High abundance of virulence gene homologues in marine bacteria

Olof P. Persson,¹ Jarone Pinhassi,¹ Lasse Riemann,¹ Britt-Inger Marklund,¹ Mikael Rhen,² Staffan Normark,² José M. González³ and Åke Hagström¹*

¹Marine Microbiology, Department of Natural Sciences, University of Kalmar, SE-39182 Kalmar, Sweden.
²Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, SE-17771 Stockholm, Sweden.
³Department of Microbiology and Cell Biology, University of La Laguna, ES-38206 La Laguna, Tenerife, Spain.

Summary

Marine bacteria can cause harm to single-celled and multicellular eukaryotes. However, relatively little is known about the underlying genetic basis for marine bacterial interactions with higher organisms. We examined whole-genome sequences from a large number of marine bacteria for the prevalence of homologues to virulence genes and pathogenicity islands known from bacteria that are pathogenic to terrestrial animals and plants. As many as 60 out of 119 genomes of marine bacteria, with no known association to infectious disease, harboured genes of virulence-associated types III, IV, V and VI protein secretion systems. Type III secretion was relatively uncommon, while type IV was widespread among alphaproteobacteria (particularly among roseobacters) and type VI was primarily found among gammaproteobacteria. Other examples included homologues of the Yersinia murine toxin and a phage-related ‘antifeeding’ island. Analysis of the Global Ocean Sampling metagenomic data indicated that virulence genes were present in up to 8% of the planktonic bacteria, with highest values in productive waters. From a marine ecology perspective, expression of these widely distributed genes would indicate that some bacteria infect or even consume live cells, that is, generate a previously unrecognized flow of organic matter and nutrients directly from eukaryotes to bacteria.

Introduction

Pathogenic bacteria have a profound impact on plants, animals and humans. Thus, understanding the origin and distribution of genes that convey pathogenicity is a central issue. These virulence genes represent virulence factors that are used by the bacteria for attachment to and degradation of eukaryotic cells. In particular, bacterial protein secretion systems are crucial for pathogenicity as they allow export of virulence factors. The molecular maps that describe the interaction between bacterial pathogens and their hosts have largely been generated through research on infectious disease and its causative agents. The extent to which homologues to such virulence factors are present in environmental bacteria is less known (Chakraborty et al., 2000; Dziejman et al., 2005; Bingle et al., 2008). Interestingly, such genes are widespread in soil bacteria (Casadevall, 2006), where they supposedly allow bacteria to interact with eukaryotes. Analogously Moran et al. (2007) suggested that aquatic heterotrophic prokaryotes could prosper at the expense of marine eukaryotes using the same principles as known pathogens.

The typical genome composition of a pathogenic bacterium consists of a core genome shared with related commensals, yet augmented with varying genetic elements that appear to fall into a few strategic categories (Hochhut et al., 2005; Gal-Mor and Finlay, 2006), for example, coding for the capacity of adhesion to host surfaces through the expression of adhesins to enable colonization, for molecules that interfere with intrinsic host cell functions, and for the ability to deliver such molecules (effector proteins) upon contact with their cognate host target. Often, the virulence genes are contained on extended genetic continuums termed pathogenicity islands (PAIs, see Schmidt and Hensel, 2004 for review). The PAIs vary in size from a few kilobases to large chromosomal regions, and their base composition and codon usage often differ from the host genome. They are frequently integrated in proximity to tRNA or insertion sequences, or are flanked by direct repeats. This implies that PAIs can move between different bacterial species...
Virulence gene homologues in marine bacteria

Results and discussion

The search and identification of virulence gene homologues in marine bacteria were based on three approaches: (i) BLAST analysis to generate amino acid sequence similarity values, (ii) classification of proteins within PFAM, TIGRFAM or COG domain structure libraries and (iii) gene arrangement, that is, conservation of operon structure of defined PAIs (see Methods section for details). We found virulence genes in 60 out of 119 analysed bacteria (Table 1, for complete list of genomes included in the study see Table S1) and, in addition, we found numerous other genes and operons encoding putative adhesins, surface structures and protein secretion, and chemotactic sensory systems, which may also play important roles in marine bacterial behaviour.

Protein secretion systems

Most pathogenic bacteria need to produce and export virulence factors, enzymes or toxins, in order to invade a host cell. To secrete nucleic acids or proteins through the outer membrane, Gram-negative bacteria apply specialized translocation systems. Predicted homologues to genes of known virulence-associated types III, IV, V and VI secretion systems were found in a large portion of the analysed marine bacteria (Table 1).

The type III secretion system (T3SS), or ‘injectisome’, exports proteins from the bacterial cytoplasm into eukaryotic cells, and helps mediating the interaction of bacterial symbionts or pathogens with their host (Cornelis, 2006). The exported effector molecules can, for example, enable tissue invasion and/or inhibition of host defences (Mota and Cornelis, 2005). We found T3SSs in six marine bacteria (Table 1), where putative T3SS genes were located in genome regions encompassing at least 17–43 genes (Sphingomonas sp. SKA58 and Vibrio alginolyticus 12G01 respectively). In type III secretion, specific ATPases are required for the actual secretion event; and the marine bacterial T3SS genome regions contained type III secretion ATPases with amino acid sequence similarities between 44% and 82% to the corresponding ATPase in Yersinia pestis CO92. A T3SS similar to that found in Y. pestis CO92 was found in V. alginolyticus 12G01, in accordance with previous reports (Park et al., 2004). The T3SSs are found in a wide variety of gammaproteobacteria, but appear less common in alphaproteobacteria (Pallen et al., 2005; Troisfontaines and Cornelis, 2005). We found putative T3SSs in three alphaproteobacteria, where previously not described. One of these systems, found associated with a large plasmid in Sphingomonas sp. SKA58, apparently shared features typical for both T3SSs and the structurally related flagellar motor.

The type IV secretion system (T4SS) is involved in DNA and/or protein transfer into prokaryotic and eukaryotic cells, and accounts for translocation of virulence factors in a number of bacterial pathogens (Cascales and Christie, 2003). Studies of the model plant pathogen Agrobacterium tumefaciens (alphaproteobacteria) have provided molecular detail on the VirB/D4 T4SS (Christie et al., 2005). Moran and colleagues (2007) found putative T4SSs in seven genome-sequenced roseobacters (some of which were also included in the present study). Among the diverse marine bacteria studied here, putative T4SSs of the VirB/D4 type were exclusively found in alphaproteobacteria (18 genomes), with one to three vir gene operons per genome (Table 1). This secretion system was amply distributed among members of the Roseobacter clade, but was also present in members of the families Erythrobacteraceae and Caulobacteraceae.
Table 1. Secretion systems and PAIs in genome sequenced marine bacteria.

| Organism                                      | Tax     | T3SS | T4SS | T5SS | T6SS | Antifeeding island | Novel PAI |
|-----------------------------------------------|---------|------|------|------|------|--------------------|-----------|
| Leeuwenhoekiella blandensis MED217            | Bact    | –    | –    | –    | –    | 1                  | –         |
| Microscilla marina ATCC 23134                  | Bact    | –    | –    | –    | –    | 1                  | –         |
| Algoraphagus sp. PR1                          | Bact    | –    | –    | –    | –    | 1                  | –         |
| Kordia algicida OT-1                          | Bact    | –    | –    | –    | –    | 1                  | –         |
| Blastopirellula marina DSM3645                 | Pla     | α    | –    | –    | –    | 1                  | –         |
| Alphaproteobacterium BAL199                    | α       | –    | –    | –    | 2    |                    | –         |
| Erythrobacter litoralis HTCC2594               | α       | –    | 1    | 1    | –    |                    | 1         |
| Erythrobacter sp. NAP1                        | α       | –    | 1    | 2    | –    |                    | –         |
| Erythrobacter sp. SD-21                       | α       | –    | 1    | –    | –    |                    | –         |
| Sphingomonas sp. SKA58                        | α       | 1    | 1    | –    | –    |                    | –         |
| Brevundimonas sp. BAL3                        | α       | –    | 1    | –    | –    |                    | –         |
| Nitrobacter sp. Nb311A                        | α       | –    | –    | –    | 1    |                    | –         |
| Fulvimarina pelagi HTCC2506                    | α       | –    | 1    | –    | –    |                    | –         |
| Stappia aggregata IAM 12614                    | α       | 1    | 1    | 1    | 1    |                    | –         |
| Pseudovibrio sp. JE062                        | α       | 1    | –    | 2    | –    |                    | –         |
| Roseobacter sp. SK209-2-6                      | α       | –    | 1    | –    | –    |                    | –         |
| Oceanicola bartsensis HTCC2597                 | α       | –    | 2    | 1    | 1    |                    | –         |
| Rhodobacterales bacterium HTCC2654             | α       | –    | 3    | –    | –    | 1                  | –         |
| Roseovarius rubinhibens ISM                    | α       | –    | 1    | –    | –    |                    | –         |
| Roseovarius sp. TM1035                         | α       | –    | 1    | –    | –    |                    | –         |
| Roseovarius sp. 217                           | α       | 3    | 1    | 1    | –    |                    | –         |
| Roseobacter sp. MED193                         | α       | –    | 2    | –    | 1    |                    | –         |
| Roseobacter litoralis Och 149                  | α       | –    | –    | –    | 1    |                    | –         |
| Phaeobacter gallaeciensis BS107                | α       | 1    | –    | –    | –    |                    | –         |
| Roseovarius sp. HTCC2601                       | α       | –    | –    | 1    | 2    |                    | –         |
| Roseobacter sp. CCS2                           | α       | –    | –    | 1    | –    |                    | –         |
| Rhodobacterales bacterium HTCC2083             | α       | –    | –    | 1    | –    |                    | –         |
| Sagittula estella E-37                        | α       | –    | 1    | –    | 1    |                    | –         |
| Oceanibulbus indolifex HEL-45                  | α       | –    | 3    | –    | –    |                    | –         |
| Sulfobacter sp. NAS-14.1                       | α       | –    | 2    | –    | –    |                    | –         |
| Roseobacter sp. GA101                          | α       | –    | –    | 1    | –    |                    | –         |
| Rhodobacteriales bacterium Y41                 | α       | –    | –    | 1    | –    |                    | –         |
| Rhodobacterales KLH11                          | α       | –    | 1    | 3    | –    | 1                  | –         |
| Pelagibacter ubique HTCC1062                   | α       | –    | –    | –    | –    |                    | –         |
| Methylophilales bacterium HTCC2181             | β       | –    | –    | 1    | –    |                    | –         |
| Betaproteobacteria sp. KB13                     | β       | –    | 1    | –    | –    |                    | –         |
| Marinomonas sp. MED121                         | γ       | –    | –    | 2    | –    |                    | –         |
| Marinobacter sp. DG893                         | γ       | –    | –    | 1    | –    |                    | –         |
| Marinobacter sp. ELB17                         | γ       | –    | –    | 1    | –    |                    | –         |
| Methylophaga sp. DMS010                        | γ       | –    | 1    | –    | –    |                    | –         |
| Alcanivorax sp. DG881                          | γ       | –    | 1    | 1    | –    |                    | –         |
| Limnobacter sp. MED105                         | γ       | 1    | 3    | 1    | –    |                    | –         |
| Moritella sp. PE36                             | γ       | –    | –    | 2    | –    |                    | –         |
| Nitrococcus mobilis Nb-231                     | γ       | –    | –    | 1    | 1    |                    | –         |
| Oceanobacter sp. RED65                         | γ       | –    | 2    | 1    | –    |                    | –         |
| Gammaproteobacterium HTCC2080                  | γ       | –    | 1    | –    | –    |                    | –         |
| Alteromonadales TW-7                           | γ       | –    | 1    | –    | –    |                    | –         |
| Pseudoalteromonas tunicata D2                  | γ       | –    | –    | 1*   | 1    |                    | –         |
| Psychromonas sp. CNPT3                         | γ       | –    | –    | 1    | –    |                    | –         |
| Reinekea blandensis MED297                     | γ       | –    | –    | 1    | –    |                    | –         |
| Sternotrophomonas sp. SKA14                     | γ       | –    | 1#   | 1    | –    |                    | –         |
| Photobacterium sp. SKA34                       | γ       | –    | –    | 1    | –    |                    | 1         |
| Vibrio angustum S14                           | γ       | –    | –    | 1    | 1    |                    | –         |
| Vibrio Fischeri MJ11                           | γ       | –    | –    | 2    | –    |                    | –         |
| Vibrio shilonii AK1                            | γ       | –    | –    | 1    | –    |                    | –         |
| Vibrio alginolyticus 12G01                     | γ       | 1    | –    | 2    | –    |                    | –         |
| Vibrio sp. MED222                              | γ       | –    | –    | 1    | –    |                    | –         |
| Vibio splendidus 12B01                         | γ       | –    | –    | 1    | –    |                    | –         |
| Vibrionales bacterium SWAT-3                   | γ       | –    | –    | 3    | –    |                    | –         |
| Vibrio campbellii AND4                         | γ       | 1    | –    | 1    | 1    |                    | –         |
| Plesiocystis pacifica SIR-1                    | δ       | –    | –    | 1    | 1    |                    | –         |

a. Lacks ImcF.
b. Lacks VirB8.
c. Lacks POTRA.
d. Lacks ShlB.
e. Lacks ImcF and ImpA.

The presence of the corresponding genomic island is indicated by digits denoting the number of occurrences; ‘–’ denotes not detected. Bact, Bacteroidetes; α, Alphaproteobacteria; β, Betaproteobacteria; γ, Gammaproteobacteria; Pla, Planctomycetes.
The two-partner secretion (TPS) system, a variant of the type V secretion system (T5SS), typically consists of a secreted TpsA protein (generally an adhesin) and a TpsB protein that facilitates the secretion of TpsA (Jacob-Dubuisson et al., 2001). In Bordetella pertussis, the causative agent of whooping cough, the TPS system secretes the filamentous haemagglutinin, which mediates adhesion to ciliated cells of the upper respiratory tract (Clantin et al., 2004). Other examples of TPS-mediated adhesion include cell-root interaction of Pseudomonas putida KT2440 (Molina et al., 2006) and the binding to red-blood cells in Proteus mirabilis (Bailey et al., 2005). Operons similar to TPS were found in 16 marine bacteria (Table 1). In Oceanobacter sp. RED65 we found two copies of TPS operons. The first TPS operon encoded two different putative haemagglutinins/adhesines, one having a similarity of 41% conserved amino acids to the filamentous haemagglutinin of B. pertussis. The second TPS operon encoded a protein with 38% similarity to HmwA filamentous haemagglutinin/adhesin of the human pathogen Haemophilus influenzae. The predicted size of the three variants of the putative excreted virulence factors in strain RED65 ranged from 80.3 to 306.1 kDa.

The type VI secretion system (T6SS) represents another pathway for protein translocation (Mougous et al., 2006; Pukatzki et al., 2006). The genes of the T6SS constitute a genomic island important for the virulence/antivirulence of a large number of animal and plant pathogens (Das and Chaudhuri, 2003). Searches specific for conserved T6SS proteins revealed the presence of putative virulence factor homologues in 23 gammaproteobacteria and 12 alphaproteobacteria (Table 1). Proteins of the T6SS operons in the marine bacteria showed amino acid sequence similarities between 29 and 85% to the corresponding T6SS proteins in Salmonella enterica ssp. I (the Sci- operon; Folkesson et al., 2002). Additionally, the planctomycete Blastopirellula marina DSM3645 harbours 12 typical T6SS genes, divided into two genome locations. The T6SS genomic islands carry a core set of genes found in almost all of the examined genomes and several additional genes (Fig. 1). Focusing on this core set of genes, the T6SS island of Roseobacter sp. MED193 showed the highest similarity to the S. enterica ssp. I T6SS genes, being 29–71% and 46–84% at the nucleotide and amino acid levels respectively (see Table S2). The closest match to the V. cholerae O1 T6SS was found in Marinomonas sp. MED121 (54–85% identical, 79–92% conserved amino acids). That the closest matches were not found in the phylogenetically closest relatives might be an indication of the T6SS genomic island as a functional unit, which may have been acquired from other species through horizontal gene transfer. Genes encoding proteins known to be exported by the V. cholerae T6SS, that is, the Hcp (SciK/M) and VgrG proteins, are found within the operon (Pukatzki et al., 2006; 2007), and had predicted homologues in all of the marine T6SS islands identified (Fig. 1). The genes vasA (sciC) and vasK (sciS), necessary for T6SS-mediated secretion in V. cholerae V52 (Pukatzki et al., 2006), belonged to the core set of genes found in all the marine T6SS islands, whereas the vasH gene, also important for T6SS function in Aeromonas hydrophila (Suarez et al., 2008), had a homologue only in six marine gammaproteobacteria (Photobacterium sp. SKA34, V. angustum S14, Marinobacter spp. DG893 and ELB17, Psychromonas sp. CNPT3 and Marinomonas sp. MED121). Taken together, our detection of T6SS in 37 out of 119 marine bacterial genomes, where previously unrecognized, corroborate and extend the findings of Bingle and colleagues (2008) that this secretion system is ‘widespread in nature and not confined to known pathogens’.

‘Antifeeding islands’

Serratia entomophila causes amber disease in larva of the grass grub Costelytra zealandica, a plant pest in New Zealand. The pathogenicity of this bacterium, for example, strain A1MO2, derives in part from an ‘antifeeding effect’
that prevents larvae from feeding (Hurst et al., 2004). The bacterial genes responsible for the antifeeding effect reside in a locus containing genes that bear similarity to phage structural genes. In the marine bacterium *Vibrio campbellii* AND4 (Fig. 2), a genomic island was detected that contained 16 out of 18 genes in the *S. entomophila* operon constituting the ‘antifeeding island’ (Hurst et al., 2004). Amino acid sequence similarities of these putative encoded virulence factors ranged from 43% to 83% (Table S3). Similar genomic islands were found in another 10 marine bacteria (Table 1, Fig. 2), which contained from 8 to 13 of the 18 genes in the *S. entomophila* operon. Notably, the genes *afp*2–4 and *afp*7, identified as essential for the antifeeding effect provided by *S. entomophila*, were present in all the ‘antifeeding island’ copies identified, although some other conserved genes were missing. It was recently shown that the phage-like gene products of the ‘antifeeding operon’ in *Photorhabdus asymbiotica* ATCC43949 form contractile ‘syringe’ structures, and it was suggested that the phage structural proteins could be used to inject toxins into eukaryotic cells (Yang et al., 2006).

**Fig. 2.** Gene organization of the ‘antifeeding’ prophage-like island in marine bacteria. Genes that are predicted homologues to *S. entomophila* ‘antifeeding’ genes are shown with the same patterns as these. Unfilled boxes indicate genes that do not have homologues in *S. entomophila*. Filled black circles above genes indicate genes that show similarity to phage structural genes. Black circles above genes of *S. entomophila* indicate genes that have been shown to be essential for the antifeeding effect of *S. entomophila*. *S. entom*: *Serratia entomophila* A1MO2 AND4, *Vibrio campbellii* AND4; MED217, *Leeuwenhoekella blandensis* MED217; HTCC2594, *Erythrobacter litoralis* HTCC2594; NB231, *Nitrococcus mobilis* Nb-231 (two loci); NB311A, *Nitrobacter* sp. Nb311A; HTCC2654, *Rhodobacteraceae bacterium* HTCC2654.

**Yersinia phospholipase-related toxin**

The rat flea is the natural vector that spreads *Y. pestis*, the causative agent for plague. In the flea, *Y. pestis* cells growing in the midgut prevent the flea from feeding and increase its attempts to bite, thereby increasing the spread of *Y. pestis*. The *Yersinia* ‘murine toxin’ (*ymt*), a phospholipase D homologue (Hinnebusch et al., 2000), is essential for infection of the flea gut. When transferred to *Escherichia coli* the *ymt* gene enables *E. coli*, which normally lacks this gene, to establish a chronic infection of the flea gut (Hinnebusch et al., 2002). Predicted homologues to the *Y. pestis* *ymt* gene were found in the marine bacteria *Marinomonas* sp. MED121 and *Vibrio* sp. MED222 with a similarity of 64–69% conserved amino acids. Indeed, some bacteria of the *Vibrionaceae* family are well known to associate to marine arthropods, for example, copepods (Huq et al., 1983), possibly indicating a role for the marine *ymt* genes in this context.

**Novel putative PAIs**

A frequently used strategy of invasive pathogenic bacteria is to interfere with actin polymerization in the eukaryotic target cell, for example, through interference with Rho GTPases or by directly targeting actin. One such inhibitor of actin polymerization is the mono(ADP-ribosyl)-transferase SpvB from the enteropathogenic bacterium *S. enterica* serovar Dublin, which is known to act both on mamalian and *Acanthamoeba polyphaga* actin polymerization (Tezcan-Merdol et al., 2005). The ability to immo-
bilize eukaryotic cells could be useful also for marine bacteria when infecting eukaryotes. Predicted homologues of the <i>S. enterica</i> SpvB protein were found in the two Vibrionaceae bacteria <i>Photobacterium</i> sp. SKA34 and <i>Vibrio angustum</i> S14. In <i>Photobacterium</i> sp. SKA34 there were three copies of the spvB-like genes (with 41–42% conserved amino acids) in a cluster of six genes, where the other three genes in the operon showed weak similarities to anthrax lethal factor (41% conserved over 360 amino acids), tetanus (53% conserved over 101 amino acids) and botulinum toxins (36% conserved over 328 amino acids) respectively. This cluster is located in a region (~80 kb, 66 genes) also containing genes with high sequence similarities (i.e. >40% conserved amino acids) to genes encoding insect toxins, autotransporter adhesins with Ig-domains, a secreted endonuclease, EmpA metallo-protease of the type found in fish-pathogen <i>V. anguillarum</i> and a haemolysin/lecithinase produced by <i>V. cholerae</i>. Also, other virulence factors, such as putative cytastatin, chitinase, collagenase and penicillin V acylase genes, as well as a PapC/FimD type usher porin involved in pili biogenesis, were found in this genomic region. <i>V. angustum</i> S14 carries a similar genomic region but with some gene rearrangements (Fig. S1), which might be due to that the region is framed and divided by several transposase and phage-type integrase genes. This suggests that the region is part of a mobile genetic element. Similar to many PAs (Schmidt and Hensel, 2004), this genomic island is located next to a cluster of tRNA genes.

### Environmental virulence genes

We found a high number of predicted virulence gene homologues in the genomes of cultured marine bacteria, indicating that bacteria carrying such genes can be readily isolated from sea water. However, this observation may not necessarily reflect the occurrence of potential virulence genes among the uncultivated majority of marine bacterioplankton. Therefore, to achieve a first estimate of the prevalence of virulence genes in the ocean we searched the environmental DNA data set generated by the Sorcerer II Global Ocean Sampling Expedition (GOS) including data from 39 marine planktonic samples, a hypersaline lagoon and a lake (Rusch et al., 2007). The GOS data set was searched for selected protein sequences from the T4SSs and T6SSs, as well as the (ADP-ribosyl)-transferase of the novel island in <i>Photobacterium</i> SKA34 and the ‘antifeeding’ island Afp2. In Table 2 we present the frequencies of these genes normalized to the metagenomic library sizes with marine samples grouped according to high (>1 µg chl a l⁻¹) and low (<1 µg chl a l⁻¹) chlorophyll a concentrations. The investigated virulence genes showed a three to four times higher abundance in productive marine waters (58 genes per 10⁹ bp) and in the hypersaline lagoon (57 genes per 10⁹ bp) compared with open ocean sites (12 genes per 10⁹ bp). The freshwater samples showed an intermediate value (22 genes per 10⁹ bp) with a notable exception in that the T6SS genes were lacking.

The frequency of the typical single-copy recA gene was used to estimate the abundance of the virulence genes as a fraction of the total number of genomes (Table 2; Karlin et al., 1995; Rusch et al., 2007). From this analysis, we estimate that the investigated putative virulence genes were present in 8–12% of the genomes from the productive marine localities and the hypersaline lagoon, but were less common in more oligotrophic marine localities and in freshwater (2–3%; Table 2). These values may be underestimates due to queries being limited to only a few selected virulence genes or overestimates due to the presence of several virulence genes/copies per bacterial genome.

### Table 2. Frequencies of putative virulence genes in the Global Ocean Sampling metagenomic data set.

| Virulence gene homologue | Aquatic environment |
|--------------------------|---------------------|
|                          | Productive sea watera | Oligotrophic sea waterb | Hypersaline lagoon | Freshwater |
| T5SS TpsB                | 42 (66)              | 5.6 (61)              | 3.3 (4)            | 12 (6)     |
| T6SS SciB                | 1.9 (3)              | 1.3 (14)              | 7.5 (9)            | 0 (0)      |
| T6SS SciS                | 8.9 (14)             | 2.9 (31)              | 41 (50)            | 0 (0)      |
| ‘Antifeeding island’ Afp2| 4.5 (7)              | 1.4 (15)              | 3.3 (4)            | 9.7 (5)    |
| (ADP-ribosyl)-transferase| 0.64 (1)             | 1.1 (12)              | 1.7 (2)            | 0 (0)      |
| Total virulence genes    | 58 (91)              | 12.3 (133)            | 57 (69)            | 22 (11)    |
| RecA                     | 765 (1203)           | 512 (5533)            | 479 (584)          | 883 (455)  |
| Total virulence genes : RecA (%) | 8 (2) | | 12 | 3 |

a. > 1 µg chl a l⁻¹

b. < 1 µg chl a l⁻¹

Marine samples were divided into productive (>1 µg chl a l⁻¹, 12 samples) and more oligotrophic (<1 µg chl a l⁻¹, 27 samples) regions based on chl a data published in Rusch and colleagues (2007). Samples from a hypersaline lagoon (Floreana Island, Ecuador) and from freshwater (Lake Gatun, Panama) were also included. Numbers give gene frequencies per billion bases sequenced. Numbers within parentheses give total number of hits in the CAMERA Global Ocean Sampling Database (http://camera.calit2.net/).

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genome. Nevertheless, the GOS subset of randomly sequenced environmental DNA support our finding from whole-genome sequences that virulence gene homologues have a widespread occurrence in marine bacteria, particularly in productive waters with high densities of algae. In this scenario, expression of virulence genes as a means to obtain resources could provide a selective advantage for opportunistic bacteria. In contrast, one would not expect to find homologues of virulence genes in typically oligotrophic marine bacteria like Pelagibacter ubique that is characterized by small size and a slow growing single-cell life strategy (Morris et al., 2002; Simu and Hagstrom, 2004; Giovannoni et al., 2005). Accordingly, we found no predicted homologues of virulence genes in any of the three sequenced strains of P. ubique (Table 1 and Table S1).

Concluding remarks

In light of our findings it is reasonable to question if presence of virulence gene homologues fits with what is known about marine bacteria. Unfortunately, a comprehensive understanding of their life strategies is largely lacking. However, some interesting patterns have emerged. For example, several of the Roseobacter clade bacteria appear to be associated with phytoplankton (Allers et al., 2007; Moran et al., 2007), and their containing T4SSs may provide a means to retrieve resources from these eukaryotes. For fast-growing gammaproteobacteria, such as Vibrio and Alteromonas species, labile organic carbon substrates, for example, amino acids or glucose, stimulates growth (Eilers et al., 2000; Pinhassi and Berman, 2003). Also organic matter released during intense algal blooms stimulates the growth of V. cholerae O1 in sea water (Mourino-Pérez et al., 2003). At this stage it can only be speculated that the use for specific protein secretion systems and other virulence gene homologues enable, or even is a prerequisite, for a fruitful transition from a free-living lifestyle to life attached to phytoplankton or crustaceans.

We screened for only a subset of known virulence genes and PAIs, and thus our data should represent a conservative estimate of the number of potential virulence genes in marine bacteria. Still, the presence of virulence genes does not necessarily imply that these bacteria are pathogens (e.g. on humans or fish). Even though our sequence analysis shows that marine bacteria have genetic homologues of virulence genes in known pathogens, the function of the expressed proteins may differ between organisms. For example, marine bacteria could use secretion systems and adhesins to attach to each other, other bacteria or to floating particles. However, it is plausible that such traits could also be useful to attach to and exploit compromised, dying or dead single-celled or multicellular eukaryotes. In so doing, virulence genes and PAIs could constitute the genetic tools for marine bacteria to cause harm to algae, and thereby contribute to explaining some of the ample records of such behaviour (Mayali and Azam, 2004). Alternatively, or in addition, ‘virulence’ genes may play an important role in protecting bacteria against protozoan predation (Matz and Kjelleberg, 2005). These authors also proposed that microbial virulence factors associated with human disease could originate from such antipredator defence.

Interestingly, it was recently suggested that host–pathogen interactions should be studied in a wider ecological and evolutionary perspective to better understand the life strategy of pathogenic bacteria (Pallen and Wren, 2007). Functions that have evolved under long time in nature may have been recruited through horizontal gene transfer to perform similar or different functions by more recently emerging pathogenic bacterial species. The precise functions of the putative virulence gene homologues reported here remain unknown until experimental genetics and physiological response experiments can confirm their native function; for example, are they used to support host association, mutualism or commensalism or infection? Whatever the case may be, our analysis show that several marine bacteria contain virulence gene homologues that could potentially be used to attach to, and take advantage of, eukaryotic cells in a manner similar to that used by known pathogens (i.e. as an intrusive way to obtain organic matter and nutrients). Such functions could be important for understanding the ecology and evolution of marine bacteria.

In conclusion, we found a widespread occurrence of virulence gene homologues in a phylogenetically diverse set of genome sequenced marine bacteria. Further, our analysis of environmental DNA suggests that several of these genes are widely distributed in the oceans. Consequently, marine bacteria may constitute a reservoir for virulence genes found in human, animal, plant and insect pathogens as previously suggested for virulence genes in soil bacteria (Casadevall, 2006). From a marine ecology perspective, expression of these genes would indicate that some bacteria infect or even consume live cells. This would result in a previously unrecognized flow of organic matter and nutrients directly from eukaryotes to bacteria (Fig. S2).

Experimental procedures

Analyses of genomes of marine bacteria

Whole-genome sequencing was done by the J. Craig Venter Institute (JCVI; sequences and strain information available at https://moore.jcvi.org/moore/) within the Gordon and Betty Moore Foundation Marine Microbiology Initiative (http://www.moore.org/marine-micro.aspx). Open reading frames were predicted and annotated using the annotation pipeline.
at JCVI, which integrates results from hidden Markov models and BLAST. Functional prediction was carried out using COG analysis and motif finding. Non-coding RNA prediction was done using tRNA-scanSE and BLAST. All automatically annotated genes of interest were inspected and verified manually by BLAST (Altschul et al., 1990), COG, PFAM and TIGRFAM analyses. If the annotation was judged to be correct, the analyses were further extended by repeating the analyses on neighbouring genes and by searching for elements (tRNA genes, IS sequences, transposons, phage/plasmid replicons and deviating GC content) indicative of mobile genetic regions. When a putative genomic island had been identified, homologous islands were screened for in other marine bacteria, where in addition to the aforementioned analysis, attention was also paid to gene order and operon structure in homologous genomic islands to verify that the putative island was indeed a conserved genomic unit.

An initial analysis was made on the genome sequences from marine bacterial isolates that we contributed to the Marine Microbiology Initiative and made available to us directly after sequencing. These genomes were: Neptunibacter caesariensis MED92, Marinomonas sp. MED121, Dokdonia sp. MED134, Polaribacter sp. MED152, Roseobacter sp. MED193, Leeuwenhoekiella blandensis MED217, Vibrio sp. MED222, Reinekea sp. MED297, Oceanobacter sp. RED65, Photobacterium sp. SKA34, Loktanella vestfoldensis SKA53 and Sphingomonas sp. SKA58. In a first step, to find potential virulence gene homologues, we screened the automatic JCVI annotation by searching for virulence and secretion system genes or by looking for signs of defect prophage and IS elements indicating the presence of mobile genetic elements.

We also conducted BLASTP searches using genes of known virulence factors from animal and human pathogens as query sequences. In particular, we looked for tyrosine kinases, ADP-ribosyltransferases, various known and suspected haemolysins using characterized virulence genes from S. enterica (e.g. spvB, hlyA) and Y. pestis (e.g. ymt, yopH). For each of the virulence gene systems analysed, the original pathogen genomes where the virulence genes are present were included in the screening as positive controls to confirm that the methodology used could detect the relevant genes. Standard BLASTP was made with BLASTP 2.2.14 with no filtering, expectancy: 10, word size: 3, scoring matrix: BLOSUM 62, gap costs: opening 11, extension 1. Virulence factor homologues were identified on the criteria of an E-value ≤ 10^-5 and sequence similarity > 30%.

Type IV secretion system. The T4SSs were screened for among the marine bacterial genomes using PF04610 (TrbB/VirB6 plasmid conjugal transfer protein) and PF04335 (VirB8 protein) and an E-value ≤ 10^-6. Genomes with positive hits for these PFAMs were manually inspected for presence of the T4SS core conserved protein classes VirB4, VirB6, VirB8, VirB9, VirB10, VirB11, VirD4 (Medini et al., 2006). Operon structures with genes for these seven proteins were thus detected using BLASTP with known VirB proteins from A. tumefaciens and PFAM and COG matches. The vir gene homologues encoding these proteins were all present in the genomes scored as T4SS positive and presented in Table 1.

Type V secretion system. We screened for TPS (or T5bSS) systems using a combination of the PFAMs PF05860 (filamentous haemagglutinin, N-terminal domain), PF08479 (polypeptide transport-associated, ShiB-type) and PF03865 (haemolysin activator HlyB) and an E-value ≤ 10^-6. Positive hits were manually inspected to confirm that the two components of the TPS system were located in adjacent open reading frames.

Type VI secretion system. The T6SSs were identified essentially as described by Bingle and colleagues (2008), using the seven T6SS-specific PFAM domains PF05591 (DUF1770), PF05936 (DUF1876), PF05943 (DUF1877), PF05947 (DUF1879), PF06596 (DUF1305), PF06761 (ImcF-related) and PF06812 (ImpA, N-terminal domain) and an E-value ≤ 10^-5. Genomes were scored as positive for T6SS based on the presence of these markers combined. Some of the genomes had two or more paralogues of the genes containing these PFAMs.

Yersinia murine toxine was searched for among the marine bacterial genomes using BLASTP with the Ymt amino acid sequence from Y. pestis strain CO92 (GenBank Accession Number NP_395420) and an E-value ≤ 10^-6 and sequence identity values > 30%.

In summary, functional annotation was based on: (i) sequence similarity values in BLAST searches over the entire length of the queried genes (unless specifically stated otherwise in the text), all sequence comparisons were made at the protein level, sometimes complemented by DNA sequence analysis, (ii) classification of proteins within the COG, PFAM or TIGRFAM domain structure libraries (Marchler-Bauer and Bryant, 2004) and (iii) gene arrangement/complement, that is, presence in the same genetic context in the marine bacteria as in known pathogens, that is, within the operon structure of defined PAIs.

Gene frequencies in the Global Ocean Sampling data

We used BLAST analysis, using virulence factor sequences from the marine bacterial genomes as queries, to find and identify putative virulence factors in the CAMERA Global Ocean Sampling Database (http://camera.calit2.net/). Proteins from the following organisms were used to search the Global Ocean Sampling data (Rusch et al., 2007): TSSS TspB homologue: Oceanobacter sp. RED65, T6SS SciB and SciS homologues: Roseobacter sp. MED193, ‘Antifeeding island’ Afp2 homologue: L. blandensis MED217, Yersinia
murine toxin homologue: *Vibrio* sp. MED222 (APD-ribosyl)-transferase homologue: *Photobacterium* sp. SKA34. Matches to *Burkholderia* were excluded from the analysis as these sequences may stem from contaminants (Rusch et al., 2007).

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Virulence gene homologues in marine bacteria

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Novel PAI in *Photobacterium* sp. SKA34 and *Vibrio angustum* S14. Genes with more than single copies, showing homologies to different classes of virulence genes, are marked in different colours (see legend below figure). The genome regions span from SKA34_08238 to SKA34_08563 and VAS14_15892 to VAS14_16212.

Fig. S2. Conceptual model of the carbon flow in the marine planktonic food web. Grey lines represent release of dissolved organic carbon as the result of several different mechanisms including virus lysis. The red lines depict possible added effects of putative virulence genes on the carbon fluxes in the marine planktonic food web.

Table S1. List of genomes included in the study. Genomes marked in boldface contained some/several of the virulence genes or PAIs that were screened for. Genomes were sequenced through the Moore Foundation Initiative in Marine Microbiology.

Table S2. Comparison of the proteins in type VI secretion systems of *Salmonella enterica* subsp. Typhimurium (SALTY), *Vibrio cholerae* (V.chol.), *Rhizobium leguminosarum* (RHIZO) and marine bacteria (*Roseobacter* sp. MED193, *Marinomonas* sp. MED121, *Vibrio* sp. MED222, *Reinekea* sp. MED297, *Oceanobacter* sp. RED65, *Photobacterium* sp. SKA34). Standard BLASTP was made with BLASTP analyses in GenBank.

Table S3. Comparison between antifeeding PAIs from *Ser- ratia entomophila* (Af1–Af18), *Photorhabdus luminescens* and a number of marine bacteria (see https://research. venterinstitute.org/moore/MultiOrganism? MULTIORGANISM_SORT_ATTR=SOR= SORT_ORGANISM for sequence access). Number in parenthesis represent % amino acid identity/% aa similarity/length in aa of matched region. The conserved domains in the table legends indicate domains detected upon BLASTP analyses in GenBank.

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