Exploration of phage-host interactions in the fish pathogen *Vibrio anguillarum* and anti-phage defense strategies

PhD thesis
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Preface
The project presented in this PhD was carried out under the supervision of Prof. Mathias Middelboe primarily at the Marine Biological Section, Department of Biology, University of Copenhagen, Denmark.

The work was done in collaboration with Prof. Lone Gram (Department of Systems Biology, Technical University of Denmark) and Dr. Sine Lo Svenningsen (Department of Biology, University of Copenhagen).

List of publication and manuscripts


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Abstract

The disease vibriosis is caused by the bacterial pathogen *Vibrio anguillarum* and results in large losses in aquaculture both in Denmark and around the world. Antibiotics have been widely used in antimicrobial prophylaxis and treatment of vibriosis. Recently, numerous multidrug-resistant strains of *V. anguillarum* have been isolated, indicating that antibiotic use has to be restricted and alternatives have to be developed. Lytic phages have been demonstrated to play an essential role in preventing bacterial infection. However, phages are also known to play a critical role in the evolution of bacterial pathogenicity development. Therefore, successful application of phage therapy in the treatment of vibriosis requires a detailed understanding of phage-host interactions, especially with regards to anti-phage defense mechanisms in the host.

Part I. As a first approach, 24 *V. anguillarum* and 13 *Vibrio* sp. strains, representing considerable temporal (20 years) and geographic (9 countries) variation in regards to their origins of isolation, and 11 vibriophages representing three different families (*Myoviridae*, *Siphoviridae*, and *Podoviridae*), were characterized with respect host range, morphology, genome size and lytic properties. Together the host range of the 11 vibriophages covered all the 37 *Vibrio* strains in the collection. In addition, the occurrence of unique susceptibility patterns of the individual host isolates, as well as key phenotypic properties related to phage susceptibility that were common to the isolated strains were studied.

Part II. *In vitro* phage-host interactions in two *V. anguillarum* strains (BA35 and PF430-3) and their corresponding phages (ΦH20 (*Siphoviridae*) and KVP40 (*Myoviridae*)), during growth in the settings of micro-colonies, biofilms, and the free-living phase were investigated, in order to explore the resistance/tolerance mechanisms responsible for the different outcomes of these two phage-host interactions. The study demonstrated large intraspecific differences in phage-protection mechanisms, as strain BA35 obtained genetic resistance through mutational changes, whereas strain PF430-3 was protected from phage infection by formation of a biofilm. These different protection mechanisms have important implications for the phage-host dynamics and efficiency of phage infection.

Part III. To gain an insight into whether bacteria of the *Vibrio* genus use quorum sensing (QS) to control their phage-susceptibility, a study of the QS-mediated interaction between phage KVP40 and *V. anguillarum* PF430-3 was carried out. The data show that QS does indeed regulate phage-host interactions in *V. anguillarum* PF430-3, potentially by using QS transcription factor VanT to repress *ompK* expression. It was demonstrated that QS controls the choice of anti-phage defense strategies in the *V. anguillarum* strain.
PF430-3, suggesting the presence of dynamic, temporary adaptations to phage infection pressure, while still securing the ability to produce a functional OmpK receptor.

In conclusion, this thesis provides a first insight into the dynamic vibriophage-host interactions, indicating the complexity of phage therapy in the treatment of vibriosis, regarding the evolution of anti-phage defense mechanisms, gene regulation, quorum sensing, biofilm formation, as well as pathogenesis. Together, these discoveries will enable us to evaluate these potential factors which regulate phage-host interactions, and on that background, to optimize phage therapeutic strategies against vibriosis with regards to the composition of applied phage cocktails, the timing of treatment, and the likely outcome of phage therapy and QS inhibiting molecules.
Resumé

**Vibrio anguillarum** er en vigtig patogen bakterie i akvakultur, hvor den forårsager sygdommen vibriose, som resulterer i fiskedød og store økonomiske tab i akvakulturindustrien i både Danmark og hele resten af verden. Typisk bruges antibiotika i behandlingen af vibriose, men forøget forekomst af antibiotika-resistente *V. anguillarum* har medført øget fokus på behovet for alternative behandlingsmetoder. Anvendelse af lytiske bakteriofager har de senere år vist sig som et potentielt alternativ til antibiotika ifm forebyggelse og behandling af fiskeinfektioner. Denne tilgang er dog også forbundet med udfordringer og begræsninger, dels fordi fagerne kan spille en rolle i bakteriers patogene egenskaber og dels fordi bakterie også udvikler resistens overfor fager. Succesful anvendelse af bakteriofager i sygdomsbehandling kræver derfor et grundigt kendskab til fag-vært interaktioner og specielt en forståelse af anti-fag forsvarsmechanismer i værtsbakterien.

Del 1. Som en første tilgang dette undersøgtes 24 *V. anguillarum* og 13 *Vibrio* sp., stammer som repræsenterede en betydelig tidsmæssige (20 år) og geografiske (9 forskellige lande) forskellige med hensyn til stedet for deres oprindelige isolation, samt 11 vibrio fager, der repræsenterede 3 forskellige morfologiske familier (*Myoviridae*, *Siphoviridae*, og *Podoviridae*). Bakteriofagerne blev karakteriseret med hensyn til "host range", morfologi, genomstørrelse og lytiske egenskaber, og tilsammen kunne de 11 fagisolater inficere alle 37 *V. anguillarum* stammer i stammesamlingen. Derudover blev mønstre i inficerbarhed overfore fagerne samt egenskaber associeret med resistens overfor disse undersøgt hos værtsstammerne.

Del 2. **In vitro** studier af fag-værtinteraktioner hos to *V. anguillarum* stammer (BA35 and PF430-3) og deres associerede fager (ΦH20 (*Siphoviridae*) og KVP40 (*Myoviridae*)) blev undersøgt under forskellige vækstfaser (mikrokolonier, biofilm og fritlevende) med det formål at kortlægge resistens/tolerance mekanismer i de to stammer. Studiet dokumenterede store intraspecifikke forskelle i de underliggende mekanismer for beskyttelse mod fager i de to stammer. Stamme BA35 udviklede genetisk resistens gennem mutationer mens stamme PF430-3 beskyttedes mod fag-infektioner ved dannelse af biofilm. Disse forskelle i beskyttelsesmekanismer havde store implikationer for fag-vært dynamik og infektions-effektivitet af de to fager.

Del 3. I dette studie undersøgtes i hvilket omfang *Vibrio anguillarum* stamme PF430-3 anvender "quorum sensing" (QS) i reguleringen af tolerance overfor faginfektion. Data påviser at QS regulerer interaktionerne mellem fag KVP40 og *V. anguillarum* PF430-3, idet QS medierer en aktivering af transkriptionsfaktor VanT som leder til en nedjustering af ekspressionen af *ompK*, som er receptor for KVP40. Arbejdet viser at QS kan
kontrollerer skift i anti-fag strategi hos V. anguillarum strain PF430-3, som afhænger af de givne vækstbetingelser. Disse skift i forsvarsmechanisme indikerer meget dynamiske tilpasninger til fag-infektionstryk i V. anguillarum PF430-3, som samtidigt sikrer at stammen kan opretholde en funktionel ompK receptor.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Abi</td>
<td>Abortion infection system</td>
</tr>
<tr>
<td>AHLs</td>
<td>N-acyl-L-homoserine lactone</td>
</tr>
<tr>
<td>AI</td>
<td>Autoinducer</td>
</tr>
<tr>
<td>CAI-1</td>
<td><em>cholerae</em> autoinducer-1</td>
</tr>
<tr>
<td>cas</td>
<td>CRISPR-associated</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered regularly interspaced short palindromic repeats</td>
</tr>
<tr>
<td>CT</td>
<td>Cholera toxin</td>
</tr>
<tr>
<td>CVEC</td>
<td>Conditionally viable environmental cells</td>
</tr>
<tr>
<td>CWD</td>
<td>Cold water disease</td>
</tr>
<tr>
<td>eDNA</td>
<td>Extracellular DNA</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>EPS</td>
<td>Extracellular Polymeric Substances</td>
</tr>
<tr>
<td>ERIC</td>
<td>Enterobacterial repetitiveelement intergenic consensus sequence</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td>ICTV</td>
<td>International Committee on Taxonomy of Viruses</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MLST</td>
<td>Multi-locus sequence typing</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin-resistant <em>S. aureus</em></td>
</tr>
<tr>
<td>OA</td>
<td>Oxolinic acid</td>
</tr>
<tr>
<td>Omp</td>
<td>Outer membrane protein</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulsed-field gel electrophoresis</td>
</tr>
<tr>
<td>qrr</td>
<td>Quorum sensing regulatory RNA</td>
</tr>
<tr>
<td>QS</td>
<td>Quorum sensing</td>
</tr>
<tr>
<td>R-M</td>
<td>Restriction-Modification</td>
</tr>
<tr>
<td>VBNC</td>
<td>Viable but non-culturable</td>
</tr>
<tr>
<td>vps</td>
<td><em>Vibrio</em> polysaccharide</td>
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Introduction

1. Aquaculture

1.1. Current status of aquaculture

A daunting challenge of our time is to ensure adequate nutritional food to the expanding human population (1). Over the past 50 years, the aquaculture industry has become one of the fastest growing industries globally, with an annual growth rate of 8.3%. The aquaculture industry not only provides healthy food for human consumption, but it is extremely important for local economies which rely heavily on the sales of high valued marine fish and crustaceans (1). According to Figure 1, at the end of 2012, aquaculture production has already reached 66.6 million tons per year (1). With declining natural fish stocks and global fisheries, aquaculture plays a vital role in feeding the planet, today and in the future.

Aquaculture is defined as farming aquatic organisms including fish, molluscs, crustaceans and aquatic plants. In addition, aquaculture farming not only involves interventions in the rearing process to enhance production, such as stock, feeding, protection from predators, but also implies stocks cultivation (1). Despite the fact that aquaculture is a well-controlled and organized industry, problems such as disease outbreaks can still occur. Disease outbreaks are considered as one of the most significant constraints to sustainable aquaculture growth (2).

1.2. Bacterial diseases in aquaculture

Fish are susceptible to a wide range of bacterial pathogens. Some of the most important opportunistic bacterial diseases are vibriosis, salmon rickettsial syndrome (SRS), furunculosis, and cold water disease (CWD), which is caused by Vibrio anguillarum, Piscirickettsia salmonis, Aeromonas salmonicida, and
Flavobacterium psychrophilum, respectively (3-5). These bacterial cells are naturally occurring in the environment but become serious pathogens when fish hosts are physiologically unbalanced, immunocompromised or exposed to other stressors, i.e., poor water quality, overstocking, and overfeeding, etc., which then allows opportunistic bacterial infections to progress (6). Once the abundance of these opportunistic pathogens reaches a threshold level, they can cause disease. Hence, farmed fish in many cases are affected by these bacterial diseases, resulting in dynamic unpredictable mortality rates, and subsequently, severe economic losses. Thus, disease management in aquaculture is becoming more complex and challenging.

1.3. Vibriosis and Vibrio anguillarum

Vibriosis, a highly fatal haemorrhagic septicaemia, is one of the most prevalent fish diseases and has been found in more than 50 fresh and salt-water fish species including various species of economic importance to the larviculture and aquaculture industry, especially during the larval and juvenile stages. These species include salmon (Salmo salar), rainbow trout (Onchorhynchus mykiss), turbot (Psetta maxima), European sea bass (Dicentrarchus labrax), gilthead sea bream (Sparus aurata) and ayu (Plecoglossus altivelis) (7). The pathogen can invade the host either via contaminated food or through the skin epithelial cells, while the infection is then triggered due to environmental factors commonly found in fish farms such as high population density or poor water quality (8). Typical external clinical symptoms of the disease include weight loss, lethargy, and development of red spots on the ventral and lateral areas of the fish, with swollen, dark skin lesions causing ulceration and bleeding (Figure 2) (9). Nevertheless, in acute pandemic infections the infection spreads so rapidly that most of the infected fish die even without evidence of any clinical symptoms (10). Previous studies have reported that V. anguillarum, as one of the most prominent fish and shellfish pathogens, is associated with vibriosis infection in aquaculture (11-13).

Figure 2. Clinical symptoms of vibriosis infection a sea bass causing haemorrhagic septicaemia on the fin and around the operculum, figure adapted from Austin (10).
\textit{V. anguillarum} is a Gram-negative, halophilic and facultative anaerobic bacterium, which grows rapidly in the range of temperatures between 15 and 30 °C on rich media containing 1.5-2% NaCl. According to Harrell’s study, \textit{V. anguillarum} was initially divided into two distinct biotypes (1 and 2) (14). However, through advances in DNA sequence technology, \textit{V. anguillarum} biotype 1 was reclassified as \textit{Listonella anguillarum}, as a result of 5S ribosomal RNA gene sequence analysis (15). Nevertheless, because of its strong similarities with other \textit{Vibrio} species, it is generally still identified as \textit{V. anguillarum} (16). Biotype 2 was reclassified as a new species, \textit{V. ordalii} (11).

\textit{V. anguillarum} is composed of 2 chromosomes (3.0 and 1.2 Mbp) with a whole genome size of about 4.2 Mbp and a G+C content of 43-46%. Most of the O1 serotype strains isolated harbor a plasmid which carries many virulence factors, such as genes affecting chemotaxis and motility, an iron uptake system, lipopolysaccharides (LPSs) and extracellular products with proteolytic or haemolytic activity (11). Recently, the genome of the O1 serotype strain \textit{V. anguillarum} 775 was sequenced. The genome annotation shows that the majority of the genes for essential cell functions and pathogenicity are located in chromosome 1. This chromosome also has 8 genomic islands, while chromosome 2 has only 2. It was found that some of these islands carried potential virulence genes (17).

\textit{V. anguillarum} strains show close similarities according to 16S rRNA sequences irrespective of time or place of isolation, suggesting 16S rRNA is highly uniform and stable (18), indicating further genetic fingerprinting tools such as enterobacterial repetitive element intergenic consensus (ERIC) PCR (19), multi-locus sequence typing (MLST) (20), and pulsed-field gel electrophoresis (PFGE) (21) are needed for surveillance purposes, monitoring outbreaks and tracking pathogen spreading.

Currently, 23 different O serotypes (O1-O23) within \textit{V. anguillarum} are discriminated, each single O serotype with its own different pathogenicity and host specificity. Among these, only serotypes O1, O2, and O3, have been reported to associate with vibriosis infection in fish (22). Within serotype O2, three host-specific sub-serotypes, O2a, O2b and O2c, have been distinguished by Rasmussen (23).
2. Bacterial communication

Bacteria have adapted to thrive in diverse ecological niches. Over the long-term evolution, they have developed sophisticated mechanisms, allowing them to perceive and respond to dynamic environmental conditions (24).

Quorum sensing (QS) is a ubiquitous cell-cell communication found in bacteria, thus far (25). By using this QS system, bacteria coordinate their cell density-dependent gene expression to act in unison, via the production, secretion and subsequent detection of extracellular signaling molecules, namely, autoinducers (25). Detection of autoinducers allows bacterial cells to distinguish different behavioral modes, i.e. from low cell densities to high cell densities (25).

2.1. Quorum sensing in Gram-negative bacteria

Most Gram-negative proteobacteria use acyl homoserine lactones (AHLs) as major autoinducers for interspecies communication, with homoserine lactone (HSL) rings carrying acyl chains of a range from C4 to C18 in length (26). The first AHL was identified in bioluminescent bacteria (V. fischeri), which have a symbiotic relationship with bobtail squid (Euprymna scolopes) (27). By using QS to activate expression of the luciferase operon to counter-illuminate itself, the squid can easily escape from predators as a cooperative symbiotic interaction between the squid and V. fischeri (25, 28, 29). The mechanism underling this phenomenon has been fully characterized. Briefly, in the V. fischeri model, LuxI synthesizes QS autoinducers, and LuxR acts as a cytoplasmic receptor for AHLs as well as transcription factor of the luxI operon (28, 30, 31). However, without AHLs ligand, LuxR is unstable and can be rapidly degraded. Since AHLs can freely diffuse across cell membranes, their concentration increases correspond to the cell density increases. By binding to the transcriptional factor LuxR, the AHLs-LuxR complexes turn on the luxICDABE expression (Figure 3) (28, 32).

![Figure 3. A canonical Gram-negative LuxI-R-type QS circuits. Red pentagons denote AHL autoinducers. Figure adapted from Ng (28).](image-url)
2.2. Quorum sensing in *V. anguillarum*

*N*-acyl homoserine lactone-mediated QS circuits have been identified in *V. anguillarum*, showing similarities to many other *Vibrio* species in previous studies (28, 33-35). These circuits contain components for multiple QS phosphoreplay system, and control QS-regulated genes via the transcription factor VanT, which is activated in response to extracellular signaling molecules (35).

In general, at low cell densities, these auto-inducers are also at low concentrations, and the membrane-binding sensors are kinases, VanN, VanQ and CqsS, which start autophosphorylation, and initiate a cascade, transferring phosphate onto the phosphotransferase VanU, which phosphorylates the $\sigma^{54}$-dependent response regulator VanO (33, 36). Upon phosphorylation, VanO is activated together with the alternative sigma factor RpoN, together with the RNA chaperone Hfp, which finally destabilizes *vanT* mRNA, repressing expression of the QS transcriptional regulator VanT (33, 36).

At high cell densities, auto-inducer concentrations reach certain thresholds with *vanT* expression induced, while VanO is inactivated (35). Specifically, binding of QS-signal molecules to the kinase sensor inhibits kinase activity, which changes the receptor function from kinase to phosphatase activity, leading to the dephosphorylation reactions and finally to inactivate VanO. Thus, QS regulatory RNA (*qrr*) is not expressed while stabilizing the expression of VanT, leading to the QS-mediated gene regulation (33, 36). Figure 4 is a model of *V. anguillarum* QS circuits.
Figure 4. *V. anguillarum* QS-circuits. Solid lines with arrows and bars represent activation and repression of gene expression, respectively. Solid lines with double arrowheads represent the transfer of phosphoryl groups to each other. At low cell densities, auto-inducers are at low concentrations. Upon phosphorylation, VanO is activated together with the alternative sigma factor RpoN, which finally destabilizes vanT mRNA, repressing expression of the QS transcriptional regulator VanT. At high cell densities, auto-inducers reach a certain threshold with *vanT* expression induced, while VanO is inactivated. OM and IM indicate outer membrane and inner membrane, respectively. The P in the circle indicates that the protein is in the phosphorylated state (35).
In addition, several N-Acyl homoserine lactone auto-inducers (N-(3-Oxodecanoyl)-L-homoserine lactone, N-(3-hydroxyhexanoyl)homoserine lactone, and N-hexanoylhomoserine lactone) (Figure 5) have been identified in stationary-phase *V. anguillarum* cell-free supernatant at concentrations of approximately 8.5, 9.5, and 0.3 nM, respectively (37).

![Figure 5](image_url)

Figure 5. Structures of three different *V. anguillarum* autoinducers present in stationary phase cell-free culture supernatant, namely, N-(3-Oxodecanoyl)-L-homoserine lactone, N-(3-hydroxyhexanoyl)homoserine lactone, and N-hexanoylhomoserine lactone.
3. Bacterial biofilm formation and its role in infections

Until recently, a bacterium cell has been considered as an individual cell without interactions with neighboring cells. This dogma has been challenged by the discovery of the cell-density dependent bioluminescence of the marine bacterium *V. fischeri*, and bacterial biofilm formation (27, 38, 39). These types of gene regulatory phenomena are generally recognized as QS (25). As discussed above QS regulates multiple bacterial social behaviors, such as bioluminescence (40), sporulation (41), competence (42), virulence (43), motility (44), and phage-host interactions (45, 46), as well as bacterial biofilm formation (47). The development of biofilm and QS are meticulously interconnected, as cooperative bacterial group behaviors in a self-produced extracellular matrix are essential for biofilm formation and development (48).

It is, therefore, necessary to better understand the mechanisms of *V. anguillarum* evolution and pathogenicity to develop better animal models and design new strategies and therapeutics for the control of vibriosis.

During vibriosis infection, bacterial growth in planktonic form is relatively rare. In natural environments and aquaculture, bacterial infection involves adhesion to the host organism in the form of a biofilm, sustaining in the persistence of the infection during the vibriosis outbreak (49, 50). Studies have shown, after the first stages of infection, *V. anguillarum* formed biofilm-like micro-colonies within the skin mucosal tissues of rainbow trout, causing chronic infection and contributing to disease persistence in the face of antibiotic treatment regimens, due to poor antibiotic penetration, nutrient limitation and slow growth, as well as adaptive stress responses (51, 52). Thus, understanding of the role of bacterial biofilm during infection should help the management of antibacterial therapy of relevant infections.

3.1. Biofilm structure

Biofilms are matrix enclosed microbial communities that can be established on a range of biological or non-biological surfaces (53). The complex mixture of hydrated polymers, known as extracellular polymeric substances (EPS), plays an important role in the function of biofilms, including retention of nutrients and water as well as protection from antimicrobial agents such as antibiotics, protozoan grazing, host immune systems and bacteriophages (53). Although polysaccharides are predominant components, EPS also contain a variety of other substances, including protein, extracellular DNA (eDNA), membrane vesicles, and other polymers. For instance, in the model organisms of strain *Enterococcus faecalis*, *Staphylococcus aureus*, *P. aeruginosa* and *V. cholerae*, eDNA has been shown essential for maintaining the stability of saturated biofilms (54-57). The structural role of eDNA in promoting biofilm formation is highly variable and depends on the bacterial strains and growth conditions as well as the stages of the biofilm. The observation of prophage-stimulated biofilm formation (58, 59), and demonstration that addition of DNase I at the
initiation stage of biofilm growth inhibited biofilm formation suggested that prophage mediated lysis results in the release of eDNA enhancing biofilm formation. However, DNase I-treated experiments did not affect phage KVP40-mediated V. anguillarum strain PF430-3’s biofilm formation, suggesting that eDNA released from phage KVP40-mediated lysis did not serve as a critical structural component in the initial stages of biofilm formation (Tan et al., unpublished). However, the results should be interpreted carefully, since further work with more controls, such as DNase I inactivation and biofilm penetration, are needed.

3.2. Biofilm development and variation in biofilm formation

Biofilm formation is a multistep process (Figure 6), first involving surface-associated bacterial attachment followed by immobilization, then combined unidirectional movement along the surface, and finally the formation of three-dimensional micro-colonies (60). Meanwhile, these sessile biofilm communities can also rapidly multiply and disperse to planktonic bacterial cells (61).

![5 stages of Biofilm Development](image)

Figure 6. Five complex development processes involving biofilm maturation. Stage 1-5, initial attachment; irreversible attachment; maturation I; maturation II; and dissemination, respectively. Figure adapted from Monroe’s study (62).

It turns out that there is a whole range of different types of biofilm development that bacteria use for a variety of purposes. For instance, V. cholerae bacterium stops biofilm formation when a certain density of bacterial cells gather together; while other bacterium such as P. aeruginosa bacterium makes biofilms when the bacterial number reaches a certain density threshold.

P. aeruginosa biofilms, which is contrasted with cholera acute infection case, cause devastating chronic lung infections in the immunocompromised hosts (63). QS signaling molecules, on the other hand, activate gene transcriptions which facilitate biofilm formation at high cell densities (64). Another well-known example of biofilm formation at high cell densities is that of E. coli, in which the physiological and morphological development of its biofilms have been well studied. In the post-exponential growth phase of E. coli, while nutrients are no longer optimal yet still available, E. coli cells stop producing flagella, and enter into stationary phase, in a process of cellular aggregation (65). This aggregation imposes diffusion
constraints on material exchanges, preventing both entering or leaving the biofilm system, which impedes, for example, antimicrobial substances and the interactions between phage receptor and phage binding proteins (66). However, biofilm sessile cells can also be detached by seeding dispersal, and these detached cells also involve the establishment of new biofilms, which is of fundamental importance to the dissemination of pathogens in the infection model (67).

In V. cholerae cases, biofilm formation is activated at low cell densities (47), in contrast to other bacterial pathogens that induce biofilm formation at high cell densities in the presence of QS signaling molecules. The ability to produce biofilm at low cell densities not only protects the V. cholerae cells against the host immune system and gastric acid, but it also defends against host internal bacteria, such as human gut microbial communities (68). Once the cell densities reach high numbers, bacterial cells stop generating biofilm, since biofilm production takes up valuable resources, which could otherwise be used in growth and cell division (47). At high cell densities, QS-mediated expression of the hap protease gene, was shown to promote release from the epithelium and escape into the environment for successful infection of new hosts (69). The same phenomenon also occurred in the cultures of V. anguillarum strain PF430-3 and phage KVP40 at high cell densities, detachment of cells from aggregates were disseminating into free-living variants, which co-existed with phage KVP40.

Potential application of treatments that promoted dispersal of aggregated cells into the planktonic phase seems attractive, as the results described in previous work indicated that even the use of pro- and anti-QS therapies that function by modulating bacterial chemical communication circuits to weaken the biofilm and lead the sessile cells into planktonic phase, where they could be efficiently killed (70-72). Also, it seems likely that the antibiotics used for biofilm infection could be significantly reduced if biofilms have already partially been disintegrated (73). Thus, detachment of biofilms will ultimately improve the therapeutic effect of biofilm control.

As discussed above, comparisons of differences among V. cholerae, P. aeruginosa and E. coli strains reveal a wide range of biofilm formation patterns, all linked to QS signaling molecules (Al-1 or Al-2) (47, 64, 74). These interspecific differences are likely dependent on the internal environment and related to the amount of competition and the necessity for dispersal.

Intraspecific variations in biofilm formation have also been observed. The biofilm formation of V. anguillarum strains PF430-3 and BA35 in a flow cell device (75) were highly different with respect to structure and stability (Figure 7). For example, an increase in biofilm formation associated with the pellicle on the top of tubes were observed in strain PF430-3; On the contrary, the biofilm stability of V. anguillarum
strain BA35 is relatively poor, as no surface pellicle in the liquid cultures could be found. According to the in vitro study of strain BA35, biofilm formation decreased after 2 days. The decrease in the level of biofilm could also be partially attributed to an earlier onset of the dispersal phase because of a rapid increase in biofilm formation, which has been observed in Hosseinidoust’s study (76). In general, biofilm stability is closely correlated with the EPS production (77), as previously visualized by use of fluorescently labeled lectins carbohydrate-containing EPS in P. aeruginosa biofilms (78). Perhaps, EPS production in V. anguillarum strain PF430-3 contributed to the formation of the characteristic colonies, which prevented early dispersing. A similar phenomenon was also mentioned in Fong’s V. cholerae study (79), by deleting vps genes which is required for biofilm formation, many of the mutants exhibited reduced capacity to produce VPS and biofilm formation.

Figure 7. Bacterial biofilm formation of V. anguillarum strain PF430-3 and BA35 after 24 h, samples were stained with LIVE/DEAD BacLight viability stain (Invitrogen) and visualized by confocal scanning light microscope.

3.3. Heterogeneity in biofilms

Bacterial biofilms are physiologically and spatially heterogeneous; even within a single species, differential spatial and temporal gene expression occurs due to chemical gradients and adaptation to the local environmental conditions (80-82). The expression of different extracellular molecules could change the surface antigen properties of the cells and thereby promote the formation and maintenance of different types of multicellular communities during the biofilm infection, including different stages between “on” and “off” status (83). These strategies generally were recognized by the function of enabling the individual cells to evade immune system or predator (84). For example, a subset of the population in the planktonic phase may accumulate mutations that enable them to produce more EPS, grow slower, become non-motile and form aggregates (80). These aggregates from the mutants may increase the adhesion capacity to the substratum and function as a physical barrier that protects against attacks and enhances the survival of the entire community. The remaining planktonic members of the community could contribute to different
benefits to the community, such as protozoa grazing, phage attack or others may be involved in cellular communication (47, 85, 86). Thus, functional specialization may be promoted through mutations or gene regulations and could also stimulate multicellular development (86).

Despite an increasing knowledge of biofilm structure and properties, many key questions still remain to be answered about essential factors regulating biofilm formations. Several environmental conditions have been proposed to influence biofilm structure. For instance, Kreft’s study (87) has shown that the stability of a biofilm is closely correlated with the EPS production as well as pilus and flagella, i.e. high EPS production resulted in a solid structure of biofilm. Similarly, Nagaoka reported that a high level of EPS production increased viscosity of the medium (88). Moreover, in Reisner’s *E. coli* K-12 biofilm models, the presence of transfer constitutive IncF plasmids were essential in inducing biofilm forming structures (89). It should be noted that the discussion here is limited to the single-species biofilm formation; additionally, solid-air biofilms and liquid-gas biofilms would probably have their distinct characteristics, which would influence cellular communications (90).

### 3.4. Diagnosis of biofilms

Detection of bacterial biofilms of *V. anguillarum*, *V. cholerae*, *S. aureus* and *P. aeruginosa* are pivotal in many diagnostic decisions. In addition, the presence of multi-drug resistance *V. anguillarum*, *V. cholerae* and *P. aeruginosa*, and methicillin-resistant *S. aureus* (MRSA) can pose intractable problems. These pathogens all form culturable biofilms, which can be studied in the laboratory, and much of the progress in our understanding of biofilm structure, regulation and treatment relates to studies of such pathogens (91, 92). Additionally, the problems of biofilms cannot be neglected, since most biofilm samples are either conditionally viable environmental cells (CVEC) or viable but non-culturable (VBNC). Due to these CVECs and VBNCS, traditional methods are inefficient in detecting and identifying certain biofilms. However, molecular methods offer a way to overcome these shortages in traditional methods. Most nucleic acid-based molecular methods for the detection and identification of bacteria begin with the extraction of DNA and/or RNA from the sample to be analyzed or directly identified by fluorescence in situ hybridization (FISH) (93). This extraction and thus diagnosis will be more efficient, and will yield more precise quantification, if the nucleic acids have not been degraded by chemical preservatives or by endonuclease enzymes (91). Also, recent studies have shown that using enrichment media containing autoinducers (AIs) improves resuscitation of dormant *V. cholerae* in environmental water samples and *S. enterica* serovar typhimurium and enterohemorrhagic *E. coli* (92, 94-96).
3.5. Treatment of vibriosis in aquaculture

Traditionally, pathogens in aquaculture have been treated with antibiotics. For instance, quinolones is still the first drug of choice for vibriosis in aquaculture environments. This may explain the oxolinic acid (OA), a 4-quinolone resistance found among *Vibrio* spp. strains isolated from diseased Atlantic cod (97). Moreover, bacterial infections mediated via biofilm formation are persistent and difficult to treat with traditional methods, due to low antibiotic penetration and non- or extremely slow-growing physiological state (98). Besides, a severe problem in the aquaculture industry these days is that even without any apparent disease symptoms, antibiotics are still used routinely and prophylactically, due to lack of sanitary barriers (99). The unconsumed fish pellets and fish feces, may add to excess antibiotic residues in the sediments at the bottom of raising pen (99). For instance, in Thailand, it has been reported by Holmström and colleagues that of 76 shrimp farmers, 56 used antibiotics (100), with more than 10 different antibiotics used prophylactically. Table 1 is an overview of the major classes of antibiotics used in the aquaculture industry adapted from Defoirdt’s study (101). Serious concerns have been raised with respect to the development of bacterial antibiotic resistances and horizontal gene transfer to human pathogenic bacteria, via antibiotic residues. As discussed above, antibiotic use has to be restricted and alternatives for treating bacterial infection must be implemented.
Table 1 (101)

The different classes of antibiotics used in aquaculture, their importance for human medicine and examples of (multi)resistant pathogenic bacteria isolated from aquaculture settings.

<table>
<thead>
<tr>
<th>Drug class</th>
<th>Importance for human medicine</th>
<th>Example</th>
<th>Resistant bacteria</th>
<th>Multiple resistance?</th>
<th>Isolated from</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoglycosides</td>
<td>Critically important</td>
<td>Streptomycin</td>
<td><em>Edwardsiella ictulari</em></td>
<td>Yes</td>
<td>Diseased striped catfish (<em>Pangasianodon hypophthalmus</em>), Vietnam</td>
</tr>
<tr>
<td>Amphenicols</td>
<td>Important</td>
<td>Florfenicol</td>
<td><em>Enterobacter</em> spp. and <em>Pseudomonas</em> spp.</td>
<td>Yes</td>
<td>Freshwater salmon farms, Chile</td>
</tr>
<tr>
<td>Beta-lactams</td>
<td>Critically important</td>
<td>Amoxicillin</td>
<td><em>Vibrio</em> spp., <em>Aeromonas</em> spp. and <em>Edwardsiella tarda</em></td>
<td>Yes</td>
<td>Different aquaculture settings, Australia</td>
</tr>
<tr>
<td>Beta-lactams</td>
<td>Critically important</td>
<td>Ampicillin</td>
<td><em>Vibrio harveyi</em></td>
<td>Yes</td>
<td>Shrimp farms and coastal waters, Indonesia</td>
</tr>
<tr>
<td>Fluoroquinolones</td>
<td>Critically important</td>
<td>Enrofloxacin</td>
<td><em>Tenacibaculum maritimimum</em></td>
<td>Yes</td>
<td>Diseased turbot (<em>Scophthalmus maximus</em>) and sole (<em>Solea senegalensis</em>), Spain and Portugal</td>
</tr>
<tr>
<td>Macrolides</td>
<td>Critically important</td>
<td>Erythromycin</td>
<td><em>Salmonella</em> spp.</td>
<td>Yes</td>
<td>Marketed fish, China</td>
</tr>
<tr>
<td>Nitrofurans</td>
<td>Critically important</td>
<td>Furalidone</td>
<td><em>Vibrio anguillarum</em></td>
<td>Yes</td>
<td>Diseased sea bass and sea bream, Greece</td>
</tr>
<tr>
<td>Nitrofurans</td>
<td>Important</td>
<td>Nitrofurantoin</td>
<td><em>Vibrio harveyi</em></td>
<td>Yes</td>
<td>Diseased penaeid shrimp, Taiwan</td>
</tr>
<tr>
<td>Quinolones</td>
<td>Critically important</td>
<td>Oxolinic acid</td>
<td><em>Aeromonas</em> spp., <em>Pseudomonas</em> spp. and <em>Vibrio</em> spp.</td>
<td>Yes</td>
<td>Pond water, pond sediment and tiger shrimp (<em>Penaeus monodon</em>), Philippines</td>
</tr>
<tr>
<td>Sulphonamides</td>
<td>Important</td>
<td>Sulphadiazine</td>
<td><em>Aeromonas</em> spp.</td>
<td>Yes</td>
<td>Diseased katla (<em>Catla catla</em>), mrigel (<em>Cirrhinus mrigala</em>) and punti (<em>Puntius</em> spp.), India</td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>Highly important</td>
<td>Tetracycline</td>
<td><em>Aeromonas hydrophila</em></td>
<td>Yes</td>
<td>Water from mullet and tilapia farms, Egypt</td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>Highly important</td>
<td>Oxytetracycline</td>
<td><em>Aeromonas salmonicida</em></td>
<td>Yes</td>
<td>Atlantic salmon (<em>Salmo salar</em>) culture facilities, Canada</td>
</tr>
</tbody>
</table>
4. Alternative disease control in aquaculture

Multiple commercial vaccines are currently used to protect fish against outbreaks of vibriosis; all of these vaccines consist of inactivated strains of both *V. anguillarum* serotypes O1 and O2 (102). However, outbreaks of vibriosis caused by serotype O2 have not been completely prevented (22). Moreover, at the larval stage, fish are more vulnerable to *V. anguillarum* infections, and the vaccine is largely ineffective as their immune system is not fully developed. Therefore, alternative antibacterial strategies are required to be identified and developed.

Global regulators such as autoinducer-1 (AI-1) and autoinducer-2 (AI-2) inhibitors of bacterial physiology have emerged as a potential approach to manipulate bacteria as discussed above (103, 104). These AIs not only have important roles in biofilm formation, virulence and antibiotic resistance, but also have secondary functions, which can be used as pro- or anti-QS/antibiotic/phage therapy synergy for both acute and persistent infection treatment in the future (103, 105-108). All these characteristics can be taken into consideration for the development of future treatment strategies for bacterial infections in aquaculture.

4.1. Bacteriophages

Bacteriophages are viruses that parasitize bacteria. Phages are the most abundant microorganisms in the ecosystem, with total numbers estimated to be more than $10^{30}$ (109). Bacteriophages are ubiquitous, and are found in marine and freshwater, soils, as well as the intestinal tracts of animals, and are estimated to be on the order of $10^7$–$10^9$ per gram dry weight of soils and feces or per milliliter of seawater (109). The ecological functions of bacteriophages also involves playing important roles in e.g. structuring bacterial diversity and succession in the ocean, promoting biogeochemical element cycling and as key drivers of horizontal gene transfer (110).

4.2. Phage classification

Bacteriophages are classified by the International Committee on Taxonomy of Viruses (ICTV) based on their morphology and types of nucleic acid. More than 5500 phages have been examined by electronic microscopy, ~96% are tailed (111). These tailed phages, which belong to the order of *Caudovirales*, can be divided into 3 families (*Myoviridae*, *Siphoviridae*, and *Podoviridae*) (Figure 8). The differences among these three families are: a long or short contractile tail (*Myoviridae*), a long non-contractile tail (*Siphoviridae*), and a short, non-contractile tail (*Podoviridae*).
4.3. Bacteriophage life cycle

Since phages need bacterial hosts to replicate, phage infection may lead to bacterial cell death (lytic life cycle) or to a lysogenic relationship, where both genomes of phage and host replicate together. Specifically, in the lytic life cycle, phage progenies are released from lysis of the bacterial host. New progenies then continue to infect more hosts. In the lysogenic life cycle, temperate phage DNA is integrated into host chromosomes and replicates along with cell division (Figure 9). However, this is not always permanent and phages can be induced from the lysogenic to the lytic life cycle, which is dependent upon environmental signals and the number of infected phages per cell (112), such as DNA damage, UV exposure, mitomycin C, hydrogen peroxide and high MOI, by causing an SOS response (113). Additionally, phages are known to play a critical role in the evolution of pathogenic bacterial species, and as it is particularly true for *V. cholerae* (114). For example, a major virulence factor of *V. cholerae*, the cholera toxin (CT), is encoded by *ctxAB* in the lysogenic phage CTXΦ (115). Likewise, cryptic prophages also were shown to help bacteria cope with adverse environments, such as cell growth, antibiotic resistance, early biofilm formation, as well as environmental stresses (116, 117).
Figure 9. Schematic picture of phage lytic and lysogenic life cycles. Phage adsorption is the first key step in phage proliferation; in order to efficiently bind to the bacterial receptor, phage receptor-binding protein (RBP) is required to specifically interact with bacterial cell surface receptor to enable intracellular DNA injection. In the lytic life cycle, phage DNA replicated separately from host genome, resulting in the destruction of the infected cell and its membrane. In the lysogenic life cycle, phage DNA is integrated into the host genome and can be transferred to daughter cells, until the lytic cycle is induced. However, a portion of these induced cells could enter an abortive lytic cycle by losing prophage and becoming non-lysogens (curing) (112).

4.4. Vibriophages

Although viruses have been enumerated, isolated and characterized from the marine environment, there is little information about the specific phage types. Previous seasonal and spatial studies about vibriophages were carried out mainly based on the *V. parahaemolyticus* hosts, such as vibriophages isolated from the Strait of Georgia (Vancouver, Canada) (118) and Tampa Bay (Florida, USA) (119), as well as *V. cholerae* prophages such as K139 (120), and filamentous phage CTXΦ, which has been shown to be linked to the bacterial pathogenicity, CT production (121).

Among the best characterized vibriophages is bacteriophage KVP40. KVP40 was originally isolated from polluted seawater off the coast of Japan using *V. parahaemolyticus* as the host (122). Phage KVP40 is a novel T4-like virulent vibriophage, with a broad-host-range, belonging to the *Myoviridae* family, and the genome size is around 244 kb, with a G+C content of 42.6%, and a National Center for Biotechnology Information (NCBI) accession number of B_KVP40 AY283928 (123). Vibriophage KVP40 is known to cause infection through the universal outer membrane protein K (OmpK) and has previously been shown to infect
more than 8 Vibrio species, including *V. anguillarum*, *V. parahaemolyticus*, *V. harveyi*, *V. natriegens*, *V. cholerae*, as well as *Photobacterium leiognathi* (Table 2) (122, 124).

Table 2. Adapted from Inoue (124)

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Phage sensitivity (^a)</th>
<th>Phage adsorption (^b)</th>
<th>OmpK-like protein (^c)</th>
<th>Phage-resistant mutants (^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. parahaemolyticus</em></td>
<td>1010</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>OmpK(^d)</td>
</tr>
<tr>
<td></td>
<td>VIB4</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VIB8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VIB9</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>V. algolyticus</em></td>
<td>VIB20</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VIB31</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VIB33</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>V. natriegens</em></td>
<td>VIB30</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>OmpK(^d), OmpK(^d)</td>
</tr>
<tr>
<td><em>V. cholerae</em> non-O:1</td>
<td>VIB5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>V. mimicus</em></td>
<td>VIB13</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>OmpK(^d), OmpK(^d), OmpK(^d)</td>
</tr>
<tr>
<td><em>V. anguillarum</em> I</td>
<td>VIB35</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>OmpK(^d)</td>
</tr>
<tr>
<td></td>
<td>VIB36</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>OmpK(^d)</td>
</tr>
<tr>
<td><em>V. splendidus</em> II</td>
<td>VIB37</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>OmpK(^d)</td>
</tr>
<tr>
<td><em>V. fluvialis</em> I</td>
<td>VIB7</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VIB18</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>OmpK(^d)</td>
</tr>
<tr>
<td><em>V. vulnific</em></td>
<td>VIB6</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>V. harveyi</em></td>
<td>VIB26</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>V. campbellii</em></td>
<td>VIB27</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>V. nereis</em></td>
<td>VIB29</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>V. pelagius</em> I</td>
<td>VIB38</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>V. costicola</em></td>
<td>VIB39</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>P. leiognathi</em></td>
<td>PHO1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>P. angusturn</em></td>
<td>PHO2</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Sensitivity to KVP40 tested by plaque formation. \(^b\) More than 10% adsorption of KVP40 as compared with 1010. \(^c\) OmpK or its homologs detected by immunoblotting. \(^d\) Phenotypes of KVP40-resistant mutants isolated. The OmpK\(^d\) like protein was defective (OmpK\(^d\)), partially defective (OmpK\(^d\)), or produced normally (OmpK\(^d\)).
4.5. **Application of phages to control fish pathogens: Phage therapy**

Due to their efficient lysis, lytic phages can potentially be used against bacterial infection, and are much more specific than commonly used antibiotics. Therefore, by using phage therapy, a specific bacteriophage could theoretically be chosen to target a specific pathogen. Because of their host specificity, they would not affect beneficial bacteria (e.g. gut flora), thus reducing the chances of opportunistic infections (125). In 2006, the United States Food and Drug Administration (FDA) approved using bacteriophages on ready-to-eat meat and poultry products in order to kill the *Listeria monocytogenes* bacteria, giving these products GRAS status (Generally Recognized as Safe) (126). Therefore, properly developed phage products can be invaluable in controlling bacterial infections in various settings.

Recently, there has been a growing number of studies on phage therapy, focusing on a variety of fish pathogens including *A. salmonicida* in brook trout, *Yersinia ruckeri* in salmon, *Lactococcus garvieae* in yellowtail, *P. plecoglossicida* in ayu, *F. psychrophilum* in rainbow trout, *V. anguillarum* in Atlantic salmon and *V. harveyi* in shrimp (127-134). These studies have demonstrated the potential of specific phages to significantly control pathogen density and, in some cases, reduce fish mortality. For instance, Silva’s study of *V. anguillarum* and vibriophage showed that the larvae mortality in the infected and treated group was similar to normal levels and significantly lower than the infected but not treated group (135). Moreover, according to Lomelí-Ortega’s study, lytic phage A3S and Vpms1 were also effective to reduce larvae mortality caused by *V. parahaemolyticus* (136). Similarly, in Vinod’s field trail experiments, treatment with bacteriophage improved larval survival and brought about decline in luminescent *V. harveyi* counts in hatchery tanks (133).

In addition, studies from *V. cholerae* (114) have also shown that lytic vibriophages may play an essential role in modulating the seasonal dynamics of cholera in endemic areas, such as the Ganges Delta region of Bangladesh and India. Specifically, the number of cholera patients increased whenever the number of lytic vibriophages in the water decreased, and cholera epidemics tended to end concurrent with large increases in the concentration of vibriophages in the water (114, 137), indicating that lytic vibriophages specific for *V. cholerae* may limit the severity of cholera outbreaks by killing susceptible bacteria present in the reservoir. Due to their selective killing of susceptible strains, vibriophages are also believed to play a key role in the emergence of new *V. cholerae* pandemic serogroups or clones (114, 138, 139).

Therefore, it seems a promising strategy to apply vibriophages to gain control of vibriosis infections in fish used for aquaculture. However, successful application of phage therapy in the treatment of vibriosis requires a detailed understanding of phage-host interactions in both planktonic and biofilm forms,
especially with regards to anti-phage defense mechanisms in the bacterial hosts, specifically, bacterium *V. anguillarum* in this Ph.D. study.
5. Bacteriophage resistance mechanisms

Predation pressure from bacteriophages is substantial, mainly because their abundance outnumbers microbial cells by an estimated 10-fold in most natural environments (140). Accordingly, bacteriophage lytic infection imposes a strong selection for bacterial mutations/tolerance providing reduced phage susceptibilities or resistance against phage infection. Bacteria have evolved a wide range of different resistance/tolerance mechanisms including 1), preventing phage adsorption; 2), cutting phage nucleic acid; 3), abortive infection through altruistic suicide; and 4), QS-mediated receptor down-regulation, which can make the host immune to the viral infection (141). Similarly, phages can also overcome bacterial resistance by adapting to new receptors, battling restriction-modification systems, evading CRISPR-Cas systems, and escaping abortive-infection mechanisms (142).

5.1. Phage receptor modification

Adsorption is a key step recognition between phage receptor-binding protein and phage receptors on the sensitive host cells (143). Most of phage receptors are presented on the bacterial cell walls, such as LamB for phage lambda, the prion outer membrane protein F and C (OmpF and OmpC) for phage T2 and T4, and moreover, the flagella protein for phage phi (144-147). A recent study showed that ϕCb13 and ϕCbK actively interact with the flagellum of the bacterium Caulobacter crescentus and subsequently attach to receptors on the cell pole. Using the flagella or pili to initiate contact with the host cell, increases the likelihood of attachment and successful infection (148). Similarly, we noticed that a large fraction of cells in the BA35+ΦH20 cultures were found to be either non-motile or with impaired mobility, which may imply that flagella play an important role in phage-host interactions. Furthermore, a specific polysaccharide was also implicated as an essential component in adsorption, for instance, phage kh and host L. lactis subsp. cremoris KH, as well as phage 1358 and L. lactis (149, 150). Additionally, according to Seed’s study, V. cholerae lipopolysaccharide O1 antigen functions as a major target of phage ICP1 (151). By using phase variation of O antigen biosynthesis, V. cholerae cells can easily generate variable expression of surface components, which is generally thought to help these organisms evade the immune system and phage predation (151). However, little is known about the requirements of these phage receptors and it is unclear if the polysaccharide was acting as a receptor or if it was facilitating reversible phage binding to a secondary receptor (152, 153).

Mutating phage receptors or producing EPS to block the interaction between phage receptor and phage receptor-binding protein to prevent phage adsorption can be the first step that bacterial cells exhibit in developing resistance/tolerance to avoiding infection (154). Previous studies in Vibrio hosts, showed that mutations or modifications of the outer membrane protein K (OmpK), led to resistance to vibriophage
KVP40 (124). Similarly, in Paper III, \textit{ompK} in-frame deletion also proved that OmpK acts as a phage binding receptor in \textit{V. anguillarum} strain PF430-3 (Figure 10). Furthermore, mutations in outer membrane protein A (OmpA) of \textit{E. coli} K-12 appear to play a role in inhibiting phage infection (155). Phage receptors, in addition to the phage attachment, were involved in the bacterial nutrient intakes, which may be responsible for the morphological changes of the colonies with small size, known as small-colony variants (SCVs) and fitness costs, as have been previously demonstrated experimentally, such as reduced abilities to take up specific nutrients (156) or reduced competitive abilities in general (157, 158). For example, mutation in the LamB phage receptor caused the inability to transport long chain maltodextrin across the outer membrane (159). In Paper II, phage ΦH20-resistant mutant of strains BA35 was found to have reduced ability to utilize a number of carbon resources in the BIOLOG GN2 test.

Additionally, Park (160) found that bacteriophage-resistant mutants of \textit{P. plecoglossicida} lacked virulence for ayu. In addition, in a successful phage therapy experiment of \textit{E. coli} infection in mice and calves, the resistant mutants isolated from the treatment were the less virulent \textit{k}^{-1} type mutants (161). In Paper III, the function of phage KVP40 binding receptor OmpK still remained unknown, but, it has been suggested to be involved in bile salt resistance and iron acquisition (162). Studies on vaccines have shown that OmpK could also function as a subunit vaccine against \textit{V. anguillarum} infection (162), because the outer membrane has been reported as selectively permeable or serves as a receptor as well as adhesions in most Gram-negative bacteria for colonization (163, 164).

Figure 10. Spot assay for testing host specificity of phage KVP40 against wild-type, QS mutants (Δ\textit{vanT} and Δ\textit{vanO}) and \textit{ompK} mutants (Δ\textit{ompK}, Δ\textit{vanT ΔompK}, and Δ\textit{vanO ΔompK}) as well. Five μl serial 100-fold dilutions of phage KVP40 lysate (1, 10^{5}; 2, 10^{3}; 3, 10^{3}; 4, 2, 10^{3} PFU ml^{-1}) were shown by spot titration onto top agar lawns of the indicated strains (Paper III).
The role of spatial refuge in stabilizing bacteria-phage interactions has been observed in many ecosystems, especially, micro-colonies and biofilms, as discussed in previous studies (165-167), and even in the marine environments, such as marine snow and sediments. Because of the low dispersal rates in the heterogeneous environments, by creating ephemeral refuge may directly/indirectly block phage receptor from phage attack by non-mutation-based mechanisms (165-168); as also shown in V. anguillarum strain PF430-3 protected against phage KVP40 infection by increasing cell aggregation and biofilm formation, allowing coexistence rather than coevolution, finally promoting the stability of phage-host systems by reducing the risk of lytic phage attacks.

5.2. Cutting phage nucleic acids

Once the nucleic acid has been injected into the bacterial cell, the restriction-modification (R-M) systems protect the bacterium by cutting invading DNA into pieces (169). When unmethylated phage DNA enters a cell harboring R-M systems, restriction enzymes, thereby, rapidly degrade the foreign genetic material functioning as a prokaryotic immune system (170). For instance, in Bacillus subtilis Marburg nonB mutated strain, nonsense mutation on ydiB was found to be related to the restriction system targeting sequence of BsuMR, which was identical to Xhol (CTCGAG) (171, 172). However, according to Krüger’s study (173), R-M systems are not always perfect, for instance, phages and plasmids can acquire host modifications to avoid restriction endonuclease, which highlights an evolutionary arms race between bacterial host and phages (142).

5.3. CRISPR/Cas bacterial immune system cleaves bacteriophage DNA

Another mechanism recently described is CRISPR (Clustered Interspaced Short Palindromic Repeats) and the CRISPR-associated (cas) genes system, in which a CRISP-cas loci was identified composing of 21-48 bp direct repeats interspaced by non-repetitive spacers (26-72 bp) of similar length (174, 175). By using this immunity system to acquire at least one new repeat-spacer unit at the 5’ end of the repeat-spacer region of a CRISPR locus that targets foreign nucleic acids, bacteria can efficiently protect themselves from including phage DNA and plasmids (175, 176).

5.4. Abortive infection (ABI) system

The study of the ABI system began 50 years ago, and even now, the mechanisms underlying the infection are still not completely understood (141). The bacterial cell can increase the chances of its own population survival by using the abortive infection system, where phage infection leads to the death of infected bacterial cells. The system is characterized by a normal infection start (i.e., the phage adsorbs and injects its DNA into the host cell), followed by an interruption of the replication, transcription or translation, leading to the release of little or no new phage progenies (141, 177). Recent studies in B. subtilis showed that the
Marburg strain remained resistant to phage SP10 due to a NonA-mediated aborted infection system, acted as a second layer of protection against phage SP10 infection, specifically, the overexpression of nonA gene terminated cell growth with reduced efficiency of colony formation and respiration activity (172).

5.5. Regulation of phage-host interactions by extracellular signaling molecules
A recent study has demonstrated a new mechanism of phage resistance in which, bacteria can coordinate their receptor gene expression upon the environmental QS signal, to avoid the risk of infection at high cell densities (45). Since phages require a host to replicate, it follows that the predation pressure is relatively higher at a high cell density status compared to sparsely populated environments. Hence, if bacterial hosts could regulate their anti-phage mechanisms based on cell densities, they could easily reduce their susceptibilities to infection during high cell densities, while avoiding the metabolic burden of maintaining elevated anti-phage defenses during growth at low cell densities. For instance, E. coli possesses the ability to use AHLS to reduce its susceptibility to at least two phages, phage λ and phage χ (45). In the phage λ case, phage receptor LamB was shown 40% down-regulation compared to the untreated controls without AHLS (45). In addition, high concentration of universal communication molecule Al-2 (100 μM) was demonstrated to induce virulence genes and transfer them via phage release in E. faecalis V583ΔABC (178). Intriguingly, a recent study from Hargreaves (179) identified Clostridium difficile phage θCDHM1 encoded an intact QS operon. Similarly, QS-associated genes have also been identified in several phages of Psedomonas spp (180, 181), and function as acylhydrolase to break down AHLS, was identified in the Iodobacter-phage ϕPLPE (182). Although it remains unknown whether any of these genes are of functional importance, it certainly opens the exciting possibility that some phages may actively interfere with the QS in the bacterial communities.

5.6. Implications of phage protection mechanisms: Phage-host coexistence and co-evolution
As complexity arises within populations of bacterial resistant mutants, it may help mutants survive better or have more offspring. If so, this complexity will be favored by natural selection and spread through the bacterial population. However, most mutations with phenotypic effects are harmful, such as reducing the competitiveness of the mutant strains, as most phage receptors involve nutrient intake or pathogenicity, a receptor-deficient mutant will have a slower growth rate or reduced virulence (160, 161, 183-185). That is, phage resistant mutants with those traits will tend to be wiped out before reproducing, taking the deleterious traits out of bacterial communities. Therefore, the non-mutation defense mechanisms among these phage hosts may suggest phage-host co-existence interactions, rather than the classical phage-host co-evolutionary arms race, known as Red-Queen theory.
The Red-Queen hypothesis was first formed by Van Valen in order to explain the “law of extinction” (186). According to the previous phage-host interaction studies, virulent phages managing to coexist with their bacterial host leads to continuous variations and selections towards the adaptation of bacterial hosts by evolving resistance to current phages and phage evolve to counter resistance. It has been reported that the arms race has a huge impact on global nutrient cycling, on climate, on the evolution of the biosphere, and on the evolution of virulence pathogens (187). However, this theory was also criticized because the evolution rates between phage and host are not symmetrical. Recent studies showed that, in soil, phages seem ahead of the bacterial hosts in the evolutionary arms race (188). In the natural environment, at each stage of the arms race, one could become extinct, and without one, phage and bacteria coexistence, therefore, does not even exist. For instance, one of the typical examples of “Cheshire Cat” ecological dynamics is the haploid phase of *Emiliania huxleyi* provided an escape mechanism that involved separation of meiosis from sexual fusion in time, to ensure that genes of dominant diploid clones were passed on to the next generation in a virus-free environment (189). Thus, it is important to understand both evolutionary and non-evolutionary mechanisms (such as gene regulation) that can regulate phage-host interactions in the future.

Uncovering anti-phage defense mechanisms is essential for understanding phage-host dynamics and for application of phages in disease control, as they reflect the remarkable diverse interactions between bacterial hosts and viruses and play a key role as agents shaping microbial community structure. As the predation pressure from phages is a key determinant of the size and composition of bacterial populations, understanding of the potential factors that govern phage-bacterial interactions will be important in any context where the goal is to control the growth of a microbial population including, for example, the treatment of bacterial infections, development of effective probiotics, production of cultured dairy products, or manipulation of the human microbiome to prevent or treat life-style diseases.
**Aims of this Ph.D. project**

The aim of this Ph.D. project was to investigate the interactions between vibriophages and their hosts *V. anguillarum*, and to ultimately improve the efficiency of phage therapy.

The objectives of this project were:

To isolate and characterize vibriophages and *V. anguillarum* and investigate the occurrence of phage susceptibility patterns. (Paper I)

To explore phage-host interactions in two *V. anguillarum* strains, and the resistance/tolerance mechanisms responsible for the different outcomes of these two different systems. (Paper II)

To determine whether AHLs-mediated Quorum Sensing regulates anti-phage defenses in *V. anguillarum* strain PF430-3. (Paper III)
A summary of this Ph.D. project

Paper I
Vibriophages and their interactions with the fish pathogen *Vibrio anguillarum*

The use of phage therapy to control bacterial pathogens may offer an effective method to reduce vibriosis infection in fish or shellfish maintained in aquaculture. Phage therapy is a promising alternative to the use of antibiotics and thus avoids the risks associated with antibiotics abuse and overuse. However, a detailed understanding of phage-host interactions is needed to evaluate the potential of this technique.

As the first approach, we collected and isolated 24 *V. anguillarum* strains and 13 *Vibrio* sp. strains, which represented considerable temporal (20 years) and geographic (9 countries) variations. Irrespective of their origins of isolation, further characterizations based on phylogenetic (16S rRNA) and physiological (BIOLOG GN2 and API 20NE) fingerprints, revealed that they were quite similar.

Ten phages were isolated from Danish water samples with different Vibrio hosts. In addition to the Danish phage isolates, a broad-host-range vibriophage KVP40 was also included in this study. Phages were further characterized by genome sizes, morphologies, and host-range analysis. Together, 11 phages represented three different families (*Myoviridae*, *Siphoviridae*, and *Podoviridae*), which covered all the host ranges of 37 Vibrio strains.

Despite the occurrence of unique susceptibility patterns of the individual host isolates, key phenotypic properties related to phage susceptibility are distributed worldwide and have been maintained in the global Vibrio community for decades. Unfortunately, the phage susceptibility pattern of the isolates did not show any relation to the physiological relationships, demonstrating that similar phage susceptibility patterns may occur across a broad phylogenetic spectrum and with large physiological differences among Vibrio communities.

Subsequently, culture experiments were conducted with two *V. anguillarum* strains (BA35 and PF430-3) and their corresponding phages (ΦH20 and KVP40), representing strong lytic potential initially followed by rapid regrowth of phage-resistant and phage-tolerant subpopulations, which is an obstacle to successful phage therapy. Different bacteriophage resistance/tolerance mechanisms underlined by these two specific phage-host interactions were not revealed in this study.
Vibriophages differentially influence biofilm formation by Vibrio anguillarum strains

In this study, we explored in vitro phage-host interactions in two V. anguillarum strains (BA35 and PF430-3) and their corresponding phages (ΦH20, Siphoviridae and KVP40, Myoviridae), respectively, during growth in the settings of micro-colonies, biofilms, as well as the free-living phase.

We sought to figure out the resistance/tolerance mechanisms responsible for the different phenomena between these two phage-host systems.

For strain BA35 and phage ΦH20, a strong phage control of the phage-sensitive population and subsequent selection for the phage-resistant mutants with fitness cost, implicates a co-evolutionary arms race may exist between bacterial hosts and their predators. This then serves as a potential mechanism to promote diversity of resistant subpopulations and phage ΦH20 through stable co-evolution and mutations.

For strain PF430-3 and phage KVP40, addition of phage KVP40 to strain PF430-3 cultures resulted in an increase in the levels of biofilm/micro-colony/aggregate formation, especially, during the initial stage. By creating physical spatial refuge between host and parasite, the phage can potentially allow long-term co-existence between strain PF430-3 and phage KVP40. In addition, we were unable to isolate any single colonies with full and permanent resistance to phage KVP40. Hence, it may be speculated that the development of this transient protection is due to the fact that phage KVP40 infects more than 8 Vibrio species using phage receptor OmpK, which is widely distributed in the Vibrio community. Thus, resistance mutations are either very rare or have significantly negative or even lethal effects on host fitness. In that case, development of alternative strategies to genetic mutations in the OmpK receptor would be of strong selective advantage, perhaps contributing to the evolution of the protection mechanism.

Overall, the specific mechanisms of phage resistance/tolerance were not identified in this study between these two phage-host systems, and further studies are needed to continue to investigate the mechanism of resistance/tolerance towards these two phages. Taken together, these data demonstrated highly variable phage protection mechanisms among Vibrio communities, emphasizing the challenges of using phages to control vibriosis in aquaculture, and adding to the complex roles of phages as drivers of prokaryotic diversity and population dynamics.
Paper III

Quorum Sensing determines the choice of anti-phage defense strategy in *Vibrio anguillarum*

Since phage and host have a long co-existence and co-evolutionary development, it is generally believed that bacteria can effectively defend against phage predation by using various means and with limited fitness cost to cope with further selection pressure. Alternating different protection strategies at different stages perhaps lessens the metabolic burden, facilitating specific subpopulations to prevail in phage-host systems. As bacterial densities vary over time from sparsely populated environments to highly dense bacterial populations, phage predation pressure is not constant. By altering their anti-phage activities upon population density (phase-dependent defense), bacteria could actively reduce their susceptibility to infection during growth at high cell densities. Vibriophage KVP40 is known to cause infection through the universal outer membrane protein K (OmpK) and has previously been shown to infect more than 8 *Vibrio* species; by using spatial refuge and a quorum sensing-mediated receptor down-regulation would decrease the metabolic burden in these *Vibrio* communities.

In this study, we revealed the roles of spatial refuge and cell-cell signaling in shaping phage-host interactions. Two *V. anguillarum* PF430-3 QS mutants were constructed, ΔvanT-locked in low cell density and ΔvanO-locked in high cell density, allowing us to explore the role of QS for protection against phage infection. Specifically, ΔvanT became more susceptible to phage KVP40 by enhancing biofilm formation, and thus creating spatial refuge, providing protection to the host against phage infection. In contrast, ΔvanO became more resistant by down-regulating the phage receptor OmpK to reduce its susceptibility to phage infection with no bacterial aggregates. Further, wild-type strain culture experiments showed a strong negative correlation between AHLs production in the supernatant and phage receptor ompK gene expression, which was consistent with previous results. The data presented here, therefore, suggest that VanO negatively regulates the expression of VanT, which in turn directly or indirectly represses the expression of essential phage receptor OmpK (Figure 1).

![Figure 11. Models for the mechanism and function of QS regulation and biofilm formation and phage receptor ompK expression in *V. anguillarum*. Solid arrows represent positive effects, while solid T bars represent negative effects.](image-url)
Future perspectives

The studies presented in this Ph.D. dissertation provide new insights into phage-host interactions of the important fish pathogen *V. anguillarum* and their vibriophages, shedding light on the relationships between phage infection pressure, biofilm development, quorum sensing and bacteriophage resistance/tolerance mechanisms in *V. anguillarum*. However, there are lots of unsolved issues in this study, which raise questions regarding future experiments.

Paper I

Thirty seven *Vibrio* strains were collected with considerable temporal (20 years) and geographical (9 countries) differences in their origins of isolation, which provides us with knowledge on spatial and temporal distributions of the pathogen which may prove useful when assessing global outbreaks of vibriosis. However, these strains are still insufficiently characterized. For instance, phage susceptibility patterns of the isolates did not show any similarities to the physiological relationships obtained from BIOLOG GN2 and 16S rRNA profiles, and thus further studies linking phage susceptibility to phenotypic and genotypic characteristics would be relevant.

First, by selecting vibriophage resistant mutants among the potential hosts, we could identify cellular components that are involved in the bacterial receptor for the specific phages, as previously shown in Davidson’s study in *B. anthracis* (gamma) phage γ receptor GamR (190). Further studies will be focused on potential phage receptor identification and will determine whether there is any correlation between phage susceptibilities and phage receptor types.

Secondly, one of the most important issues in phage therapy is the ability to obtain phages capable of infecting the causal pathogen; hence, the isolation step is a critical element. The isolation of vibriophages is considered relatively difficult, and there is a need for new and more efficient isolation methods.

Thirdly, physical and chemical factors, such as temperature, acidity, and ions have shown to affect phage persistence (191). A better understanding of storage and stability of phage stock, i.e. mechanisms and rates of phage decay, are also essential for a successful future phage therapy, not only for those interested in pharmaceutical and agricultural applications, but also for laboratory phage studies.

Lastly, most bacterial pathogens contain prophages or phage remnants integrated into bacterial chromosomes (192). As these prophages may carry virulence genes, it is essential to obtain a better understanding of the content and genetic properties of prophages integrated in *V. anguillarum* strains. During the lytic phage cycle, prophage induction can be triggered, which makes lytic phage stock
contaminated with the induced prophages that were previously resident in the host strains. Thereby, the data we obtained from the host range assay could be related to the prophage from the host of the lytic phage. The argument of prophage here suggests that phage host range can be optimized by using prophage free hosts for lytic phage proliferation in the future. We are currently whole genome sequencing our full collection of *V. anguillarum* and vibriophages, which will allow such a detailed analysis of prophage contents.
**Paper II**

The studies from Paper II demonstrated that two closely related *V. anguillarum* strains BA35 and PF430-3 displayed highly variable phage protection mechanisms, during growth in the settings of micro-colonies, biofilms, as well as the free-living phase.

In general, biofilm stability is closely correlated with EPS production (87), and we hypothesized that EPS production in strain PF430-3 contributed to the formation of the characteristic micro-colonies, which protected the strain PF430-3 from phage infection by creating spatial refuge. Therefore, further experiments on EPS quantification and identification remain to be carried out. Previous studies on *P. aeruginosa* have shown fluorescently labelled lectins could be used in combination with epifluorescence microscopy, allowing the visualization and characterization of carbohydrate-containing EPS in biofilm matrices (78). This could lead us to the next step in determining and quantifying EPS production. Moreover, *vps* in-frame deletion mutants could be constructed to test whether phage-induced biofilm/aggregate formation is mediated by EPS production at the genetic level. Additionally, to better understand the genetic regulatory network, bacterial transcriptomes could provide us with the bacterial strain’s immediate genetic response to phage exposure, enabling an assessment of the transcriptional pattern, such as genes involved in polysaccharide production and flagella assembly (193). Furthermore, it is relevant to determine whether phage KVP40-induced aggregates are related to host strain PF430-3 or to the phage KVP40 and could be tested by combining phage KVP40 with susceptible *V. anguillarum* strains which have been characterized in Paper I.

As previously mentioned, isolating vibriophages is tedious and isolation of a natural vibriophage which is both specific to a certain host and expresses a relevant depolymerase is thus likely very low. Therefore, using synthetic biology to engineer our broad-host-range phage KVP40 to express the most effective EPS-degrading enzymes specific to the *Vibrio* targeting biofilms may be a way forward, as has been successfully conducted in an *E. coli* study (194). This would allow us to develop a diverse library of biofilm-dispersing phages rather than rely on isolating vibriophages from the environment.

Albeit the specific mechanism of resistance of strain BA35 was not identified in Paper II, the observation that a large fraction of phage resistant mutants of BA35 were found to be either non-motile or having severely impaired motility, which implies that flagella may play an important role in the infection of *V. anguillarum* strain BA35 by phage ΦH20. This was consistent with recent observations that the flagellum was involved in the initial infection of *C. crescentus* and *S. enterica* serovar typhimurium phages (148, 195). Future work on whether phage-resistant mutants BA35 have mutations or down-regulations in genes involved in both flagella and potential phage receptors should be carried out. Lastly, time-shift experiments
(196) will be conducted to gain new insights into the underlying processes of antagonistic co-evolution between BA35 and phage ΦH20, if any.
Paper III

It is well known that mutations to phage resistance frequently have maladaptive pleiotropic effects (197, 198). It should be advantageous to avoid maintaining constantly elevated anti-phage defenses, if bacteria would benefit from subjecting their anti-phage defenses to QS-control. Thus, future studies will focus on whether QS-mediated OMP down-regulation gives rise to a reduction in relative fitness to elucidate the regulatory pathway connecting QS-signaling to OMP down-regulation in V. anguillarum. Then it should be possible to construct mutants, in which regulation of the relevant OMPs is uncoupled from QS, without affecting the remainder of the QS regulon. The relative fitness of these mutants relative to the otherwise isogenic parent strains will be measured in the absence of vibriophage.

Ultimately, to fully characterize the regulatory pathway underlying QS-mediated OmpK down-regulation mechanisms, which have not been identified in Paper III, we will determine whether QS transcription factor VanT binds directly to the promoter of putative targets, such as ompK and vps. Specifically, we will set up a VanT Electrophoretic mobility shift assay (EMSA) experiment to test our hypothesis. Using this approach would allow us to potentially expand the currently modest number of described targets mediated by VanT in the literature.

Finally, the evidence that QS regulates phage receptor expression may potentially be used actively by quenching QS signaling, hence preventing receptor down-regulation. However, in the case of V. cholerae, at low cell densities, when the concentrations of the extracellular AIs are low, the expressions of virulence factor toxin-co-regulated pilus, CT and biofilm formation are activated; at high cell densities, the accumulation of two QS autoinducers (CAI-1 and AI-2) repress the expression of virulence factors and biofilm formation (43, 104). If the close-related V. anguillarum strains behave similarly, it would be problematic to synergize phage therapy and quorum quenching to prevent vibriosis infection. Further, it would be interesting to target potential phage receptors which are not regulated by QS signaling molecules, and by combining specific phages and QS molecules (CAI-1 and AI-2) may offer a synergistic antimicrobial effect to prevent vibriosis infection in aquaculture industries.
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Vibriophages and Their Interactions with the Fish Pathogen Vibrio anguillarum

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Vibriophages and Their Interactions with the Fish Pathogen *Vibrio anguillarum*

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*Vibrio anguillarum* is an important pathogen in aquaculture, responsible for the disease vibriosis in many fish and invertebrate species. Disease control by antibiotics is a concern due to potential development and spread of antibiotic resistance. The use of bacteriophages to control the pathogen may offer a non-antibiotic-based approach to reduce vibriosis. A detailed understanding of the phage-host interaction is needed to evaluate the potential of phages to control the pathogen. In this study, we examined the diversity and interactions of 11 vibriophages, 24 *V. anguillarum* strains, and 13 *Vibrio* species strains. Together, the host ranges of the 11 phages covered all of the tested 37 *Vibrio* sp. host strains, which represented considerable temporal (20 years) and geographical (9 countries) differences in their origins of isolation. Thus, despite the occurrence of unique susceptibility patterns of the individual host isolates, key phenotypic properties related to phage susceptibility are distributed worldwide and maintained in the global *Vibrio* community for decades. The phage susceptibility pattern of the isolates did not show any relation to the physiological relationships obtained from Biolog GN2 profiles, demonstrating that similar phage susceptibility patterns occur across broad phylogenetic and physiological differences in *Vibrio* strains. Subsequent culture experiments with two phages and two *V. anguillarum* hosts demonstrated an initial strong lytic potential of the phages. However, rapid regrowth of both phage-resistant and phage-sensitive cells following the initial lysis suggested that several mechanisms of protection against phage infection had developed in the host populations.

Vibriosis is one of the most prevalent and devastating diseases in marine aquaculture, causing substantial mortality and economic losses in both fish and shellfish cultures worldwide. The disease is caused by the marine pathogen *Vibrio* (*Listonella*) *anguillarum*, which infects >50 different species of fish and shellfish, with severe implications for the marine fish-rearing industry. Vaccines against vibriosis have been developed and have been widely successful in grown fish. However, the larval stage is especially vulnerable to *V. anguillarum* infections, as the immune system is not fully developed and vaccines are not effective. Traditionally, antibiotics have been widely used in antimicrobial prophylaxis and treatment of vibriosis in aquaculture, despite concern about the development and dispersal of antibiotic resistance in the pathogen community. Tetracycline and quinolones are still the first drugs of choice, and multiple cases of antibiotic resistance have been reported for *V. anguillarum* in aquaculture environments. Therefore, there is a need for the development of alternative methods to control and prevent *V. anguillarum* infection in aquaculture.

Bacteriophages are natural and abundant biological entities in the marine environment, playing important roles in, e.g., structuring bacterial diversity and succession in the ocean and promoting biogeochemical element cycling, and being key drivers of horizontal gene transfer. The strong lytic potential of bacteriophages against specific bacterial hosts has led to an increasing interest in the therapeutic use of lytic phages to control pathogenic bacterial infections in aquaculture. There has been a growing number of studies on phage therapy focusing on a variety of fish pathogens, including *Aeromonas salmonicida* in brook trout, *Yersinia ruckeri* in salmon, *Lactococcus garvieae* in yellowtail, *Pseudomonas plecoglossicida* in ayu, *Flavobacterium psychrophilum* in rainbow trout, *V. harveyi* in shrimp, and *V. anguillarum* in salmon. These studies have demonstrated the potential of specific phages to significantly control pathogen density and, in some cases, reduce fish mortality. Therefore, there is growing evidence that phages can be applied to control diseases in aquaculture.

The use of bacteriophages to control specific pathogens is complicated by the phenotypic and genotypic complexity of both phage and host communities, which may consist of multiple co-existing strains with different host range and susceptibility patterns. In order to establish efficient phage control of a diverse host community, a large and well-characterized collection of phages is required which, in combination, covers a broad range of hosts.

In this study, a collection of *Vibrio* sp. strains and vibriophages was isolated from Danish aquaculture farms and characterized along with a collection of 20 *V. anguillarum* strains and one lytic *V. anguillarum* phage of different temporal and geographic origins. Patterns in phage and host phenotype, genotype, morphology, and phage-host ranges and interactions were studied to establish a collection of well-characterized vibriophages and to evaluate their potential for controlling the pathogen.

**MATERIALS AND METHODS**

**Bacterial strains.** The collection consisted of 20 *V. anguillarum* strains provided by Technical University of Denmark (DTU, Denmark); *V. anguillarum* strain PF430-3, derived from strain PF4, which was originally

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isolated in Chile (16) but was reisolated after long-term (2 weeks) culturing; and 16 Vibrio sp. strains recently isolated from Danish aquaculture farms (Table 1). The 16 recent Danish isolates were obtained from 18 water samples collected from two fish farms (Venøsund and Maximus, Denmark) between May 2012 and August 2012. Aliquots of water samples (200 ml) were mixed with 200 ml 2X LB medium (12106; MO-BIO) and agitated at room temperature for 24 h. For isolation of Vibrio sp. strains, 100-μl subsamples from serial dilutions were plated on thiosulfate citrate bile salt sucrose agar (TCBS; 221872; Difco) incubated at 30°C for 24 h. Single colonies were then isolated and subsequently pure cultured by re-isolation on bile salt sucrose agar (TCBS; 221872; Difco) incubated at 30°C for 24 h. For isolation of V. anguillarum, 200 ml samples collected from two fish farms (Venøsund and Maximus, Denmark) were mixed with 200 ml 2XLB medium (557904; Difco) and agitated at room temperature for 24 h. For isolation of Vibrio sp. strains recently isolated from Danish aquaculture farms, 200 ml 2XLB medium (557904; Difco) was incubated at 95°C, 1 min at 51°C, and 2 min at 72°C and final extension at 72°C for 7 min. PCRs were verified using 1% agarose gels stained with ethidium bromide. PCR products were subsequently sequenced in both forward and reverse directions (Beijing Genomic Institute [BGI]).

A phylogenetic analysis based on neighbor joining was performed on 16S rRNA gene sequences. The original sequences were manually corrected, trimmed, and aligned using the BioEdit sequence alignment tool (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Each of the compiled sequences was compared to the available databases using the basic local alignment search tool (BLAST) to determine phylogenetic affiliations. Mega 5.1 software (http://www.megasoftware.net/) was used to align all 37 sequences. Overlapping ends were removed to allow direct comparison of sequences with the same length (695 bp). The phylogenetic relationship was bootstrapped 1,000 times, and the gaps were treated by pairwise deletion.

### 16S rRNA sequencing
Bacterial genomic DNA was extracted using a commercial genomic DNA purification kit (Clontech). The 16S rRNA gene was amplified by universal primers 27f (5’-AGAGTTTGATCCTGGCTCAG-3’) and 1492r (5’-GGTACCTTGTACGACTT-3’). PCR was performed in a 24-μl reaction mixture, which contained genomic DNA template, 10X PCR buffer, 2 mM deoxynucleoside triphosphates (dNTPs), primers (12.5 μM each), MgCl₂ (25 mM), hot start polymerase, and water. PCR amplification was carried out on a thermal cycler (Applied Biosystems) for 5 min at 95°C, 35 cycles of 45 s at 95°C, 1 min at 51°C, and 2 min at 72°C, and final extension at 72°C for 7 min. PCRs were verified using 1% agarose gels stained with ethidium bromide. PCR products were subsequently sequenced in both forward and reverse directions (Beijing Genomic Institute [BGI]).

### Phenotypic analysis of bacterial strains
Biochemical tests were performed using an API 20NE system (bioMérieux) according to the instructions.
tions of the manufacturer. The inoculum was distributed into test strips which were then incubated at 30°C for 48 h. Biochemical reactions were read based on color development, translated into numerical codes, and interpreted with the API 20NE database.

The sensitivity of the strains to the vibriostatic compound agent 0129 (2,4-diamino-6,7-diisopropylpteridine phosphate) (Oxoid) was determined by the disk diffusion method (17) at both 10 and 150 μg per disk with incubation for 24 h at 30°C.

Biolog GN2 MicroPlates (Biolog) containing 95 different carbon sources were used to test the ability of the strains to use different carbon sources. Briefly, pure cultures of bacterial strains were grown on LB agar plates for 24 h at 30°C and subsequently transferred to 0.9% saline buffer (9 g NaCl in 1 liter distilled water) and washed twice to remove medium (8,000 × g for 10 min). From this bacterial suspension (ca. 10^8 CFU ml^-1), 100-μl aliquots were inoculated into each well in duplicate plates and incubated in the dark at 30°C for 48 h. Data were analyzed based on visual inspection of tetrazolium violet development as an indicator of metabolic activity.

**Bacteriophage isolation.** The water samples for bacterial isolation were also used for propagation of phages for subsequent isolation and purification of new vibriophages. Four different protocols were used (18).

(i) A 100-ml water sample was sterile filtered (0.22 μm; Millipore) and mixed with 100 ml 2× LB medium containing a mixture of 5 random *Vibrio* strains (Table 1) to stimulate proliferation of phages specific to the added strains. After 24 h of incubation at room temperature, bacteria in the lysates were killed by chloroform (20 μl ml^-1), followed by centrifugation (10,000 × g, 10 min) to remove bacterial debris. (ii) One-hundred-ml unfiltered water samples were mixed with 100 ml 2× LB broth and incubated at room temperature for 24 h to stimulate proliferation of phages specific to cooccurring *Vibrio* species host strains in the water sample. Aliquots of enrichment samples (50 ml) were then treated with chloroform and centrifuged as described above, and the supernatant was mixed with individual *Vibrio* strains (Table 1) in a second round of enrichment as described above. (iii) Five-ml water samples were filtered (0.22 μm pore size), and the filtrate was diluted and used directly for plaque assay (19) without previous enrichment. (iv) Two-hundred-ml water samples were filtered (0.22 μm), and phages were then concentrated using 30-kDa centrifugation filters (Amicon ultra 15 protein; Millipore) and stored at 4°C until further use.

The presence of *Vibrio*-specific phages in the water samples described above was tested against all 37 *Vibrio* sp. strains by spotting 5-μl samples on lawns of individual strains using the double-layer agar method (18). In the case of cell lysis in the spotted area, phages were scraped off the plate and transferred to SM buffer (50 mM Tris-Cl, pH 7.5, 99 mM NaCl, 8 mM MgSO4, 0.01% gelatin). These phage concentrates were then diluted, and the presence of phages was verified by plaque assay (19). For purification of individual phages, single plaques were isolated with a sterile pipet and transferred to SM buffer. This procedure was repeated at least three times to ensure purification of individual phages.

To obtain high-titer phage stocks, 5 ml SM buffer was added to plates with confluent lysis, and the top agar was shredded with an inoculation loop. The mixture was transferred to a sterile tube and incubated on a shaker for at least 2 h at room temperature to release phage particles and then centrifuged (10,000 × g, 10 min, 4°C). The supernatant was filtered through a 0.22-μm membrane (Millipore), and the phage stock was stored at 4°C in the dark. In addition to the phage isolates obtained from Danish aquaculture farms, a broad-host-range vibriophage (KVP40) (20) was kindly provided by the Hellenic Centre for Marine Research.

**Phage host range and EOP.** The host range of each phage isolate was determined using a spot assay against all of the available *Vibrio* sp. strains (Table 1). Aliquots of 5 μl phage stock (phage titer between 10^6 and 10^8 PFU ml^-1) were spotted on lawns of the potential host strain prepared as 4 ml top agar inoculated with 500 μl bacterial cultures in mid-log phase (ca. 10^8 CFU ml^-1). After 24 h of incubation at 30°C, the spots were assessed by the clarity of plaques and scored as transparent, turbid, or no inhibition. To provide a quantitative measure of the phage lytic potential, the efficiency of plating (EOP) for each phage on each phage-sensitive strain was determined by measurement of the number of PFU produced on individual hosts from a given phage stock, as quantified by plaque assay (19).

**Purification of bacteriophage genome DNA.** Aliquots (500 μl) of high-titer (ca. 10^9 PFU ml^-1) bacteriophage stocks were further concentrated by centrifugation (1,000 × g, 25 min) in 30-kDa ultrafiltration Amicon Microcon spin devices (Millipore), and then the membrane was washed three times with 50 μl 100× Tris-EDTA buffer (TE buffer; 10 mM Tris-Cl, 1 mM EDTA, pH 8) (1,000 × g for 18 min each). After the washing, the phages were eluted with 20 μl 100× TE buffer by centrifugation of the inverted membrane into a new tube (300 × g for 5 min). To denature phage proteins and release the phage DNA, the concentrated phages were heated to 70°C for 10 min and then cooled on ice for subsequent determination of phage genome size. To confirm the concatemer phenomena of a number of phages (phage ΦH2, ΦH8, and ΦH20), additional extraction of DNA from these phages was performed using a QIAprep spin M13 kit (Qiagen) according to the manufacturer’s instructions.

**Phage genome size.** The bacteriophage genome sizes were determined by pulsed-field gel electrophoresis (PFGE) using the CHEF-DR III pulsed-field electrophoresis system (Bio-Rad) (14). Phage DNA was run using 1% agarose gels in 0.5× Tris-borate-EDTA (TBE) buffer (10× TBE, 890 mM Tris, 20 mM EDTA, 890 mM boric acid, pH 8.3) with two different instrument settings for small and large genome sizes. For the large genome sizes (>30 kb), switch times were ramped from 50 to 90 s for 24 h and a voltage of 6 V/cm, whereas switch times ranged from 0.5 to 5 s and a total run time of 15 h for small genomes (<30 kb). An 8- to 48-kb molecular size DNA marker and a Lambda DNA ladder (Bio-Rad) were used as standards. The gels were stained with 0.01% (vol/vol) SYBR green I (Invitrogen) in 0.5× TBE for 1 h in the dark and then washed with distilled water for 30 min prior to visualization of the bands using the ChemiDoc MP imaging system (Bio-Rad).

**Bacteriophage morphology by TEM.** For transmission electron microscopy (TEM) analysis of phage isolates, the phage stocks were further concentrated by ultracentrifugation (100,000 × g, 90 min, 20°C; 70,1T1; Beckman), followed by resuspension of the pellet in 100 μl SM buffer. Formvar-carbon-coated copper grids were placed on top of the phage concentrates, allowing the phages to adsorb for 20 min. The grids were then stained with 10 μl 2% sodium phosphotungstate (pH 7.4; 0.02-μm pore-size filters) for 2 min. Excess stain was removed by touching the edge of the grids with filter paper, and the grids were washed with several drops of distilled water and allowed to dry on the filter paper for 15 min. The grids were observed with a JEM-2100 transmission electron microscope (JEOL) operated at 80 kV.

**One-step growth curve.** To characterize phage life cycle characteristics, one-step growth experiments were performed for two of the phage isolates using marine broth (MB; 2216; Difco) at a low multiplicity of infection (MOI; i.e., the initial ratio of phage particles to host cells), following a protocol modified from that of Ellis and Delbrück (21). For phage ΦH20, 1 ml of exponentially growing bacteria (10^5 CFU ml^-1) was mixed with 100 μl lytic phage stock (10^8 PFU ml^-1; MOI of 0.1) and incubated at room temperature for 20 min to allow the phages to adsorb to the host according to procedures described by Hidaka and Tokushige (22). Bacteria were then pelleted (7,000 × g, 10 min), and the nonadsorbed phages in the supernatant were discarded. The pellet was then resuspended in 1 ml MB and diluted to an appropriate phage titer (10^4 PFU ml^-1). Two 100-μl subsamples of culture were collected at regular intervals for 60 min for enumeration. In one subsample, free phages were immediately quantified by plaque assay (18). The other subsample was pretreated with 10 μl chloroform to measure the release of intracellular phages and then centrifuged at 10,000 × g for 1 min prior to plaque enumeration. For phage KVP40, the same procedure was applied, except that for this phage, the stock was diluted to 10^7 PFU ml^-1, reducing the MOI to 0.01.
Susceptibility of *V. anguillarum* to phage infection at different MOIs. The lytic potentials of two selected phages, H20 and KVP40, against *V. anguillarum* were examined at MOIs of 0.1, 1, and 10 in liquid MB cultures during infection of *V. anguillarum* BA35 and PF430-3, respectively. Serial dilutions of the phage stocks (ca. 10^2 to 10^9 PFU ml^{-1}) were inoculated in duplicate 50-ml mid-log-phase cultures of host bacteria in MB at a density of ~10^9 CFU ml^{-1}. For each experiment, duplicate control cultures without phage addition were also established. The effects of phage lysis on host cell density were monitored by regular measurements of the optical density at 600 nm (OD600) during the 24-h incubations. Phage abundance was quantified in one of the experiments (MOI, 0.1) from the number of PFU obtained on lawns of host strains by plaque assay (19) periodically over 24 h. At various time points, individual colonies were isolated from the cultures on TCBS plates and subsequently purified through 2 to 3 rounds of reisolation of single colonies to determine changes in phage susceptibility in the host community during incubation.

Statistical analysis. For all of the dendrograms, UPGMA (un-weighted-pair group method with arithmetic mean) construction experiments and Dice coefficients were calculated between pairs of each strain or phage and grouped by UPGMA and 100 bootstrap replicates using the dendrogram construction utility DendroUPGMA (Biochemistry and Biotechnology Department, Rovira i Virgili University, Tarragona, Spain) (http://genomes.urv.cat/UPGMA/index.php).

RESULTS

Isolation of *Vibrio* strains. A total of 16 Vibrio sp. strains isolated from Danish aquaculture were susceptible to phage KVP40; therefore, these 16 strains were tentatively classified as closely related *Vibrio* sp. strains. Of the total of 37 strains examined, 35 were sensitive to the vibriostatic agent O129 at both 10 and 150 μg, whereas two isolates (VSP8 and VSP9) were resistant to O129 at concentrations of up to 150 μg. The 37 Vibrio sp. strains used in this study covered a considerable spatial variability, with isolates originating from the Mediterranean Sea (Spain and Italy), the Baltic Sea (Finland and Germany), the North Sea (Norway and United Kingdom), the Atlantic Ocean (United States), the south Pacific Ocean (Chile), and inner Danish waters; the range in time of isolation covered ~20 years (23).

Identification of *Vibrio* strains. Based on 16S rRNA gene similarity, 24 of the isolates were presumptively identified as *V. anguillarum* according to NCBI BLAST analysis (NCBI BLAST; http://www.ncbi.nlm.nih.gov/BLAST/) (Table 1), including the entire DTU collection, the Chilean strain (PF430-3), and 3 Danish water isolates (VA1, VA2, and VA3), whereas the remaining 13 Danish isolates yielded identification as *V. cyclotrichus*, *V. splendidus*, *V. shilonii*, and *Vibrio* spp. (Table 1). A phylogenetic tree based on the 16S rRNA gene sequence analysis showed three main groups: group 1 (*V. anguillarum*), containing 20 DTU strains, strain PF430-3, and three Danish *V. anguillarum* isolates (VA1, VA2, and VA3); group 2 (*V. splendidus* and *V. cyclotrichus*), containing six Danish water isolates (VSP1, VSP2, VSP3, VSP8, VSP10, and VSP12); and group 3 (*V. shilonii* and *Vibrio* spp.), containing the remaining strains (VSP4, VSP5, VSP6, VSP7, VSP9, VSP11, and VSP13) (see Fig. S1 in the supplemental material).

API 20NE fingerprinting. Eleven different identities (Table 1) were generated from the 37 strains using API 20NE test. For all of the strains, positive results were obtained for potassium nitrate, D-glucose, esculin ferric citrate, 4-nitrophenyl-β-D-galactopyranoside, malic acid, and oxidase reactions, and negative reactions were found for urea, capric acid, adipic acid, and phenylacetic acid, whereas the remaining 11 biochemical tests gave various results (data not shown).

Biolog fingerprint. The phenotypic diversity of and relationship between the 37 strains were explored by studying the differences in carbon resource utilization, which showed a clustering of the strains into three primary phenotypic groups (Fig. 1). Cluster 1 contained 24 strains, including all of the strains in the DTU collection, the PF430-3 strain, and three of the Danish isolates (VA1, VA2, and VA3), whereas the remaining 13 Danish isolates were grouped into clusters 2 and 3 (Fig. 1). Overall, the number of substrates used varied from 54 (strain BA35) to 22 (strain VSP10) of the 95 tested substrates, with the highest number of available substrates found in cluster 1 (33 to 54) and the lowest in cluster 2 (22 to 38). All of the 22 substrates used by strain VSP10 could also be used by all other strains, suggesting that the utilization of these 22 substrates was a fundamental property of *Vibrio* strains.

Bacteriophage enrichment and isolation. Ten different bacteriophage isolates (Table 2) were obtained from Danish waters using different enrichment protocols. Phages H20, H8, H9021, and H9021 were isolated by following protocol I, using a mixture of *Vibrio* sp. strains as hosts for enrichment, whereas phages H8, H9021, and H9021 were isolated as H1, H7, and H9021-1 were isolated using a single *Vibrio* sp. strain for enrichment (protocol II).

Phage genome sizes, as determined by PFGE, showed large differences among the 11 phages and covered a 20-fold range, from 11 kb to 244 kb (Table 2). An interesting phenomenon was the systematic observation of several distinct bands appearing on the PFGE gels for phage H20, H8, and H9021 despite up to 5 rounds of phage purification from a single plaque. For example, PFGE analysis of phage H20 revealed seven bands, ranging from 50 to 291 kb, with each band size increasing by 50 kb, and phage H8, which for which two bands of 50 kb and 100 kb, respectively, were observed (Fig. 2; also see Fig. S2 in the supplemental material).

Host range and EOP. The host range and lytic potential of the 11 vibriophages were tested against 24 *V. anguillarum* and 13 *Vibrio* sp. strains by spotting 5 μl of a serially diluted phage stock onto agar containing the potential host strain. Together, the collection of phages was able to infect all 37 strains; however, there were large differences in host range pattern and lytic efficiency among the phages (Fig. 3). Six phages (H2, H8, H9021, DS4-7, DS4-18, and DS4-18) exhibited a narrow host range, infecting only the host used for its isolation and perhaps one or two additional strains. Phages H1, H7, H4, H5, and KVP40, on the other hand, were characterized by broader host ranges covering both local host isolates and isolates obtained from geographically distant locations. Conversion of the phage host range pattern into a dendrogram using the Dice coefficients comparing algorithm showed some distinct clusters of the phages and bacterial strains (Fig. 3). Generally, phages with the same genome size had similar host ranges, for example, the H1 and H7 pair and the H4 and H5 pair, which had pairwise identical genome sizes and also clustered together according to host range (Fig. 3). Interestingly, the phages with the largest genomes, i.e., H1, H7 (194 kb), and KVP40 (244 kb), also had the largest host range, infecting many of the same hosts, despite the fact that they were isolated from Danish (H1 and H7) and Japanese (KVP40) locations (24). Phage susceptibility pattern, on the other hand, did not show obvious connection to the other genotypic or phenotypic relationships.
Phage morphology. Phage morphology was examined by TEM for all 11 phages (Table 2 and Fig. 4). According to the International Committee on Taxonomy of Viruses (ICTV), three morphological groups of phages were represented by the 11 phages: *Siphoviridae* ($\Phi H_8$ and $\Phi H_{20}$), with flexible tails, *Podoviridae* ($\Phi 2E-1$, and $\Phi S4$-$18$), with short tails, and *Myoviridae* ($\Phi H_1$, $\Phi H_4$, $\Phi H_5$, $\Phi H_7$, $\Phi S4$-$7$, and KVP40), possessing an icosahedral head and contractile tails with tail fibers and relatively

FIG 1 Phenotype dendrogram derived by UPGMA cluster analysis of all 37 strains in the Biolog GN2 profiles. The data were scored and compared by using Dice coefficient algorithm. The scale bar indicates the dissimilarity.
large genomes (25) (Fig. 4 and Table 2). Interestingly, some vibriophages with different genome sizes, e.g., ΦH2 and ΦS4-18, belong to the same family.

**Phage-host interactions.** Life cycle characteristics were determined for phages ΦH20 and KVP40 from one-step growth curves during incubation with their host strains BA35 and PF430-3, respectively. Despite different morphologies and genome sizes, phages ΦH20 and KVP40 had quite similar burst sizes (70 and 80 PFU per cell, respectively) and identical eclipse (20 min) and latent periods (25 min).

The parallel long-term (24 h) infection experiments with phages ΦH20 and KVP40 and the hosts BA35 and PF430-3, respectively, at different MOIs also showed similar lytic potential of the two phages, with a strong dependence on the initial MOI for phage control of the host population (Fig. 5). In batch cultures at an MOI of 0.01, both phage KVP40 and ΦH20 showed rapid propagation during the first 10 h of incubation, resulting in phage titers stabilizing around 10^{10} and 10^{9} PFU ml^{-1}, respectively (Fig. 5). The host growth rates in the control cultures were 0.241 ± 0.002 h^{-1} and 0.231 ± 0.007 h^{-1} for PF430-3 and BA35, respectively, and both strains reached stationary phase after 10 h. In the phage-amended cultures, phages were able to control growth of the host for 5 to 6 h followed by regrowth of the host population.

Accordingly, decreasing the initial MOI resulted in a delayed and less pronounced decrease in the OD_{600} (Fig. 6). Regrowth occurred in all cultures, and after 20 h the OD_{600} stabilized at 0.6 to 0.7, corresponding to 65 to 75% of the final OD_{600} in the control cultures. In addition to the OD_{600} measurements, bacterial isolates were obtained during the incubations for further analysis of changes in phage susceptibility in the host population (Fig. 6). In the BA35-plus-ΦH20 cultures, the occurrence of the bacteriophage-resistant isolates varied considerably over time and between treatments. According to the spot test results, the emergence of the bacteriophage-resistant bacteria was faster at high than at low MOI. Within the first hour after incubation, resistant strains were isolated at an MOI of 10, whereas resistant phenotypes were not detected at MOIs of 1 and 0.1. At high MOI, only resistant isolates were obtained after 7 h of incubation. At the lower MOIs, resistant isolates dominated during the regrowth phase (7 h). However, partially sensitive bacteria (i.e., reduced sensitivity compared to the sensitive wild-type strain) were isolated from 8 h onwards and constituted 40 to 50% of the isolates after 24 h. All of the resistant and partially sensitive strains isolated from the phage-amended BA35 culture after 24 h formed smaller colonies with lower growth rates on TCBS plates than those seen in the control cultures without phages. For the partially sensitive and resistant BA35-derived isolates, a second round of purification and subsequent susceptibility test were carried out. Interestingly, all of the partially sensitive strains returned to their sensitive phenotype after purification in the absence of phages, whereas all of the completely resistant isolates remained resistant.

In the parallel experiment with *V. anguillarum* strain PF430-3 and phage KVP40, no completely resistant isolates were obtained from any of the treatments during the incubation, and the colony...
morbidity did not change over time. Instead, all of the isolates were sensitive or partially sensitive strains, and after 8 h all obtained isolates were fully sensitive to KVP40. In contrast to the control cultures, visible biofilm was formed in the KVP40-amended cultures, and some isolates formed aggregates when subsequently grown in liquid culture. For the aggregating and partially sensitive PF430-3-derived isolates, a second and third round of purification and subsequent spot assay showed that all strains returned to the original phenotype and were fully sensitive to phage KVP40.

DISCUSSION

Successful application of phage therapy in the treatment of vibriosis requires detailed knowledge of the diversity and distribution of the phage susceptibility properties of Vibrio sp. pathogens associated with vibriosis as well as a collection of well-characterized phages that covers host diversity. In this study, we characterized 11 vibriophages and 37 Vibrio sp. strains (Table 1 and 2), covering considerable temporal and spatial variability. Further, two V. anguillarum strains, BA35 and PF430-3, and the specific phages ΦH20 and KVP40 were selected for more detailed studies of phage lytic potential and phage-host interactions.

**Diversity of V. anguillarum strains.** The V. anguillarum strains examined in the present study (Table 1) showed close similarity according to 16S rRNA gene sequences irrespective of time or place of isolation. Thirty-seven of the isolates which grouped together covered a period of isolations of >20 years and a wide range of geographical locations (Chile, Spain, Italy, Germany, United Kingdom, Norway, Finland, Denmark, and the United States) and fish species (turbot, trout, sea bass, and salmon). This suggests that the 16S rRNA gene phylogeny of V. anguillarum is highly uniform and stable in time and space. Thus, the method is unable to discriminate between potentially pathogenic and non-pathogenic V. anguillarum strains or resolve differences in V.

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**FIG 3** Host ranges of 11 phages (columns) against 37 Vibrio sp. strains (rows). Black boxes indicate clear plaques, gray boxes indicate turbid plaques, and white boxes indicate no infection. Numbers express the efficiency of plating, i.e., the fraction of infectivity of the phage stock on a given host compared to the infectivity on the original host of isolation (isolation efficiency of the host was 100%). Dendrograms were derived by UPGMA cluster analysis. The data were scored and compared by using the Dice coefficient algorithm. The scale bar indicates the dissimilarity.
anguillarum communities across spatial and temporal scales. Similarly, genetic fingerprinting methods (enterobacterial repetitive-element intergenic consensus sequence [ERIC] PCR) showed that Vibrio sp. isolates from coastal British Columbia did not group according to geography, suggesting genetic homogeneity among the environmental strains (26). Previous studies have, however, revealed a large degree of microheterogeneity in 16S rRNA genes within Vibrio sp. strains (27), limiting the use of 16S rRNA genes for phylogenetic studies in Vibrionaceae. Higher taxonomic resolution has been obtained using multilocus sequence analyses (MLSA) of six protein-coding genes (28); however, this method also focuses on variations in housekeeping genes and is applicable mainly for identification of Vibrio strains at the species level.

The V. anguillarum isolates clustered in distinct groups based on the Biolog GN2 profiles and differed from the other identified clusters of Vibrio strains. In contrast, the phage susceptibility pattern of the V. anguillarum isolates did not show any connection to the species or physiological relationships obtained from Biolog profiles. In fact, phage susceptibility patterns did not even group according to the species clusters defined for the 37 strains. This suggests that phenotypic traits related to sensitivity to phages are not resolved by the methods used in this study. This supports previous studies of Cellulophaga baltica (29), V. parahaemolyticus (15), F. psychrophilum (14), and Escherichia coli (30), which have all demonstrated a high degree of phenotypic diversity in host susceptibility at the strain level that was uncoupled from the overall genetic relationships among the host isolates. Thus, our results confirm highly variable patterns in phage sensitivity even within relatively narrow phylogenetic and phenotypic groups of bacteria, and at the same time they demonstrate similar susceptibility patterns across a broad phylogenetic scale in Vibrio spp., emphasizing the complexity of phage-host interactions in this group.

As observed for the physiological fingerprints, there was overlap in the phage susceptibility patterns for V. anguillarum strains isolated across the large temporal and spatial scales and from different fish species represented in the current study. For example, the strains 90-11-286 and VA3, which were isolated from Danish rainbow trout and salmon farms, respectively, with 20 years between isolations, were both infected by the same 3 phages (ΦH4, ΦH7, and KVP40). Similarly, strains VIB88 and BA35, isolated from rainbow trout farms and salmon farms in Germany and the United States, respectively, both were infected by phages ΦH4 and ΦH5, isolated from a Danish turbid farm 20 years later. This demonstrates that the occurrence of unique susceptibility patterns of individual isolates, key phenotypic properties related to phage susceptibility are distributed worldwide and maintained in the global V. anguillarum community for decades.

**Phage isolation and diversity.** The 10 vibriophages isolated from Danish fish farms all had unique host ranges when tested against the collection of 37 Vibrio strains. All three major morphological families, Myoviridae, Podoviridae, and Siphoviridae, were represented, and the phages ranged in genome size from 11 to 194 kb, distributed in 6 distinct groups (Table 2). The phage isolates showed large differences in host specificity, and together the Danish phages were able to infect and lyse 30 of the 37 strains, covering most of the phylogenetic, geographical, and temporal variation that characterized the host isolates. This is interesting, as it suggests that vibriophage infective properties are not restricted to local cooccurring host communities but rather widely distributed across large spatial and temporal scales, as also suggested by previous observations of an ocean-scale distribution of genetically related vibriophages (31).

The two broad-host-range phages isolated in this study (ΦH1 and ΦH7) both belonged to the Myoviridae family and had relatively large genomes (194 kb). This is consistent with the host range pattern observed for F. psychrophilum phages, where the large-genome phages (>90 kb) had substantially broader host ranges than small-genome phages (<12 kb) (29). In accordance with this, the previously isolated large-genome vibriophages KVP40 (244 kb) (32) and ϕpp2 (246 kb) (33) also have very broad host ranges covering several species of Vibrio. KVP40 is known to cause infection through the common outer membrane protein OmpK and has previously been shown to infect eight Vibrio species, including V. anguillarum, V. parahaemolyticus, and V. cholerae (20). The presence of several copies of genes encoding proteins associated with phage tail or tail fibers in the KVP40 genome suggested an increased flexibility in host range adaptation, increasing host range (32). Despite the fact that KVP40 was originally isolated in Japan >20 years ago (24), it still infected approximately 40% of the Vibrio sp. isolates obtained from Danish

**FIG 4** TEM pictures of selected phages. Phages were stained with 2% sodium phosphotungstate. Scale bar, 200 nm.
aquaculture, confirming its cross-species host range, and it maintained infective properties across large temporal and spatial scales. Thus, the characteristic of the new phage isolates ΦH1 and ΦH7 adds to the emerging view of a global distribution of large-genome, broad-host-range vibriophages.

Generally, vibriophages were widespread in Danish aquaculture, as phages were isolated from all of the investigated farms independently of outbreaks of vibriosis. However, the fact that phages could not be isolated without previous propagation in enrichment cultures indicated that densities of specific vibriophages

FIG 5 (A) Optical density (OD<sub>600</sub>) in cultures of *V. anguillarum* BA35 amended with phage ΦH20 at MOIs of 0 (control), 0.1, 1, and 10 and a corresponding abundance of PFU ml<sup>−1</sup> in the treatment at an MOI of 0.1. (B) Optical density (OD<sub>600</sub>) in cultures of *V. anguillarum* PF430-3 amended with phage KVP40 at MOIs of 0 (control), 0.1, 1, and 10 and the corresponding PFU abundance in the treatment at an MOI of 0.1. Error bars represent standard deviations from all experiments carried out in duplicate.
FIG 6 Phage susceptibility properties of *V. anguillarum* isolates obtained from cultures enriched with phages at different MOIs. (A to C) *V. anguillarum* strain BA35 plus phage ΦH20 at an MOI of 10 (A), 1 (B), or 0.1 (C). (D to F) *V. anguillarum* strain PF430-3 plus phage KVP40 at an MOI of 10 (D), 1 (E), or 0.1 (F). “Sensitive” indicates that isolates were fully sensitive to the phage. “Resistant” indicates that isolates were not susceptible to phage ΦH20 infection in the spot test. “Partially resistant” indicates that isolates had a reduced susceptibility to the phage. “Aggregates” indicates that isolates were forming aggregates, which protected against phage infection.
were relatively low in the fish farms. Some samples from which Vibrio sp. hosts were isolated did not harbor their corresponding phages, suggesting that specific hosts and lytic phages did not always co-exist in the same environment. However, it should be noted that the filtration of the water samples and chloroform treatment prior to phage isolation may have resulted in the loss of phages (18, 34).

In all isolate-based studies of phage diversity, the results are biased by the choice of host strains used for isolation, as each individual strain will only target a subset of the infective phages present in the sample. In the current study, broad-host-range phages ΦH1 and ΦH7 were isolated using strains from the global Vibrio species collection, whereas the more host-specific phages, such as Φ2E-1, Φ54-18, and Φ54-7, were isolated using a co-occurring Vibrio sp. host obtained from the same sample. As the evolution of a broad host range in phages is believed to be accompanied by a fitness cost, i.e., a reduced infective efficiency in its original hosts (35), it is likely that the observed differences in phage isolation pattern reflect different phage properties. Therefore, we speculate that highly specific phages are efficient, opportunistic phages which propagate rapidly in response to the growth of their specific hosts and will dominate in samples where their host is present. Consequently, these phages are the most likely to be isolated using cooccurring hosts as target strains. Broad-host-range phages, on the other hand, may follow a K-strategy (36), maintaining lower densities but for longer periods; therefore, they may be more likely to be pulled out from a sample using non-cooccurring or rare hosts. Due to these differences in isolation pattern, we do not know to what extent the isolated phages are representative of the local phage community.

PFGE provides a good discrimination for phage genome sizes and clearly demonstrates a large variability in genome size within the group of vibriophages. Several phage genomes, including ΦH20, ΦH8, and ΦH2, yielded several bands in PFGE analysis, representing multiplications of its own genome size. These bands may represent concatemers of the phage genome produced during DNA replication, as previously demonstrated in λ phage (37). Also, concatemer formation of phage DNA has been observed as a result of DNA cleavage during heating and subsequent reassembly at ambient temperature (38). The possibility of coinfection of other phages still cannot be ruled out; however, we did not see different phage morphotypes during TEM analysis of single isolates. A previous report of different phage morphotypes associated with multiple bands on a PFGE gel, however, suggested that coinfection of more than one phage may be an explanation for the occurrence of several genome sizes on a PFGE gel of a purified phage (29). Further genomic analyses are required to elucidate the origin of the multiple bands obtained in the PFGE analyses.

Phage-host interactions. The in vitro bacterial challenge assay with V. anguillarum strains BA35 and PF430-3 demonstrated significant but temporary phage control of both strains which was strongly dependent on the initial MOI. Even at the highest MOI, the host bacteria were not completely eliminated and regrowth of cells following phage infection suggested the emergence of phage-resistant strains after 5 to 6 h of incubation. Lytic bacteriophages have previously been shown to select for the emergence of resistant strains in culture studies with marine heterotrophic bacteria (39–41), where phage-resistant strains rapidly replaced the sensitive wild type following exposure to phages.

Isolation and characterization of bacterial isolates during the experiments confirmed that completely resistant strains, and strains with reduced sensitivity, dominated the bacterial population after 7 h in the culture containing strain BA35 and phage ΦH20. Interestingly, however, phage-resistant strains were not isolated after exposure of strain PF430-3 to phage KVP40, as has also been observed in other phage infection studies with hosts such as Salmonella enterica (42), S. enterica serovar Oranienburg (43), and E. coli O157:H7 (44). In the current experiment with strain PF430-3, phage exposure led to the formation of bacterial aggregates, and all of the isolated colonies had maintained full sensitivity to the phage after purification. These results suggest that different mechanisms were responsible for the regrowth of cells in the presence of phages following lysis of the initial sensitive population. The BA35 and phage ΦH20 interaction results in a succession toward phage-resistant strains, whereas the strain PF430-3 apparently is protected against phage KVP40 infection by aggregation. The underlying mechanism providing bacterial protection against phages via aggregation, allowing the coexistence of lytic phages and sensitive strains, is not revealed in the present study. However, previous studies have shown that phage-induced lysis can promote the formation of bacterial biofilms (45), which may offer protection against phage infection via the exopolysaccharide (EPS) matrix covering the biofilm (46) or the presence of bacterial refuges in the spatially heterogeneous environment (47). More recently, Høyland-Kroghsbo et al. (48) found that N-acyl-t-homoserine lactone (AHL) quorum-sensing signals in E. coli caused a transient reduction in the phage adsorption rate and suggested this as a mechanism allowing coexistence of lytic phages and sensitive hosts. Such mechanisms may also have contributed to the observed coexistence of sensitive strains of PF430-3 and the lytic phage KVP40 in the current study.

Potential for phage therapy in aquaculture. One of the main challenges in using bacteriophages to control pathogens in aquaculture is to cover the diversity and temporal and spatial variations in pathogen composition, as well as the phage resistance that may rapidly develop. A detailed characterization of phage properties and understanding of phage-host interactions are essential requirements for the successful application of phage-based pathogen control. The local occurrence of both specific and broad-host-range phages which, in combination, were able to infect all of the tested host strains, covering large geographical and temporal scales, suggested that vibriophage-host systems are ubiquitously distributed. In a phage therapy context this is promising, as it suggests the possibility of establishing a phage library with broad application against V. anguillarum communities worldwide. However, the fast development of phage resistance during exposure to specific phages and the indication that different mechanisms provided resistance in different hosts also emphasized that the susceptibility to phages is a dynamic property in the Vibrio sp. hosts. Consequently, phage resistance mechanisms and dynamics and the implications for host properties are important to address in future exploration of phage control of V. anguillarum in aquaculture.

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Figure S1. Neighbour-joining tree based on 16S rRNA gene sequencing showing the phylogenetic relationship between 37 strains listed in table 1 and *V. harveyi* (AY747308.1, NCBI) and *V. parahaemolyticus* (FJ594056.1, NCBI). Phylogenetic relationships were bootstrapped 1,000 times. The scale bar represents the number of nucleotide substitutions per site.
Figure S2: PFGE analysis of all the 11 phage genomes. Ladders indicate DNA size in standards (kb)
Vibriophages differentially influence biofilm formation by *Vibrio anguillarum* strains

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Running title: The interaction of vibriophages and biofilms
Abstract

*Vibrio anguillarum* is an important pathogen in marine aquaculture, responsible for vibriosis. Bacteriophages can potentially be used to control bacterial pathogens; however, successful application of phages requires a detailed understanding of phage-host interactions at both free-living and surface-associated growth conditions. In this study, we explored *in vitro* phage-host interactions in two different strains of *V. anguillarum* (BA35 and PF430-3) during growth in micro-colonies, biofilms, and free-living cells. Two vibriophages, ΦH20 (*Siphoviridae*) and KVP40 (*Myoviridae*), had completely different effects on the biofilm development. Addition of phage ΦH20 to strain BA35 showed efficient control of biofilm formation as well as in liquid cultures. The interactions between BA35 and ΦH20 were thus characterized by a strong phage control of the phage-sensitive population and subsequent selection for phage-resistant mutants. Addition of phage KVP40 to strain PF430-3 resulted in increased biofilm development, especially during the early stage. Subsequent experiments in liquid cultures showed that addition of phage KVP40 stimulated the aggregation of host cells, which protected the cells against phage infection. By the formation of biofilms, strain PF430-3 created spatial refuges that protected the host from phage infection and allowed coexistence between phage-sensitive cells and lytic phage KVP40. Together the results demonstrate highly variable phage protection mechanisms in two closely related *V. anguillarum* strains, thus emphasizing the challenges of using phages to control vibriosis in aquaculture, and adding to the complex roles of phages as drivers of prokaryotic diversity and population dynamics.
**Introduction**

*Vibrio anguillarum* is a marine pathogenic bacterium causing vibriosis, a fatal hemorrhagic septicemia, which contributes to significant mortalities in fish and shellfish aquaculture worldwide (1-3). The persistence of *Vibrio* pathogens in aquaculture has been attributed to their ability to form biofilms with increased tolerance to disinfectants and antibiotics (4, 5). Moreover, the first stage of infection involves biofilm-like micro-colonies in the skin tissue, causing chronic infection (5).

Recently, bacteriophages have been suggested as potential agents of pathogen control in aquaculture, and the controlling effects of phages have been explored for a number of fish pathogens (6-8). Successful application of phages to reduce vibriosis-related mortality has been demonstrated (9, 10). The capabilities of some phages to produce depolymerases, which hydrolyze extracellular polymers in bacterial biofilms, have made the use of bacteriophages particularly relevant in the treatment of biofilm-forming pathogens, as demonstrated in biofilms of *Pseudomonas aeruginosa* (11), *Escherichia coli* (12), and *Staphylococcus aureus* (13).

Apart from the potential physical and chemical barrier provided by biofilms, the use of bacteriophages to control pathogens is challenged by the development and selection for phage-resistant or phage-tolerant subpopulations. A broad range of resistance mechanisms have evolved in bacteria (14) and the lytic infection of phage-susceptible bacteria has shown to drive a functional and genetic diversification of the host population based on the rapid emergence of phage-resistant mutants (15, 16). A deeper understanding of temporal and spatial dynamics of phage-biofilm interactions and the development of phage-resistance in these systems is therefore essential for assessing the potential of using phages in disease control.

In this study, we quantified the influence of phages on biofilm formation in two strains of *V. anguillarum*, both during the initial stages of attachment and micro-colony formation and in more developed biofilms. The results showed large intraspecific differences in phage-biofilm interactions
of two *V. anguillarum* phage-host systems, and revealed the presence of highly different protective mechanisms against phage infection, adding further complexity to the use of phages to control vibriosis.

**Materials and Methods**

**Bacterial strains and bacteriophages**

Bacteriophages and hosts used in this study are listed in Table 1. Two *V. anguillarum* strains were used in this study: strain PF430-3, originally isolated from salmonid aquaculture in Chile (17) and strain BA35, originally isolated from sockeye salmon in USA (18). Phage KVP40 which infects PF430-3 is a broad-host-range phage originally isolated from Japan (19, 20) and phage ΦH20, infecting BA35, was isolated from Danish aquaculture (21).

**Effects of phages on initial bacterial attachment and micro-colony formation**

To quantify the effect of phages on micro-colony formation of the two strains, 10 µl of diluted mid-log-phase cultures (10^6 CFU ml⁻¹) was filtered onto 20+ replicate 25 mm 0.2 µm polycarbonate filters and incubated on LB agar in 6-well plates (22). Two ml phage stock (ca. 10^8 PFU ml⁻¹) was added to half the filters, the other half serving as controls with addition of 2 ml SM buffer (50 mM Tris-Cl, pH 7.5, 100 mM NaCl, 8 mM MgSO₄, 0.01% gelatin). Duplicate filters of phage amended and controls were collected every hour for 8 h, transferred to a 25 mm filtration manifold (Millipore) and stained with 0.5 % SYBR Gold (Invitrogen) for 10 min, followed by 3 x rinsing with Milli-Q water. The filters were then mounted on a microscope slide and stored frozen until quantification by epifluorescence microscopy using Cell M image analysis software (Olympus) for determination of colony area.

**Phage-biofilm interactions**

Effects of phage ΦH20 and KVP40 on the formation and disruption of *V. anguillarum* biofilms were monitored for the two strains BA35 and PF430-3 in two different experimental approaches: In the
pre-treated experiment, duplicate sets of polypropylene plastic tubes (Sarstedt) with 5 ml marine
broth (MB, Difco) were inoculated with 100 μl overnight bacterial culture and 100 μl phage stock
(final concentration 10^7 CFU ml^-1 and 10^6 PFU ml^-1, multiplicity of infection, MOI=0.1) simultaneously.
In the post-treated experiments, the bacteria were allowed to grow in 5 ml MB for 10 days after
inoculation to establish a biofilm. After 10 day incubation, the liquid was removed and the tubes
were rinsed with MB. Then 5 ml MB was added along with 100 μl phage stock (final concentration
10^6 PFU ml^-1), and the tubes were incubated for another 7-8 days.

In both experiments, the tubes were incubated at 30 °C and duplicate tubes were sacrificed daily for
measurement of biofilm formation, and density of free-living bacteria and phages. Measurements of
OD600nm were used to estimate free-living bacterial abundance and determination of phage
concentration in the free-living phase was done by plaque assay (23). Biofilm was quantified
according to O'Toole (24) with some modifications. Briefly, the liquid was removed, and tubes were
rinsed twice with artificial seawater (ASW, Sigma). Then 6 ml 0.4% crystal violet (Sigma) was added
to each tube and after 15 min, stain was removed. Tubes were washed with tap water in order to
remove excess stain and left to dry for 5 min. Six ml of 33% acetic acid (Sigma) was added and left
for 5 min to allow the stain to dissolve. The absorbance was measured at OD595nm.

In addition to the measurements above, we also quantified the biofilm-associated infective phages
in the post-treated experiment after disruption of the biofilm by sonication. First, the liquid was
removed and the tube rinsed 3 times with ASW. Then, 6 ml ASW was added to completely cover the
biofilm, and the tube was sonicated for 10 s at 80 amplitude (Pulse on 1 s and Pulse off 1 s, Misonix
sonication bath) to dislodge bacteria and phages from the biofilm matrix. Serial dilutions were
performed and phage concentration was quantified by plaque assay (23).

**Phage-host interactions in the free-living phase: detection of aggregate formation**

The effects of phages ΦH20 and KVP40 on the growth and aggregate formation of their respective
host strains, BA35 and PF430-3, during free-living growth in liquid cultures were examined.
Overnight cultures were inoculated in 50 ml MB with their respective phage at a MOI of 0.1. The flasks were incubated at room temperature with agitation; samples were collected for determination of the presence of cell aggregates by epifluorescence microscopy. For epifluorescence microscopic analysis of cell aggregation, subsamples were filtered onto 0.45 μm polycarbonate membrane filters (Whatman) and stained with SYBR Gold (0.5%, Invitrogen) for 10 min. Bacteria and phages on the filters were distinguished based on their dimensions and/or their relative brightness.

**Phage susceptibility and physiological fingerprint of bacterial isolates**

In order to determine changes in phage susceptibility, a total of ~180 cells were isolated from phage-treated micro-colony experiments, pre-treated biofilm experiments and free-living samples and analyzed for phage susceptibility pattern. For colony isolation, samples were in all cases plated onto Thiosulfate Citrate Bile Salt Sucrose agar (TCBS, Difco) and incubated for 24 h. From these plates, single colonies were isolated and purified by 3 rounds of re-isolation and then transferred to 4 ml MB for subsequent phage susceptibility analysis. Phage susceptibility of the isolated strains was determined by spot tests (21).

BIOLOG GN2 Micro-Plates (BIOLOG) containing 95 different carbon sources were used to test the ability of selected phage-amended isolates to use different substrates following manufacturer’s instructions (21). For strain PF430-3, the procedure was slightly modified as bacterial lysate (bacterial culture subjected to phage-induced lysis) and aggregates were collected by centrifugation at a lower speed (3,000×g, 5 min), and washed twice with 0.9% saline buffer.

**Adsorption experiments with fluorescently labelled phages**

Phage adsorption to selected isolates was tested by adding SYBR Gold labelled phages to cultures followed by visual inspection phage binding to the cells using epifluorescence microscopy, following the protocol of Kunisaki (25) with some modifications. Aliquots of 600 μl phage lysate were digested by addition of DNase I (1 μl DNase I +3 μl RDD buffer, Qiagen) at 37°C for 2 h and then stained with
SYBR Gold (final concentration 5×, Invitrogen) overnight at 4 °C. Ten μl chloroform was then added to inactivate DNase I. The phage stock was then further filtered through a 30 kDa ultrafiltration spin devices (Millipore) at 1,000×g for 90 min to remove the free SYBR Gold. The SYBR Gold-labelled phages were added to the mid-log wild-type cells and phage lysate, respectively, at a MOI of approximately 10 and incubated at room temperature for 20 min. Samples were pelleted at 12,000×g for 3 min, and the pellet was re-suspended in a small volume of SM buffer, mixed with pre-heated 0.5% agarose (w/v) (Bio-rad) around 45°C and transferred to gel-coated slide glass.

Statistical analysis
All statistics were performed using the Student's paired t-test (two-tailed) (OriginPro 8.6) to determine the significance of the differences between phage amended and control cultures. Differences were considered to be significant if $P<0.05$.

Results

Initial biofilm formation and short-term effects of phages

The two *V. anguillarum* strains formed micro-colonies with highly different morphologies in the absence of their corresponding phages. Strain BA35 formed simple single-layered micro-colonies, where individual cells could easily be identified whereas strain PF430-3 formed complex 3-dimensional volcano-shaped structures (Fig. 1). Phage ΦH20 showed an efficient control of micro-colonies BA35 according to the microscope observations, reducing total colony area from 41,000 µm² per mm² filter on the control filters to 5,000 µm² per mm² filter after 6 h incubation, and leaving mainly single cells on the filters following phage exposure (data not shown). For strain PF430-3, the individual colonies were unaffected by phage exposure, whereas single cells were lysed by phage KVP40, leaving mainly colonies on the filters 6 h post addition (Fig. 1).

Effects of phages on biofilm formation
Establishment and growth of biofilm produced by the two *V. anguillarum* strains BA35 and PF430-3 in cultures pre-treated with phage ΦH20 and KVP40, respectively, was examined over a long-term experiment in parallel to control cultures without phages. The OD<sub>600nm</sub> of free-living bacteria in the control cultures reached maximum values of 0.72±0.01 and 0.98±0.01 for BA35 and PF430-3, respectively, after 2 days (Fig. 2A and 2C). A gradual minor decrease was then observed for BA35, whereas the density of PF430-3 decreased to ~50% of the maximum value at day 9 (Fig. 2A and 2C).

The presence of the phages had different effects on the free-living cell density of the two strains. For strain BA35, the density reached a level of 70% of the control cultures after 2 days and remained at this level (only significantly lower at day 3 (p=0.025) and 6 (p=0.009)) throughout the experiment (Fig. 2A). In the cultures with strain PF430-3, addition of phage KVP40 significantly reduced the optical density of the culture (0.008≤p≤0.027 when comparing the OD values at individual time points from day 2 onwards) to approximately 25% of the corresponding values in the control cultures (Fig. 2C). Phage concentration reached peaks of around 1.3×10<sup>10</sup>±3.5×10<sup>8</sup> and 3.4×10<sup>8</sup>±7.1×10<sup>6</sup> PFU ml<sup>-1</sup> for ΦH20 and KVP40, respectively at day 1 (Fig. 2A and 2C). Interestingly, in the PF430-3+KVP40 cultures, phage concentrations decreased significantly (p=0.007) from day 1 to day 2 then stabilizing around 10<sup>6</sup> PFU ml<sup>-1</sup> (Fig. 2C). Likewise, the concentrations phage ΦH20 decreased significantly (p=0.0055) from day 1 to day 5 and then remained at a titer above 2.2×10<sup>9</sup>±2.8×10<sup>8</sup> PFU ml<sup>-1</sup> (Fig. 2A).

The biofilm development in response to phage addition also showed completely different patterns in the two strains with either inhibition (BA35) or stimulation (PF430-3) of biofilm formation relative to control cultures. In the BA35+ΦH20 pre-treated cultures, phage ΦH20 effectively prevented biofilm formation, causing a 2-fold decrease in the biofilm biomass relative to the control level (Fig. 2B). In the PF430-3+KVP40 pre-treated cultures, phage KVP40 addition resulted in almost a 3-fold increase relative to the control (Fig. 2D). In the control cultures, there was a steady increase in biofilm density...
for strain PF430-3, whereas a fast initial establishment of biofilm of strain BA35 was gradually reduced during the 7-day period (Fig. 2B and 2D).

To assess the effect of phages on fully matured biofilms, 10-day biofilms were challenged with their corresponding phages measuring the same parameters as above. As in the pre-treatment experiments, phage addition significantly reduced the BA35 biofilm (3-4 fold reduction) within 2 days ($p=0.046$, when comparing OD values from day 1 and 2) and slightly stimulated the production of biofilm in PF430-3, relative to controls (Fig. 3B and 3D).

For the free-living populations, the input of nutrients associated with the addition of phages (and corresponding addition of medium in the controls) caused an increase in bacterial concentration in the control cultures (Fig. 3A and 3C). Similar to the pre-treated cultures, the OD$_{600nm}$ decreased over time in the control cultures, with the strongest decrease observed in the PF430-3 cultures. The presence of phages, however, imposed a significant 4-5 fold decrease in concentration of free-living cells in both experiments ($0.003 \leq p \leq 0.046$, when comparing the OD values at individual time points from day 2 onwards), except for day 5 (Fig. 3A and 3C).

The concentration of free-living phages reached a peak around $3 \times 10^9 \pm 2.8 \times 10^7$ and $5.8 \times 10^8 \pm 2.8 \times 10^7$ PFU ml$^{-1}$ for $\Phi H20$ and KVP40, respectively, after 24 h (Fig. 3A and 3C) followed by a gradual decrease in the concentration of phage KVP40 (Fig. 3C). The biofilm-associated concentration of phage KVP40 constituted less than 10% of the free-living population and decreased dramatically after the peak (Fig. 3D).

**Effects of phage KVP40 on bacterial aggregation in liquid cultures**

In the BA35 and BA35+H20 cultures, no aggregate formation was observed and phage addition reduced the density of free-living cells relative to the control as a result of cell lysis (Fig. 4). Motility of BA35 seemed to be affected by phage addition as cells in the phage-added cultures had reduced or lost motility after 24 h (Supplementary video 1 and 2). In the PF430+KVP40 cultures, on the other
hand, aggregates of multi-layered cell clusters embedded in a matrix were observed after 24 h (Fig. 4 and Supplementary video 3 and 4).

**Bacteriophage resistance and physiological fingerprint**

For strain BA35, all the isolates obtained from the micro-colony experiment were resistant to phage infection and approximately 30 % of the free-living and biofilm-associated isolates were sensitive to ΦH20 (Table S1). The resistant colonies were small-colony variants on TCBS plates. In contrast, all the isolates from KVP40-exposed PF430-3 cultures were fully or partially susceptible to phage KVP40 and no completely KVP40-resistant mutants were isolated (Table S1). Colony morphologies of PF430-3 isolates were similar to the wild-type PF430-3. Spot assays showed that all partially resistant isolates regained full sensitivity to phage KVP40 after purification by re-streaking of single colonies on agar plates.

The phenotypic diversity and implications of resistance for the metabolic properties of the isolates following phage exposure were explored by obtaining a metabolic fingerprint of selected isolates using BIOLOG GN2 assay (Fig. S1). For strain BA35, the phage-resistant isolate had lost the ability to use 10 of the 54 substrates that could be metabolized by wild-type BA35 cells, corresponding to a 19% reduction of the capacity substrate utilization. In contrast, none of the PF430-3 isolates obtained after phage exposure showed any changes in physiological fingerprint according to the BIOLOG profile (Fig. S1).

To further examine the mechanisms of resistance/tolerance against phages in the two strains, SYBR Gold-labelled phages ΦH20 and KPV40 were incubated with wild-type cells and phage lysate. Fluorescently labeled phage ΦH20 attached to the sensitive cells of the strain BA35 (Fig. 5A), whereas no phage-attachment was observed in phage-resistant mutants (Fig. 5B). In the parallel experiment with fluorescently labelled KVP40 phages, the phages were immobilized in the aggregates and did not attach to the cell surface (Fig. 5C and 5D).
Discussion

**Phage effects on micro-colony formation and initial biofilm development**

The microscopic observations of micro-colony formation of cells attached to a polycarbonate filter surface revealed that the two strains formed highly different types of micro-colonies, with large implications for the phage-host interactions. The phage-resistant isolates obtained from BA35 cultures exposed to $\Phi H20$ maintained resistance upon re-culturing, suggesting that resistance was a result of genetic mutations which prevented phage infection. In contrast to the strong lytic effect of $\Phi H20$ and corresponding reduction in micro-colony formation of strain BA35, phage KVP40 did not significantly affect the size of individual PF430-3 micro-colonies. A possible explanation could be that the complex 3-dimensional structure of PF430-3 micro-colonies provided protection from phage infection by creating physical barriers, once formed.

**Long-term effects of phages on biofilm formation from liquid cultures of different V. anguillarum strains**

The strong controlling effects of phage $\Phi H20$ on the strain BA35 biofilm observed both in the pre-treated and post-treated conditions supported the microscopic observations that the BA35 biofilm is a relatively simple structure which is easily accessible for phage adsorption. Also in the free-living phase, phage $\Phi H20$ was able to control the phage susceptible BA35 population, but at these conditions, phage-resistant mutants replaced the sensitive population within the first 24 h. The interactions between $\Phi H20$ and strain BA35 thus seemed very similar for attached and free-living cells, suggesting that the flat and smooth biofilm structure did not offer protection against phage infection.

In contrast, addition of phage KVP40 to strain PF430-3 resulted in an enhanced biofilm formation in both experiments, and particularly during the early stage of biofilm development. At the same time, the free-living bacterial population was efficiently controlled and maintained at an OD level of $<25\%$
of the maximum density in the control cultures, in both the pre-treated and post-treated PF430-3 experiments. This suggested a coupling between the phage-mediated decrease in density of free-living cells and the corresponding cell aggregate formation and subsequent increase in biofilm formation.

**Potential mechanisms of phage protection in *V. anguillarum* strains**

While the dominance of phage resistant strains following phage exposure suggested mutational changes as the mechanism of phage protection in strain BA35, the interaction between strain PF430-3 and phage KVP40 pointed towards aggregation and biofilm formation as a protection against phage infection. This mechanism of protection was supported by a number of observations: 1), addition of fluorescently labelled KVP40 phage to aggregates and free-living cells of PF430-3 showed that KVP40 was trapped in the aggregate matrix and did not adsorb to the host cells embedded in the aggregates; 2), the complex 3-dimensional structure of the micro-colonies observed for PF430-3 was also consistent with the hypothesis that the aggregation provided a refuge for phage-sensitive bacteria (26-28); and 3), the presence of a fraction of free-living phage-sensitive cells outside the aggregates may in part be explained by the entrapment of phages in the aggregates which reduced the phage encounter rate, possibly allowing co-existence of small populations of free-living phages and free-living sensitive cells.

In combination with the reduced phage-sensitivity found in a number of the isolates, the immobilization of free-living cells in aggregates and subsequent attachment to surfaces provides an efficient refuge for cells exposed to phage attack, thus adding to the multiple and complex protection mechanisms found in bacteria (14, 29, 30). Whether the aggregation is a direct response to phage exposure, e.g. through quorum sensing (QS)-mediated gene regulation which is well known from *V. anguillarum* (31-33), or is an indirect effect of phage-lysis of free-living cells, thus adding a strong positive selection pressure for aggregate-forming phenotypes needs to be elucidated (30).
It should be noted that successful phage amplification depends not only on the host cell lysis, but also on the phages able to bind to the susceptible bacterium. Polysaccharide depolymerases have been reported in various phages, which allow phages to access phage receptors in biofilm-associated cells (34). Consequently, the generality of protection mechanisms observed in the present study is unknown, as other phages may be able to infect the biofilm-forming strain PF430-3. It is often assumed that bacteriophage resistance is associated with a cost (35, 36) due to reduced abilities to take up specific nutrients (37) and reduced competitive abilities in general (38, 39). The BIOLOG fingerprint of resistant isolates of strain BA35 supported such a link between phage-resistance and reduced physiological performance, as the resistant mutants had a reduced ability to utilize a number of substrates. The absence of observed physiological changes in the phage-exposed isolates of strain PF430-3 relative to the wild-type strain, on the other hand supported the suggestion that phage tolerance in strain PF430-3 was not caused by mutational changes but rather was due to protection by aggregation. It should be emphasized however, other fitness costs than those resolved by the BIOLOG assay may have been present in strain PF430-3.

Interestingly, the free-living isolates obtained from KVP40-amended PF430-3 cultures, which had reduced phage susceptibility, all recovered full sensitivity after re-growth in the absence phage KVP40. This indicated that the phage tolerance was a transient phenotype, as has also been observed in other phage infection studies including hosts such as *V. cholerae* (40), *S. enterica* (41), *S. oranienburg* (42), and *E. coli* O157:H7 (41, 43), and suggests that down-regulation of phage receptor gene expression may also play a role in phage susceptibility in strain PF430-3 (44).

In conclusion, our data demonstrated completely different mechanisms of protection against phages in two strains of *V. anguillarum* and showed that these intraspecific differences strongly influenced the outcome of the phage-host interactions with respect to 1) the ability of phages to control free-living host populations; 2) the ability of phages to control the development and stability of biofilm; 3) the phage-driven diversification of bacterial populations; and 4) the co-existence and co-evolution of
Implications for phage-control of the pathogen

In a phage therapy context, the interaction between strain BA35 and phage ΦH20 suggests that phage control of strain BA35 potentially would prove useful, since the phage efficiently inhibited biofilm formation both on short and longer time scale as well as proved efficient in disrupting already established biofilms. The phage-derived stimulation of biofilm formation in strain PF430-3 may, on the other hand, suggests that phage KVP40 would be inefficient in controlling this strain in aquaculture, and perhaps rather stimulate its pathogenic impact by inducing biofilm formation.

Our study thus confirms that successful application of phage therapy in the treatment of biofilm requires detailed understanding of phage-host interactions, and emphasizes that the complexity and diversity of phage-host interactions even within the same species of pathogen is a challenge for future use of phages in disease control.

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References


Figure Legends

Figure 1. Fluorescent microscopic images of micro-colony formation of strain BA35 (left two columns) and PF430-3 (right two columns) at different time points in the absence and presence of phage ΦH20 and KVP40, respectively. Samples were stained with 0.5% SYBR Gold for 10 min.

Figure 2. Culture density and biofilm formation of *V. anguillarum* strain BA35 and PF430-3 in cultures pre-treated with phage ΦH20 and KVP40, respectively, and control cultures. A) Optical density (OD<sub>600nm</sub>, left y-axis) of free-living cells (strain BA35) in the presence and absence of phage ΦH20, and the corresponding free-living phage ΦH20 concentration (PFU ml<sup>-1</sup>, right y-axis); B) Strain BA35 biofilm formation in the presence and absence of phage ΦH20, quantified by crystal violet (OD<sub>595nm</sub>, left y-axis); C) Optical density (OD<sub>600nm</sub>, left y-axis) of free-living cells (strain PF430-3) in the presence and absence of phage KVP40 and the corresponding free-living phage KVP40 concentration of (PFU ml<sup>-1</sup>, right y-axis); and D) Strain PF430-3 biofilm formation in the presence and absence of phage KVP40, quantified by crystal violet (OD<sub>595nm</sub>, left y-axis). Error bars represent the actual ranges from all experiments carried out in duplicate.

Figure 3. Culture density and biofilm formation of *V. anguillarum* strain BA35 and PF430-3 in cultures post-treated with phage ΦH20 and KVP40 respectively, and control cultures. A) Optical density of free-living cells (strain BA35) (OD<sub>600nm</sub>, left y-axis) in the presence and absence of phage ΦH20, and the corresponding phage free-living ΦH20 concentration of (PFU ml<sup>-1</sup>); B) Strain BA35 biofilm formation in the presence and absence of phage ΦH20, quantified by crystal violet (OD<sub>595nm</sub>, left y-axis) and corresponding biofilm-associated phage concentration (PFU ml<sup>-1</sup>, right y-axis); C) Optical density (OD<sub>600nm</sub>, left y-axis) of free-living cells (strain PF430-3) in the presence and absence of phage KVP40, and the corresponding free-living phage KVP40 concentration of (PFU ml<sup>-1</sup>, right y-axis); and D) Strain PF430-3 biofilm formation in the presence and absence of phage KVP40, quantified by crystal violet (OD<sub>595nm</sub>, left y-axis) and the corresponding biofilm-associated phage concentration of (PFU ml<sup>-1</sup>, right y-axis). Error bars represent the actual ranges from all experiments carried out in duplicate.

Figure 4. Fluorescence microscopic examination of aggregate formation in *V. anguillarum* strains BA35 and PF430-3 in the presence and absence of phages. No aggregate formation was observed in BA35 and BA35+ΦH20 cultures, and phage addition reduced the concentration of free-living cells relative to the control. In the PF430+KVP40 cultures, phage-induced aggregates were observed.

Figure 5. Visualization of phage adsorption by SYBR Gold-labelled phages under phase-contrast epifluorescence microscope. A, wild-type BA35 cells with SYBR Gold-labelled phage ΦH20 (phase-contrast+fluorescence); B, BA35 phage lysate with SYBR Gold-labelled phage ΦH20 (phase-contrast+fluorescence); C, PF430-3 phage lysate with SYBR Gold-labelled phage KVP40 (phase-contrast); and D, PF430-3 phage lysate with SYBR Gold-labelled phage KVP40 (phase-contrast+fluorescence).
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Table S1. Susceptibility of isolates of *V. anguillarum* strains BA35 and PF430-3 to phages ΦH20 and KVP40, respectively, obtained from microlony experiment, pre-treated free-living culture experiment and biofilm experiment. ND, not done.

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Supplementary Figure S1. Carbon substrate utilization of different *V. anguillarum* strains (BA35 and PF430-3). BA35 WT, BA35 wild-type; BA35 R, BA35 resistant isolate; PF430-3 WT, PF430-3 wild-type; PF430-3 S, PF430-3 Sensitive isolate; and PF430-3 L, PF430-3 bacteria and bacteriophage lysate.

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Quorum sensing determines the choice of anti-phage defense strategy in *Vibrio anguillarum*

Demeng Tan\textsuperscript{a}, Sine Lo Svenningsen\textsuperscript{b}, Mathias Middelboe\textsuperscript{a}\textsuperscript{*}

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Abstract

Selection for phage resistance is a key driver of bacterial diversity and evolution, and phage-host interactions may therefore have strong influence on the genetic and functional dynamics of bacterial communities. In this study, we found that an important, but so far largely overlooked, determinant of the outcome of phage-bacterial encounters in the fish pathogen Vibrio anguillarum is bacterial cell-cell communication, known as quorum sensing. Specifically, V. anguillarum PF430-3 cells locked in the low-cell-density state (ΔvanT mutant) express high levels of the phage receptor OmpK; resulting in a high susceptibility to phage KVP40, but achieve protection from infection by enhanced biofilm formation. By contrast, cells locked in the high-cell-density state (ΔvanO mutant) are almost completely unsusceptible due to quorum-sensing-mediated down-regulation of OmpK expression. The phenotypes of the two quorum sensing mutant strains are accurately reflected in the behavior of wild-type V. anguillarum, which (i) displays increased OmpK expression in aggregated cells compared to free-living variants in the same culture, (ii) displays a clear inverse correlation between ompK mRNA levels and the concentration of N-acyl homoserine lactone quorum-sensing signals in the culture medium, and (iii) survive mainly by one of these two defense mechanisms, rather than by genetic mutation to phage resistance. Taken together, our results demonstrate that V. anguillarum employs quorum sensing information to choose between two complementary anti-phage defense strategies. Further, the prevalence of non-mutational defense mechanisms in strain PF430-3 suggests highly flexible adaptations to KVP40 phage infection pressure, possibly allowing the long-term coexistence of phage and host.
Importance

Comprehensive knowledge on bacterial anti-phage strategies and their regulation is essential for understanding the role of phages as drivers of bacterial evolution and diversity. In an applied context, development of successful phage-based control of bacterial pathogens also requires detailed understanding of the mechanisms of phage protection in pathogenic bacteria. Here we demonstrate for the first time the presence of QS-regulated phage defense mechanisms in the fish pathogen *V. anguillarum* and provide evidence that QS regulation allows *V. anguillarum* to alternate between different phage protection mechanisms depending on population cell density. Further, our results demonstrate the prevalence of non-mutational defense mechanisms in the investigated *V. anguillarum* strain, which allow flexible adaptations to a dynamic phage infection pressure.
**Introduction**

*Vibrio anguillarum* is a marine pathogenic bacterium which causes vibriosis in numerous fish and shellfish species leading to high mortalities and economic losses in aquaculture worldwide (1). The use of bacteriophages to control bacterial infections in aquaculture has gained increased attention in the past years and successful application of phages to reduce vibriosis-related mortality has been demonstrated (2). Development of a phage-based treatment is, however, challenged by the wide variety of anti-phage defense strategies observed in bacterial hosts (3). There is clear evidence that genetic mutation, normally causing disruption or modification of phage receptors in the host membrane, plays an important role in preventing phage infection in some cases (4-8). Such genetic changes may impose a fitness cost to the host cell as disruption of phage receptors can reduce the uptake of certain substrates (9-11). Alternative defense mechanisms which do not involve mutational changes have been described and also play a role in *V. anguillarum* (Tan et al., unpublished). Aggregate formation and production of exopolysaccharides was suggested to provide protection against infection in *V. anguillarum* strain PF430-3 (7, Tan et al., unpublished). Recently, a more flexible protection mechanism was discovered in *Escherichia coli*, involving a temporary down-regulation of phage receptor production in response to N-acyl-L-homoserine lactone (AHL) cell-cell signaling molecules (12). This mechanism is controlled by quorum sensing (QS), i.e., the ability of bacteria to regulate gene expression according to population density via the production and subsequent detection of extracellular signaling molecules (13).

Little is still known about the mechanisms of phage protection in natural *Vibrio* communities. The universal outer membrane protein K (OmpK) has previously been shown to be the infection site for vibriophage KVP40, which infects more than 8 *Vibrio* species, including *V. anguillarum*, *V. parahaemolyticus*, *V. harveyi* and *V. cholerae* (14, 15). Mutations in this protein have been reported in *V. parahaemolyticus* R4000 upon exposure to KVP40 (14), but were not detected in KVP40-amended cultures of *V. anguillarum* PF430-3 (7, Tan et al., unpublished). Thus, other mechanisms for preventing infection by KVP40 in the *Vibrio* community may also be prevalent.

AHL-mediated QS circuits have been identified in many *Vibrio* species including *V. anguillarum* (13, 16, 17). *V. anguillarum* controls QS-regulated genes via the transcription factor VanT, which is activated in response to extracellular signaling molecules (18, 19). At low autoinducer
concentrations (i.e. low cell density), the response regulator VanO becomes activated by phosphorylation, and represses the expression of VanT. At high cell densities, autoinducer concentrations increase and bind to membrane-bound receptors (18, 19). At a certain threshold concentration, VanO is dephosphorylated, and VanT expression is induced, allowing gene regulation within the QS regulon (18, 19). Several N-Acyl homoserine lactone autoinducers have been identified in stationary-phase *V. anguillarum* spent culture supernatant (20).

As densities of bacterial populations vary from sparsely populated environments to highly dense populations in nutrient-rich environments, and phages require a bacterial host in order to multiply, phage predation pressure is not constant and may be expected to correlate with the density of the bacterial host population, among other factors. Thus, bacteria could potentially benefit from altering their anti-phage strategies depending on the perceived population density, thereby minimizing the metabolic burden often associated with resistance by genetic mutation (3, 9, 21, 22).

In this study, we identified a potent anti-phage defense mechanism in *V. anguillarum*, and show that different anti-phage strategies prevail at different population densities. At high cell density conditions, QS-mediated down-regulation of the OmpK receptor reduced phage adsorption and rendered individual cells almost unsusceptible to phage infection. At low cell density conditions, on the other hand, OmpK expression was unaffected by QS and the individual cells were fully susceptible to infection. However, at these conditions, we have shown in a previous study that aggregation of the cells prevents phage from reaching the OmpK receptor (7, Tan et al., unpublished). In neither case was phage protection associated with *ompK* mutation. Overall, the study shows that QS controls the choice of anti-phage defense strategy in the examined *V. anguillarum* strain PF430-3 suggesting the presence of dynamic, temporary adaptations to phage infection pressure, while still securing the ability to produce a functional OmpK receptor. In a phage therapy context, these results are highly relevant, as a combination of phage-based and anti-QS targeted treatments could enhance the efficiency of phage control of vibriosis.

**Results**

We have recently demonstrated that addition of phage KVP40 to *V. anguillarum* strain PF430-3 resulted in increased cell aggregation and biofilm formation, which provided protection against
phage infection (7, Tan et al., unpublished). However, at high cell densities, detachment of cells from aggregates into free-living variants were observed, which co-existed with phages (Tan et al., unpublished), suggesting that alternative defense mechanisms also played a role under these conditions. Isolation and subsequent re-culturing of these free-living cells in the absence of KVP40 showed that the re-cultured cells had regained sensitivity to phage KVP40 (7), suggesting that the initial protection was not caused by mutational changes but rather a temporary protection mechanism. To test if an extracellular factor might be involved in the regulation of the phage-induced aggregation phenotype, we examined the effect of adding cell-free spent culture fluid from high-cell-density cultures of strain PF430-3 to freshly inoculated cultures of the same strain in the presence or absence of phage KVP40 by phase-contrast microscopy (Fig. 1, left panels). Intriguingly, the phage-induced aggregation phenotype of PF430-3 was completely inhibited by the presence of the cell-free spent culture fluid, suggesting that an extracellular factor(s), possibly a QS signaling molecule, is involved in the regulation of phage defense in *V. anguillarum* PF430-3.

**Synthetic AHL-induced down-regulation of phage production**

*V. anguillarum* have been shown to produce at least three different AHL QS autoinducers, namely *N*-\((3\text{-hydroxyhexanoyl})\) homoserine lactone, *N*-\((3\text{-oxodecanoyl})\) homoserine lactone, and *N*-hexanoylhomoserine lactone (20). To further evaluate the role of QS for phage-host interactions in *V. anguillarum*, the effect of synthetic AHL addition on phage KVP40 production was quantified in strain PF430-3 (Fig. S2). The results showed that the presence of synthetic AHL autoinducers in the medium reduced phage KVP40 production by 1.5 to 2-fold after 2 h relative to control cultures without AHL addition (Fig. S2, squares). In parallel cultures grown without phage addition, cell growth was unaffected by AHL addition (Fig. S2, circles).

**KVP40 efficiency of plating on QS mutants of PF430-3**

In order to examine the effects of QS on the interaction between phage KVP40 and *V. anguillarum* PF430-3, two otherwise isogenic QS mutants were constructed which represent cell behavior at high (\(\Delta\text{vanO}\)) or low (\(\Delta\text{vanT}\)) cell densities, respectively. Since the phosphorylated VanO protein represses the QS regulator VanT at low cell densities, \(\Delta\text{vanO}\) displays full expression of VanT at all densities, and is therefore locked in a high-cell-density phenotype (18). By contrast, a \(\Delta\text{vanT}\) mutant has lost the ability to regulate QS-associated functions and is therefore locked in a low-
cell-density phenotype. Use of the two extreme QS-phenotypes of ΔvanT and ΔvanO mutants in the current study allowed us to explore the role of QS-mediated phage protection in V. anguillarum PF430-3. We first tested the infectivity of phage KVP40 on the wild-type V. anguillarum PF430-3 strain, and the constructed mutants ΔvanT, ΔvanO, ΔompK, ΔvanT ΔompK, and ΔvanO ΔompK by examining the efficiency of plating (EOP, the number of plaques obtained on each host from a given phage input). Relative to the wild-type strain, the phage susceptibility of the ΔvanT mutant had increased by 53% (p < 0.05, n=2), and the plaque morphology had changed from turbid to clear (Fig. S3A). Additionally, the ΔvanO mutant had become less susceptible to phage infection with a reduction of 37%, (p<0.01, n=2) relative to wild-type (Fig. S3B). As expected, ΔompK, ΔvanT ΔompK, and ΔvanO ΔompK had all become resistant to KVP40 confirming that OmpK is the phage receptor (Fig. S3A).

Phage-host interactions in liquid cultures

Further assessments of QS-mediated regulation of phage infectivity in 10 h infection experiments with phage KVP40 and 3 hosts (wild-type, ΔvanT, and ΔvanO), showed strong effects of the mutational changes on phage-host interactions. Phage KVP40 reduced cell density in the wild-type and ΔvanT cultures within 10 h incubation by 60% and 75%, respectively, relative to the OD of control cultures without phages, with significantly lower OD values in the ΔvanT culture than in the wild-type culture (0.62 and 0.88 after 10 h, respectively) (p<0.01, n=2; Fig. 2A). In the ΔvanO culture, on the other hand, OD was not significantly affected by phage addition (Fig. 2A). In accordance with these results, we observed rapid phage propagation in the wild-type and ΔvanT cultures where KVP40 abundance stabilized at ~10^{10} PFU ml^{-1}, whereas the phage production was ~100-fold lower in the ΔvanO culture (Fig. 2B). We note that the minimum OD values of 0.33 and 0.15 after 5 h in the phage-treated wild-type and ΔvanT cultures, respectively, was followed by a regrowth of cells during the remainder of the incubation (Fig. 2A), despite the abundance of KVP40 phage.

Aggregation of cells in the two QS mutants following phage KVP40 exposure was assessed by phase-contrast microscopy (Fig. 1, center and right panels; see also Fig. S1). Similar to the wild-type shown in Figure 1, addition of phages led to increased aggregation in the ΔvanT mutant cultures. In fact, at 24 h most cells occurred in large aggregates in the ΔvanT mutant cultures,
while a fraction of free-living cells was still detectable in the wild-type culture (Fig. 1 and Fig. S1). In the ΔvanO culture, on the other hand, phage addition did not result in formation of aggregates (Fig. 1 and Fig. S1), suggesting that the QS pathway is involved in the regulation of the phage-induced aggregation phenotype. Furthermore, the addition of cell-free spent culture fluid, which prevented aggregation of the wild-type culture, did not prevent aggregation of the ΔvanT mutant cultures upon exposure to phage, suggesting that the inhibitory factor present in the cell-free culture fluid prevents aggregation via the QS pathway (Fig. 1). However, addition of the synthetic AHL autoinducers did not mimic the inhibitory effect of adding cell-free spent culture fluid on phage-induced aggregation in wild-type PF430-3 (data not shown).

**Effects of phage KVP40 and QS on biofilm formation**

Based on the indications of phage-driven stimulation of biofilm formation in the short term liquid culture experiments, a more systematic quantification of the effects of phage KVP40 on biofilm formation in the three strains was performed. In the control cultures without phages, the biofilm formation of the wild-type and ΔvanT strains increased for 5 days and then remained constant at a level of OD_{595nm} = 1.5-1.75, as measured by crystal violet staining of the biofilm. In ΔvanO cultures, the biofilm developed at a slower rate but reached the same end point as the other strains after 8 days (Fig. S4A). Pretreatment of the cultures with KVP40 enhanced biofilm formation in all 3 strains. The OD_{595nm} in wild-type and ΔvanT cultures reached values of 2.8 after 3 days, whereas OD_{595nm} in the KVP40-treated ΔvanO cultures increased gradually during the incubation to a maximum of 2.2 after 8 days (Fig. S4A). Phage concentration in the liquid above the biofilm increased rapidly and stabilized around 10^9 PFU ml^{-1} in both wild-type and ΔvanT cultures after 1 day. In the ΔvanO+KVP40 cultures, phage concentration increased at a slower pace until day 3, where it also stabilized at ~10^9 PFU ml^{-1} (Fig. S4B).

Together, the results from these experiments demonstrate a reduced susceptibility to phage KVP40 in the ΔvanO mutant despite the fact that this mutant aggregates less and forms biofilm more slowly than the wild type, suggesting that *V. anguillarum* PF430-3 may obtain protection against KVP40 via a separate mechanism in the high-cell-density QS mode mimicked by the ΔvanO mutant.

**Phage adsorption rate**
To address the question of how ΔvanO mutant cells reduce their susceptibility to KVP40, we first measured the rate of adsorption of KVP40 to the wild-type and QS mutants host cells. The adsorption rate of wild-type, ΔvanT, and ΔvanO was calculated as $6.7 \times 10^{-10} \pm 8.49 \times 10^{-11}$ ml$^{-1}$ min$^{-1}$, $8.1 \times 10^{-10} \pm 9.2 \times 10^{-11}$ ml$^{-1}$ min$^{-1}$, and $3.8 \times 10^{-10} \pm 1.4 \times 10^{-11}$ ml$^{-1}$ min$^{-1}$, respectively. Thus, the rate of adsorption of phage KVP40 to ΔvanO was significantly lower than that observed for the wild-type ($p<0.04$, n=2) and ΔvanT ($p<0.02$, n=2) strains.

**ompK expression in cultures of wild type and QS mutant cells**

To further examine the mechanism underlying the differences in adsorption rates among wild-type and QS mutants (ΔvanT and ΔvanO), and to link these differences to QS regulation, we quantified the expression of phage KVP40 receptor ompK in 9 different cultures (wild-type, ΔvanT, ΔvanO, wild-type+KVP40, ΔvanT+KVP40, ΔvanO+KVP40, ΔompK, ΔvanT ΔompK, and ΔvanO ΔompK) by quantitative real-time PCR (qPCR). The relative ompK expression levels of wild-type and ΔvanT strains were approximately 4 times higher than the relative ompK expression in the ΔvanO strain suggesting a down-regulation of ompK, when the cells are locked in the regulatory state mimicking high cell densities (Fig. 3A). Purification and separation of outer membrane proteins by SDS-PAGE (Fig. 3B) using three ompK mutants (ΔompK, ΔvanT ΔompK, and ΔvanO ΔompK) as negative controls confirmed the presence of the OmpK receptor (26 kDa) in wild-type and ΔvanT strains and the QS-mediated OmpK receptor down-regulation in the ΔvanO strain (14). In agreement with the gene expression data, OmpK appeared to be slightly more abundant in the ΔvanT strain than in the wild-type strain. SDS-PAGE outer membrane protein analysis also demonstrated that other outer membrane proteins than OmpK, which potentially function as phage receptors, were down regulated in the QS mutant ΔvanO as well (Fig. 3B, OmpK-indicated by arrowhead).

**ompK mRNA levels in the free-living and aggregated cell fractions**

We have shown above that the ΔvanT mutant displays increased aggregation, and contains high levels of ompK, whereas the ΔvanO mutant displays very little aggregation, but instead reduces its susceptibility to phage KVP40 by expressing low levels of OmpK. Next, we verified whether these two phenotypes are also correlated in the wild-type PF430-3 strain. We fractionated cultures (wild-type+KVP40, ΔvanT+KVP40 and ΔvanO+KVP40) into a free-living and an aggregate fraction by centrifugation, and compared relative ompK mRNA levels in the two fractions using qPCR (Fig.
The analysis revealed that *ompK* gene expression varied significantly between aggregated and free-living cells in the wild-type strain, with approximately 2-fold higher *ompK* mRNA levels in the bacteria located in aggregates than in the fraction containing free-living cells (Fig. 4). Moreover, it should be noted that the applied centrifugation procedure does not result in complete separation of the aggregates from the free-living cells; hence the difference in *ompK* mRNA levels between the two fractions is likely underestimated. Interestingly, *ompK* mRNA levels were also higher in the aggregated fraction of the ΔvanO mutant cells than in the corresponding free-living fraction, although the *ompK* levels in both fractions were still much reduced compared to the wild-type or ΔvanT cultures. Thus, even in the absence of a functional QS pathway, free-living cells express less *ompK* mRNA than aggregated cells, which could either reflect the existence of a redundant VanO-independent regulatory mechanism, or simply reflect a phage-mediated enrichment of cells with low *ompK* mRNA expression among the surviving free-living cells. Unfortunately, we were unable to collect free-living cells from ΔvanT cultures grown in the presence of phage, as any free-living cells were lysed by the phage and the remaining cells were all aggregated. However, in agreement with the data shown in Figure 3A (*ompK* mRNA levels in the presence of phage KVP40), *ompK* mRNA levels were up-regulated in the ΔvanT aggregates even compared to the wild-type aggregated fraction.

**Temporal changes in *ompK* expression and AHLs production in wild type *V. anguillarum* PF430-3**

In order to determine whether *ompK* expression was in fact repressed by a cell density-dependent factor secreted by the *V. anguillarum* PF430-3 wild-type strain, simultaneous measurements of *ompK* expression and extracellular AHL levels were performed during growth of the wild-type strain in batch culture in the absence of phages. Interestingly, phage receptor *ompK* expression and AHL levels were significantly negatively correlated (r=0.811, \( p \leq 0.001 \), n=2) during the first 12 h. Thus, in accordance with the previous experiment, *ompK* expression was 3-4 times higher at the low cell density around 2 h, than at high-cell density at 6 h (Fig. 5). AHL production reached a maximum at 6 h and decreased after 8 h, concomitant with an increase in *ompK* expression. After 16 h a sharp decline in *ompK* expression was observed.

**Examination of potential fitness costs in ΔompK mutants**
Throughout our studies of the interaction of phage KVP40 with *V. anguillarum* strain PF430-3, we did not once observe a single case of resistance to phage infection due to mutation of *ompK*. By contrast, cells that survived exposure to phage KVP40 always multiplied to produce offspring that had regained phage sensitivity (7, Tan et al., unpublished). Therefore, we speculate that mutation of *ompK* may be associated with a significant fitness cost. The physiological role of OmpK is not known, but BIOLOG GN2 physiological fingerprints of the wild-type strain and the Δ*ompK* mutant showed that the Δ*ompK* mutant lost the ability to utilize glucose-1-phosphate, glucose-6-phosphate, cis-aconitic acid, L-alaninamide, and L-alanine as growth substrates, suggesting that indeed OmpK may play an important role for *V. anguillarum* metabolism (Fig. S5). We were, however, unable to detect a change in the relative abundance of the Δ*ompK* mutant compared to wild-type after 8h of growth in mixed culture. Thus, loss of the OmpK receptor does not directly affect the competitive ability of strain PF430-3 under our experimental conditions (Fig. S6).

**Discussion**

Given the recent findings that bacteria may employ QS to reduce phage receptor expression during conditions of high infection risk (12), we aimed at exploring the role of QS in regulating susceptibility to the broad-host-range phage KVP40 in *V. anguillarum* strain PF430-3 through down-regulation of the phage receptor OmpK. The reduced susceptibility to phage KVP40 in *V. anguillarum* strain PF430-3 after addition of cell-free supernatant or synthetic AHLS, provided the first indications of a QS-regulated mechanism of phage protection. This was further supported by our subsequent studies of phage susceptibility in the two constructed *V. anguillarum* QS mutants, Δ*vanT* and Δ*vanO*. These results showed that phage susceptibility and adsorption was reduced in the Δ*vanO* and enhanced in Δ*vanT* relative to the wild type, directly confirming that QS played a key role in the protection against KVP40 infection. The >100–fold higher phage production and 3-fold lower bacterial density in cultures of Δ*vanT* relative to Δ*vanO* after phage addition thus emphasized that QS signaling in the high-cell-density phenotype mediated an efficient protection against phage infection.

Measurements of the *ompK* expression further resolved the underlying mechanisms of QS-mediated phage protection. Thus, the significant (4-fold) reduction in *ompK* expression in the Δ*vanO* and the verification that an OmpK receptor deficient mutant (Δ*ompK*) was resistant to
phage KVP40 provided direct evidence for QS-mediated down-regulation of *ompK* expression as an important mechanism for protection against phage infection in *V. anguillarum*.

Interestingly, our results also showed that down-regulation of *ompK* expression was not the only QS-regulated phage defense mechanism in the investigated *V. anguillarum* strain. Cell aggregation and formation of a biofilm in response to phage addition in the wild-type strain suggested that the transformation from a free-living life form to growth in a biofilm was also a mechanism of protection against phage infection, as also supported by a previous study (Tan et al., unpublished). The exact mechanism by which phages induce cell aggregation, however, remains to be discovered.

While the production of extracellular polymers by the host to create a physical barrier against phage infection has been suggested previously (3, 23), the current results suggest that this mechanism is QS-controlled in *V. anguillarum*. Addition of phage KVP40 strongly stimulated cell aggregation and biofilm formation in the wild-type and low-cell-density mutant (Δ*vanT*), whereas no aggregation was observed in the high-cell-density mutant (Δ*vanO*). Hence the data suggest that cell aggregation and biofilm formation is an important phage defense mechanism at low cell densities where QS regulation of *ompK* expression is inefficient. Consequently, we show that *V. anguillarum* PF430-3 alternates between these two types of phage defense mechanisms and that QS regulates the choice of strategy by up-regulating one mechanism (removal of OmpK receptor) while down-regulating another mechanism (cell aggregation) at high cell densities. Whether the overall reduction in OmpK receptors at high-cell-density conditions results in a general decrease in the number of receptors per cell in the population, thus reducing the encounter rate between phages and OmpK receptors, and/or if OmpK down-regulation generates a fraction of cells completely without receptors (i.e. resistant cells) is not known. However, the presence of turbid plaques in the Δ*vanO* mutant upon exposure to KVP40 in spot assays indicated that some of the cells were, in fact, temporarily fully resistant to the phage. The presence of two distinct phage defense mechanisms in wild-type cells was confirmed by the observation that *ompK* expression was reduced in free-living cells relative to cells embedded in aggregates. Together with the significant negative correlation between extracellular AHL concentration and *ompK* expression during growth of the wild-type strain in batch cultures, this demonstrated the dynamic nature of phage defense and emphasized that the obtained results were not restricted to the extreme phenotypes of the QS mutants. Interestingly, *ompK* expression was in fact enhanced in phage-
amended wild-type and $\Delta$vanT cultures relative to control cultures without phages. OmpK is a porin-like protein which has been suggested to be involved in bile salt resistance, as well as iron acquisition (24). We show here that OmpK also plays a role for the ability of V. anguillarum to use glucose-1-phosphate, glucose-6-phosphate, cis-aconitic acid, L-alaninamide, and L-alanine as growth substrates. We speculate therefore that up-regulation of ompK in aggregated cells may be an adaptation to stimulate nutrient uptake in an aggregate environment where supply of specific nutrients may be limited, or for enhancing the bile salt resistance of the cell aggregates inside the host. However, in the short-term competition experiment we carried out, the $\Delta$ompK mutant was able to reach the same density as the wild-type strain in a mixed culture. Further studies are needed to determine the physiological role(s) of OmpK and hence the cost of its repression or loss.

We note that in the wild-type cultures of V. anguillarum PF430-3, AHLs accumulate as the culture grows to high cell densities, but they largely disappear later in stationary phase (Fig. 5). This phenomenon has been observed previously in cultures of Yersinia pseudotuberculosis and Pseudomonas aeruginosa, grown in LB medium, and was found to be caused by pH-dependent lactonolysis (25). We found, however, that the pH of the marine broth cultures used here remained stable around 7.6 for at least 13 hours (data not shown). Alternative explanations for the disappearance of the AHLs in stationary phase are the possible production of an AHL lactonase enzyme as reported in Bacillus spp. (26) or the uptake of AHLs for use as a source of energy, carbon and nitrogen, as reported for Variovorax paradoxus (27). Additional studies are required to determine the mechanism responsible for AHL removal in V. anguillarum strain PF430-3.

One surprising observation from the current study was that QS reduced biofilm formation (Fig. S4). Biofilm formation is in many bacterial pathogens found to be up-regulated by QS and stimulated at high cell density promoting virulence (28). This has also been observed in V. anguillarum (strain NB10), where a low-cell-density mutant ($\Delta$vanT) showed significantly lower biofilm formation than the wild-type strain (29). The reason for this contrasting effect of QS on biofilm formation in PF430-3 is not clear. V. anguillarum strain NB10 showed limited susceptibility to phage KVP40 and produced turbid plaques (data not shown), suggesting that KVP40 is not an important predator on that strain; hence other anti-phage strategies may be favored. VanT expression is known to regulate physiological responses required for survival and stress response (18), and in the
investigated strain PF430-3, this seems to include shifting from aggregation to ompK down regulation in response to increased cell density. In addition, since total protease activity correlates with VanT expression in *V. anguillarum* strain NB10 (29), it may be speculated that at high cell densities, when VanT is fully expressed, VanT activates protease activity to promote the release of cells from aggregates, in a manner similar to the action of the VanT homologue HapR in *V. cholerae*, where QS also down-regulates biofilm formation (30, 31). By QS-mediated ompK gene regulation, aggregate-associated bacteria may thus have evolved a mechanism of detaching a subpopulation of free-living cells with reduced phage susceptibility which can survive in phage dense environments, thus allowing further spreading of vibriosis infections. In any case, the results support previous indications of large differences in phage defense strategies among different strains of *V. anguillarum* (Tan et al., unpublished) and emphasize the need for further exploring whether the mechanisms described here are a general phenomenon in bacteria or are limited to a subset of *V. anguillarum* strains.

Phages are known to be key drivers of bacterial evolution by selecting for phage-resistant mutants, and it has been proposed that phage-host interactions lead to either an arms race dynamics of antagonistic host and phage co-evolution, or a fluctuating selection dynamics involving frequency-dependent selection for rare host and phage genotypes (32, 33). In this study, however, we found evidence for an alternative to these scenarios, as the defense mechanisms identified do not involve mutational changes or complete elimination of phage susceptible host cells. The described anti-phage mechanisms in *V. anguillarum* thus represent more flexible adaptations to dynamic changes in phage and host densities, adding to the complexity of phage-host co-evolutionary interactions and phage-driven genetic and phenotypic changes in bacterial populations. Since mutational changes often result in a loss of fitness for the host (9, 10), the mechanisms described here represent a potential fitness advantage for the host. It may be speculated that the development of this temporary protection mechanism in *V. anguillarum* PF430-3 is related to the fact that the OmpK receptor is widely conserved among *Vibrio* and *Photobacterium* species, and thus probably executes an important function(s) in the cell. It is likely, therefore, that mutations in ompK could have significantly negative effects on host fitness in natural habitats, in which case development of alternative phage-defense strategies that leave the ompK gene intact would be of strong selective advantage. Further studies are needed, however, to confirm this hypothesis.
Successful application of phage therapy in the treatment of vibriosis requires detailed knowledge of the phage-host interactions and the regulation of anti-phage strategies in Vibrio. Our results add to the suite of known phage defense mechanisms and their regulation in marine bacteria and further emphasizes that the complexity of phage-host interactions pose a challenge for future use of phages in disease control. On the other hand, the evidence that QS regulates phage receptor expression may potentially be used actively by quenching QS signaling, hence preventing receptor down-regulation. In support of that, QS inhibitors have been shown to impede expression of virulence factors (34) and recently the addition of modified T7 phages producing quorum-quenching enzymes have resulted in inhibition of biofilm formation, suggesting that the use of QS-inhibitors may be a promising strategy in future antimicrobial therapy (35).

**Materials and Methods**

**Bacterial strains and bacteriophages**

The bacterial strain *V. anguillarum* PF430-3 was originally isolated from salmonid aquaculture in Chile (36), and phage KVP40 which infects PF430-3 is a broad-host-range phage originally isolated from Japan (37) (Table S1). Basic characterization of phage KVP40 and its interactions with PF430-3 has been provided recently (7). All the constructed mutant strains derived from *V. anguillarum* PF430-3 wild-type strain and plasmids used in this study are listed in supplementary Table S1. *E. coli* S17-1 (*λpir*) was used as the donor strain for transfer of plasmid DNA into *V. anguillarum* PF430-3 by conjugation (29, 38). Antibiotics were used at the following concentration: 100 μg ml⁻¹ ampicillin, 25 μg ml⁻¹ chloramphenicol (for *E. coli*), and 5 μg ml⁻¹ chloramphenicol (for *V. anguillarum*) (29, 38).

**DNA manipulation and mutant construction**

For mutant constructions, in-frame deletions were made in the vanT, vanO, and ompK genes by allelic exchange as first described by Milton and Croxatto (29, 38). The upstream DNA sequence of the vanT gene was amplified from wild-type *V. anguillarum* PF430-3 by PCR using primers vT1 and vT2, which introduced a BglII site. The downstream DNA sequence of vanT was amplified using primers vT3 and vT4, which introduced a ScaI site. These two fragments, which contain a 15-nt overlap of identical sequence, were used as template for a second PCR using primers vT1 and vT4.
The PCR product was digested with *BglII* and *SacI* and cloned into the *BglII* and *SacI* sites of pDM4, creating pDM4vanT. In-frame deletion of vanT in PF430-3 was confirmed by PCR using primers vT1 and vT4 and subsequent gel electrophoresis. To construct plasmid pDM4vanO and pDM4ompK, and vanO and ompK mutants, we used the same method described for vanT above. The primers are listed in supplementary Table S2.

**Examination of fitness loss in the ompK mutant**

In an attempt to search for potential functions of phage receptor OmpK and hence implications of its down regulation or loss, BIOLOG GN2 Micro-Plates (BIOLOG) containing 95 different carbon sources were used to test the ability of wild-type strain and ΔompK mutant to use different substrates following the manufacturer’s instructions. Further, the potential reduction in competitive abilities of the ΔompK mutant was examined in competition assays. Briefly, wild-type and ΔompK bacterial cells were mixed in a ratio of 1:1 in sterilized seawater and then quantified over time. Subsamples were collected every 1-2 h for 8 h and diluted and plated on LB plates. Subsequently, 20 colonies were picked from each time point and identified as either wild-type or ΔompK by PCR with Primers 1340 F and 1340R listed in Table S2.

**Effects of cell-free supernatant enrichment on KVP40 phage infection**

Cell-free supernatant from wild-type *V. anguillarum* PF430-3 cultures was prepared from a mid-log-phase culture of *V. anguillarum* PF430-3 in Marine Broth (MB), which was centrifuged (10,000×g for 10 min), sterile filtered (0.2 μm, Millipore) and subsequently added to freshly inoculated cultures of wild-type, ΔvanT and ΔvanO, with and without phage KVP40, for examination of the potential effects of *V. anguillarum* PF430-3-produced autoinducer molecules on the lytic effects of the phage KVP40. Images were obtained after 24 h incubation.

**Effects of synthetic AHLs on phage production in *V. anguillarum* PF430-3 wild type**

Synthetic AHLs (obtained from Sigma and Nottingham University), *N*-hexanoylhomoserine lactone (C6-HSL), *N*-(3-hydroxyhexanoyl)homoserine lactone (3-hydroxy-C6-HSL) and *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C10-HSL) were added to *V. anguillarum* PF430-3 to examine their effects on phage susceptibility. Briefly, the AHLs were dissolved in acidified ethyl acetate (0.1% v/v acetic acid), mixed and added to a glass test tube to a final concentration of 10
μM and placed at room temperature for complete evaporation of the solvent, as described previously (12, 39). For the control cultures without AHLs, test tubes containing only ethyl acetate were prepared in parallel. Overnight cultures of wild-type cells were pelleted and resuspended, and aliquots of 100 μl cell suspension were transferred to 5 ml 3% NaCl buffer in tubes containing AHLs or the solvent control, and incubated at 30 °C for 1 h. The phage susceptibility assay was then initiated by addition of phage KVP40 at an average phage input (API) of 1. Parallel control cultures without phages were also established. Samples were collected every hour for 7 h for quantification of possible effects of AHL on cell growth in the absence of phage (colony forming units, CFU) and on phage production (plaque forming units, PFU).

**Phage-host interactions and effects on cell aggregation in wild-type and QS-mutants**

The susceptibility of each strain (wild-type, ΔvanT, ΔvanO, ΔompK, ΔvanT ΔompK, and ΔvanO ΔompK) to phage KVP40 was gauged by the efficiency of plating. Briefly, a fixed quantity of phage obtained from the same phage stock (KVP40) was mixed with identically grown bacterial cells of each genotype, and the plaque forming units were quantified by plaque assay (40). The experiments were performed in triplicate.

The lytic potential of phage KVP40 against wild-type and QS-mutants (ΔvanT and ΔvanO) was tested at an API of 1 in duplicate 100 ml liquid MB cultures and parallel control cultures without phage. The effect of phage-mediated lysis on host cell density was monitored by regular OD 600nm measurement over the 10 h incubation. Phage concentration was quantified by plaque assay periodically (40).

For visual inspection of cell densities and aggregate formation, additional aliquots were collected at 24 h, and visualized by phase-contrast microscopy using an oil immersion objective (Olympus BX61). The images shown in Figure 1 and Figure S1 represent random fields on the microscope slide from the 24 h samples.

**Effects of phage addition on biofilm formation in wild-type and QS-mutants**

Biofilm formation was monitored in wild-type, ΔvanT, and ΔvanO cultures following addition of phage KVP40 using methods described previously (41) with some modifications. Briefly, 13 ml polypropylene plastic tubes filled with 5 ml MB were inoculated with 100 μl overnight bacterial
inoculum and 100 μl phage stock (10^6 CFU ml^-1 and 10^6 PFU ml^-1, API=0.01) and incubated without shaking along with parallel control cultures without phages. For each experiment, duplicate tubes were examined daily for quantification of phage abundance in the liquid phase (40) and biofilm biomass was quantified by crystal violet staining using a standard protocol (42). For biofilm quantification, the liquid was removed, and tubes were rinsed twice with artificial seawater (ASW, Sigma). The biofilm was stained with 0.4% crystal violet (Sigma) for 15 min and the tubes were washed with tap water to remove excess stain. An aliquot (6 ml) of 33% acetic acid (Sigma) was added and left for 5 min to allow the stain to dissolve. The absorbance was measured at OD_{595nm}.

**Bacteriophage adsorption rate**

The rates of adsorption of phage KVP40 to wild-type, ΔvanT, and ΔvanO cells were determined by mixing phage KVP40 and exponentially growing host cultures at an API of 0.01 and incubating at room temperature for 32 min with agitation. Aliquots were removed periodically, centrifuged at 18,000×g at 4°C for 2 min, and the supernatants were immediately diluted to prevent additional adsorption. The un-adsorbed phage particles were quantified by plaque assay (40).

**Outer membrane protein K (OmpK) preparation and SDS-PAGE analysis**

OMP preparation was made essentially according to Wang (43) with some modifications. Briefly, cells were pelleted from 25 ml overnight cultures of all the strains (wild-type, ΔvanT, ΔvanO, ΔompK, ΔvanT ΔompK, and ΔvanO ΔompK), resuspended in 4.5 ml water, and sonicated on ice (100 amplitude, 3 min). To solubilize the cytoplasmic membranes, sarkosyl (N-lauroyl sarcosine) was added to a final concentration of 2%, and incubated at room temperature for 30 min. To pellet the outer membranes, the mixture was ultra-centrifuged (400,000 rpm for 1 h at 4 °C, SW 55 Ti, Beckman). The pellet was washed with ice-cold water and ultra-centrifuged again (400,000 rpm for 30 min at 4 °C, SW 55 Ti, Beckman). The pellet was resuspended in 100 μl 100 mM Tris-HCl (pH 8) and 2% SDS buffer, and the proteins were separated on a 10% SDS-polyacrylamide gel and stained with Coomassie blue.

**Fractionation of V. anguillarum cultures in aggregate and free-living cell fractions**
In order to examine *ompK* gene expression patterns in aggregates and free-living cells, respectively, cultures were fractionated by centrifugation and *ompK* expression was determined in the aggregate and non-aggregate fractions. Cultures of *V. anguillarum* wild-type and QS mutants were grown in MB in the presence of phage KVP40 at an API of 1 at RT for 12 h, and the aggregates were collected by centrifugation (1,000×*g*, 10 min). The supernatant was transferred to a new tube and centrifugation was repeated. After the second round of centrifugation, the free-living cells in the supernatant was transferred to a new tube and collected by centrifugation at 10,000×*g* for 10 min. Pellets of cell aggregates and free-living cells were collected and stored at -80 °C for subsequent RNA extraction.

**AHL signal levels in cultures of wild-type *V. anguillarum* PF430-3.**

An overnight culture of the wild-type strain was diluted in MB to the final OD$_{600nm}$ of 0.04 and grown at 30 °C for 20 h with agitation. Samples were taken every 2 h for determination of AHL production and for extraction of RNA for *ompK* mRNA quantification. AHL biosynthesis was assayed using *E. coli* SP436 reporter strain (44). *E. coli* SP436 harbors a plasmid-borne fusion of the *V. fischeri lux* operon to *gfp*, and responds to AHL by expressing green fluorescence protein (GFP) as described previously (44). Briefly, 50 μl cell-free supernatant was mixed with 150 μl LB medium and 10 μl of an overnight culture of SP436 (100 μg ml$^{-1}$ ampicillin), and further incubated at 30 °C for 5 h with agitation using a FLUOstar Omega microtiter plate reader (BMG Labtech) with gain setting of 1150.

**RNA extraction and cDNA synthesis**

For quantification of *ompK* expression in the wild-type and QS mutants (*ΔvanT* and *ΔvanO*), RNA was extracted from cells pelleted after 9 h incubation in MB for the experiments shown in Figure 5, or as described above for the experiments shown in Figure 3 and 4. Total RNA was extracted using Trizol (Invitrogen) and chloroform extraction as described previously (45). Genomic DNA was removed by adding DNase I according to the manufacturer’s protocol (Fermentas). RNA was stored at -80 °C until further use.

Reverse transcription was performed with the Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit as described by the manufacturer (Thermo). The first cDNA strand was obtained
using random hexamer primers with 1000 ng of DNase I-treated RNA. Control samples (-RT) were treated identically except that the reverse transcriptase enzyme was omitted.

**Real-time PCR gene expression quantification**

The relative expression levels of *ompK* and *recA* (as the endogenous control) were determined by quantitative RT-PCR performed in a CFX96 real-time PCR detection system (Bio-Rad), using SsoAdvanced SYBR Green Supermix (Bio-Rad), and the gene-specific primers listed in supplementary Table S2 (46). qPCR reactions were set up in 25 μl containing 0.2 μM of primers, and SYBR Green qPCR mix with the following program, 30 s at 95 °C for denaturation, followed by 40 cycles of (4 s at 95 °C, 30 s at 55°C). Melting curve analysis was performed from 65 °C to 95 °C with an increment of 0.5 °C for 5 s. The comparative \(C_T\) method was used for relative quantification of RNA (47).

**Acknowledgements**

The study was supported by the Danish Council for Strategic Research (ProAqua project 12-132390) and the EU-IRSES-funded project AQUAPHAGE (269175). We thank Debra Milton, Umeå University, for providing plasmid pDM4 and *E. coli* S1-17 and Pantelis Katharios, Hellenic Centre for Marine Research, for providing *V. anguillarum* PF430-3 and vibriophage KVP40. We are grateful to Nina Molin Høyland-Kroghsbo for many helpful discussions.

**References**


**Figure Legends**

Figure 1. Visualization by phase-contrast microscopy of *V. anguillarum* strains PF430-3 (wild-type, ΔvanT and ΔvanO) in the presence or absence of phage KVP40 in either fresh Marine Broth or cell-free spent culture fluid obtained from a mid-log phase culture of wild-type *V. anguillarum* PF430-3.

Figure 2. (A) Optical density (OD\textsubscript{600nm}) of cultures of *V. anguillarum* wild-type and QS mutants (ΔvanT and ΔvanO) in the presence or absence of phage KVP40 at API of 1 were measured at 1 h intervals over a 10-h period incubation. (B) Corresponding abundance of PFU ml\textsuperscript{-1} were quantified by plaque assay over a 10-h period incubation in wild-type+KVP40, ΔvanT+KVP40 and ΔvanO+KVP40 cultures, respectively. Error bars represent standard deviations from all experiments carried out in duplicate.

Figure 3. (A) *ompK* gene expression of wild type and QS mutants (ΔvanT and ΔvanO) in the presence or absence of phage KVP40. (B) SDS-PAGE analysis of the outer membrane protein K (OmpK) with Coomassie blue staining. Lane M, protein marker is shown on the right; lane 1-6, wild-type, ΔvanT, ΔvanO, ΔompK, ΔompK ΔvanT, and ΔompK ΔvanO, respectively. Phage receptor *ompK* mRNA transcript levels were quantified by real-time PCR. “Relative gene expression” corresponds to the level of *ompK* mRNA after normalization to the level of *recA* mRNA in the same sample. Error bars represent standard deviations (duplicate samples).

Figure 4. Fractionation experiment. For bacterial aggregates (A), samples were harvested at 1,000×g for 10 min. For free-living bacteria (F), samples were harvested at 10,000×g for 10 min. Phage receptor *ompK* mRNA transcript levels were quantified by real-time PCR. Relative gene expression was normalized against the gene expression level of *recA*. Error bars represent standard deviations (duplicate samples).

Figure 5. AHL assay and *ompK* expression quantification in the wild-type strain over time. Aliquots of cultures were withdrawn every 2 h during growth for measurement of AHL production and *ompK* gene expression by RT-PCR as described in the legend to Figure 3. Error bars represent standard deviations (duplicate samples).
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
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**Table S2 Primers**

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Figure S1. Examination of phage-host interactions (wild-type+KVP40, ΔvanT+KVP40, and ΔvanO+KVP40) in marine broth under phase-contrast microscopy after 24 h incubation. Images represent random fields obtained from microscopic slides prepared after 24 h incubation of the indicated V. anguillarum strain with phage KVP40.
Figure S2. Phage KVP40 production and bacterial growth upon exposure to synthetic AHLs. Shown are phage concentrations in the cell-free supernatant of cultures incubated with (closed symbols) or without (open symbols) 10 μm AHLs over the course of 7 h (squares). AHLs were added at time = -1h, and phage were added at an API of 1 at time = 0 h. To determine whether the AHLs affected bacterial growth in the absence of phage, the number of colony forming units (CFU) in parallel cultures +/- AHL but without phage addition were quantified (circles). Error bars represent standard deviations of experiments carried out in duplicate.
Figure S3. (A) Spot assay for testing host specificity of phage KVP40 against wild type, QS mutants (ΔvanT and ΔvanO) and ompK mutants (ΔompK, ΔvanT ΔompK, and ΔvanO ΔompK) as well. Five μl serial 100-fold dilutions of phage KVP40 lysate (1, 10⁵; 2, 10⁴; 3, 10³; 4; 10² PFU ml⁻¹) were shown by spot titration onto top agar lawns of the indicated strains. (B) Plaque assay quantification by using wild type and QS-mutants (ΔvanT and ΔvanO) as host, respectively. Error bars represent standard deviations (duplicate samples).
Figure S4. (A) Biofilm formation in *V. anguillarum* wild-type and QS mutants (ΔvanT and ΔvanO) pretreated with phage KVP40 (open symbols) and in controls without phage KVP40 addition (closed symbols) quantified by crystal violet (OD_{595nm}) staining during 8 days incubation. (B) Viable phage KVP40 recovered from free-living phases of wt+KVP40, ΔvanT+KVP40 and ΔvanO+KVP40, respectively, were quantified by plaque assay for 7 days. Error bars represent standard deviations from all experiments carried out in duplicate. It should be noted that the quantification of KVP40 most likely represent an underestimation of the actual phage production as only detached phage particles were counted. It is likely therefore, that the actual phage concentration was higher in the wild-type and ΔvanT cultures than in the ΔvanO cultures as more phages were likely trapped in the more developed biofilm of those cultures.
**Figure S5.** Carbon substrate utilization (BIOLOG GN2) between wild-type strain and ΔompK mutant. +, positive reaction at 24 h; -, negative reaction after 48 h.

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Figure S6. Competition assay between wild-type (black) and ΔompK (grey) at a ratio of 1 was performed. Colonies were picked at intervals and subsequently identified by PCR. Each bar represents (percent plotted in y axis) the fraction of the mixture.