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ORIGINAL ARTICLE

Populations, not clones, are the unit of vibrio pathogenesis in naturally infected oysters

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Disease in oysters has been steadily rising over the past decade, threatening the long-term survival of commercial and natural stocks. Our understanding and management of such diseases are of critical importance as aquaculture is an important aspect of dealing with the approaching worldwide food shortage. Although some bacteria of the *Vibrio* genus isolated from diseased oysters have been demonstrated to be pathogenic by experimental infection, direct causality has not been established. Little is known about the dynamics of how the bacterial population hosted by oysters changes during disease progression. Combining experimental ecology, a high-throughput infection assay and genome sequencing, we show that the onset of disease in oysters is associated with progressive replacement of diverse benign colonizers by members of a phylogenetically coherent virulent population. Although the virulent population is genetically diverse, all members of that population can cause disease. Comparative genomics across virulent and nonvirulent populations identified candidate virulence factors that were clustered in population-specific genomic regions. Genetic analyses revealed that one gene for a candidate virulent factor, a putative outer membrane protein, is necessary for infection of oysters. Finally, analyses of oyster mortality following experimental infection suggest that disease onset can be facilitated by the presence of nonvirulent strains. This is a new form of polymicrobial disease, in which nonpathogenic strains contribute to increase mortality.

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Introduction

Vibrios have been associated with successive mortality outbreaks of oyster beds (*Crassostrea gigas*) in France that have resulted in losses of up to 100% of production (Samain, 2008). Given the near monoculture of *C. gigas* in Europe, there is an urgent need to understand the epidemiology of these outbreaks, particularly the role of vibrios in the diseases. To date, it has been difficult to determine whether vibrios resident in diseased oysters are mutualistic, opportunistic or pathogenic owing to a lack of diagnostic tools for distinguishing pathogenic from nonpathogenic strains, the fact that individual animals can harbor multiple bacterial genotypes and limitations inherent within the experimental systems available for studying infection. Consequently, it has been difficult to conclusively identify bacterial genotypes or genes that are linked to virulence in oysters.

In the last few years, significant progress has been made in understanding the population structure and diversity of vibrios (Thompson *et al.,* 2005; Hunt *et al.,* 2008). Despite their enormous microdiversity, these organisms fall into well-defined genetic clusters that have similar resource preferences. These clusters have been hypothesized to correspond to populations that act as cohesive ecological units, that is, ecological populations (Hunt *et al.,* 2008). However, a link between ecological populations and pathogenicity has not been demonstrated, and it is unclear whether pathogenicity is a trait primarily linked to clones or to populations comprising a large number of distinct genotypes.

Experimental infections of oysters, which have been performed for a limited number of bacterial strains, have allowed identification of a few factors that contribute to virulence, namely a metalloprotease (Le Roux *et al.,* 2007; Labreuche *et al.,* 2010)

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and the outer membrane protein OmpU (Duperthuy *et al.,* 2010, 2011). However, knowledge of the absence/presence of these genes is not sufficient for determination of a strain's pathogenicity (Saulnier *et al.,* 2010). Furthermore, the laboratory analyses previously used to define virulence-linked loci do not capture the complexity of infection within the natural environment. Oysters are typically injected with a single bacterial strain, whereas in their natural environment animals are typically colonized by a diverse assemblage of vibrios (Gay *et al.,* 2004a; Wendling *et al.,* 2014). This diversity may contribute to virulence; in fact, experimental infections have demonstrated that some strains are moderately virulent when injected into animals individually, and display heightened virulence in mixed experimental infections (Gay *et al.,* 2004b).

Oyster vibrioses studied in the laboratory also do not accurately model the natural infection process. Numerous attempts to kill oysters by immersion in vibrio-contaminated sea water have proven unsuccessful, and have necessitated reliance on infection via injection (Gay *et al.,* 2004a; Le Roux *et al.,* 2007; Duperthuy *et al.,* 2011). It is possible that infection in the wild is aided by vibrios' association with and attachment to other organisms and particles; a recent study has reported that marine aggregates facilitate retention of nanoparticles (including bacteria) by suspension-feeding bivalves (Froelich *et al.,* 2012). Thus, growth of oysters in an environment in which bacteria are not simply in a planktonic form may yield a more accurate understanding of the factors that contribute to virulence.

In the present study, we investigate the oyster disease ecology of microdiverse *Vibrio* genotypes using a new, field-based approach. We take advantage of recently developed specific pathogen-free spats of *C. gigas* that become naturally infected when placed in an oceanic environment (Petton *et al.,* 2013). In addition, we use these standardized animals for high-throughput experimental infections. We show that pathogenicity can be ascribed to a cluster of genetically related strains that coincides with a previously defined ecologically cohesive population. Genes specific to this population likely reflect the selective pressure associated with population specialization, and we demonstrate that one of them is required for pathogenicity.

Materials and methods

Strains, plasmid collections and culture conditions

In May 2011, specific pathogen-free oyster spats were transferred to a farming area to allow infection (Supplementary Methods). An oyster set (designed as sentinel) was maintained in the field to monitor the first mortality onset and determine the cumulative mortality rates occurring naturally after 1 month. At the first mortality report, infected animals were reintroduced in the laboratory to reveal the disease. Each day, from a pool of 10 living oysters, vibrios were isolated on selective media (thiosulfate-citrate-bile salts-sucrose agar (TCBS), Difco, BD, Le pont de Claix, France) and re-streaked two times before genotyping using *gyrB* partial sequence (Supplementary Methods). The strains used for the genomic analyses are described in Table 1. Other bacterial strains are described in Supplementary Table S1. Vibrio isolates were grown in Zobell or Zobell agar, Luria-Bertani (LB) or LB agar (LBA) + NaCl 0.5 M at 20 °C. *Escherichia coli* strains were

Table 1 Strains sequenced by HTS in this study

Virulence	*Strain*	*Origin*	*Contig number*	*Genome size (Mb)*	*CDSs*	*Accession number*	*Apparent clonality*
Vir +	J2-9	This study	174	5.79	5495	PRJEB5876	J2-13
	J5-4	This study	189	5.86	5589	PRJEB5877	
	J5-5	This study	165	5.81	5543	PRJEB5878	
	J5-15	This study	92	5.65	5345	PRJEB5879	
	J5-19	This study	145	5.61	5255	PRJEB5880	
	J5-20	This study	145	5.74	5462	PRJEB5882	J5-6
	LGP7^T	Oyster mortalities, 2001	122	5.64	5413	PRJEB5883	J5-23, J5-24, J5-28, LGP15
	LGP8	Oyster mortalities, 2001	177	5.58	5427	PRJEB5884	
	LGP107	Oyster mortalities, 2001	86	5.49	5249	PRJEB5885	LGP108
Vir −	J2-1	This study	69	5.36	4941	PRJEB5886	J2-24, J2-28
	J2-3	This study	54	5.52	5063	PRJEB5887	
	J2-4	This study	84	5.67	5285	PRJEB5888	
	J2-6	This study	64	5.38	4934	PRJEB5889	J2-18
	J2-8	This study	98	5.39	4969	PRJEB5890	
	J2-12	This study	158	5.55	5177	PRJEB5891	
	J2-14	This study	65	5.67	5244	PRJEB5892	
	J2-15	This study	74	5.57	5158	PRJEB5893	J2-7
	J2-17	This study	95	5.42	4977	PRJEB5894	J2-10
	J2-26	This study	74	5.42	4981	PRJEB5895	J2-30
	J2-29	This study	117	5.66	5301	PRJEB5896	
	J2-31	This study	255	5.73	5437	PRJEB5897	

Abbreviations: CDS, coding DNA sequence; HTS, high-throughput sequencing.

grown in LB or on LBA at 37 °C. Chloramphenicol (12 μg ml^{-1}), spectinomycin (100 μg ml^{-1}), thymidine (0.3 mM) and diaminopimelate (0.3 mM) were added as supplements when necessary. Induction of the P_{BAD} promoter was achieved by the addition of 0.2% L-arabinose to the growth media and, conversely, was repressed by the addition of 1% D-glucose.

Genome sequencing, assembly and annotation
A total of 34 strains (Table 1) were sequenced using the Illumina HiSeq 2000 technology (Plateforme de génomique de l'Institut Pasteur, Paris, France) with ~50-fold coverage (Supplementary Methods). Contigs were assembled *de novo* using Velvet (Zerbino and Birney, 2008) and genome assembly was improved by contig mapping against the LGP32 reference genome (Le Roux *et al.,* 2009). Computational prediction of coding sequences together with functional assignments were performed using the automated annotation pipeline implemented in the MicroScope platform (Supplementary Methods) (Vallenet *et al.,* 2013).

In silico analyses
A dedicated precomputing repository (marshalling) was created to perform comparative genomic and phylogenomic analyses. Orthologous proteins were defined as reciprocal best hit proteins with 80% MaxLrap and a minimum of 60% identity cutoff (Daubin *et al.,* 2002). The nucleic acid sequences were aligned using Muscle (Edgar, 2004) and filtered by BMGE (Block Mapping and Gathering with Entropy; Criscuolo and Gribaldo, 2010). Phylogenetic trees were built using the parallel version of PhyML applied to maximum-likelihood algorithm and GTR model as parameters (Guindon *et al.,* 2010). A first phylogenetic analysis of concatenated nucleic acid sequences derived from 3229 shared proteins from the 34 genome sequences suggested the clonality of some isolates within our collection (Table 1). This was confirmed by average nucleotide identity value of >99.5% and an accessory genome of <150 coding DNA sequences between isolates. Consequently, only 21/34 isolates were considered as distinct strains.

Vector construction and mutagenesis
Alleles carrying an internal deletion were cloned into a suicide vector using the Gibson method (New England Biolabs, Genopole, Evry, France) (Supplementary Methods). The R6K γ-*ori*-based suicide vector encodes the *ccdB* toxin gene under the control of an arabinose-inducible and glucose-repressible promoter, P_{BAD}(Le Roux *et al.,* 2007). Matings between *E. coli* and *Vibrio* were performed at 30 °C as described previously (Le Roux *et al.,* 2007) (Supplementary Methods). Selection of the plasmid-borne drug marker (chloramphenicol resistance) resulted in integration of the entire plasmid in the chromosome by a single crossover. Elimination of the plasmid backbone resulting from a second recombination step was selected by arabinose induction of the *ccdB* toxin gene. Mutants were screened by PCR and are described in Supplementary Table S1. For complementation experiments, the Gibson assembly method was used to clone the *R-5.7* gene under a constitutive promoter (P_{LAC}) in pMRB plasmid known to be stable in vibrios (Le Roux *et al.,* 2011). This plasmid was then transferred to *Vibrio* by conjugation as described previously.

Virulence studies using oysters
Bacteria were grown under constant agitation at 20 °C for 24 h in Zobell media. Then, 100 μl of the culture (10^6 colony-forming units (CFUs)) pure or diluted were injected intramuscularly into oysters. The bacterial concentration was confirmed by conventional dilution plating on Zobell agar. After injection, the oysters were transferred to aquaria (20 oysters per aquarium of 2.5 l) containing 1 liter of aerated 5 μm filtered sea water at 20 °C, kept under static conditions for 24 h.

Results and discussion

Disease is associated with progressive replacement of nonvirulent vibrios by genetically related virulent strains
Specific pathogen-free oysters were exposed to natural sea water in the field during a mortality outbreak and then returned to the laboratory after 15 days. On each subsequent day, 10 oysters were killed and bacteria were isolated from the tissue (Supplementary Methods). Mortalities started at day 3, reached 50% at day 5 and then ceased (Figure 1a, red bars). The cumulative mortality after 5 days in the laboratory was similar to the extent of mortality observed for a subset of the same batch of oysters maintained in the field for 1 month. We speculate that this mesocosm allows development of disease to proceed more rapidly in the lab.

Roughly 30 of the bacterial isolates from each day were characterized by partial sequencing of a protein-coding gene (*gyrB*). Phylogenetic analysis allowed the grouping of 162/173 isolates in 6 clades (designated a to f) with a bootstrap value of >70% (Figure 1b). These clades were matched with named species using type strains *V. mediterranei* (a), *V. chagasii* (b), *V. lentus* (c), *V. splendidus* (d), *V. cyclitrophicus* (e), *V. crassostreae* and *V. gigantis* (f). Clades b to f belong to the Splendidus super-clade (Sawabe *et al.,* 2013). Strains isolated at the beginning of the experiment were mainly related to clade d (36%) and clade f (39%) (Figure 1a). The clade f strains increased to as high as 77% when the mortalities started but returned to

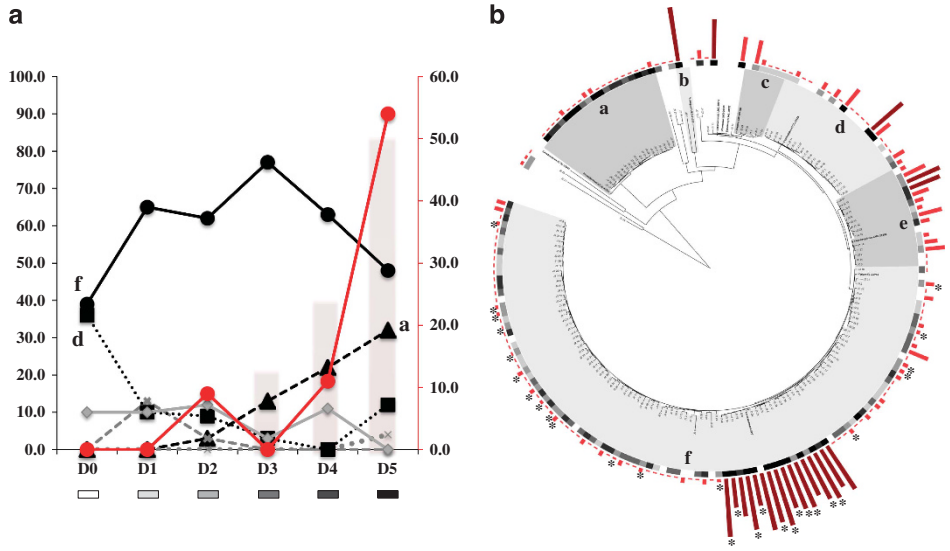

Figure 1 Bacterial population dynamics during oyster infection. (**a**) Specific pathogen-free oysters were transferred to open sea water for 2 weeks and then transferred to the laboratory to study *Vibrio* dynamics during the disease expression. Mortalities (pink bar) were recorded daily (D0 to D5) and expressed in percentage (right y axis). D, day. Lines indicate the percentage of strains (left y axis) belonging to the phylogenetic clades described in (**b**). The black lines correspond to the three most represented clades (clade f: circle; clade d: square; and clade a: triangle) whereas gray lines (clade e: plain; clade c: large dots; and clade b: small dots) correspond to less abundant genotypes. The red line corresponds to the strains inducing >50% mortality (right y axis). (**b**) Date of isolation and virulence superimposed on the phylogeny of bacterial isolates inferred by maximum-likelihood analysis of partial *gyrB* gene sequences, with outer and inner rings indicating the % of mortalities obtained 24 h after oyster injection (brown bars >50%; red bars <50%) and the day of isolation (D0 in white, D1–4 in gray gradient and D5 in black, as in (**a**) below the x axis) respectively. Clades a, b, c, d, e and f were obtained with a bootstrap value of 99%, 94%, 92%, 100%, 100% and 71% and contain the type strains of *V. mediterranei*, CIP 10320^T (clade a), *V. chagasii*, R-3712^T (clade b), *V. lentus*, CECT 5110^T (clade c), *V. splendidus*, LMG 4042^T (clade d), *V. cyclitrophicus*, LMG 21359^T (clade e), *V. crassostreae*, LGP7^T and *V. gigantis*, LGP13^T (clade f). Asterisks indicate the strains sequenced in this study.

their starting level by the final day. The prevalence of clade d strains declined to 12%, whereas clade a strains increased from 0% to 32% when mortality reached 50%.

To address the pathogenic potential of individual strains, we used an injection model of infection that enables more reliable and rapid infection in the laboratory. Among the 173 isolates individually introduced into specific pathogen-free oysters, 143 (83%) were found to induce <20% mortality (Figure 1b and Supplementary Figure S1). Twenty strains induced >50% mortality and were consequently classified as virulent (vir+). The majority of vir+ strains (75%) clustered into a subclade of (f) that contains the *V. crassostreae* type strain (LGP7^T) (Figure 1b) (Faury *et al.,* 2004). This type strain and other conspecifics were isolated from an oyster mortality event in 2001 and demonstrated to be pathogenic for oysters (Gay *et al.,* 2004a).

The genetic cluster of virulent strains coincides with an ecological population

We investigated whether the vir+/vir− subclades of (f) coincides with any of the ecological populations described by Polz and collaborators (Hunt *et al.*, 2008). As the Vibrio ecological population structure analysis was based on hsp60 sequences, this genetic marker was used to compare the populations (Supplementary Figure S2). The vir+ and vir− strains were found to be included in distinct clades corresponding to ecologically differentiated populations. These two populations were reported to show preferences for either zooplankton or large particles (Hunt *et al.*, 2008).

It is possible that the association between vir+ strains and particles/plankton is important for natural infection of oysters by these pathogens. Recent studies have reported the central role of marine aggregates in facilitating colonization of *Crassostrea virginica* oysters by *Vibrio vulnificus* and showed that differences in the ability to incorporate into these aggregates may play a role in the bacterial population disparity observed within oysters (Froelich *et al.*, 2012). Thus, identifying the microhabitats of *C. gigas* pathogens may facilitate development of an experimental infection model mimicking the natural route of infection, for example, through the use of adapted polymeric substrates. Such approach would also allow for a better understanding of the mode of transmission and primary target tissues or organs for these pathogens.

Comparative genomic analyses reveal limited gene clusters that distinguish vir+ and vir− populations

To identify genetic features that distinguish the vir+ population from vir− strains within clade f, we used high-throughput sequencing (Supplementary Methods) to analyze the genomes of 34 isolates (Figure 1b and Table 1). Of these isolates, 21 appeared to be distinct strains (Table 1): 9 virulent

strains (6 from the present study and 3 from a 2001 mortality outbreak) and 12 avirulent strains. The core genome phylogeny (3229 genes) shows that the strains split into two lineages consistent with the pathogenicity status (Figure 2). The average nucleotide identity was 92–93% between populations, whereas within vir+ and vir− lineages, the average nucleotide identity values ranged between 97.8% and –99.9% and between 96.6% and 97.3%, respectively. Despite a strong clonal frame in the core genome, we detected extensive genetic diversity in the flexible genome. Strain-specific gene numbers varied from 1000 to 1700 and from 100 to 1200, respectively, at inter- and intra-population levels, which is two times higher than the flexible genome diversity observed in *V. cholerae* (Supplementary Figure S3).

We performed comparative analyses to identify sequences present in the vir+ and absent from the vir− population. A total of 101 genes were found to be specific to vir+ strains, 53% of which were localized in 7 distinct regions (designated R-1 to 7) (Supplementary Table S2). Four regions (R-2, -3, -4 and -5) are involved in drug resistance and metabolic function, suggesting adaptation of strains to local competitive or environmental pressure. The R-6 region encoding for arylsulfatase may have an important scavenging function in removing sulfate groups from exogenous substrates such as macroalgal polysaccharides and providing carbon sources (Cohen *et al.,* 2007; Mann *et al.,* 2013). The R-1 region is homologous to the widespread colonization island (also named *tad* gene cluster), which encodes adhesive pili, and was demonstrated as essential for biofilm formation, colonization and pathogenesis in numerous bacteria (Tomich *et al.,* 2007). The R-1 region also encodes the PhoPQ two-component system that controls a variety of processes including resistance to antimicrobial peptides (Otto, 2009). The antimicrobial peptides in concert with reactive oxygen species play a crucial role in the invertebrate immune system (Bachere *et al.,* 2004). Interestingly, in the R-4-specific locus, we identified genes encoding a catalase and a superoxide dismutase putatively implicated in reactive oxygen species resistance (Ibarra and Steele-Mortimer, 2009). Finally, the R-7 region carries genes encoding putative transposases and proteins of unknown function.

Reciprocally, we identified 193 genes present in all vir− strains but absent from all vir+ strains, half of which were localized in 14 regions (Supplementary Table S3). Two regions are putatively involved in phosphonate transport (Yu *et al.,* 2013). Altogether, our data suggest that ecological specialization, possibly through differential association with hosts and/or particulate material, results from gene acquisition conferring function as scavenging, drug resistance, adhesion and host immune response survival.

Clade f vir+ strains encode a putative outer membrane protein that is necessary for virulence

We assessed the importance of vir+-specific loci for *V. crassostreae* virulence using a genetic knockout approach. Deletion of regions R-1, -2, -4, -5 or -7 in strain J2-9 did not impair bacterial growth in culture media, but deletion of R-5 resulted in a threefold decrease in mortalities induced after bacteria injection (Figure 3a). The importance of R-5 was confirmed using two additional strains (J5-5 and LGP8) belonging to the vir+ population (Figure 3a). Among the 32 genes localized in the R-5 region of J2-9, only 8 genes were present in all vir+ strains and absent from all vir− strains (Figure 3b and

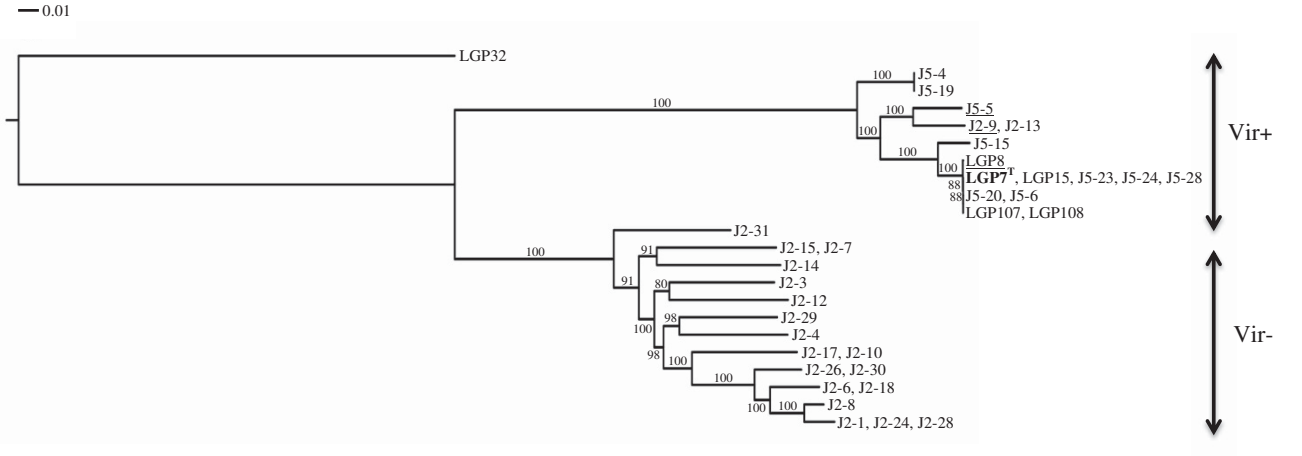

Figure 2 Phylogenetic analysis based on concatenated alignments of nucleic acid sequences of core genes of strains sequenced in this study. J2-x and J5-x strains were isolated in this study at days 2 and 5 respectively. LGPx strains were isolated in 2001. LGP7^T is the type strain of *V. crassostreae* species. The strain LGP32 was used as an outgroup. Trees were built by the maximum-likelihood method (GTR substitution model, NNIs, γ4, invariant site) based on sequences aligned using Muscle and filtered with BMGE. Branch lengths are drawn to scale and are proportional to the number of nucleotide changes. Numbers at each node represent the percentage value given by bootstrap analysis of 100 replicates. Strains in which the deletions of the R-5 region were made are underlined.

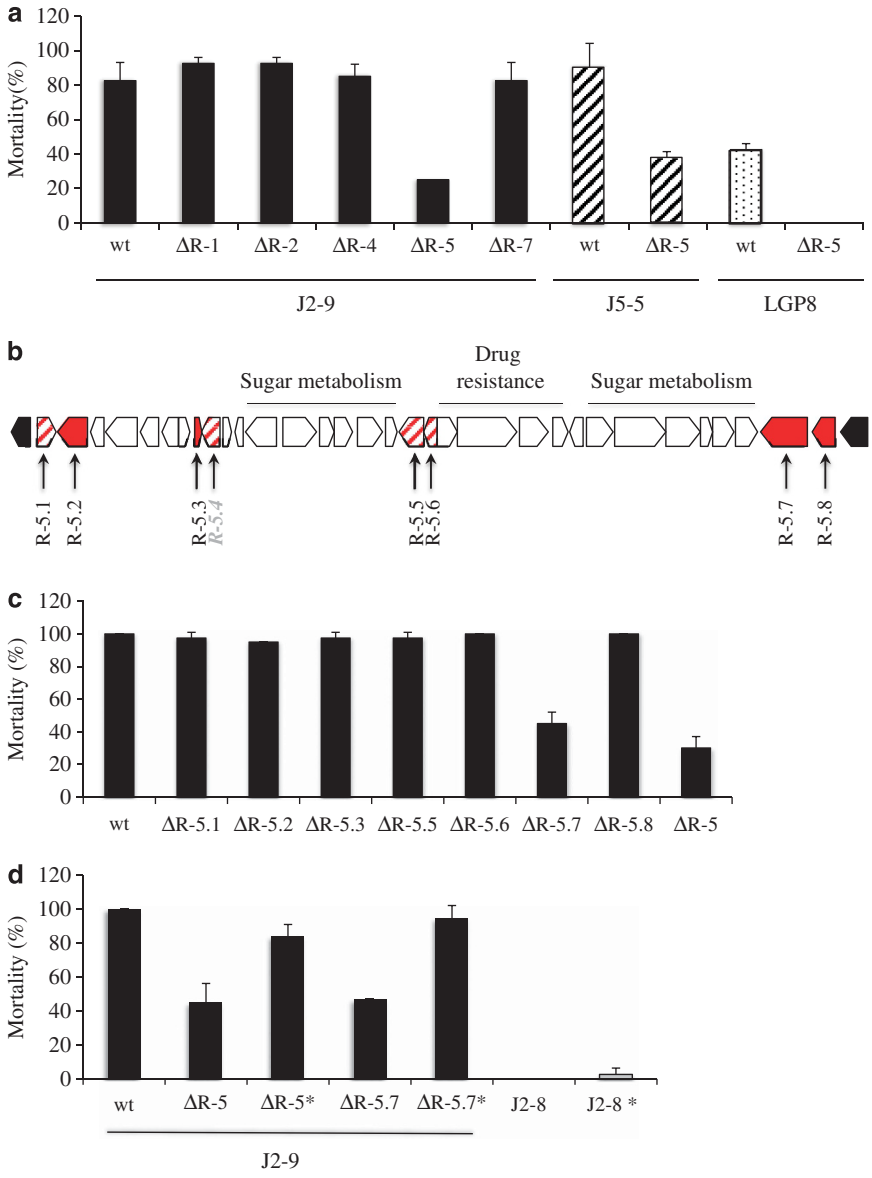

Figure 3 Oyster mortality in response to experimental infection with *V. crassostreae* wild-type (wt) strains and derivatives. A total of 10^6 CFUs of the tested strains ((**a**) ΔR-1 to -7 for deleted regions 1 to 7 respectively; (**c**) ΔR-5.1 to -8 for deleted genes 5.1 to 8 in the region R-5; (**d**) J2-9 and derivatives ΔR-5, ΔR-5.7 or J2-8 carrying (indicated with a star) or not the expression vector pMRB-P_{LAC}*R-5.7*) were intramuscularly injected into oysters ($n = 20$, in duplicate). Mortality (%) was assessed after 24 h. (**b**) Organization of the genomic region R-5. Genes in red are found in all vir+ strains and absent in all vir− strains (plain: exported unknown protein; hatched: regulators). Genes in white were also found in vir− strains. Genes in black indicate the region boundaries that have been targeted for the deletion of the entire region (locus tag of the J2-9 strain: VRSK9J2v1_ 73037 and 730270). Arrows indicate genes targeted for single-gene mutagenesis; we were unable to establish a deletion in R-5.4 (gray, italics).

Supplementary Table S2). Analyses of mutants lacking individual genes revealed that the R-5.7 gene accounts for the contribution of R-5 to virulence (Figure 3c). When constitutively expressed *in trans*, R-5.7 was sufficient to restore the virulence of the mutants *ΔR5.7* and *ΔR5* (Figure 3d). Thus, R-5.7 is the only gene necessary for the contribution of R-5 to virulence. On the other hand, the expression of R-5.7 *in trans* in a vir− strain was not sufficient to induce a virulent phenotype (Figure 3d). These complementation experiments confirm that *R-5.7* gene is necessary but not sufficient for the pathogenicity. The R-5.7 gene (labeled VRSK9J2v1_730268 in J2-9, Supplementary Table S2) is predicted to encode a 798 amino-acid exported protein with a theoretical molecular mass of 89 kDa. No functional domains within the protein could be identified using InterProScan, PFAM or Figfam, but Psort predicted R-5.7 to encode an outer membrane protein. Blast analysis revealed the presence of R-5.7 orthologous genes in several genomes of vibrios belonging to Splendidus, Orientalis and Photobacterium clades (Supplementary Figure S4), although not in LGP32, a *V. splendidus*-related

strain previously demonstrated to be pathogenic for oysters (Le Roux *et al.,* 2009).

It is notable that of the 81 genes analyzed by deletion only one was found to be necessary for *V. crassostreae* pathogenicity. This finding suggests that the primary role of these population-specific genes may be unrelated to virulence. It is possible that these shared genes are simply a 'fossil' of the common ancestry. On the other hand, the vir+ strains may be specifically adapted to a particular environmental niche where these genes are beneficial. In such a scheme, oysters may be considered as an alternative habitat for the vir+ population rather than their principal environmental niche. This hypothesis is in accordance with previous data demonstrating that *V. crassostreae* was associated with both algal detritus and zooplankton (Preheim *et al.,* 2011; Szabo *et al.,* 2012). Further mapping of virulent strains onto *V. crassostreae* ecological populations may enable determination of the microhabitats from which oyster pathogens emerge and provide more insight into the nature of populations that serve as reservoirs of pathogens.

Nonvirulent strains may facilitate the disease

Naturally infected oysters initially contain a large proportion of avirulent strains, but these are progressively replaced by a virulent population that comprises ~50% of the bacterial isolates at the point of maximal mortalities (Figure 1a). The low prevalence of vir+ strains in the early infection process could reflect a contribution of the nonvirulent strains to the development of disease. To address this question, the vir+ strain J2-9 (*V. crassostreae*) was injected into oysters at various doses, either alone or in combination with the nonvirulent strains J2-8 (clade f) or J2-20 (*Shewanella* sp.) (Figure 4). When injected alone, reduction of the injected dose of J2-9 from 10^6 to 4×10^4 CFUs (via dilution in culture media) significantly reduced oyster mortality (~90% vs 5% mortality), even if the infection was allowed to progress for a longer time. In contrast, when J2-9 was injected at 4×10^4 CFUs following serial dilutions with pure cultures of vir- strains (J2-8 or J2-20), so that the final CFUs per inoculum were 10^6, mortality rates were markedly higher than with J2-9 alone (cumulative mortalities of 70% for J2-9 diluted in J2-8 and 60% for J2-9 diluted in J2-20). Thus, the presence of nonvirulent bacteria dramatically increases the virulence of low doses of J2-9, suggesting that there are genotype-independent effects of bacterial density upon virulence.

Thus, although vir− strains are not sufficient for pathogenesis, they clearly have some features (as yet undetermined) that contribute either directly or indirectly to virulence. One possibility is that vir− strains provide resources lacking by the vir+, enabling the vir+ strains to act as 'cheaters', as seen in some analyses of siderophore synthesis and utilization (Cordero *et al.,* 2012). An alternate role for the vir− strains may be to generate a sufficient bacterial load, either to overcome host defenses or to induce expression of virulence factors that are regulated by quorum sensing (Bassler, 2002). Notably, autoinducer synthases (CsqA, LuxM and LuxS), which initiate the quorum sensing signaling cascades, appear to be encoded by both vir+ and vir− strains. In the future, we will investigate the importance of quorum sensing pathways in virulence, as well as explore additional means by which vir− strains contribute to the disease in order to better understand this process of density-dependent pathogenesis.

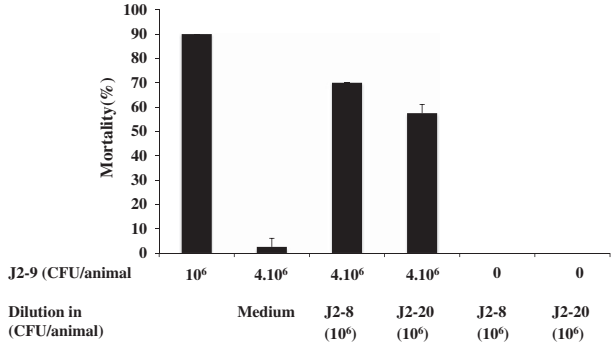

Figure 4 Oyster mortality in response to experimental infection with vir+ strain in the presence of vir− strain cultures. The J2-9 (*V. crassostreae*, vir+) was intramuscularly injected into oysters (two aquaria of $n = 20$) in pure culture (10^6 CFUs per animal), diluted (4×10^4 CFUs per animal) with culture media or with a pure culture of vir− strains J2-8 (clade f, vir−) or J2-20 (*Shewanella* sp.). As a negative control, a pure culture (10^6 CFUs per animal) of J2-8 or J2-20 was also injected. Mortality (%) was assessed after 24 h.

Conclusion

Our results demonstrate the consistency of the virulent population that also corresponds to a previously identified ecologically cohesive genotypic cluster. In the future, delineation of ecological populations together with experimental infections should allow the determination of populations with high or low risk of pathogenicity, the microhabitats from which oyster pathogens emerge and, consequently, which populations serve as reservoirs of pathogens. Hence, it may be possible to develop diagnostic tools at the taxonomic level as soon as population-specific genes are targeted.

Conflict of Interest

The authors declare no conflict of interest.

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Author Contributions

AL, TV, BP, YL and FLR performed experiments; DG and AC performed the *in silico* analyses; YL and FLR designed experiments, interpreted results and wrote the paper; AL, DG and TV contribute equally to this work. The manuscript has been seen and approved by all authors. The material represents an original result and has not been submitted for publication elsewhere.

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