage but became 7–15 times more sensitive to solar radiation. Among 27 independently obtained pandemic strain isolates 10 (37%) carried the prophage (García *et al*., 2009). Resistance to VP58.5 was less abundant, among 77 isolates 11 (14%) was resistant (Data not shown). The killing of the pandemic strain by infection with VP58.5 and the increased sensitivity to solar radiation of the surviving lysogenized strains may have a significant role in reducing the survival and propagation of the *V. parahaemolyticus* pandemic strain in the coastal sea. Moreover, based on the observation that none of the 28 isolates of the other DGREA groups tested was sensitive to this phage the presence of bacteriophage VP58.5 could possibly not be advantageous for the pandemic strain when competing with other *V. parahaemolyticus* strains.

Taken together, our analyses suggest that the rise of pandemic *V. parahaemolyticus* after its arrival in the Chilean southern coast and its subsequent fall after several years were due to successful colonization of local shellfish, and was likely helped by higher-than-usual seawater temperatures and amounts of solar radiation. Successful colonization and growth in the face of competition from an abundant and diverse population of closely related bacteria may have been helped by the presence of phages such as VP93. The decline of the pandemic strain may have been aided by the presence of phage VP58.5, which can kill the pandemic strain and renders the cells that become resistant by lysogenization more sensitive to solar radiation. The permanence of the pandemic strain in shellfish did not differ from that of the various *V. parahaemolyticus* strains, which were detected for a few summers and later disappeared. However, since we observed that some strains reappeared after several years without detection, we do not exclude the possibility of the repetition of large outbreaks caused by the pandemic strain, as from 2004 to 2009.

These data support the idea that bacterial species are composed of a highly diverse population including very different strains, some of which may be human pathogens. The dynamics of the bacteria that compose the population are determined by a conjunction of biological and physical environmental factors; in this particular case, the bacterial cycle seems to have been modulated by temperature, solar exposure and interaction with bacteriophages.

Experimental procedures

Strains

Vibrio parahaemolyticus RIMD 2210633 (also called VpKX) was obtained from the Research Institute for Microbial Diseases, Osaka University, Osaka, Japan. *Vibrio parahaemolyticus* RIMD 2210633 (also called VpKX) was obtained from the Research Institute for Microbial Diseases, Osaka University, Osaka, Japan. PMC57.5 corresponds to the pandemic strain isolate obtained in Chile and selected as prototype of this group for Chilean isolates. This isolate was employed in most experiments the Chilean pandemic strain. The environmental strains, identified by the prefix PMA (Table 1), were obtained from shellfish samples taken during January and February of 2010 and 2011. Mussels were situated at Quillaipe (41°52′S, 72°75′W) and were distributed over the intertidal area. Isolates from preceding years have been described previously (Fuenzalida *et al*., 2006; 2007; Harth *et al*., 2009; García *et al*., 2009).

PCR and DGREA

Samples from shellfish were obtained and analysed as described previously (Fuenzalida *et al*., 2006). Briefly,

samples of shellfish soft tissue were enriched for *V. parahaemolyticus* in three-tube serial dilutions in alkaline peptone water for assessment of bacterial load by the probable number method (Kaysner and DePaola, 2004); tubes with bacterial growth were tested for *tlh, tdh* and *trh* by multiplex PCR (Bej *et al*., 1999). Total and pandemic *V. parahaemolyticus* loads were calculated according to the number of tubes positive for *tlh* and for *tdh* and *trh* respectively. Positive enrichment tubes were plated on CHROMagar *Vibrio* (CHROMagar Microbiology, Paris, France), and bacterial colonies with mallow colour were purified and the isolate was characterized. DGREAs were performed as described previously (Fuenzalida *et al*., 2006). Each of the DGREA patterns found in 2010 and 2011 was compared with patterns described in previous years, and when similarities were observed, their identities were evaluated by comparing the patterns obtained during the same electrophoresis run.

Littoral mussel temperatures

Internal temperature of mussels living in natural banks in the intertidal zone of Bahia Quillaipe were registered simultaneously, on a sunny day, at intervals of 20–30 min during the tidal decline, beginning at the moment the mussels emerge the seawater. Ten mussels were analysed each time. The days the measurements were performed air temperature was between 15°C and 17°C; seawater temperature varied according the distance from the shore line, water next to the shore reached temperatures around 20°C while seawater 10 or more metres away from the shoreline was between 15°C and 17°C.

Environmental data

Average seawater temperatures from 2004 to 2011 were obtained from records from the Hydrographic and Oceanographic Service from the Chilean Navy database (http://www.shoa.cl); these measurements were performed each hour at a depth of 5 m. Solar exposition data for Bahía Quillaipe for December, January and February 2003–2011 were provided by the sensor MODIS-AQUA (NASA) and were processed with the SEADAS 6.2.0 software.

Co-culture of the pandemic strain of V. parahaemolyticus *(PMC57.5) and a non-pandemic strain (PMA112) with phage VP93*

Co-cultivation of strains PMC57.5 and PMA112 began with a 1:100 dilution of the corresponding stationary-phase cultures. The co-culture was incubated at 37°C with constant stirring. Samples were taken from the culture at the specified intervals to determine the number of colony-forming units (cfu). To identify the strain that generated each colony, the colonies were dissolved in 500 µl of culture medium and boiled for 10 min; 1 µl of this lysate was used for PCR with primers for *tlh*, which is present in both strains, and for *tdh*, which is present only in the pandemic strain PMC57.5. The primers used for *tlh* identification were 5′-AAAGCGGATTATGCA GAAGCACTG (sense) and 5′-GCTACTTTCTAGCATTTTCT CTGC (antisense). The primers used for *tdh* identification were 5′-GTAAAGGTCTCTGACTTTTGGAC (sense) and 5′-TGGAATAGAACCTTCATCTTCACC (antisense). To assay the growth of PMC57.5 and PMA112 in the presence of phage VP93, the culture was infected with VP93 in early exponential phase with a multiplicity of infection of 10. An equal volume of fresh culture medium (the volume depended on the desired multiplicity of infection) was added to the control cultures without phage.

Sensitivity of different strains of *V. parahaemolyticus* to the phages VP93 and VPC58.5 was determined using the standard method of double agar and micro drops of the corresponding phage (Clokie and Kropinski, 2009).

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