Isolation, Characterisation and Application of Bacteriophages in Aquaculture

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Doctor of Philosophy

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To my ever supportive and loving parents

Declaration

This thesis has been composed in its entirety by the candidate. Except where specifically acknowledged, the work described in this thesis has been conducted independently and has not been submitted for any other degree.

Zinan Xu

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List of Abbreviations

16S rRNA	16 subunit of ribosomal RNA
x g	gravitational force (multiples of gravity)
bp	base pair
CFU	colony forming unit
DNA	deoxyribonucleic acid
e.g.	for example
et al.	"*et alia*" and others
i.e.	"*id est*" that is
kbp	kilo base pairs
kDa	Kilo Dalton
LC_{50}	lethal concentration 50%
Mb	million base pairs
min	minute
mL	millilitre
mM	milli-Molar
MOI	multiplicity of infection
nm	nanometre
OD	optical density
PFU	plaque forming unit
RNA	ribonucleic acid
sp.	species
spp.	species pluralis (multiple species)
subsp.	subspecies
UK	United Kingdom
µL	microlitre
USA	United States of America
US$	United States dollars
UV	ultraviolet
V	volt
v/v	volume/volume
w/v	weight/volume

Abstract

The increasing incidence of infections due to antibiotic resistant bacteria has led to renewed interest in bacteriophages (= phages) and phage therapy. Although phage therapy has been applied to control bacterial diseases in plants, poultry, livestock and humans, its application in aquaculture is still relatively limited. The emergence of phage-resistant bacterial mutants has been considered to be one of the major limitations of phage therapy. This study aimed to (i) isolate and characterise phages; (ii) select phages and their bacterial hosts to set up *in vivo* phage therapy models with aquaculture animals, and estimate the efficiency of phage therapy; (iii) investigate the generation and characteristics of phage-resistant mutants, and thus estimate the consequence of applying phage therapy when phage-resistant mutants emerge; and (iv) discuss the prospects for application of phages in aquaculture.

Two *Vibrio* isolates and their phages were isolated from a Scottish marine fish farm. Based on the results of conventional phenotype testing and 16S rRNA gene sequencing analysis, the two vibrios, V9 and V13, were identified as *Vibrio splendidus* and *Vibrio cyclitrophicus*, respectively. The bacterial characteristics including morphology, temperature and salinity range of growth, production of extracellular enzymes, and the possession of virulence genes were examined. According to the morphological characteristics observed using transmission electron microscopy by negative staining, phage PVS9 of *V. splendidus* V9 was identified as a myophage, while phage PVC13 of *V. cyclitrophicus* V13 was identified as a siphophage. The phages could only lyse one bacterial host strain and their genomic DNA was double stranded with a size of ~46 kb. The two *Vibrio* isolates were found to be non- or of low virulence to rainbow trout, goldsinny wrasse and *Artemia* in pathogenicity experiments. Thus an *in vivo* phage therapy model could not be set up using these *Vibrio* isolates and their phages.

Two phages pAS-3 and pAS-6 were isolated using the *Aeromonas salmonicida* subsp. *salmonicida* Hooke strain as the host. Phages pAS-3 and pAS-6 had a similar genome

size of ~50 kb, and the same relatively narrow host range within *A. salmonicida* subsp. *salmonicida* strains. The siphophage pAS-3 formed clear plaques and inhibited *A. salmonicida* Hooke growth *in vitro* completely for at least 18 hours when using MOI = 1,000, whereas the podophage pAS-6 formed turbid plaques and weakly inhibited Hooke growth. Rainbow trout exposed by intraperitoneal injection with 0.1 mL of the raw phage preparations at a concentration of 10^8 PUF mL^{-1} showed no adverse effects over 14 days. In the phage therapy trial, fish were firstly injected with 1 x 10^2 CFU $fish^{-1}$ of *A. salmonicida* Hooke, then immediately injected with phage preparations of pAS-3 and pAS-6, respectively, using MOI = 10,000. Compared with the control group (which did not receive phage treatment), phage treated groups showed a delay in the time to death, and lower mortalities. However, the mortalities and time to death between phage treated and non-treated groups were not significantly different.

Phage-resistant mutants of pathogenic *A. salmonicida* strain Hooke were induced by repeatedly challenging with phage pAS-3. One of the mutants, termed HM, was chosen to compare the characteristics with the parental wild-type strain Hooke. Test results including the formation of 'smooth' colonies on TSA, autoagglutination negative, the formation of creamy colonies on Coomassie Brilliant Blue agar, and the degradation of a thick/furry layered structure on the cell surface indicated a deficiency of the A-layer in the phage-resistant mutant HM. Therefore, it was deduced that the A-layer either directly acted as the receptor of *A. salmonicida* phage pAS-3, or was affected indirectly by the change of an unknown phage receptor. The greater wax moth larvae model was used to compare the virulence of the phage-resistant mutant HM and the parental wild-type strain Hooke, as it is an ethically acceptable animal model, which has the advantages of being low cost and convenient for injection, and is also a recognised alternative model for bacterial pathogens of fish. The results showed that virulence of the phage-resistant mutant HM did not decline in the greater wax moth larvae model compared with that of the parental wild-type strain Hooke.

In conclusion, different approaches were used to isolate and characterise phages from different aquaculture environments for potential use in phage therapy. A rainbow trout model was set up using pathogenic *A. salmonicida* strain Hooke and two *A.*

salmonicida phages pAS-3 and pAS-6. The use of phage treatment led to lower cumulative mortalities and delay to the time of death, although the differences between the groups were not significant, futher work is required to determine if these phages have potential in phage therapy. The consequence of applying phage therapy when phage-resistant mutants emerge was estimated based on their characteristics and virulence, and no decline in virulence of the phage-resistant mutant from this study indicates the importance of fully testing the virulence of phage-resistant mutants before carrying out large scale field trials of phage therapy. It appears feasible to use phage therapy as an alternative approach to control bacterial infections in aquaculture, but further studies are required to focus on improving effectiveness, and also to overcome the concrete limitations and hurdles in application and commercialisation. Moreover, a broader range of applications of phages in aquaculture should be explored.

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Chapter 1

General Introduction

1.1 Aquaculture

1.1.1 Definition of aquaculture

Aquaculture is an ancient farming activity, and may be traced back to China more than 4,000 years ago, when common carp (*Cyprinus carpio*) were raised for food in fresh water ponds (Pillay & Kutty, 2005). The term, aquaculture, is derived from the Latin ‘aqua’ and ‘colere’, which mean ‘water’ and ‘to care for’, respectively. Aquaculture has been defined in many ways: macro-aquaculture includes all activities where it is aimed at producing, processing and marketing aquatic animals and plants from fresh, brackish and sea water. The most current and internationally accepted definition has been given by the Food and Agriculture Organization (FAO) of the United Nations (UN), as ‘the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants. Farming implies some sort of intervention in the rearing process to enhance production, such as regular stocking, feeding, and protection from predators’ (FAO, 1995).

1.1.2 Global aquaculture

Aquaculture is a globally important and is currently the fastest growing food producing sector, which provides almost half of all fish for human consumption. In 2012, global aquaculture production attained another all-time high of 90.4 million tonnes (US$ 144.4 billion), including 66.6 million tonnes of food fish (US$ 137.7 billion) and 23.8 million tonnes of aquatic algae (mostly seaweeds, US$ 6.4 billion) (FAO, 2014). In addition, 22, 400 tonnes of non-food products (US$ 222.4 billion), such as pearl oysters and seashells for ornamental and decorative uses, was also reported collectively by some countries (FAO, 2014). China is the largest aquaculture producer who is diversified in terms of aquaculture species and farming systems (FAO, 2014). In 2012, Asia accounted for about 88 % of world aquaculture production by volume, and China contributed 61.7 % in the world total farmed food fish production, followed by India, Vietnam, Indonesia and Bangladesh as the top five countries (FAO, 2014). As at 2012, there has been 567 species registered in FAO statistics as the species produced in aquaculture, including finfishes (354 species, with 5 hybrids), molluscs (102),

crustaceans (59), amphibians and reptiles (6), aquatic invertebrates (9), and marine and fresh water algae (37). Carp is the most produced fish in the world, due to the vast scale of aquaculture in Asia. Salmonids are the most produced mariculture fish, whose biggest producer is Norway (FAO, 2014; Lillicrap, *et al.*, 2015).

1.1.3 Aquaculture in Scotland

Aquaculture is an increasingly important industry for Scotland, helping to sustain economic growth in the rural and coastal communities of the North and West. Involving the farming or culturing of fish, molluscs, crustaceans and seaweed, aquaculture produces the most valuable food exports of Scotland (http://www.gov.scot/Topics/marine/Fish-Shellfish). The aquaculture industry in Scotland is led by Atlantic salmon (*Salmo salar*) farming, and Scotland is currently the largest producer of farmed Atlantic salmon in the EU and third largest globally, but also produces significant quantities of rainbow trout (*Oncorhynchus mykiss*) and mussels (*Mytilus* spp.) (http://www.scotland.gov.uk/Topics/marine/Fish-Shellfish/FactsandFigures).

In 2014, Scotland produced 179,022 tonnes of Atlantic salmon, 5,882 tonnes of rainbow trout, 66 tonnes of halibut (*Hippoglossus hippoglossus*), 48 tonnes of brown trout (*Salmo trutta*), 5 tonnes of lumpsucker (*Cyclopterus lumpus*), and 0.1 tonnes of wrasse (*Labridae*) (Munro & Wallace, 2015), as well as 7,683 tonnes of mussels, 3,392,000 shells of Pacific oysters (*Crassostrea gigas*), 242,000 shells of native oysters (*Ostrea edulis*), 18,000 shells of queen scallop (*Chlamys opercularis*) and 48,000 shells of scallop (*Pecten maximus*) (Munro & Wallace, 2015). The Scottish aquaculture industry has plans for expansion of aquaculture with the aim of increasing marine finfish (including farmed Atlantic salmon) production sustainably to 210,000 tonnes, and shellfish (particularly mussels) production to 13,000 tonnes sustainably by 2020 (Scotland's National Marine Plan, the Scottish Government, 2015).

1.1.4 Bacterial diseases of fish

Cultured fish sustains greater stresses than fish in the wild (Dallaire-Dufresne *et al*., 2014). High stocking densities, low levels of dissolved oxygen, environmental pollution and inappropriate management practices are associated with the onset of diseases in aquaculture (Barton & Lwama, 1991; Dallaire-Dufresne *et al*., 2014). Infectious diseases, which may be caused by bacteria, viruses, parasites and fungi concentrated in culture systems, may lead to mortality and significant economic disruptions (Diana *et al*., 2013). A wide variety of bacterial pathogens are involved in salmonid diseases, including *Aeromonas hydrophila* (haemorrhagic septicaemia; fin/tail rot), *A. salmonicida* (furunculosis; ulcer disease), *Flavobacterium psychrophilum* (rainbow trout fry syndrome, RTFS), *Flexibacter* spp. (gill disease, black patch necrosis), *Mycobacterium* spp. (mycobacteriosis), *Pseudomonas* spp. (generalised septicaemia/haemorrhagic ascites/ulceration), *Renibacterium salmoninarum* (bacterial kidney disease; Dee disease, corynebacterial kidney disease), *Streptococcus* spp. (streptococcicosis), *Vibrio* spp. (vibriosis) and *Yersinia ruckeri* (enteric redmouth disease, ERM) (Austin & Austin, 2012). Typical *A. salmonicida* and *Vibrio* spp. are the focus of this study.

1.2 *Aeromonas salmonicida* subsp. *salmonicida*

1.2.1 The organism

Aeromonas salmonicida belongs to the genus *Aeromonas*, family *Aeromonadaceae*, order *Aeromonadales*, and class *Gammaproteobacteria*. It is composed of five subspecies: *salmonicida*, *achromogenes*, *masoucida*, *smithia*, and *pectinolytica* (Beaz-Hidalgo & Figueras, 2013). The isolates, which are identified as *A. salmonicida* subsp. *salmonicida*, are generally referred to as typical *A. salmonicida* that is related with the systemic infection in salmonids commonly referred to as furunculosis. The four other subspecies and the isolates that cannot be classified in any of the five subspecies are generally considered as atypical *A. salmonicida* that often cause ulceration in non-salmonids, such as cyprinids and including some marine flatfish (Burr & Frey, 2007; Austin & Austin, 2012; Dallaire-Dufresne *et al*., 2014).

Aeromonas salmonicida subsp. *salmonicida* (= *A. salmonicida*) is characterized as Gram-negative, non-motile, and facultative anaerobic bacilli (short rods), normally associated with the production of a brown, water soluble, diffusible pigment when grown in medium containing tyrosine or phenylalanine (Dallaire-Dufresne *et al*., 2014). The optimum growth temperature of *A. salmonicida* is between 22 and 25 °C. However, even growing *A. salmonicida* in this optimum temperature range may lead to the loss or inactivation of some virulence genes (Ishiguro *et al*., 1981; Stuber *et al*., 2003; Daher *et al*., 2011). Therefore, it has been recommended to culture *A. salmonicida* below 20 °C (Dallaire-Dufresne *et al*., 2014). When cultured on agar plates at 18 °C, colonies of *A. salmonicida* are pinhead size after 18-24 hours, and then become circular, convex, and entire with a diameter of 1-2 mm after 3 days. An interesting characteristic of *A. salmonicida* is the ability to form different colony types, classified as rough, smooth and the intermediate type (= G-phase). This phenomenon was attributed to the presence (rough) or absence (smooth) of an extracellular layer (A-layer) as demonstrated by electron microscopy (Austin & Austin, 2012).

1.2.2 Virulence factors

A number of virulence factors that are related with the diseases caused by *A. salmonicida* have been studied, including an extracellular layer (= A-layer), a type III secretion system (TTSS) and a variety of extracellular molecules (Daher *et al*., 2011; Beaz-Hidalgo & Figueras, 2013).

The A-layer is the additional layer, external to the cell wall. It is a complex protein structure associated with lipopolysaccharides (LPS) (Dallaire-Dufresne *et al*., 2014). The A-layer protein is encoded by a single chromosomal gene, termed *vap*A (Belland & Trust, 1985; Reith *et al*., 2008), and its assembly requires the presence of O-polysaccharide chains (Dooley *et al*., 1989). The A-layer is responsible for bacterial autoagglutination, adherence to host tissues and resistance to phagocytosis. In addition, this layer may protect the cell from serum complement and the action of protease (Austin & Austin, 2012). In some cases, the A-layer acts as a phage receptor

(Rodgers *et al*., 1981; Ishiguro *et al*., 1984), while in others, it shields phage receptors in the outer membrane (Ishiguro *et al*., 1981). Normally, the A-layer can be detected using Coomassie Brilliant Blue agar (CBBA) (Evenberg *et al*., 1985) and Congo Red agar (Ishiguro *et al*., 1985). The A-layer positive strains form dark blue and red-coloured colonies on CBBA and Congo Red agar, respectively, whereas the A-layer negative strains appear white (Buller, 2004). The production of the A-layer can be related to the culture temperature of *A. salmonicida*, and it was considered that growing the bacterium above 20 ℃ may lead to the loss of the A-layer protein (Daher *et al*., 2011). Even though many studies have proven the association between the possession of an A-layer and virulence (Ishiguro *et al*., 1981; Kay *et al*., 1981; Han *et al*., 2011), there are exceptions. For example, virulent A-layer negative isolates (Ellis *et al*., 1988; Bernoth, 1990), and non-virulent A-layer positive isolates (Olivier, 1990) have been reported.

The function of the TTSS in *A. salmonicida* is to secrete and translocate toxins (effectors) from the bacterial cytoplasm to the host cell (Burr *et al*., 2002; Stuber *et al*., 2003). The vast majority of the proteins required for the formation and regulation of the TTSS are encoded by the tandem of genes located on the large pAsa5 plasmid, while the effector, i.e. the ADP-ribosylating toxin (AexT), is encoded by the *aexT* gene located on the chromosome (Reith *et al*., 2008). Both Daher *et al*. (2011) and Tanaka *et al*. (2013) demonstrated that the expression of the TTSS is temperature-dependent, supported by the evidence that growing *A. salmonicida* at temperatures above 22 ℃ caused the re-arrangement of the plasmid pAsa5, which resulted in the loss of the TTSS region and virulence. Studies that used mutant deficient strains have revealed that the structural genes of the TTSS are essential for the virulence of *A. salmonicida* (Burr *et al*., 2005; Dacanay *et al*., 2006). In addition, Fast *et al*. (2009) suggested that both the structural proteins of the TTSS and the effector proteins probably have an impact on the survival of *A. salmonicida* within the host immune cells.

Aeromonas salmonicida produces a large variety of extracellular molecules as virulence factors, including proteases, lipases and haemolysin (Ellis *et al*., 1988; Dallaire-Dufresne *et al*., 2014). These enzymes are more likely related in the infection process especially in the degradation of the host tissues (Daher *et al*., 2011). The

haemolysin in *A. salmonicida* is correlated with lesion development and rapid death in rainbow trout (Ellis *et al*., 1988; Beaz-Hidalgo & Figueras, 2013). A 64-kDa serine protease, named AspA, can liquefy the muscle tissues and cause furuncles on the infected fish (Fyfe *et al*., 1986). The glycerophospholipid–cholesterol acyltransferase (GCAT) lipase hydrolyses membrane phospholipids, resulting in lysis of fish erythrocytes and also binds to LPS to form a high molecular weight toxin (Lee & Ellis, 1990; Vipond *et al*., 1998). However, in the study of Vipond *et al*. (1998), differences were not recorded in virulence between the GCAT or AspA mutants and the parental strain in an Atlantic salmon challenging model.

1.2.3 Furunculosis

Furunculosis (Figure 1.1) is a systemic disease of salmonids, and is characterised by high mortality and morbidity (Burr *et al*., 2005; Austin & Austin, 2012). The disease spreads through contact with infected fish or simply by exposure to the water contaminated with *A. salmonicida* (Dallaire-Dufresne *et al*., 2014). The name 'furunculosis' is derived from the presence of furuncles or boils that develop on the skin and musculature of fish, which are affected by the sub-acute or chronic form of the disease (Austin & Austin, 2012). However, furunculosis is a complex disease, which develops different clinical manifestations depending on the age, species, and health conditions of fish, and even environmental conditions, especially temperature (Boyd *et al*., 2003). The sub-acute or chronic form of furunculosis is more common in older fish, which often have furuncles (Austin & Austin, 2012). The infections are characterised by the clinical signs of lethargy, slight exophthalmia, blood-shot fins, bloody discharge from the nares and vent, and multiple haemorrhages in the muscle and other tissues, as well as haemorrhaging in the liver, swelling of the spleen, and kidney necrosis (Austin & Austin, 2012). This form of the disease is usually of slow-onset with low rates of mortality. The acute form of furunculosis happens more commonly in growing and adult salmonids, which causes a rapid and general septicaemia accompanied by melanosis, inappetance, lethargy, and haemorrhages at the base of the fins as well as the abdominal wall, viscera and heart (Austin & Austin, 2012). Such infections usually

are of sudden onset, and cause high mortalities in two to three days (Boyd *et al*., 2003; Burr *et al*., 2005; Austin & Austin, 2012).

Figure 1.1 A furuncle caused by *Aeromonas salmonicida* subsp. *salmonicida* on the surface of a rainbow trout. Adapted from Austin & Austin (2012).

1.2.4 Prophylaxis and therapy

Vaccines have been generally successful in farmed Atlantic salmon (Håstein *et al*., 2005; Gulla *et al*., 2015). At present, all farmed Atlantic salmon in Norway, approximately 320 million fish per year, are vaccinated prior to sea transfer (Romstad *et al*., 2013). Different vaccine formulations and methods of administration have been attempted on salmonids with varying degrees of protection (Villumsen *et al*., 2015). The oil-adjuvanted vaccines, administrated by intraperitoneal (i.p.) injection, are the most common type of vaccines used in the aquaculture industry, and have achieved high levels of protection in trout and salmon with heightened and prolonged antibody response (Håstein *et al*., 2005; Villumsen *et al*., 2012; Romstad *et al*., 2013). However, application of oil-adjuvanted vaccines has also shown a number of adverse effects, including the presence of lesions at the injection sites, intra-abdominal adhesions, pigmentation, granulomas, autoimmunity, spinal deformities, transitory anorexia and reduction in growth, as well as long-term inflammation (Midtlyng, 1996; Mutoloki *et al*., 2004; Sørum & Damsgård, 2004; Berg *et al*., 2006; Haugarvoll *et al*., 2010; Satoh *et al*., 2011).

Applying chemotherapeutic agents (antibiotics) is another invaluable way for stopping heavy mortalities during outbreaks of furunculosis (Austin & Austin, 2012). Quinolone antibiotics, principally oxolinic acid and flumequine are used most frequently to treat furunculosis (Giraud *et al*., 2004). However, using chemotherapy leads to the rapid emergence of antibiotic resistant bacteria, even multiple resistant strains (Inglis *et al*., 1991; McIntosh *et al*., 2008; Reith *et al*., 2008; Kim *et al*., 2011; Vega-Sánchez *et al*., 2014). This can result in the failure of chemotherapy for controlling disease and also the possible release of bioactive compounds into the aquatic environments (Giraud *et al*., 2004). Other concerns associated to the application of antimicrobial drugs are the limited number of antibiotics licensed for use in aquaculture, and the potential retention of the compounds in fish products destined for human consumption (Midtlyng *et al*., 2011; Austin & Austin, 2012; Coscelli *et al*., 2015). However, in the European Union (EU) drug residues is not important in fish now, as the Council Directive (EEC) no. 82/2001 provides a general withdrawal period (a period between treatment of food-producing animals and production of foodstuff) of 500 °C-day for the depletion of residues (Esposito *et al*, 2007).

An alternative approach is to use phage therapy as a means of biological control of disease. Phages can be used as therapeutic or prophylactic agents (Nakai & Park, 2002). However, so far, only a few attempts have been made on the experimental application of phages to control furunculosis (Imbeault *et al*., 2006; Verner-Jeffreys *et al*., 2007; Kim *et al*., 2015).

1.3 Vibrios

The vibrios are Gram-negative curved rod-shaped bacteria that are fermentative, catalase and oxidase positive, motile by polar flagella, usually sensitive to the vibriostatic agent (O/129), and mostly have a requirement for sodium chloride (Austin, 2010). The genus *Vibrio* consists of more than 100 species grouped into 14 clades that are widespread in aquatic environments, including marine, fresh water, and estuaries worldwide (Romalde *et al*., 2013). In the last ten years, more than fifty new species

have been described in the genus *Vibrio*, attributed to the introduction of new molecular techniques in bacterial taxonomy, and the increasing number of environmental studies that have contributed to improve knowledge about vibrios and their phylogeny (Romalde *et al*., 2013). Some *Vibrio* spp. are important for natural systems, e.g. the carbon cycle (Johnson, 2013), and some are found in the natural flora on the surface and/or in the gastrointestinal tract of marine organisms like fish, shellfish, shrimp, plants, and zooplankton (Thompson *et al*., 2004). However, a number of *Vibrio* spp., e.g. *V. anguillarum*, *V. harveyi* and *V. tapetis*, are also pathogens that cause diseases in aquatic animals (e.g. fish, crustacea and molluscs), and some others, e.g. *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus*, can infect humans (Daniels & Shafaie, 2000; Austin, 2010; Austin & Austin, 2012; Romalde *et al*., 2013). The epizootic bacterial diseases caused by the genus *Vibrio*, referred to as vibriosis, have brought enormous economic loss to the worldwide aquaculture industry (Liu *et al*., 2013a).

1.3.1 *Vibrio splendidus*

Vibrio splendidus is a component of the normal aquatic bacterial microbiota (Lopez & Angulo, 1995). On the other hand, *V. splendidus* has been found to be a pathogen causing diseases in aquatic animals. Studies have reported the pathogenicity of *V. splendidus* strains associated with turbot (*Scophthalmus maximus*), cod larvae (*Gadus morhua*), rainbow trout and corkwing wrasse (*Symphodus melops*) (Angulo *et al*. 1994; Gatesoupe *et al*., 1999; Jensen *et al*., 2003; Reid *et al*., 2009). An aerolysin-like enterotoxin is an important virulence factor linked to damage of the intestinal tract and mortalities in cod and turbot larvae (Macpherson *et al*., 2012). Moreover, *V. splendidus* is an important pathogen causing massive mortality in reared molluscs, including oysters (*Crassostrea gigas*), clams (*Ruditapes decussates*) and Yesso scallop (*Patinopecten yessoensis*) (Gómez-León *et al*., 2005; Duperthuy *et al*., 2011; Liu *et al*., 2013b). An extracellular metalloprotease (Vsm) and outer membrane protein U (OmpU) are the virulence factors contributing to the infections caused by *V. splendidus* in molluscs. Vsm is the major toxicity factor in the extracellular products (ECPs) inducing high mortality of oysters (Binesse *et al*., 2008). OmpU can inhibit the function

of antimicrobial peptides, and function as porins to attach and invade immune cells (Duperthuy *et al*., 2010; Duperhuy *et al*., 2011). In addition, *V. splendidus* is also associated with skin ulceration syndrome in juvenile sea cucumber (*Apostichopus japonicus*) in China (Li *et al*., 2010; Yan *et al*., 2014).

1.3.2 *Vibrio cyclitrophicus*

Vibrio cyclitrophicus can degrade and utilize polycyclic aromatic hydrocarbons (PAHs) (Hedlund & Staley, 2001). Based on multilocus sequence analysis (MLSA) using eight housekeeping genes (*gapA, gyrB, ftsZ, mreB, pyrH, recA, rpoA*, and *topA*), *V. cyclitrophicus* is grouped into the Splendidus clade of the *Vibrionaceae* (Sawabe *et al*., 2013). *Vibrio cyclitrophicus* has been isolated from the haemolymph of healthy wild-captured spider crabs (*Maja brachydactyla*) (Gomez-Gil *et al*., 2010) and reared clams (*Ruditapes philippinarum*) in Spain (Beaz-Hidalgo *et al*., 2008a). However, so far it has only been proven to be the pathogen of sea cucumber skin ulceration disease (Deng *et al*., 2009). Austin *et al*. (2005) considered *V. cyclitrophicus* to be non- or low virulent to rainbow trout. In addition to pathogenicity, *V. cyclitrophicus* enhances the total organic carbon (TOC) reduction rate in organically enriched sediment below fish farms, when co-inoculated with *Capitella* sp. I (Wada *et al*., 2008); is associated with biocorrosion processes in harbour zones (Boudaud *et al*., 2010); and also produces eicosapentaenoic acid (EPA) in laboratory culture conditions (Elrazak *et al*., 2013).

1.4 Bacteriophages

Bacteriophages (= phages) are a subgroup of prokaryotic viruses that specifically invade bacterial cells. They have been observed in great numbers and constitute the largest of all virus groups (Ackermann & Prangishvili, 2012). The total number of phages in the biosphere has been estimated at over 10^{30} particles (Ackermann, 2011). Moreover, novel phages are continually reported in investigations of the environment and industrial fermentations at a rate of approximately 100 per year (Ackermann, 2007). Phages occupy all those habitats where bacteria thrive (Skurnik & Strauch, 2006; Wittebole *et al*., 2014): they occur in the oceans and fresh water, the rhizosphere, on plants and food, in industrial fermentations, and on the outside and in the body

cavities of humans and animals (Ackermann & Prangishvili, 2012). In addition, most cultivated bacteria harbour complete or defective prophages (Ackermann, 2007). However, the main habitats of phages are the oceans and topsoil (Ackermann, 2011). Since 1959, 6,196 phages have been examined by electron microscopy (Ackermann & Prangishvili, 2012). The phages belong to 10 families, and infect members of 163 bacterial genera, most of which are the Firmicutes and γ-proteobacteria (Ackermann & Prangishvili, 2012). Phages are the most ubiquitous organisms on earth and play important roles in maintaining the natural abundance and distribution of microorganisms (Mathur *et al*., 2003; Sulakvelidze, 2011). On the level of ecosystems, phages can be key contributors to the mineralization of nutrients by lysing host bacterial cells, because they contribute to the primary ecological process of soils, including decomposition and decay (Allen & Abedon, 2013). In aquatic environments, phages potentially impact global carbon cycling by short-circuiting the movement of carbon and energy to heterotrophic bacteria rather than from cyanbacteria to consumer eukaryotes (Wilhelm & Suttle, 1999; Allen & Abedon, 2013). Phages also contributed as the model organisms to the development of molecular biology (Sulakvelidze, 2011), e.g. phages were used to identify the basis of genetic material, and that three nucleotides code for an amino acid (Clokie *et al*., 2011).

1.4.1 Phage taxonomy

The most important properties for phage classification are morphology, the nature of nucleic acid and physico-chemical properties of the virion, now increasingly complemented by genomic data (Ackermann, 2011). The start of phage classification was in 1967, when Bradley defined six basic morphotypes of phages by gross morphology and the type of nucleic acid, corresponding to the present phage families *Myoviridae*, *Siphoviridae*, *Podoviridae* *Microviridae*, *Leviviridae* and *Inoviridae*, respectively (Ackermann & Prangishvili, 2012). According to the VIIIth report of the International Committee on Taxonomy of Viruses (ICTV), phages now are classified into one order and 10 families (Figure 1.2) (Ackermann, 2011).

Over 96 % of phages are tailed and all of them belong to the order *Caudovirales*, which comprises three large phylogenetically-related families: *Myoviridae*, *Siphoviridae* and *Podoviridae*. All of the phages in the three families have icosahedral heads and double stranded (ds) linear DNA, but members in different families have differences in morphologies, specifically regarding their tails. Phages in the *Myoviridae* have contractile tails, whereas phages in *Siphoviridae* and *Podoviridae* have long non-contractile and short tails, respectively (Ackermann, 2011). Myoviruses are often the most commonly isolated phages from natural marine viral communities and are typically lytic, having relatively broad host ranges; they tend to be larger than other groups and include some of the largest and most highly evolved tailed phages; their tails consist of a neck, a contractile sheath, and a central tube. Siphoviruses are frequently isolated from seawater, often have a relatively broad host range, and many are capable of integrating into the host genome. They are the most numerous of tailed phages and constitute by far the largest virus family (over 3600 descriptions, or 57.3 % of all viruses); their tails are simple, noncontractile, flexible or rigid tubes. Podoviruses are also typically lytic and have very narrow host ranges, and they are less commonly isolated from seawater; they may be more related to siphoviruses than to myoviruses; their tails are short and noncontractile (Suttle, 2005; Ackermann, 2009; Ackermann & Prangishvili, 2012).

The remaining phages of polyhedral, filamentous or pleomorphic morphology are classified into 7 families separated by profound differences in nucleic acid content and structure. All families are small and well defined, among which both the nature of nucleic acid and particulars are quite varied. They contain double-stranded or single-stranded (ss) DNA or RNA. The virions from four of the families contain lipids and two of them have lipoprotein envelopes (Figure 1.2) (Ackermann, 2007; 2011).

Shape	Order or family	Nucleic acid, particulars, size	Example
	Caudovirales	dsDNA (L), no envelope	
	Myoviridae	Tail contractile	T4
	Siphoviridae	Tail long, noncontractile	λ
	Podoviridae	Tail short	T7
	Microviridae	ssDNA (C), 27 nm, 12 knoblike capsomers	φX174
	Corticoviridae	dsDNA (C), complex capsid, lipids, 63 nm	PM2
	Tectiviridae	dsDNA (L), inner lipid vesicle, pseudo-tail, 60 nm	PRD1
	Leviviridae	ssRNA (L), 23 nm, like poliovirus	MS2
	Cystoviridae	dsRNA (L), segmented, lipidic envelope, 70–80 nm	φ6
	Inoviridae	ssDNA (C), filaments or rods, 85–1950 x 7 nm	fd
	Plasmaviridae	dsDNA (C), lipidic envelope, no capsid, 80 nm	MVL2

C, circular; L, linear.

Figure 1.2 Overview of bacteriophage families. Adapted from Ackermann (2011).

1.4.2 Phage replication cycles

Phages rely on their living bacterial host cells to replicate. On the basis of the different relationships between phages and their bacterial hosts, there are two major life cycles: lytic and lysogenic, and a more sporadically way through pseudolysogeny (Fortier & Sekulovic, 2013) (Figure 1.3).

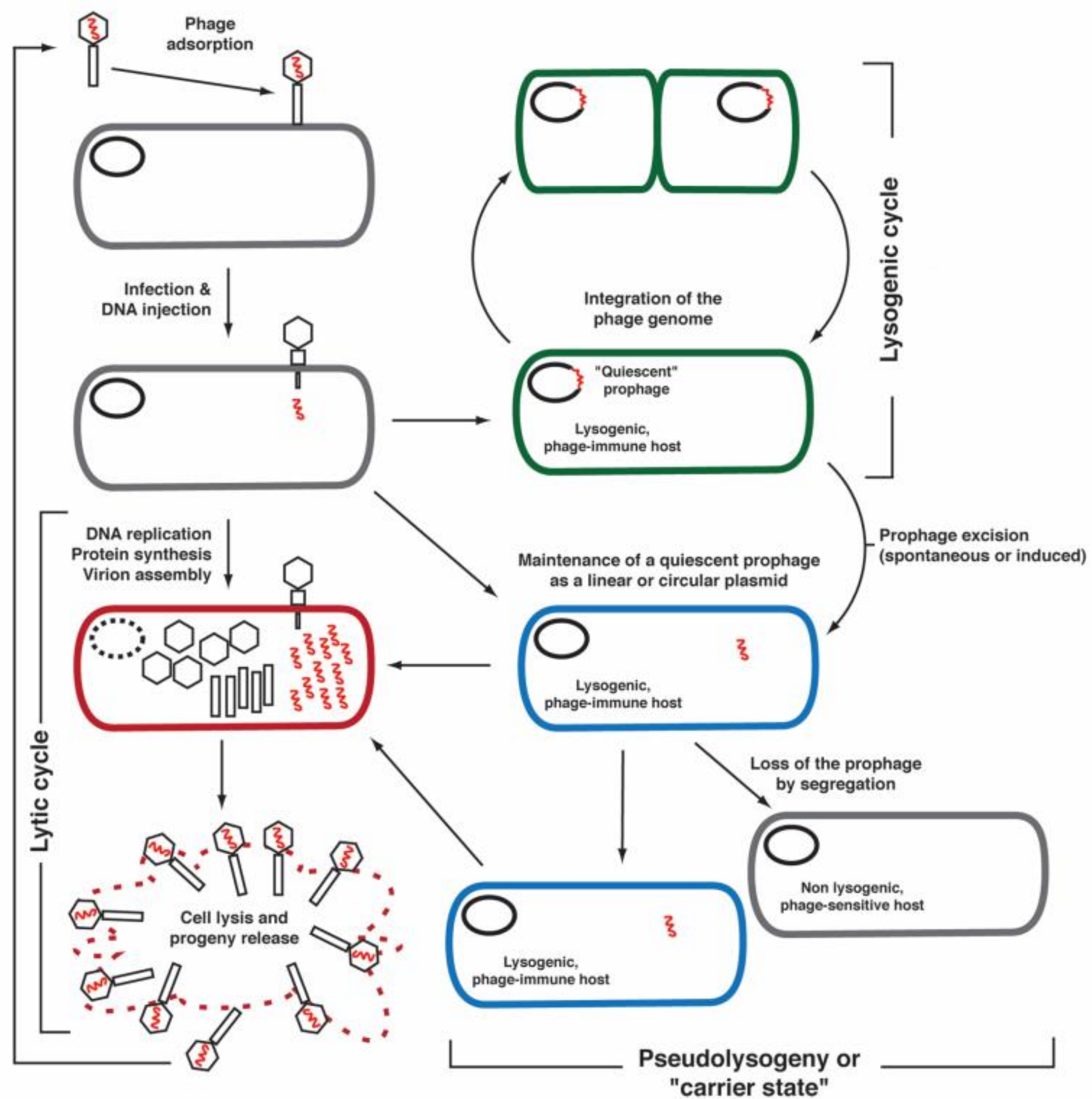

Figure 1.3 Three different life ways (lytic cycle, lysogenic cycle and pseudolysogeny) of bacteriophages. Adapted from Fortier & Sekulovic (2013).

1.4.2.1 Lytic cycle

The phages in a lytic cycle are usually termed as lytic or virulent phages. In this situation, phages replicate their genome and express the proteins by using host cell resources and energy, and producing new progeny viruses, which are released through host cell lysis into the environment where they can infect new bacterial cells (Almeida *et al*., 2009). The general steps in the process consist of contact, adsorption, injection or penetration of nucleic acid, replication and expression of the nucleic acids, virion assembly, release of new phage particles and transmission (Weinbauer, 2004).

Initially, phages adsorb to specific receptor proteins on the bacterial host surface or surface structures, e.g. flagella, pili, bacterial capsules and different parts of lipopolysaccharide (Weinbauer, 2004). Some phages may also use enzymes to breakdown capsule-like materials on the bacterial surface in a drill-like manner to reach the cell wall of the bacterium (Skurnik & Strauch, 2006). A given phage or group of phages can only adsorb to the particular sites on the surface of bacterial cells (Ackermann & DuBow, 1987). This phenomenon determines that most of the known phages have relatively narrow host ranges (Ackermann & DuBow, 1987). Following adsorption, phages use the enzymes in their tails or capsids to make the bacterial cell wall penetrable, and then inject their nucleic acid into the host cell cytoplasm, whereas the capsid remains outside the cell. In the next process, the phage genome directs and takes advantage of host cell resources and mechanisms to complete their own gene expression, genome replication, formation of capsid proteins and tails, and assembly of the genome and capsid into intact virions. Finally, the progeny phages are released from the host cell (Figure 1.3). In tailed phages, two kinds of enzymes are involved in the procedure of release: peptidoglycan hydrolases (endolysins) which break down peptidoglycan in the cell wall, and holins which permeabilize the plasma membrane and allow the endolysins to break down the peptidoglycan (Ackermann, 1999; Weinbauer, 2004; Gunawardhana, 2009). The phase of a phage infection from adsorption to host cell lysis is termed the latent period. The phase of the latent period before the capsid and genome are assembled into mature phages is called the eclipse period. The number of virions released per cell into the extracellular environment is called the burst size (Abedon, 1989; Ackermann, 1999; Weinbauer, 2004).

The capability to destroy bacteria is the basis of using lytic phages as therapeutic or prophylactic agents (Almeida *et al.*, 2009). However, there is a special completion of the lytic cycle, called chronic infection, where phages can be released by budding or extrusion without killing the host cells (Ackermann & DuBow, 1987; Ackermann & Prangishvili, 2012). The extracellular stage of all the phages ends with the deactivation of the phage or a new infection (Weinbauer, 2004).

1.4.2.2 Lysogenic cycle

The phage in a lysogenic cycle is called a lysogenic or temperate phage. In this relatively dormant stage, after been injected into to the host cell, the phage genome integrates into a host cellular replicon, such as the chromosome, a plasmid, another phage genome or exists as a self-replicating plasmid. At this stage, the phage genome is termed a prophage. It replicates along with the host cell and is passed into daughter cells (Williamson *et al*., 2001; Dalmasso *et al*., 2014). Most cultivable bacterial strains contain inducible prophages (Ackermann & Prangishvili, 2012). According to Freifelder (1983), and more than 90 % of phages are temperate.

A 'lysogenic decision', whether or not to establish a prophage state, is made after phage infection (Ackermann & DuBow, 1987). Prophages may spontaneously re-enter the lytic pathway or can be induced by a number of physical or chemical agents, such as UV radiation, mitomycin C, hydrogen peroxide or polyaromatic hydrocarbons (Williamson *et al.*, 2001; Weinbauer, 2004), pollution, changes in temperature, salinity, nutrient concentration (Cochran *et al.*, 1998; Weinbauer & Suttle, 1999; McDaniel & Paul, 2005) and antibiotics (Fortier & Sekulovic, 2013). The expression of lytic genes following damage to the host genome by any of the above mechanisms can initiate the lytic cycle (Williamson *et al.*, 2001).

Both phages and bacterial hosts may benefit from lysogeny. Phages benefit by using this approach to overcome adverse environmental and biological conditions, including extremes of water temperature, and the low host density during nutrient starvation. Conversely, lysogenic bacteria gain specific advantages from their relationship with phages that include expanded metabolic capabilities, antibiotic resistance, toxin production, and promoting bacterial evolution. The most common result is homoimmunity, which provides resistance to superinfection by the same or similar strains of phages. These effects may occur through unspecified mechanisms or the process of conversion, whereby prophage genes are expressed in the lysogens, or the bacterial host genes are modulated to express by the prophage (Williamson *et al.*, 2001; Fortier & Sekulovic, 2013).

1.4.2.3 Pseudolysogeny

Pseudolysogeny (i.e. false lysogen or phage-carrier state) is described as a phage-host cell interaction in which the nucleic acid of the phage neither establishes a long-term and stable relationship (i.e. lysogeny) nor leads to a lytic response, but rather the phage nucleic acid simply resides within the cell in a non-active state (Ripp & Miller, 1998). In this situation, the phage nucleic acid does not integrate into the host cellular replicons, and does not replicate and segregate into all progeny cells equally following host reproduction. This results in a constant production of the phage coexisting with high host abundance (Ackermann, 1987).

Unlike lysogeny, pseudolysogeny cannot be induced to the lytic cycle by using chemical or physical stimulated agents (Baess, 1971; Wommack & Colwell, 2000). Ripp & Miller (1998) suggested that pseudolysogeny is an environmental condition in which starved bacterial cells coexist in an unstable relationship with infective viruses, and it is a legitimate strategy for environmental phages to adapt to survive periods of starvation of their host organisms. Under the condition, host cells cannot provide enough energy for the phage to initiate either a true lysogenic or lytic state. However, once the level of nutrient availability to the host cell increases, the phage acquires the necessary energy to allow gene expression, leading to either the establishment of a state of true lysogeny or replication and expression of the viral genome (Ripp & Miller, 1997). This leads to virion formation and lysis of the host cell (Ripp & Miller, 1997). The regulation of pseudolysogeny and its effects for the host are still not well understood (Weinbauer, 2004). Another plausible speculation is that pseudolysogeny is a rapid response to environmental changes, or an evolutionary step towards the more stable lysogenic state (Wommack & Colwell, 2000).

1.4.3 Vibriophages

Vibriophages are a subset of bacteriophages, which infect bacteria belonging to the genus *Vibrio*. The start of the interest in vibriophages was due to their occurrence in some important human vibrio pathogens, including *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus* (Gunawardhana, 2009). The first vibriophage, which was discovered

by d'Herelle in 1922 from stools of a patient, was a phage of *V. cholerae* (Chatterjee & Maiti, 1984).

So far, vibriophages have been found in marine and fresh waters, sediments, plankton, molluscs, crustaceans, sewage and aquaculture environments (Ackermann *et al.*, 1984; Comeau *et al.*, 2006; Comeau & Suttle, 2007; Al-Fendi *et al*., 2014; Surekhamol *et al*., 2014; Yingkajorn *et al*., 2014). According to Ackermann *et al.* (1984), vibriophages are very heterogenous and include some morphologically interesting features. For example, vibriophage VPI exhibits the characteristic feature of feathery collar appendages, VP3 has a head with spherical projections, and phage 4996 tends to form pairs by aggregation of tail fibres. Some vibriophages have fragile tails: upon contraction the tail sheaths become loose and slide along the tail tubes then finally fall off. By 2007, 240 vibriophages had been isolated. Most of these phages are tailed, belong to the families *Myoviridae*, *Siphoviridae* and *Podoviridae*, and possess double-stranded linear DNA. The rest are filamentous, belonging to the family *Inoviridae*, and have single-stranded circular DNA (Ackermann, 2007). Studies on the phages of *V. cholerae*, *V. parahaemolyticus*, *V. harveyi*, *V. vulnificus*, *V. anguillarum* and *V. alginolyticus* have investigated their characterisation, classification and genome sequence, phage-host interaction, phage typing, prophage involvement in bacterial virulence as well as phage therapy (Oakey & Owens, 2000; Bhowmick *et al*., 2009; Bastias *et al.*, 2010; Campos *et al*., 2010; Wei *et al*., 2010; Faruque & Mekalanos, 2012; Li *et al*., 2013; Nigro *et al*., 2012; Maina *et al*., 2014; Tan *et al*., 2014; Zhang *et al*., 2014).

1.4.4 Phages hosted by *Aeromonas* spp.

Compared with vibriophages, the number of *Aeromonas* phages that have been isolated is relatively small. According to Ackermann (2007), in total 43 *Aeromonas* phages have been isolated. All of the phages are tailed, and the majority (33 phages) belong to the family *Myoviridae*, whereas only 7 and 3 phages belong to the families *Siphoviridae* and *Podoviridae*, respectively (Ackermann, 2007).

Early studies focused on phage typing, phage receptors, and the relations between phage sensitivity and the virulence of host cells. The majority of the work was done with *A. salmonicida*, the causal agent of furunculosis in fish, and its phages (Ishiguro *et al*., 1981; Rodgers *et al*., 1981; Chart *et al*., 1984). In contrast, current studies focus more on phage therapy in aquaculture (Imbeault *et al*., 2006; Verner-Jeffreys *et al*., 2007; Jun *et al*., 2013). More recently, further *Aeromonas* phages have been isolated, characterised, and even complete genomes sequenced, with the purpose of choosing the best fit candidates against the infections caused by pathogenic *Aeromonas* species, including *A. salmonicida* and *A. hydrophila* (Kim *et al*., 2012a; b; c; d; Shen *et al*., 2012).

1.5 Phage therapy

Phage therapy involves using intact phages or phage enzymes (lysins) as bioagents for the treatment or prophylaxis of bacterial infectious diseases (Matsuzaki *et al.*, 2005). This introduction will focus on therapy using intact phages. In most cases, lytic phages are applied in phage therapy, since they replicate quickly, and can either lyse all the bacterial cells or control their population to the level that the host immune system can clear the infection (Skurnik & Strauch, 2006; Richards, 2014). After nearly a century of investigation and practice, phage therapy has been increasingly used to control harmful bacteria in diverse fields, including human and animal health, aquaculture, agriculture, marine natural species recovery and the food processing industry (Nakai & Park, 2002; Skurnik & Strauch, 2006; Balogh *et al*., 2010; Cof fey *et al*., 2010; Kutter *et al*., 2010; Atad *et al*., 2012; Oliveira *et al*., 2012; Friedman *et al*., 2014).

1.5.1 Brief history of phage therapy

Not long after the discovery of phages in 1917, d'Herelle immediately realised their potential therapeutic utility as antibacterial agents, and did the first tests with animal models in the laboratory as well as field (Summers, 2001). He successfully used phages against avian typhoid in chickens, dysentery in rabbits, and haemorrhagic septicaemia in water buffaloes, which were caused by *Salmonella gallinarum*, *Shigella dysenteriae* and *Pasteurella multocida*, respectively (Summers, 2001). With evidence of therapeutic effectiveness, d'Herelle extended his phage trials to humans. The phage

preparations were ingested by d'Herelle and his coworkers to confirm safety, and then were successfully used to treat bacillary dysentery, bubonic plague, cholera and wound infections (Sulakvelidze *et al.*, 2001; Summers, 2001). As a result of d'Herelle's success, companies and institutions in many countries, including France, the USA, Poland, the former Soviet Union and India, commenced commercial production of phages as therapeutic agents (Sulakvelidze *et al.*, 2001).

However, following the discovery and widespread application of antibiotics in the 1940s, together with a poor understanding of phage biology, difficulties in making highly purified phage preparations, and problems of setting standardisation, phage therapy was neglected in Western Europe and North America. However, the former Soviet Union and some other Eastern European countries did not stop studying phages for their potential therapeutic utility, and the Institute supported by Georgyi Eliava and d'Herelle in Tbilisi has been one of the main centres for such work (Sulakvelidze *et al.*, 2001; Summers, 2001; Gross, 2011).

In the 1980s, the success of a series of studies on phage treatment for *E. colidiarrhea* in calves (Smith & Huggins, 1983; Smith *et al*., 1987a; b) was conducted by Williams Smith and his colleagues in the UK, and this rekindled interest in phage therapy in the West. This prompted other researchers to investigate the possibility of using phages against antibiotic-resistant bacteria causing human infections (Sulakvelidze *et al.*, 2001). The majority of the following phage therapy reports in humans were from research groups in Eastern Europe. For example, in Poland phage therapy was successful in treating septicaemia, cerebrospinal meningitis in a newborn, skin infection and recurrent abscesses, and in the former Soviet Union during 1963-1964, *Shigella* phages administrated orally to control dysentery in 17,044 children achieved a 2.3-fold reduction in diarrhoeal diseases compared to the placebo group (Sulakvelidze *et al.*, 2001).

Nowadays, many companies and universities/ institutes are working on phage therapy for humans and animals using current standards of clinical and microbiological research (Almeida *et al.*, 2009). The phase I and II clinical trials of phage therapy for

chronic otitis due to antibiotic-resistant *P. aeruginosa* have been successfully completed in the UK by BioControl® Ltd. A phage product, named LMP-102 bacteriophage preparation, consisting of a cocktail of six phages, has already been approved by the U.S. Food and Drug Administration to spray onto meat and poultry as an antimicrobial agent against *Listeria monocytogenes*. A Dutch food safety company, EBI Food Safety, has achieved GRAS (Generally Recognised as Safe) status for its *Listeria* product LISTEX™ P100, by the U.S. Department of Agriculture (USDA) and FDA. Other companies that are active in the development of phage products, including Novolytics Limited, Phico Therapeutics and Biophage Pharma Inc., are all reported to be developing phage products against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Clostridium difficile* (Housby & Mann, 2009; Ryan *et al*., 2011; Wittebole *et al*., 2014).

1.5.2 Phage therapy in aquaculture

Aquaculture is the fastest-growing food-producing sector worldwide (Bostock *et al.*, 2010). Bacterial infections have been increasingly recognised as a significant constraint to aquaculture production and trade (Rao & Lalitha, 2015). Although vaccination seems the ideal method to prevent infectious diseases, it has been only applied in some fish species and to a minority of diseases with varying levels of success (Richards, 2014). Moreover, vaccines may not be effective to the fish at their larval stages, and to invertebrates where a specific immune system does not exist (Carrias *et al*., 2012). Antibiotic treatment is a rapid and effective method to treat bacterial infection, but long-term and frequent usage has resulted in an increasing frequency of drug-resistant bacterial strains and ineffectiveness of such treatments (Pereira *et al*., 2011; Richards, 2014). Moreover, the toxicity and residues of antibiotics in aquatic food products and the surrounding environment is another severe drawback for use (Oliveira *et al*., 2012). Now in the UK only three antibiotics are licensed for use in fish farming (http://www.gov.scot/Resource/0040/00408269.pdf), which means the choices of antibiotics for fish farmers to access are very limited. Therefore, it is urgent to develop safer and more environmental-friendly methods to control bacterial infections in aquaculture.

Using lytic phages, i.e. phage therapy, as bioagents for the treatment or prophylaxis of bacterial infectious diseases is one of the particularly promising approaches. The first attempt at using phage therapy was reported in 1981 from Taiwan by Wu and colleagues, who found the loss of pathogenicity of *A. hydrophila* to loach (*Misgurnus anguillicaudatus*), when the pathogen was infected by phage Ah1 before it was injected into the fish (Wu *et al*., 1981). Then in the late 1990s, a Japanese research group successfully used phages to treat *Lactococcus garvieae* infection in yellowtail (*Seriola quinqueradiata*) (Park *et al.*, 1997; 1998; Nakai *et al.*, 1999) and *P. plecoglossicida* infection in ayu (*Plecoglossus altivelis*) (Park *et al.*, 2000; Park & Nakai, 2003). Nowadays, research work related with the application of phage therapy against bacterial infections in aquaculture has been conducted worldwide, and has involved more bacterial pathogens and aquaculture animals (Table 1.1). In addition, using phages against bacterial contamination has been investigated in the processing of aquaculture food products (Jun *et al*., 2014; Rong *et al*., 2014). However, there is a possibility of 'negative' study results not being published thus leading to a publication bias of 'positive' results. Studies also have examined phage dispersal and survival in animals and in the environment, the ecological effects on bacterial community structure, and have developed guidelines for use in fish and shellfish (Pereira *et al*., 2011; Madsen *et al*., 2013; Richards, 2014).

1.5.3 Advantages and limitations of phage therapy

Compared with antibiotic treatments, phage therapy has a number of advantages.

Phages are usually highly specific to a single species or strain of bacteria. Therefore, phage therapy will only cause minimal disruption to the normal intestinal flora in animals, and exert low impact on the natural microflora (Loc-Carrillo & Abedon, 2011). In contrast, many antibiotics have broad spectra activity, which leads to non-specific killing of both pathogens and members of the normal flora (Loc-Carrillo & Abedon, 2011). This results in the potential for altering the composition of the natural flora,

and could lead to secondary or superinfections (Loc-Carrillo & Abedon, 2011; Golkar *et al.*, 2014).

Phage therapy is considered safer than antibiotics as no serious complications have been reported during or after phage treatments in humans (Sulakvelidze *et al.*, 2001). However, some mild side effects (e.g. fever) have occasionally been reported due to the release of endotoxins from bacteria when they are lysed by phages inside the human body (Gunawardhana, 2009). In contrast, antibiotic treatments often cause side effects, including intestinal disorders, allergies (sometimes even fatal) and secondary infections (Sulakvelidze *et al.*, 2001; Gunawardhana, 2009; Golkar *et al.*, 2014).

Phages replicate and multiply in the environment where host bacteria are present. Moreover, phages are self-replicating as well as self-limiting. They replicate exponentially as bacterial numbers increase, may decline when bacteria number decreases, and finally may be at undetactablely low numbers after the host bacteria are removed (Almeida *et al.*, 2009). Thus, only small and less frequent phage doses may effectively kill the targeted pathogens at the site of infection, and will not continue to persist after treatment. Although antibiotics can be metabolised and eliminated, it is still possible that the residue can accumulate in tissues (Skurnik *et al.*, 2007). Moreover, antibiotics travel throughout the body and cannot concentrate at the infection site. To compensate for the loss in other parts of the body, high concentrations and repeated doses of antibiotic are needed (Sulakvelidze *et al.*, 2001; Summers, 2001; Skurnik *et al.*, 2007; Golkar *et al.*, 2014).

Bacteria can develop resistance to phages during the period of phage therapy, but it is usually possible to select and purify a new phage having lytic activity against the phage-resistant bacterial mutants within a few days or weeks. This is because phages are co-evolving with their host bacteria, and outnumber bacteria in the environment tenfold (Morrison & Rainnie, 2004; Almeida *et al.*, 2009). In contrast, development of a new antibiotic is usually very time-consuming and expensive in demonstrating safety and efficacy and conforming to quality standards (Huys *et al.*, 2013; Golkar *et al.*,

2014). Using engineered phages or phage enzymes, or a cocktail of phages with different bacterial receptors may overcome the problems of resistance (Golkar *et al*., 2014; Viertel *et al*., 2014). Moreover, phage-resistant bacteria can have reduced or lost virulence, because the receptors used by some phages to attach to bacteria may be associated with virulence (Smith & Huggins, 1983; Skurnik & Strauch, 2006; Capparelli *et al*., 2010a).

However, there are some limitations that should be considered before using phage therapy:

The specificity and narrow host ranges of phages makes it difficult to use the viruses against highly diverse bacterial variants. In this situation, before carrying out phage therapy, the nature of the pathogen and confirmation of phage susceptibility are required. However, use of a mixture of phages, i.e. phage cocktails, can overcome this limitation (Sulakvelidze *et al.*, 2001; Loc-Carrillo & Abedon, 2011; Lu & Koeris, 2011).

Phage particles may be rapidly cleared from the body by host defence systems, including neutralisation by antibodies (Almeida *et al.*, 2009) leading to a reduction in the number of phages available at the infection sites (Merril *et al.*, 1996). These problems may be resolved by selecting phage mutants that are able to remain in the circulatory system (Merril *et al.*, 1996) or giving repeated administration of the same phage to the organism (Barrow & Soothill, 1997).

Some lytic as well as temperate phages may act as vectors for transferring genes to bacteria, which can confer resistance to antibiotics and encode virulence (Almeida *et al.*, 2009). This may result in enhanced virulence and transformation of non-pathogenic to pathogenic strains (Almeida *et al.*, 2009). Careful screening of suitable phages, sequencing of phage genomes to avoid transmission of virulence genes, and construction of genetically modified mutant phages are all possible methods to address this issue (Matsuzaki *et al.*, 2005; Skurnik & Srauch, 2006; Loc-Carrillo & Abedon, 2011).

Table 1.1 Studies of phage therapy in disease control in aquaculture.

Pathogen	Disease	Animal	Phage Administration	Treatment effective	Success
Aeromonas hydrophila	Haemorrhagic septicaemia	Cyprinid loach (*Misgurnus anguillicaudatus*)	I.p.; oral	Both of the i.p. and oral administration improved the survival of the phage-treated loaches (Jun *et al*., 2013).	Yes
A. salmonicida	Furunculosis	Rainbow trout (*Oncorhynchus mykiss*)	I.m.	Phage treatment with a MOI of 10,000 showed notable protective effects with increased survival rates and mean times to death (Kim *et al*., 2015).	Yes
	Furunculosis	Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*)	I.p.; oral; bath	No adverse effect was observed. However, using a combination of three phages by i.p. injection only delayed the death, but didn't affect the end result. No protection was offered by any of the phage treatments (i.p., oral or bath) (Verner-Jeffreys *et al*., 2007).	No
	Furunculosis	Brook trout (*Salvelinus fontinalis*)	Water-borne	Delayed the onset of infection in fish by 7 days (Imbeault *et al*., 2006).	Yes

Edwardsiella ictaluri	Edwardsiellosis	Ayu (*Plecoglossus altivelis*)	I.p.	Higher protection was observed in fish that were first injected with phages and then 1 hour later injected with the pathogen, whereas the fish that were first injected with the pathogen and then the phages only showed delayed mortality compared with the control (Mahmoud & Nakai, 2012).	Yes
Flavobacterium columnare	Columnaris disease	Catfish (*Clarias batrachus*)	I.m.; bath; oral	Phage treatment led to disappearance of gross clinical signs, negative bacteriological test, detectable phage and 100 % survival in experimentally infected fish (Prasad *et al*., 2011).	Yes
F. psychrophilum	Cold water disease	Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*)	I.p.	Mortality decreased when phages were present (Castillo *et al*., 2012).	Yes
Lactococcus garvieae	Lactococcosis	Yellowtail (*Seriola quinqueradiata*)	I.p.; oral	Both i.p. and orally administered phage prevented fish from experimental *L. garvieae* infection (Nakai *et al*., 1999).	Yes

Pseudomonas aeruginosa	Ulcerative lesions	Catfish (*Clarias gariepinus*)	On-spot treatment	Phage therapy effectively cured the ulcerative lesions of the infected fish in 8-10 days of treatment, with a sevenfold reduction of the lesion with untreated infection control (Khairnar *et al*., 2013).	Yes
P. plecoglossicida	Bacterial haemorrhagic ascites disease	Ayu (*Plecoglossus altivelis*)	Oral	Phage-receiving fish showed high protection against infection with the pathogen (Park & Nakai, 2003).	Yes
Streptococcus iniae	Streptococcosis	Japanese flounder (*Paralichthys olivaceus*)	I.p.	Mortalities of fish receiving phages were significantly lower than the control fish without phage-treatment (Matsuoka *et al*., 2007).	Yes
Vibrio alginolyticus	Skin ulceration and viscera ejection	Sea cucumber (*Apostichopus japonicus*)	Immersion	Phage treatment increased the survival of sea cucumbers to 73, 50 and 47 % when it was used with a MOI of 10, 1 or 0.1, respectively, whereas the no phage treatment group only had 3 % survival (Zhang *et al*., 2015).	Yes

V. anguillarum	Vibriosis	Atlantic salmon (*Salmo salar*)	Water-borne	Phage treatment increased the survival of fish to 100 % when it was used with a MOI of 1 and 20, versus less than 10 % of survival in the absence of the phage (Higuera *et al*., 2013).	Yes
V. harveyi	Luminous vibriosis	Shrimp (*Penaeus monodon*)	Immersion	Phage therapy clearly improved shrimp larval survival in the presence of the pathogen (Karunasagar *et al*., 2005; 2007)	Yes
V. parahaemolyticus	Vibriosis	Whiteleg shrimp (*Litopenaeus vannamei*) larvae	Immersion	MOI = 0.1 was enough to counteract the infection, and an early application (at 6 hours post-infection) was effective to avoid mortality (Lomelí-Ortega & Martínez-Díaz, 2014).	Yes
	Vibriosis	Brine shrimp (*Artemia franciscana*)		A single dosage of the phage was effective to eliminate the adverse effects of the pathogen. However, when the phage treatment was delayed, it was ineffective to control the mortality (Martínez-Díaz & Hipólito-Morales, 2013).	Yes

MOI: multiplicity of infection; i.p.: intraperitoneal; i.m.: intramuscular.

1.6 Study Aims

The aims of this research were:

1. To develop the methods for isolation, identification and characterisation of lytic vibriophages and their *Vibrio* hosts, and evaluate the potential for setting up phage therapy models by using these vibriophages and their hosts (Chapter 2).
2. To isolate and characterise lytic *A. salmonicida* phages from the fresh water aquaculture environment, and investigate the effect of these phages on *A. salmonicida* growth *in vitro* as well as estimate the efficiency of phage therapy against *A. salmonicida* infections in a rainbow trout model (Chapter 3).
3. To estimate the risk of phage-resistant bacterial mutants generated during the process of phage therapy against *A. salmonicida* infections, by inducing phage-resistant mutants of *A. salmonicida in vitro* and comparing the physicochemical characteristics and virulence with that of the wild-type strain (Chapter 4).
4. To discuss the prospects for application of phages in aquaculture (Chapter 5).

Chapter 2

Isolation, Identification and Characterisation of Two Lytic Vibriophages and Their Hosts from the Aquaculture Environment

2.1 Introduction

Vibrios are ubiquitous and abundant in aquatic environments including marine, fresh water, and estuaries, worldwide. The genus *Vibrio* consists of more than 100 species grouped into 14 clades (Romalde *et al*., 2013). Most *Vibrio* spp. are a likely component of the normal aquatic bacterial micro biota contributing to the carbon cycle (Johnson, 2013), and some are the natural flora on the surface and/or in the gastrointestinal tract of marine organisms such as fish, shellfish, shrimps, plants, and zooplankton (Thompson *et al*., 2004). However, a number of *Vibrio* spp., e.g. *V. anguilarum*, *V. salmonicida (= Aliivibrio salmonicida)*, *V. harveyi* and *V. tapetis*, are bacterial pathogens that cause diseases in aquatic animals (e.g. fish, crustaceans and molluscs) (Daniels & Shafaie, 2000; Austin, 2010; Austin & Austin, 2012; Romalde *et al*., 2013). The epizootic bacterial diseases caused by the genus *Vibrio*, named vibriosis, have brought enormous economic loss to the worldwide aquaculture industry (Liu *et al*., 2013a); vibriosis can occur suddenly and sometimes leads to the death of the entire infected population (Thompson *et al*., 2004).

Vibriophages are a subset of bacteriophages, which occupy all those habitats where vibrios thrive. So far, vibriophages have been found in marine and fresh waters, sediments, plankton, molluscs, crustaceans, sewage and aquaculture environments (Ackermann *et al.*, 1984; Comeau *et al.*, 2006; Comeau & Suttle, 2007; Al-Fendi *et al*., 2014; Surekhamol *et al*., 2014; Yingkajorn *et al*., 2014). Studies on the phages of *V. cholerae*, *V. parahaemolyticus*, *V. harveyi*, *V. vulnificus*, *V. anguillarum* and *V. alginolyticus* have included their characterisation, classification and genome sequence, phage-host interaction, phage typing, prophage involvement in bacterial virulence, as well as phage therapy (Oakey & Owens, 2000; Bhowmick *et al*., 2009; Bastias *et al.*, 2010; Campos *et al*., 2010; Li *et al*., 2013; Nigro *et al*., 2012; Tan *et al*., 2014; Zhang *et al*., 2014).

In this study, lytic vibriophages were isolated along with their *Vibrio* hosts from a same marine fish farm environmental sample. The study aimed to: (i) develop methods for the isolation of lytic vibriophages and their bacterial hosts from the same sample; (ii)

identify and characterise the phages and their hosts; (iii) investigate the pathogenicity of the *Vibrio* hosts using different *in vivo* models; and (iv) evaluate the potential for setting up phage therapy models by using these vibriophages and their hosts.

2.2 Materials and methods

All the media and chemicals were purchased from Oxoid (Basingstoke, UK), and Sigma-Aldrich (Basingstoke, UK), respectively, unless otherwise stated.

2.2.1 Sample collection and processing

A mixed sample (100 mL) of water and sediment was collected using a 250 mL sterile laboratory capped bottle (Fisherbrand, UK) from the outlet pond of a Scottish marine fish farm in May 2011. The water temperature and salinity were 12 °C and 30 ‰, respectively.

The sample was blended using a sterile wooden stick for 10 min, and then incubated at 15 °C for 3 hours to allow free bacteria and phages to move into the liquid component. During the incubation period, the mixed sample was inverted frequently to increase the disruption of particulate material to distribute the bacteria and phages throughout the water and sediment mixture. After incubation, the sample was transferred into sterile 50 mL centrifuge tubes (VWR, UK) and centrifuged at 1,000 x *g*, 4 °C for 5 min (Sigma 4K15C centrifuge) to precipitate the sediment particles. The supernatant was then carefully pipetted into sterile tubes and stored at 15 °C.

2.2.2 Isolation of *Vibrio* spp.

The supernatant from above was diluted in sterile 3 % (w/v) NaCl to 10^{-1}, 10^{-2} and 10^{-3}, before 100 µL volumes were spread evenly over thiosulphate citrate bile salts sucrose agar plates, supplemented with 3 % (w/v) NaCl (TCBS+N) plate, in triplicate. After drying for 15 min on the bench, the plates were sealed with parafilm and incubated at 15 °C for 7 days and checked for bacterial growth.

Single colonies of bacteria were picked according to their colony morphology (colour, shape, size) and sub-cultured several times on TCBS+N plates to obtain purified cultures, which then were numbered and preserved at - 70 ℃ using microorganism preservation beads (Technical Service Consultant Ltd, UK).

2.2.3 Enrichment and isolation of vibriophages

The method of enrichment was modified from Van Twest & Kropinski (2009). Ten millilitres of supernatant (obtained from Section **2.2.1**) was mixed well with the same volume of double-strength tryptone soya broth (TSB; Oxoid) supplemented with 3 % (w/v) NaCl (TNB), and incubated in a Kuhner shaker (ISF-1-W, Switzerland) at 15 ℃, 140 rpm for 24 hours, aiming for phage enrichment. After incubation, 0.5 mL of chloroform was added into the tube, which was then inverted for 10 min, to disrupt the bacterial cells and release any phages. After centrifugation at 4,000 x *g*, at 4 ℃ for 15 min, the supernatant was carefully transferred; filter sterilised by passage through 0. 20 µm low protein binding membrane (cellulose acetate) filters (Minisart®, Sartorius Stedim, UK), and stored at 4 ℃.

The double agar overlay plaque assay of Kropinski *et al.* (2009) was used for phage isolation, with minor variations. The top agar was made of TNB supplemented with 0.3 % (w/v) agar, and kept in a water bath at 30 ℃ after being sterilized. The bottom agar was made of tryptone soya agar (TSA, Oxoid) with 3 % (w/v) NaCl (TNA), and ~15 mL was poured into each 90 mm diameter sterile plastic petri dish to form the underlay base. The bacteria that were isolated (from Section **2.2.2)** were used as the potential hosts for phage isolation. For each bacterial isolate, 200 µL of an overnight broth culture was mixed with 200 µL of the phage suspension in a sterile bijoux bottle. The mixture was incubated at room temperature (~22 ℃) for 5-10 min to allow the phages to adsorb the host cells. Five milliliters of the molten, cooled to 30 ℃ top agar were added into the bijoux, which then was inverted 5 times and immediately poured over the solid layer of medium in a petri dish. Then the plate was left standing for 15 min to form a thin overlay. In total, 1 mL of the phage suspension was plated (into 5 plates) for each bacterial isolate. The plates that were only plated with each bacterial isolate, but no phage suspension, were used as the blank control for plaque

examination. All the plates were incubated at 15 °C for 24 hours, and examined for the presence of plaques in the bacterial lawn. The plaques were indicative of the presence of lytic phages.

On the basis of plaque size and morphology, single plaques were picked with sterilised pipette tips or Pasteur pipettes and transferred into bijoux bottles containing 5 mL volumes of phage salt-magnesium (SM) buffer (50 mM Tris-HCl, pH 7.5; 100 mM NaCl; 8.1 mM $MgSO4 \cdot 7H_2O$ and 0.01 % gelatin) and inverted several times. A serial dilution (10^0, 10^{-1} and 10^{-2}) was made for each phage suspension, and a 10 µL volume of each dilution was plated with the same bacterial isolate (used as the indicator bacterium in the subsequent experiments) from which the plaque had been acquired, using the double agar overlay plaque assay described above. This procedure was repeated at least three times to ensure the purity of each phage. The phage suspension from the final purification was filter sterilised by passing through a sterile 0. 20 µm filter, and used for the subsequent experiments.

For phage propagation and also the preparation of phage stock, a liquid broth method adapted from Fortier & Moineau (2009) was used. A 10 µL volume of the purified phage suspension was inoculated into 10 mL of the indicator culture in TNB with an OD_{600} of 0.10 to 0.20, and incubated with shaking at 15 °C, 140 rpm overnight. The phage suspension was harvested by centrifugation and filtration, as before. For long-term storage, the resulting phage suspension was mixed well with an equal volume of glycerol to obtain a final concentration of 50 % (Ackermann *et al.,* 2004) and stored at - 70 °C. For short-term storage, the filtered phage suspension was stored at 4 °C without glycerol supplementation. The titre of each filtered phage suspension was determined using the double agar overlay plaque assay.

2.2.4 Identification and characterisation of the bacterial hosts

2.2.4.1 Conventional tests

The conventional bacterial identification tests used in this study included Gram stain, motility, oxidase, oxidation/fermentation (O/F), O/129 (10 and 150 µg), were performed following routine procedures (Frerichs & Miller, 1993). The biochemical profiles were determined using the API 20E rapid identification system (BioMerieux, Basingstoke, UK) and following the manufacturer's instructions. The incubations for O/F and API 20E assays were performed at 15 °C, and the readings were recorded every 24 hours for 3 days.

2.2.4.2 Identification by 16S ribosomal RNA gene sequencing

Bacterial DNA was extracted following a boiling lysis method which was adapted from Queipo-Ortuno *et al.* (2008). The bacterial overnight broth culture was pelleted by centrifugation at 12,000 *x g* for 2 min, and then washed once in sterile 3 % (w/v) NaCl. The bacterial pellet was resuspended in molecular biology-grade water (Milli-Q®), heated at 95 °C for 10 min, followed by immediately cooled on ice, and centrifuged at 13,000 *x g* for 1 min to remove bacterial debris. The supernatant containing bacterial DNA was stored long-term at - 20 °C or at 4 °C for immediate analysis by Polymerase Chain Reaction (PCR).

The primer set 20F (5'-AGAGTTTGATCCTGGCTCAG-3'; equivalent to positions 8-27 in *Escherichia coli* 16S rRNA gene) and 1500R (5'-GGTTACCTTGTTACGACTT-3'; equivalent to positions 1510-1492 in *E. coli* 16S rRNA gene) were used for the amplification of the 16S rRNA gene from genomic DNA of the bacterial isolates (Weisburg *et al.,* 1991). The PCR amplification was carried out in a total volume of 50 µL, containing 25 µL of PCR Master Mix (Thermo Scientific, UK), 10 pmol of each primer, 50 ng of template DNA, and nuclease-free water to volume. The genomic DNA template was substituted with nuclease-free water or *E. coli* genomic DNA for negative or positive control, respectively. The PCR reaction was performed in a GeneAmp PCR System 9700 thermal cycler (ABI, USA), and consisted of an initial denaturation at 95 °C for 2 min followed

by 30 cycles of denaturation at 95 ℃ for 1 min, annealing at 54 ℃ for 1 min, and extension at 72 ℃ for 1.5 min. Then, the reaction was completed by extension at 72 ℃ for additional 5 min. The resulting PCR amplicons were examined by electrophoresed on 1.2 % agarose (Invitrogen, UK) gel containing 0.5 µg mL^{-1} ethidium bromide at 80 V for 1 hour, and visualised under UV light using a trans illuminator (Syngene, BioImaging, UK). The expected size of the PCR products was approximately 1500 bp.

The amplified products were purified using a QIA quick Kit (Qiagen, UK) following the manufacturer's protocol. The purified PCR products were sequenced at the University of Dundee (The Genetics Core Services Unit, School of Medicine) using a ABI 3730 DNA sequencer with the amplification primer set 20F and 1500R. The obtained partial 16S rRNA gene sequences were analysed using the Basic Local Alignment Search Tool (BLAST) algorithm at the National Centre for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2.4.3 Phylogenetic analysis

A phylogenetic tree was constructed based on the 16S rRNA gene sequences of the isolated bacterial strains, 15 other *Vibrio* spp. strains from Splendidus clade, one *V. harveyi* strain and one *V. campbellii* strain (used as a outgroup) by the neighbour-joining (N-J) algorithm using the MEGA 4.1 software.

2.2.4.4 Morphology observation by Transmission Electron Microscopy

A drop of dense bacterial suspension in 3 % (w/v) NaCl was deposited on a 200 square mesh Formvar-coated copper grid for 1 min at room temperature. Excess liquid was drained off using a filter paper, and a drop of 2 % phosphotungstic acid (pH 7.2; EMscope) was added as stain for 1 min. Excess stain was blotted, and the grid was air dried, followed by examined under an FEI Tecnai Spirit GR Biotwin Transmission Electron Microscope (TEM).

2.2.4.5 Temperature and salinity range for growth

Single colonies were either streaked onto TNA plates which were incubated at 4 °C, 15 °C, 22 °C, 28 °C, 30 °C and 37 °C, or inoculated in 5 mL of TSB with different NaCl concentrations of 0.5, 1, 2, 3, 4, 5, 6, 7, 8 and 9 % and incubated at 15 °C. The observation for bacterial growth were recorded every 24 hours for 7 days.

2.2.4.6 Extracellular enzymatic activity

To determine the production of extracellular enzymes, the bacterial cultures were inoculated onto TNA plates which were supplemented with the following substrates: 5 % (w/v) skimmed milk (Marvel) for protease, 5 % (v/v) sheep blood (Oxoid) for haemolysin, 1 % (v/v) Tween 80 (Sigma Aldrich) for lipase and 0.1 % (w/v) gelatin (Oxoid) for gelatinase, respectively. The plates were then incubated at 15 °C for 3-5 days. The positive results for protease, haemolysin and gelatin (following the addition of saturated ammonium sulphate) showed clear zones around the bacterial colonies, whereas for lipase positive a zone of precipitation of salts of fatty acids shows around the bacterial colony.

2.2.4.7 Detection of virulence genes

According to the gene sequences of pathogenic *V. splendidus* strain LGP32 (Le Roux *et al*., 2009), two primer sets were designed to detect the genes of two virulence factors from the genome DNA of isolate V9, respectively: V-vsmF (5'-CCAACAGAGCCTCGTCG-3'; equivalent to positions 111-127 in *V. splendidus vsm* gene) and V-vsmR (5'-TGCTTTAGCGTGGTTTAGT-3'; equivalent to positions 594-576 in *V. splendidus vsm* gene) for the metalloprotease gene *vsm*, V-ompuF (5'-TGCTACTGGTGCTAACG-3'; equivalent to positions 45-61 in *V. splendidus ompU* gene) and V-ompuR (5'-TCACCGAATGTACCGC-3'; equivalent to positions 329-314 in *V. splendidus ompU* gene) for the outer membrane protein gene *ompU*. The PCRs were performed using the same annealing temperature 49 °C. The type strain *V. splendidus* NCIMB 1^T was used as the positive control. The PCR products were detected by electrophoresis, and then purified and sequenced with the same method as described above.

2.2.5 Pathogenicity of host bacteria

2.2.5.1 Passages in rainbow trout (*Oncorhynchus mykiss*)

Prior to performing the challenge studies, to enhance virulence, the *Vibrio* strains were passaged through healthy fish three times by intraperitoneal (i.p.) injection with a high bacterial concentration as per standard practice at the Institute of Aquaculture, University of Stirling. The bacteria strain was inoculated onto 5 % (v/v) sheep blood agar, and then a single colony was aseptically picked and cultured in broth overnight (~ 18 h). The bacterial suspension was centrifuged, washed once, and re-suspended in 0.5 mL sterile 0.85 % saline to get a high bacterial concentration (> 10^9 CFU mL^{-1}). One hundred microlitres of this bacterial suspension was injected by intraperitoneal (i.p.) into a healthy rainbow trout in the size of ~ 18 g. The bacteria were recovered from the anterior kidney and spleen of the moribund or dead fish by direct plating of the organ onto agar. If morbidity or mortality did not happen, the fish were sacrificed on the second day post injection, and sampled for bacterial recovery. This passage was repeated twice, and the isolate recovered from the 3rd passage was purified and confirmed the identity using the methods described above. Then the bacterial strain was ready for the challenge studies. If a bacterial strain did not result in the passage fish becoming moribund or dead in all the three passages then this strain was considered to be not pathogenic to rainbow trout, and would not be used in the challenge studies.

All the fish challenge experiments were carried out in the Aquatic Research Facility (ARF), University of Stirling. Fish were kept in the tanks with a continuous flow-through water system, except for the goldsinny wrasse challenge, where the fish were kept in 3.5 % artificial sea water with 80 % water changed every two days. In all the fish challenges, water temperature was controlled at 15 °C ± 2 °C with a 12 hours light : 12 hours dark cycle and aeration applied. The experimental fish were fed with commercial diets once daily.

2.2.5.2 Challenge study in rainbow trout

The bacterial inoculum for challenges was prepared as follows using the ex-passage strains: A single colony was inoculated into 20 mL of TNB, and cultured for 18 hours at 140 rpm, 22 °C. The culture was then centrifuged at 3,500 × *g* for 20 min at 4 °C, and washed once using 0.85 % sterile saline. The pellet was finally re-suspended and adjusted using sterile 0.85 % saline to get OD_{600} = 1.0, which gave approximately 10^8 CFU mL^{-1}. Then 10-fold serial dilutions were performed to get the aimed concentrations for injections. The actual concentrations of the injected bacterial preparations were calculated using Miles & Misra viable bacterial colony count method (Miles *et al*., 1938).

This challenge used two groups of 5 fish per group. One group was challenged by i.p. injection with a bacterial concentration of 10^7 CFU $fish^{-1}$ approximately. The other group was injected in the same way but with sterile 0.85 % saline as the control. The fish were kept for 21 days, and checked four times daily for any gross clinical signs of disease, and morbidity/mortality.

Any dead or moribund fish were removed from the tanks, but only freshly dead, moribund fish and 50 % of the survivors from each group were sampled for bacterial recovery and histopathology. The sampled fish were first examined for external and internal clinical signs of disease as observed grossly. Anterior kidney and spleen were sampled for bacteria by inserting a sterile loop into the organs and streaking onto TNA plates, which then were sealed with parafilm and incubated at 15 °C. The plates were kept for one week, and checked for bacterial growth and purity. Organs, including gills, brain, spleen, liver, heart, kidney, intestine and muscle with any lesions were fixed in 10 % neutral buffered formalin (NBF) for histopathology tests.

2.2.5.3 Challenge study in goldsinny wrasse (*Ctenolabrus rupestris*)

According to Austin *et al*. (2005), different administration methods of bacteria in challenge can affect the virulence of bacterial pathogens, and cause different clinical signs. Thus, in the challenge using goldsinny wrasse, both intraperitoneal and

intramuscular injections were applied. Eighteen goldsinny wrasse (2-3 g) from a Scottish marine fish farm were randomly put into 6 groups of 3 fish per group. Three groups were injected with the bacterial preparations of V9, V13 and sterile 0.85 % saline by i.p., respectively. The other three groups were injected with the same preparations, but by intramuscular (i.m.) below the dorsal fin. Groups were injected with sterile 0.85 % saline as the controls for i.p. and i.m. injection, respectively (Table 2.1). The bacterial preparations were prepared as described above, aiming to give 10^6 CFU $fish^{-1}$. This dose was lower than the one used in the rainbow trout challenge study, as the wrasse were much smaller in size than the rainbow trout

The fish were kept for 14 days, and checked for any morbidity/mortality, gross clinical signs of disease, and inflammation or lesions around the injection site. Because the fish were small, they were only sampled and examined external and internal clinical signs of disease, and bacterial recovery by inserting a sterile loop into the abdominal cavity and streaking onto TNA plates. These were incubated at 15 °C for one week, and checked for bacterial growth and purity.

Table 2.1 Experimental design of the challenge study in goldsinny wrasse.

Group No.	Treatment	No. of fish
1	i.p. with sterile 0.85 % saline	3
2	i.p. with V9 preparation	3
3	i.p. with V13 preparation	3
4	i.m. with sterile 0.85 % saline	3
5	i.m. with V9 preparation	3
6	i.m. with V13 preparation	3

i.p. = intraperitoneal, i.m. = intramuscular, % = percentage.

2.2.5.4 Challenge study in *Artemia* (brine shrimp) nauplii model

Disease-free *Artemia* cysts (Waterlife, Longford, UK) were hatched in sterile artificial seawater (salinity = 25 ‰) at 28 °C with constant aeration (fine bubble air stone) and fluorescent light for 24 hours. An overnight culture of the bacterial isolates were pelleted by centrifugation at 3,500 × *g*, washed once and adjusted to a concentration ~ 10^7 CFU mL^{-1} for infection using the same sterile artificial seawater for *Artemia* hatching. The reason for using the bacterial concentration of 10^7 CFU mL^{-1} for infection was that the concentration of 10^6 CFU mL^{-1} was used as the highest dose by the researchers in their studies of estimating the pathogenicity of vibrios to *Artemia* nauplii (Soto-Rodriguez *et al*., 2003; Austin *et al*., 2005; Defoirdt *et al*., 2007), so that a higher dose, i.e. 10^7 CFU mL^{-1}, was chosen for this study. Hatched *Artemia* nauplii were transferred to 20 mL of sterile artificial seawater, V9 inoculum and V13 inoculum, respectively, with 50 nauplii in each group. Nauplii were mixed well with bacteria for 5 min and then one *Artemia* nauplii was transferred with 250 µL of the according inoculum, using sterile filter pipettes, to one well on a 96-well flat-bottom plate (Sarstedt, UK). The 96-well plates were sealed and incubated at 22 °C for 3 days, and the survival was recorded every 24 hours. *Artemia* nauplii in sterile artificial seawater without adding bacteria were used as the control.

2.2.6 Characteristics of the vibriophages

2.2.6.1 Morphology observation by TEM

The filtered propagated phage suspension was used for morphology observation by TEM following negative staining method (Ackermann, 2009a). Briefly, phage suspension was purified by washing with 0.1 M ammonium acetate (pH 7) three times using a centrifuge (IEC Micromax, US) at 25,000 *x g*, 60 min each time. In the staining processes, 10 µL of the purified phage sample was added onto a 200 square mesh Formvar-coated copper grid, after 1 min adsorption, the excess liquid was drained off with filter paper before 10 µL of 2 % phosphotungstate (pH 7.2) added onto the grid to stain for 1 min. Excess liquid was drained off with filter paper again, and the grid was finally air dried instantly. The grids with stained phage samples were observed under a

Philips (FEI) CM120 Biotwin Transmission Electron Microscope (TEM). The diameters of the phage heads and the lengths of the tails were determined based on the measurments from 5 phages.

2.2.6.2 Host range test

In the phage host range test, 39 *Vibrio* strains which were either from the laboratory collection, or purchased from the National Collection of Industrial, Marine and Food Bacteria (NCIMB), and 12 unidentified bacterial strains (isolated in 2.2.2) were used (Table 2.2). The spot test method from Ra'l & H'bert (2009) was followed. Briefly, 200 µL of an overnight culture of the test bacterial strain was mixed with 5 mL of the molten top agar, and then poured to a plate containing bottom agar. After the top agar solidified, 10 µL of each sterile propagated phage supernatant was spotted over the top layer, and allowed to absorb. All the plates were incubated at 22 °C overnight. The original phage bacterial hosts were used as the positive control for bacterial strains, and sterile phage SM buffer was used as the negative control for phages. The formation of plaques was checked on the following day. The experiment was repeated twice.

Table 2.2 *Vibrio* spp. strains used for phage host range test.

Identification	Collection Number	Source
V. splendidus	NCIMB 1^{T}	NCIMB, Aberdeen
	VIB 498	Laboratory collection Rainbow trout, Australia
	VIB 502	Laboratory collection Salmon, Norway
	VIB 503	Laboratory collection Salmon, Norway
	VIB 505	Laboratory collection, Sea bass, Norway
	VIB 506	Laboratory collection, Sole, Norway
	VIB 507	Laboratory collection Turbot, Norway
	VIB 594	Laboratory collection Seawater, Sweden
	VIB 595	Laboratory collection Seawater, Sweden
	VIB 683	Laboratory collection Oyster, Spain
	VIB 684	Laboratory collection Oyster, Spain
	VIB 685	Laboratory collection Oyster, Spain
V. cyclitrophicus	LMG 21359^{T}	NCIMB, Aberdeen
	LMG 21580	Laboratory collection
V. anguillarum	VIB 01	Laboratory collection
	VIB 11	Laboratory collection, Sea bass, Greece
	VIB 5	Laboratory collection
	VIB 118	Laboratory collection

	VIB 119	Laboratory collection
	VIB 227	Laboratory collection
	VIB 549	Laboratory collection
	VIB 550	Laboratory collection
	VIB 551	Laboratory collection
	B03064	Laboratory collection
V. alginolyticus	LMG 4408^T	Laboratory collection
V. cincinnatiensis	LMG 7891^T	Laboratory collection
V. harveyi	NCIMB 1280^T	Laboratory collection
	VIB 645	Laboratory collection, Sea bass, Tunisia
	0802	Laboratory collection
V. hollisae	CIP 101886^T	Laboratory collection
V. natriegens	LMG 10935^T	Laboratory collection
V. nereis	LMG 3895^T	Laboratory collection
V. ordalli	LMG 13544^T	Laboratory collection
	VIB 02	Laboratory collection
V. parahaemolyticus	LMG 2850^T	Laboratory collection
V. proteolyticus	LMG 3772^T	Laboratory collection
V. salmonicida	MT 1584	Laboratory collection
V. tapetis	102	Laboratory collection, Crab, Ireland
	127	Laboratory collection
Unidentified strains	V1 to V14	This study

LMG = Laboratorium voor Microbiologie, Ghent, Belgium; NCIMB = National Collection for Industrial, Marine and Food Bacteria, Aberdeen.

2.2.6.3 Phage genome analysis

As all the known tailed phages have double stranded linear DNA as their genomes (Ackermann, 2011), in this study, according to the morphology, the two virbiophages were assumed to have DNA genomes. A phenol-chloroform method modified from Pickard (2009) was used for phage DNA extraction. Briefly, 600 µL of sterile propagate phage lysate was transferred into a 1.5 mL Eppendorf, and 6 µL of 1 mg mL^{-1} DNase I and 2.6 µL of 12.5 mg mL^{-1} RNase A were added into the phage lysate, and incubated at 37 ℃ for 30 min to remove bacterial genomic contaminants. In a further incubation at 58 ℃ for 1 hour, 15 µL of 20 % SDS and 6 µL of 10 mg mL^{-1} proteinase K were added to the Eppendorf tube. Then the phage sample was extracted with 0.5 mL of phenol:chloroform:isoamyl alcohol (25:24:1), and spun at 14,000 x *g* for 15 min to separate the phases. The top aqueous phase was removed into a fresh tube, and the above extraction step was repeated once, followed by a further extraction step using chloroform:isoamyl alcohol (24:1). The aqueous phase was transferred to a new Eppendorf tube, and 45 µL of 3 M sodium acetate (pH 5.2) and 500 µL of 100 % isopropanol were added to precipitate the DNA at room temperature for 30 min. The tube was spun at 14,000 *x g* for 20 min and then the DNA pellet was washed twice with 70 % ethanol prior to air drying. Finally, the phage DNA was resuspended with 50 µL of nuclease-free water and stored at 4 ℃.

For analysing the phage genome size, five restriction endonucleases *Hin*d III, *Xba* I, *Spe* I, *Eco*R I and *Eco*R V (purchased from Thermo Scientific, UK) were used to digest the phage genome DNA, respectively. The reaction was carried out in 20 µL volume and incubated overnight at 37 ℃. Restricted DNA products were analysed using 0.8 % agarose gel electrophoresis and visualised using UV light. Lamada DNA/*Hin*d III Marker (Thermo Scientific) was used as the comparison. The electrophoretogram was analysed by using Quantity One® (BIO-RAD) software to determine the sizes of restricted DNA fragments.

2.3 Results

2.3.1 Bacterial isolates from marine fish farm

In total, 14 pure bacterial cultures were isolated and preserved in cryopreservant at -70 ℃ according to their colony morphology on TCBS+N plates, and referred to as V1 to V14. Two of these isolates, V9 and V13, were chosen as the indicator bacteria for phage assays, as plaques were observed on their lawns during phage isolation, but not the lawns of other isolates. Further identification and characterisation of V9 and V13 were carried out.

2.3.2 Phenotypic characterisation of *Vibrio* spp. V9 and V13

On TCBS+N plates, the colonies of V9 were round, smooth and green, whereas the colonies of V13 were yellow (Figure 2.1a). On TNA plates, both V9 and V13 formed round, smooth and creamy colonies, but the colonies of V13 were pinkish (Figure 2.1b). Isolates V9 and V13 were Gram negative, motile, oxidase positive rods that were facultative anaerobic and sensitive to the vibriostatic agent O/129 (10 and 150 µg). The API 20E profiles of V9 and V13 were 0066104 and 3062125, respectively (Table 2.3).

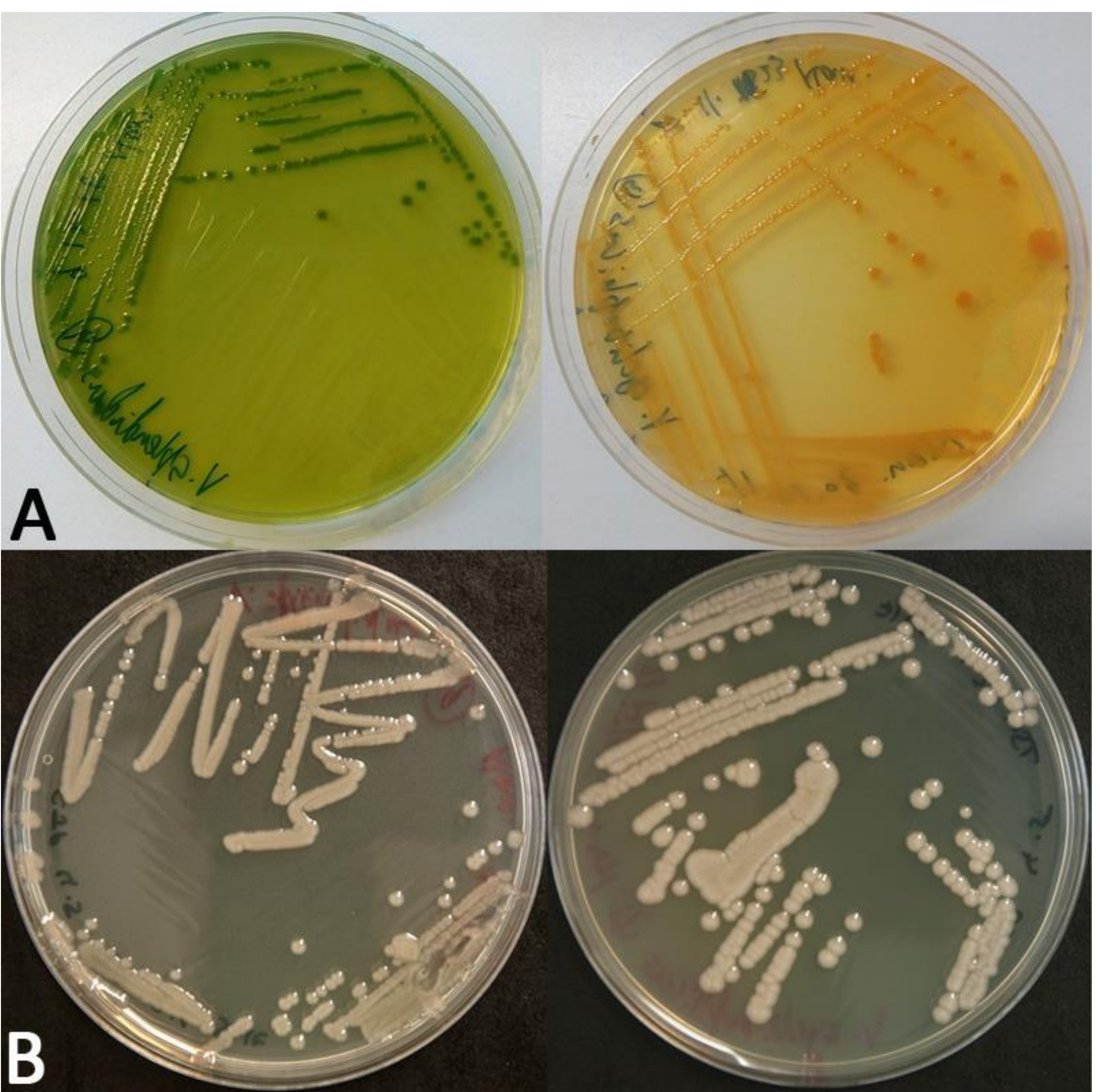

Figure 2.1 Colony morphology of *Vibrio* spp. cultures on the plates of thiosulphate citrate bile salts sucrose agar supplemented with 3 % (w/v) NaCl (A) and tryptone soya agar with 3 % (w/v) NaCl (TNA) (B). The plates growing *V. splendidus* V9 are on the left, and the plates growing *V. cyclitrophicus* V13 are on the right.

Table 2.3 API 20E results of *Vibrio* spp. isolates V9 and V13.

Test	V9	V13
O-Nitrophenyl- β-D-Galactosidase (ONPG)	-	+
Arginine dihydrolase (ADH)	-	+
Lysine decarboxylase (LDC)	-	-
Ornithine decarboxylase (ODC)	-	-
Citrate (CIT)	-	-
H_2S production	-	-
Urease (URE)	-	-
Tryptophane deaminase (TDA)	+	+
Indole (IND)	+	+
Voges-Proskauer (VP)	-	-
Gelatinase (GEL)	+	+
Glucose (GLU)	+	-
Mannitol (MAN)	+	+
Inositol (INO)	-	-
Sorbitol (SOR)	-	-
Rhamnose (RHA)	-	-
Saccharose (SAC)	-	+
Melibiose (MEL)	-	-
Amygdalin (AMY)	-	+
Arabinose (ARA)	-	-

+: positive; -: negative; S: sensitive.

2.3.3 16S rRNA gene sequences and phylogenetic tree

By sequencing and homology searching using nucleotide blast, a 1362 base sequence of the 16S rRNA gene from V9 was obtained, and showed 100 % similarity with the partial sequences from *V. splendidus* LGP32 chromosome 1 (FM954972) and *V. splendidus* isolate PB1-10rnD 16S rRNA gene (EU091328). In the same way, a 1369 base sequence of the 16S rRNA gene from V13 was obtained, and showed 100 % similarity with the partial sequences from *V. cyclitrophicus* JM-2012 16S rRNA gene (KF488567) and *V. cyclitrophicus* HS8 16S rRNA gene (KJ396067). Therefore, V9 and V13 were identified as *V. splendidus* and *V. cyclitrophicus,* respectively.

A phylogenetic tree was constructed with 16S rRNA gene sequences of V9, V13 and 17 other *Vibrio* spp. strains. Both of V9 and V13 were grouped into the Splendidus clade of vibrios: V9 clustered into the branch of *V. splendidus* strains (including the type strain *V. splendidus* LMG 4042^{T}) and V13 clustered into the branch of *V. cyclitrophicus* strains (including the type strain *V. cyclitrophicus* LMG 21359^{T}), which were distinguished from other *Vibrio* spp (Figure 2.2).

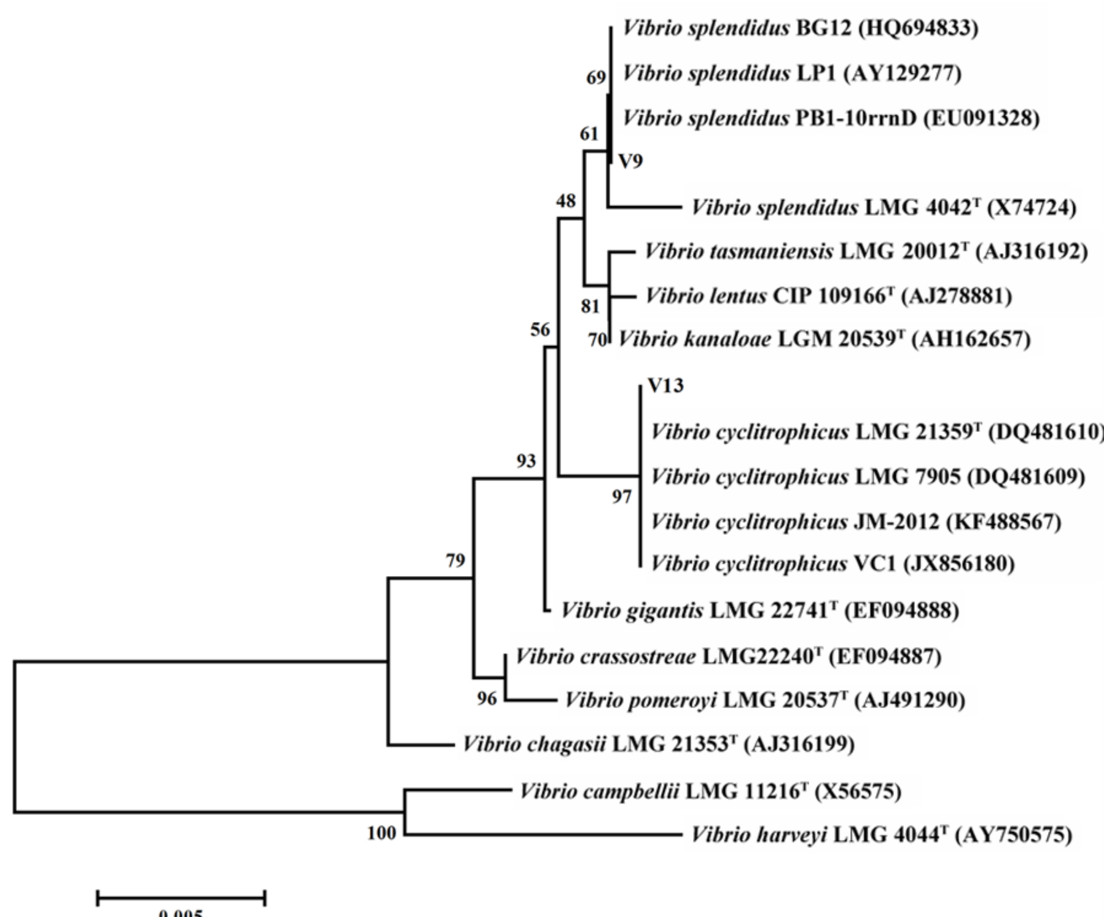

Figure 2.2 Phylogenetic tree constructed by the neighbour-joining method based on the partial 16S rRNA gene sequences of the isolated bacterial strains V9, V13 and 17 other *Vibrio* spp. strains (NCBI accession numbers are indicated in parentheses). *Vibrio campbellii* LMG 11216^{T} and *V. harveyi* LMG 4044^{T} were used as the outgroup. Numbers at nodes indicate the bootstrap percentages derived from 1000 samples. Bar=0.5 % sequence divergence.

2.3.4 Morphology of *V. splendidus* V9 and *V. cyclitrophicus* V13

According to the observation by TEM, *V. splendidus* V9 and *V. cyclitrophicus* V13 shared similar cell morphology: regularly rod-shaped with a slight curve; approximately 1.5-2.0 µm long and 0.5 µm wide in size; with a single polar flagellum. (Figure 2.3).

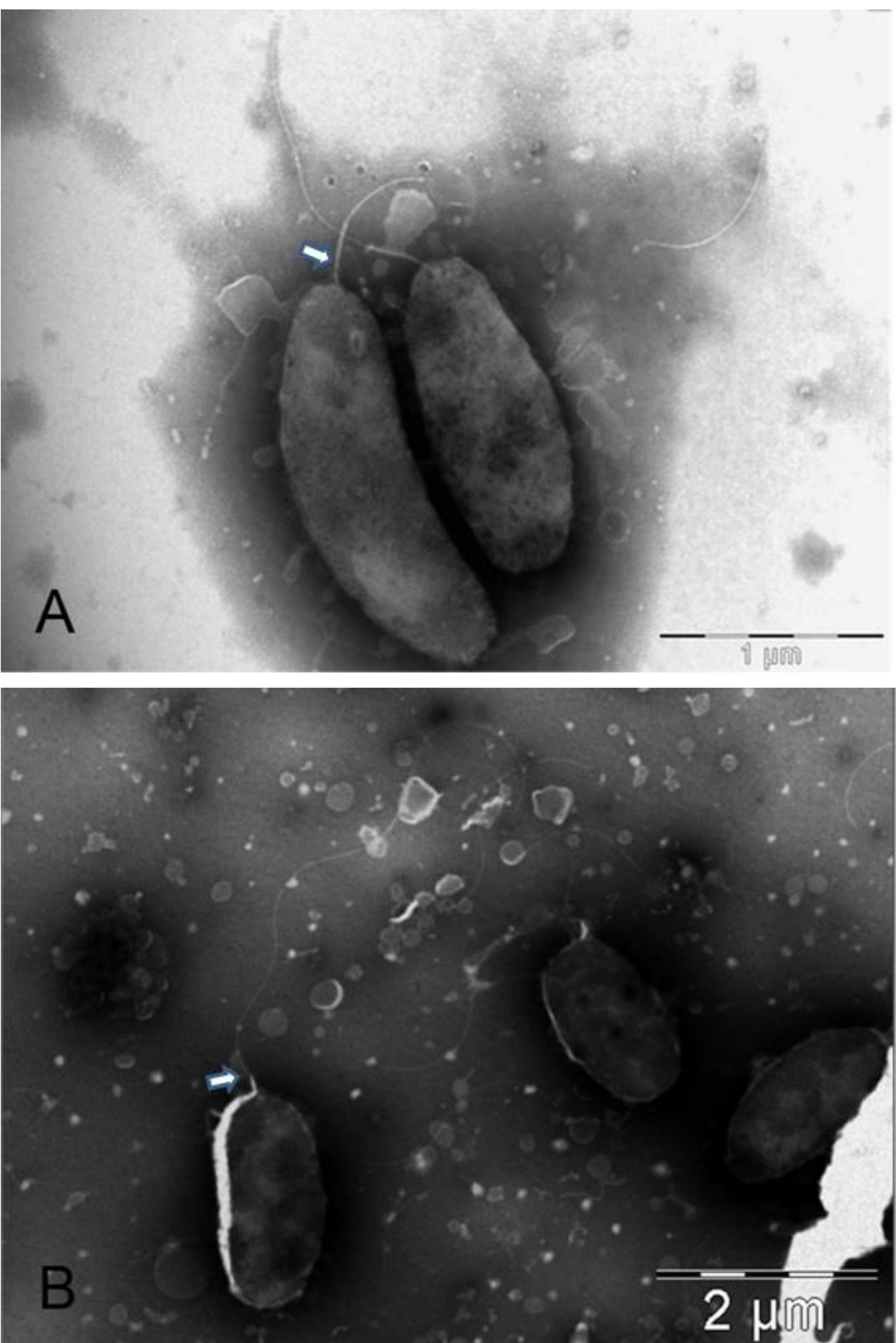

Figure 2.3 Bacterial cell morphology under transmission electron microscope. (A) *Vibrio splendidus* V9; (B) *V. cyclitrophicus* V13. Arrows point out the flagella.

2.3.5 Extracellular enzymes

Both *V. splendidus* V9 and *V. cyclitrophicus* V13 showed haemolysin, gelatinase, lipase and protease activities. *Vibrio splendidus* V9 secreted β haemolysin by presenting complete lysis of blood cells, whereas *V. cyclitrophicus* V13 secreted α haemolysin by presenting incomplete lysis of blood cells. After adding saturated ammonium sulphate to precipitate protein, clear zones appeared around both of the colonies of V9 and V13 on the gelatin plate. Zones of precipitation of salts with fatty acids appeared around both of the colonies of V9 and V13 on the tween 80 plate. A clear zone also appeared around the colony of V13 on the skim milk plate, whereas only a very thin clear ring was around the V9 colony, which showed a weak positive result (Figure 2.4).

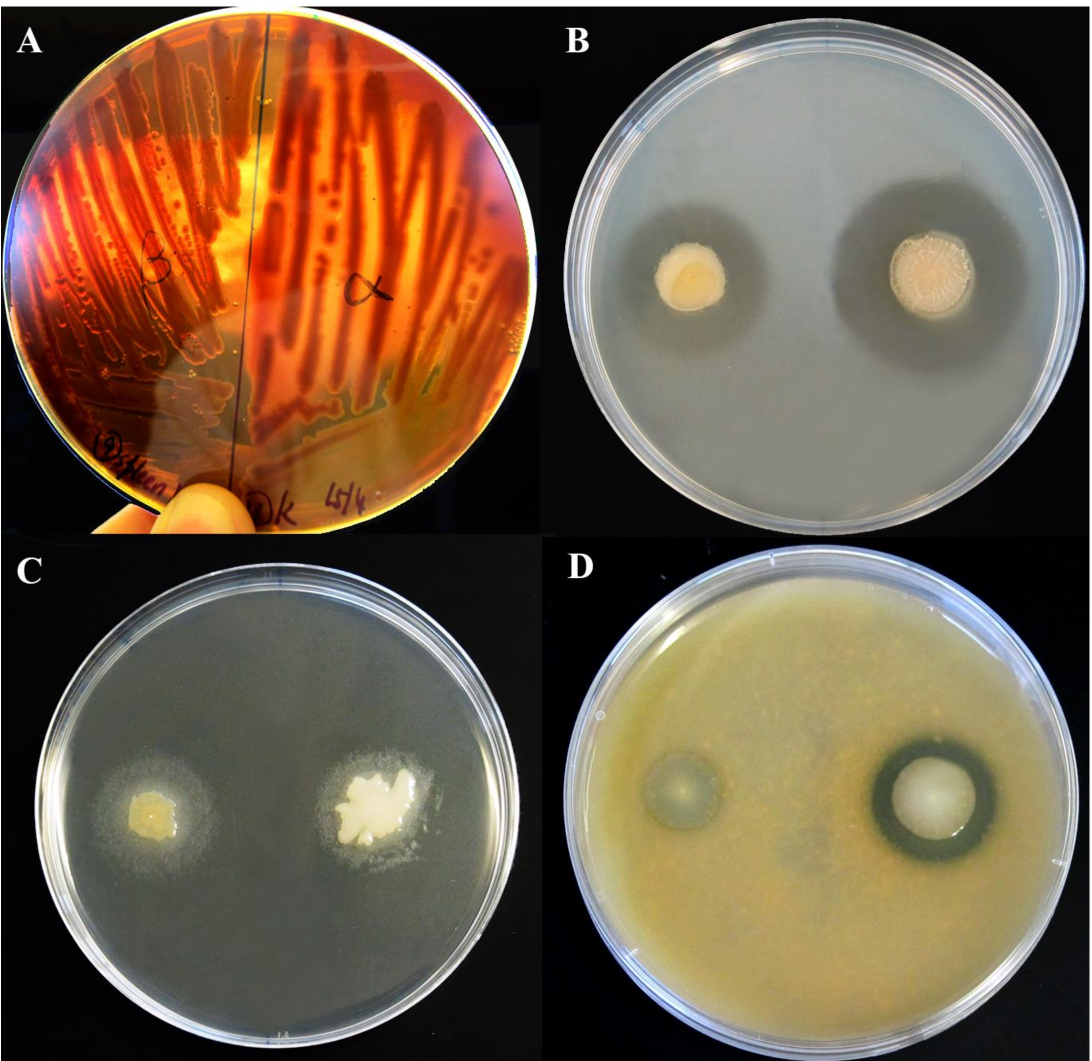

Figure 2.4 Extracellular enzymatic activity tests of (A) haemolysin, (B) gelatinase, (C) lipase and (D) protease. The colonies of *Virbio splendidus* V9 are on the left side, and the colonies of *V. cyclitrophicus* V13 are on the right side of the plates.

2.3.6 Temperature and salinity range of growth

Vibrio splendidus V9 was observed to grow on TNA plates at all the incubation temperatures, i.e. 4 to 37 ℃, on different days. *Vibrio cyclitrophicus* V13 grew on TNA plates at all the incubation temperatures since day 1**.**

Vibrio splendidus V9 grew in 0.5 % to 7 % (w/v) NaCl, whereas *V. cyclitrophicus* V13 grew in 0.5 % to 9 % (w/v) NaCl.

2.3.7 Virulence genes

The virulence genes, *vsm* and *ompU*, were detected by PCR to confirm the possible pathogenicity of the *V. splendidus* isolate V9. Neither *vsm* nor *ompU* was amplified from the genomic DNA of V9. Both of the genes were amplified from the *V. splendidus* type strain NCIMB 1^T (Figure 2.5), and identities of the PCR products were confirmed by sequencing and homology searching using nucleotide blast of NCBI.

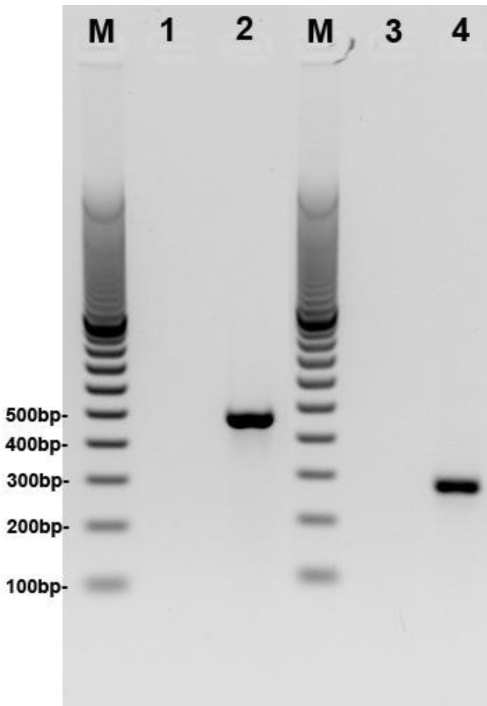

Figure 2.5 Detection of virulence genes *vsm* and *ompU* in *Virbio splendidus* strains: M. 100 bp DNA Ladder; Lanes 1. Product of *vsm* amplified from *V. splendidus* isolate V9; 2. Product of *vsm* amplified from *V. splendidus* NCIMB 1^T; 3. Product of *ompU* amplified from *V. splendidus* isolate V9; 4. Product of *ompU* amplified from *V. splendidus* NCIMB1^T.

2.3.8 Pathogenicity to rainbow trout

After three passages, *V. splendidus* V9 and *V. cyclitrophicus* V13 were recovered from the fish organs and their identities were confirmed. None of the three fish used in the passages of V13 showed morbidity/mortality or any clinical signs of disease. Thus, V13 was considered to be non-pathogenic to rainbow trout, and not included in the challenge study. The first two fish for the passages of V9, did not die nor show morbidity, whereas the third fish was moribund 24 hours post injection, and showed clinical signs of disease including sluggish behaviour, inappetence, darkened pigment, and distended abdomen. On sampling, ascetic fluid in the abdominal cavity, swollen spleen, swollen intestine containing yellow fluid, haemorrhaging on kidney were observed. Based on this result, V9 was used in the following challenge study in rainbow trout.

In the challenge study, according to the viable bacterial colony count, the concentration of V9 preparation injected into the fish was 6×10^7 CFU $fish^{-1}$. Within the 21 days of the challenge, none of the fish died or were moribund, nor showed any abnormal behaviour. At the end of the experiment, no external or internal clinical signs of disease were observed from the sampled fish including the control group, and no bacteria grew on any of the TNA plates sampled from the fish organs. From these results, *V. splendidus* V9 was considered to be non- or of low virulence to rainbow trout and the samples collected for histopathology were not processed further.

2.3.9 Pathogenicity to goldsinny wrasse

Based on the counting of viable bacterial colonies, the concentration of V9 and V13 preparations injected into the fish were 2×10^6 CFU $fish^{-1}$ and 3×10^6 CFU $fish^{-1}$, respectively. No morbidity nor mortality, nor any abnormal behaviour was observed within the 14 days of the challenge. At the end of the experiment, only one fish from the i.p. injection group of *V. cyclitrophicus* V13 was slightly swollen around the injection site on the abdomen, while the rest of the fish did not show any external nor internal clinical signs of disease. No bacteria grew on any of the TNA plates sampled

from the fish abdominal cavity, including the plate sampled from the fish mentioned above with a swollen abdomen.

2.3.10 Pathogenicity to *Artemia* nauplii

During this challenge study, no dead *Artemia* nauplii were found in the control group; two dead *Artemia* nauplii were found in the V9 challenge group at 24 hour and 72 hour, respectively; one dead nauplius was found in the V13 challenge group at 72 hour (Table 2.4).

Table 2.4 The numbers of dead *Artemia* nauplii in each treatment group in pathogenicity experiment (group size = 50), bacterial infection concentration ~ 10^7 CFU mL^{-1}.

	24 h	48 h	72 h	Total
Control group	0	0	0	0/50
V9 challenge group	1	0	1	2/50
V13 challenge group	0	0	1	1/50

2.3.11 Phages isolated from the marine fish farm

Using double agar overlay plaque assay with the 14 bacterial isolated from the same marine fish farm sample, two phages were isolated. One of the phages was hosted by *V. splendidus* V9 and named PVS9, the second was hosted by *V. cyclitrophicus* V13 and named PVC13. Both of PVS9 and PVC13 formed clear plaques on bacterial lawns (Figure 2.6).

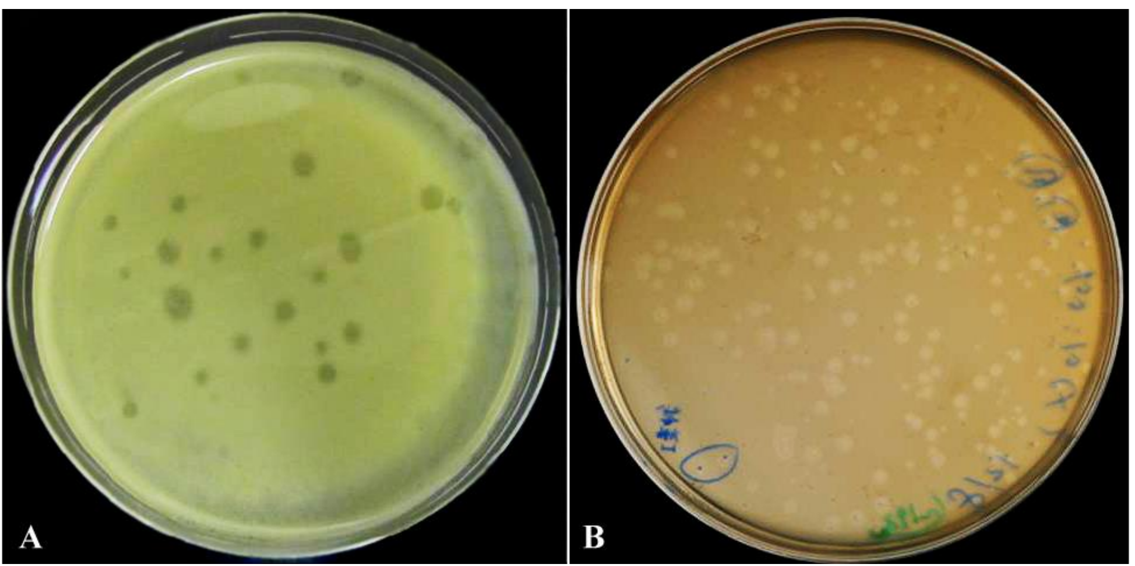

Figure 2.6 Double agar overlay plaque assay. (A) The plaques of phage PVS9 on the bacterial lawn of *Vibrio splendidus* V9; (B) The plaques of phage PVC13 on the bacterial lawn of *V. cyclitrophicus* V13.

2.3.12 Phage morphology

Both PVS9 and PVC13 were tailed phages and belonged to the order of *Caudovirales*. PVS9 showed a hexagonal head (diameter approximately 60 nm) with a neck, a contractile sheath, and a central tube (approximately 120 nm long), which are the morphological characteristics of family *Myoviridae* (Ackermann, 2009b). PVC13 showed the morphological characteristics of family *Siphoviridae* (Ackermann, 2009b): a hexagonal head (diameter approximately 55 nm) with a noncontractile and relatively flexible tail (approximately 120 nm long). A baseplate with spikes was also observed on PVC13 (Figure 2.7).

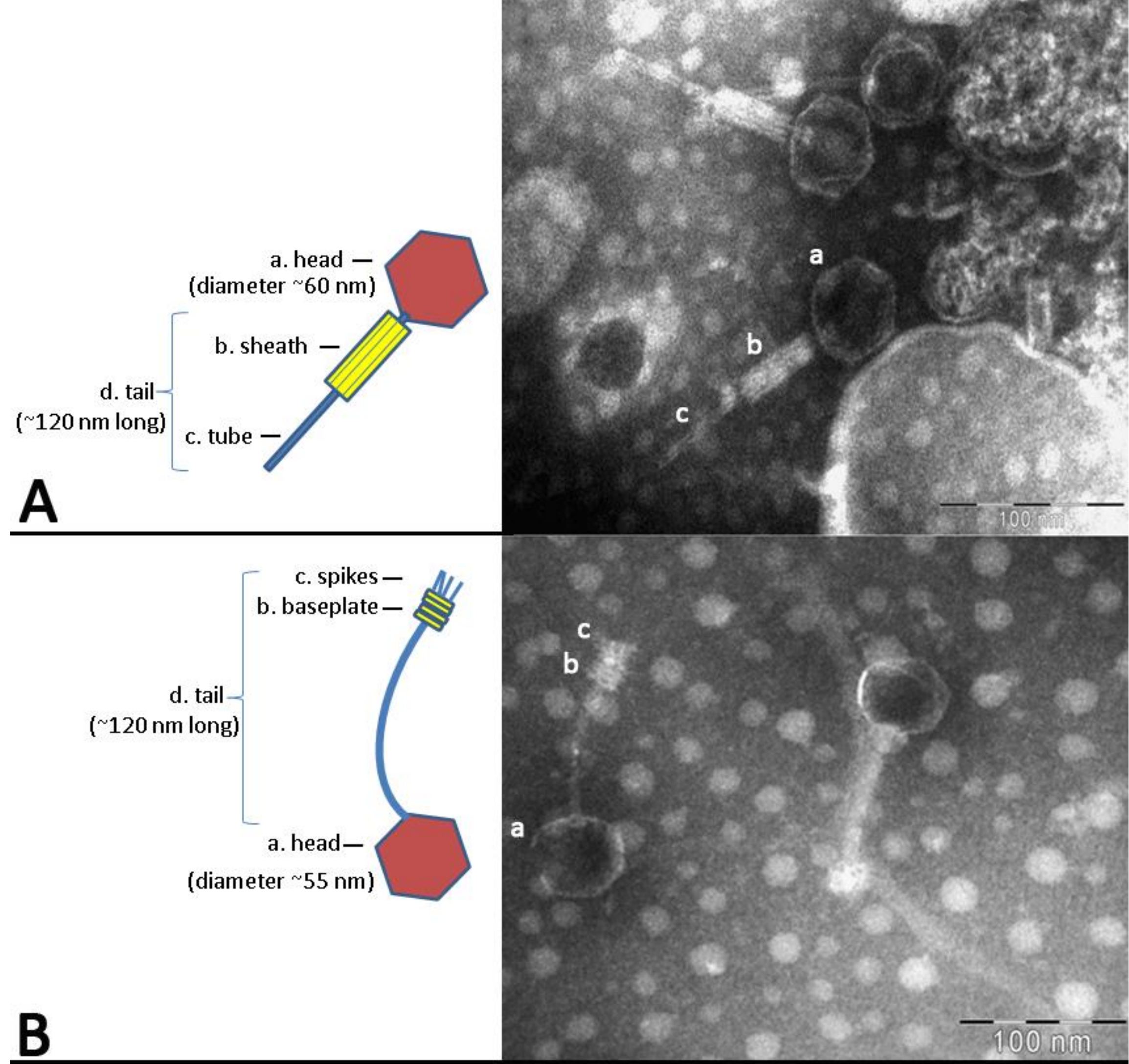

Figure 2.7 Phage morphology under transmission electron microscope and descriptions in schematic. (A) *Vibrio splendidus* phage PVS9 with the morphological characteristics of family *Myoviridae*; (B) *V. cyclitrophicus* phage PVC13 with the morphological characteristics of family *Siphoviridae*.

2.3.13 Phage host range

In the host range test, neither of the vibriophages, PVS9 or PVC13, formed plaques on any of the tested bacterial strains, including twelve *V. splendidus* strains, two *V. cyclitrophicus* strains, twenty-five strains from other *Vibrio* spp., and twelve unidentified strains isolated from the same sample with the phages in this study. Among all the tested bacterial strains, phages PVS9 and PVC13 only specifically infected and lysed *V. splendidus* V9 and *V. cyclitrophicus* V13, respectively.

2.3.14 Phage genome

Using the phenol-chloroform method, the genome DNA of *V. splendidus* phage PVS9 and *V. cyclitrophicus* phage PVC13 was extracted. When being digested with the five restriction endonucleases, only the PVC13 DNA/*Hin*d III product showed four clear bands on electrophoretogram (Figure 2.8). None of the restriction endonucleases could digest the genome DNA of PVS9. However, the genome DNA of PVS9 showed a very similar position with that of the PVC13 on the electrophoretogram. Analysed by Quantity One®, the sizes of the four clear bands from PVC13 DNA/*Hin*d III product were approximately 22.1 kb, 9.9 kb, 8.5 kb and 5.5 kb, with the sum of 46 kb.

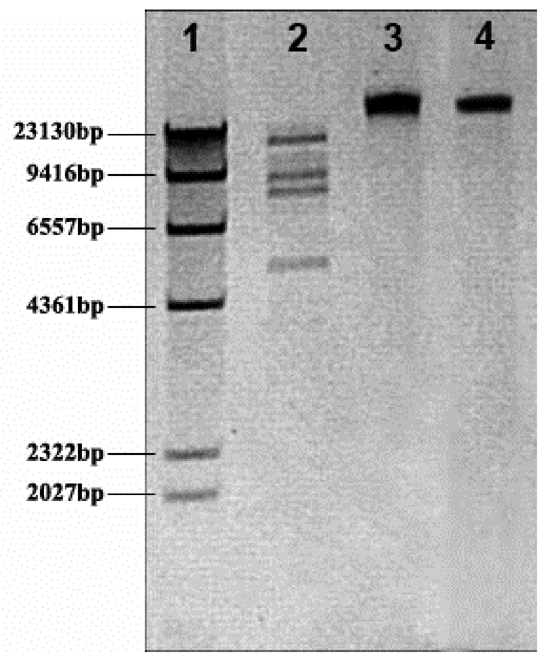

Figure 2.8 Electrophoretogram showing the genome DNA of vibriophages PVC13 and PVS9, and the PVC13 DNA/*Hin*d III product: 1. Lamada DNA/*Hin*d III Marker; 2. PVC13 DNA/*Hin*d III product; 3. genome DNA of PVC13; 4. genome DNA of PVS9.

2.4 Discussion

Phages occupy all the habitats where their bacterial hosts thrive (Skurnik & Strauch, 2006; Wittebole *et al*., 2014). Therefore, phages and their bacterial hosts were isolated from the same sample. In this study two *Vibrio* isolates and their phages were isolated from a same environmental sample from a Scottish marine fish farm. Others have used this strategy for the successful isolation of phages for phage therapy (Vinod *et al*., 2006; Alagappan *et al*., 2010; Cohen *et al*., 2013).

So far, there have been very few reports of *V. splendidus* or *V. cyclitrophicus* phages. The study here successfully isolated the lytic phages of these two *Vibrio* species. According to their morphological characteristics, both of PVS9 and PVC13 belonged to the tailed phage order *Caudovirales*, and the families *Myoviridae* and *Siphoviridae*, respectively. Tailed phages overwhelmingly predominate the currently isolated phages (Ackermann, 2011). The situation is the same for vibriophages, where 227 phages are tailed phages out of a total of 240 isolates up to 2007 (Ackermann, 2007). Ackermann *et al.* (1984) considered that vibriophages are very heterogenous and some have interesting morphological characteristics. In this study, *V. cyclitrophicus* phage PVC13 had the structure of a baseplate with spikes, which is believed to be a device for host-recognition and irreversible adsorption to start an infection (Sciara *et al*., 2010; Yamashita *et al*., 2011). The baseplate-spike adsorption devices are common in phages of the *Myoviridae* family, and those of coliphage T4 have been extensively studied (Thomassen *et al*., 2003; Rossmann *et al*., 2004; Sciara *et al*., 2010). In contrast, the devices are rarely observed in *Siphoviridae* phages, and only a small group of lactococcal phages have similar structures (Labrie *et al*., 2008; Campanacci *et al*., 2010; Sciara *et al*., 2010). The *V. splendidus* myophage PVS9 had a fragile tail, because phages missing tail sheaths and separated single sheaths were observed in the TEM images (data not shown). This may due to the movements of contraction, which lead to tail sheaths becoming loose then sliding along and finally off the tails of the phages (Ackermann *et al.*, 1984).

Some of the reported vibriophages have fairly broad host ranges. Vibriophage KVP40 can infect at least 8 *Vibrio* spp. including *V. parahemolyticus, V. alginolyticus, V. anguillarum, V. cholera, V. fluvialis, V. mimicus, V. natriegens* and *V. splendidus* (Inoue *et al*., 1995). A *Siphoviridae V. harveyi* phage lysed all of the 50 *V. harveyi* strains tested in one study (Vinod *et al*., 2006). Here, *V. splendidus* phage PVS9 and *V. cyclitrophicus* phage PVC13 only specifically infected and lysed *V. splendidus* V9 and *V. cyclitrophicus* V13, respectively, which indicated that both of these phages may have very narrow host ranges. However, because of the lack of *V. cyclitrophicus* strains, only three were used in the host range test. A larger selection of *V. cyclitrophicus* strains

would need to be tested to confirm this result. A narrow host range may be related to using a receptor that rarely presents on most of the bacterial surface. However, the method for phage enrichment and isolation used here should benefit phages that have relatively broad host ranges, so they can use a larger number of hosts for propagation. The case of PVS9 and PVC13 was contrary to this hypothesis; they could not even lyse other *Vibrio* isolates acquired from the same sample. The reasons for acquiring such narrow host range phages rather than broad host range phages may be because most of the phages in the original environment were in a status of lysogenicity, which would result in very low detectable levels of free phages; and the use of the indicator bacterial hosts that were isolated from the same sample with the phages.

Steward *et al*. (2000) suggested that the typical genome DNA sizes of marine phages are in the range from 26 kb to > 200 kb. The genome of *V. cyclitrophicus* phage PVC13 was determined as ~ 46 kb. As it could be digested by at least one restriction enzyme (*Hin*d III), the nature of its nucleic acid was double stranded DNA. However, the genome DNA of *V. splendidus* phage PVS9 could not be digested by any of the five restriction enzymes. So far, all reported tailed phages have linear double stranded DNA genomes (Ackermann, 2011). Moreover, the genome DNA of PVS9 showed a very similar position with that of the PVC13 on the electrophoretogram. Therefore, it is likely that the genome DNA of myophage PVS9 is also linear double stranded with a size of 46 kb approximately. Five restriction enzymes (*Hin*d III, *Xba* I, *Spe* I, *Eco*R I and *Eco*R V) that have been frequently used in experiments of genetic engineering (Green & Sambrook, 2012), especially in digesting bacterial or plasmid DNA, were chosen to digest the genome DNA of PVS9 and PVC13 here. However, both of the phage genome DNA showed resistance to these restriction enzymes, as PVC13 DNA could only be cut by *Hin*d III and PVS9 DNA could not be cut by any of the five enzymes. The reason of this result could be that the DNA of PVS9 and PVC13 had evolved faster than the bacterial defence systems, so that phage DNA could not be recognised and cleaved by the bacterial restriction system that would lead to a successful infection (Tock & Dryden, 2005).

For the identification of bacterial hosts, conventional phenotype testing was initially performed. With characteristics including Gram negative, motile, oxidase positive, facultative anaerobic, curved-rod shape, polar flagella, growing on TCBS, and sensitive to the vibriostatic agent O/129, both V9 and V13 were classified into the genus *Vibrio* (Buller, 2004). However, due to the phenotypic diversity of *Vibrios*, the results of the API 20 E profile of the two isolates V9 and V13 could not be used to identify to species level (according to the API 20 E database from Buller, 2004). Thereafter, 16S rRNA gene sequence analysis, a more robust, reproducible and accurate method for bacterial identification (Clarridge, 2004) was applied. Homology searching using nucleotide blast identified V9 as *V. splendidus* and V13 as *V. cyclitrophicus*. Following phylogenetic analysis was also applied by constructing a phylogenetic tree. From the results, it can be seen that 16S rRNA gene sequence analysis gave more certain and reliable results than the conventional phenotype testing in this case, and using of phylogenetic tree presented a more clear relationship between V9, V13 and other *V. splendidus*-related species. Therefore, it is suggested that 16S rRNA gene sequence analysis is an easier, more direct, and less time consuming method for bacterial identification compared with the conventional phenotype testing.

Both *Vibrio* isolates V9 and V13 showed a relatively wide range of growth temperature (4 - 37 ℃ for both) and salinity (0.5 % - 7 % for V9, 0.5 % - 9 % for V13), which suggests that they can survive in various environmental conditions, and could exist worldwide. In the extracellular enzymatic activity tests, both V9 and V13 showed positive results (some were strong, and some were weak) for the detection of haemolysin, gelatinase, lipase and protease, which have been considered as important or potential virulence factors in *Vibrio* spp. (Zhang & Austin, 2000; Baffone *et al*., 2001; Soto-Rodriguez *et al*., 2003; Zhang & Austin, 2005). However, the possession of these virulence factors does not guarantee an isolate to be pathogenic. It must be confirmed using *in vivo* animal challenges. In this study, three methods were used to estimate the pathogenicity of the *vibrio* isolates to different potential animal hosts: detection of virulence genes for bivalves, challenge in potential animal hosts directly for fish, and challenge in animal model for crustaceans.

In previous studies, extracellular metalloprotease Vsm and outer membrane protein OmpU were characterised as important virulence factors of pathogenic *V. splendidus* in infections of bivalves (Le Roux *et al*., 2007; Binesse *et al*., 2008; Duperthuy *et al*., 2010; Duperthuy *et al*., 2011; Liu *et al*, 2013b), but no evidence showed they are related with the infections of fish. The Vsm protein is the major toxicity factor in the extracellular products (ECPs) of pathogenic *V. splendidus* (Le Roux *et al*., 2007; Binesse *et al*., 2008), and ompU contributes to host antimicrobial peptide resistance and plays a role in the attachment and invasion of oyster haemocytes (Duperthuy *et al*., 2010; Duperthuy *et al*., 2011). *Vibrio splendidus* LGP32, which possesses the virulence genes of both *vsm* and *ompU*, is an oyster pathogen associated with the summer mortality syndrome that dramatically affects the production of *Crassostrea gigas* oysters worldwide (Gay *et al*., 2004a; b; Duperthuy *et al*., 2011). In contrast, a *V. splendidus* strain closely related to LGP32 that lacked the metalloprotease was avirulent to oysters (Binesse *et al*., 2008), and an *ompU* deletion mutant derived from LGP32 gives lower oyster mortalities compared to the wild-type parent strain (Duperthuy *et al*., 2010). Liu *et al*. (2003b) used PCR to detect the virulence genes *vsm* and *ompU* in 43 *V. splendidus* isolates isolated from lesions of moribund Yesso scallops (*Patinopecten yessoensis*). Thirty-four isolates were either *vsm* or *ompU* positive, eight isolates were neither *vsm* nor *ompU* positive, and only one isolate named JZ6 showed positive results for both of *vsm* and *ompU* detection. In their challenge studies, *V. splendidus* TX2, one of the isolates which were both *vsm* and *ompU* negative, and JZ6 were involved in both injection and immersion challenges to Yesso scallops. JZ6 gave a cumulative mortality rate of 80 % and 45 % in injection and immersion challenges, respectively, which were significantly higher than that of TX2 and blank control. The TX2 isolate only gave 15 % and 8.33 % cumulative mortality rates in injection and immersion challenges, respectively, which were no significant differences with that of the blank control (Liu *et al*, 2013b). From these studies, the possession of *vsm* and/or *ompU* genes can be considered as very important characteristics of pathogenic *V. splendidus* isolates to bivalves. In our study, two primer sets were designed according to the sequences of *V. splendidus* LGP32 to detect the virulence genes *vsm* and *ompU* in *V. splendidus* isolate V9, which showed negative results for both of the genes detection. Therefore, V9 was considered to be probably non- or low virulent to

bivalves, and further investigation using animal challenge study was considered as not necessary to be carried out, which saved experimental resource, labour and time.

The *V. cyclitrophicus* V13 isolate was deemed to be non-pathogenic to rainbow trout following passage experiments and was therefore not involved in the further challenge study. Injection of *V. splendidus* V9 into fish caused the third passaged fish to be moribund with clinical signs. In the following challenge study, however, when isolate V9 was injected into rainbow trout at a concentration of 6×10^7 CFU fish^{-1} by intraperitoneal injection none of the challenged fish died or was moribund. No external nor internal clinical signs were observed from the sampled fish, and no bacteria were recovered from the fish organs. The results suggested that only when injected with a very high dose (> 10^8 CFU fish^{-1}) can *V. splendidus* V9 have a chance to cause disease and lead to death in rainbow trout. However, when injected with 6×10^7 CFU fish^{-1}, which was a high concentration, but comparatively lower than the dose used in passage >10^8 CFU fish^{-1}, could not cause disease. This indicates that *V. splendidus* V9 has a very low virulence to rainbow trout. The rainbow trout model was used by Austin *et al*. (2005) to assess the pathogenicity of *Vibrio* isolates. Two members from the Splendidus clade, *V. fortis* and *V. kanaloae*, showed high virulence to rainbow trout, and both had an $LD_{50} < 10^2$ cells fish^{-1}. However, the majority of the Splendidus clade including *V. cyclitrophicus* were deemed to be non- or low virulent due to few clinical signs only occurring with the highest dose (10^6 cells fish^{-1}) injected, and cultures could not be recovered at all or only poorly at the end of the experiment. The finding of *V. cyclitrophicus* as non- or low virulent in rainbow trout is consistent with the results from the study of Austin *et al*. (2005). In the study of Angulo *et al*. (1994), a *V. splendidus* biotype I isolate was pathogenic to juvenile rainbow trout with a LD_{50} of 2.2×10^4 CFU fish^{-1} by intraperitoneal injection. According to Angulo *et al*. (1994), a useful characteristic to differentiate *V. splendidus* biotype I and II isolates is their reaction on TCBS agar: biotype I strains form yellow colonies on TCBS agar, whereas biotype II strains form green colonies. In the current study, *V. splendidus* V9 formed green colonies on TCBS agar, thus it is more closely related to biotype II strains. This may be a further reason that the V9 isolate showed low virulence to rainbow trout. In another study (Bergh & Samuelsen, 2007), 0.2 mL of an $OD_{600} = 2.0$ culture of

V. splendidus LP1 was injected intraperitoneally into unvaccinated Atlanic salmon (*Salmo salar*) smolts. No mortality or gross pathological changes were observed, and no bacteria were recovered from the kidneys. However, the biotype of *V. splendidus* LP1 was not mentioned.

In the challenge using goldsinny wrasse, with the injection concentrations of 10^6 CFU fish^{-1}, no morbidity/mortality, nor any clinical signs of diseases were observed in any of the groups. Only one fish from the i.p. injection group of *V. cyclitrophicus* V13 was a bit swollen around the injection site on the abdomen, but no bacteria were recovered from the fish. Both *V. splendidus* V9 and *V. cyclitrophicus* V13 were deemed as non-pathogenic to goldsinny wrasse in this case. Studies have proved the pathogenicity of *V. splendidus* to Corkwing wrasse (*Symphodus melops*) (Samuelsen *et al*., 2000; Samuelsen *et al*., 2003; Bergh & Samuelsen, 2007). However, so far there is no evidence showing that *V. splendidus* can cause disease in goldsinny wrasse. Birkbeck & Treasurer (2014) found that *V. splendidus* and closely related bacteria dominated the intestinal bacterial flora of goldsinny wrasse larvae, and that they could be pathogenic if the general fitness of larvae was depressed. This was one of the reasons that why goldsinny wrasse was chosen for the pathogenicity test of *Vibrio* spp. V9 and V13. And another reason for this is that goldsinny wrasse is smaller in size than other species of wrasse. In the bath challenge of goldsinny wrasse reported by Bergh & Samuelsen (2007), no significant differences in cumulative mortality were found between *V. splendidus* challenged and unchallenged groups. So far, there have been no reports about the pathogenicity of *V. cyclitrophicus* to wrasse.

Artemia is an excellent model organism for studying disease in crustaceans, as it can easily be cultured in gnotobiotic conditions (Sung *et al*., 2009). In the study of Soto-Rodriguez *et al*. (2003), *Artemia franciscana* nauplii were used to estimate the pathogenicity of luminous vibrios. *Artemia* nauplii were exposed to 10^5 to 10^6 CFU mL^{-1} bacterial inoculations for 1 hour and then kept for 48 hours, and the pathogenic isolates caused significant mortality rates from 35 % to 80 % (Soto-Rodriguez *et al*., 2003). Austin *et al*. (2005) used rainbow trout and *Artemia* nauplii to determine the pathogenicity of 56 isolates representing 26 species of vibrios. *Artemia* nauplii were

incubated in bacterial concentrations of 10^6 cells mL^{-1} for 4 days, and the high virulent isolates caused 75 % -100 % mortality rates (Austin *et al*., 2005). In the current study, bacterial concentrations of 10^7 CFU mL^{-1} were used to incubate *Artemia* nauplii for 48 hours. At the end of the challenge, only 4 % and 2 % mortality rates were caused by *V. splendidus* V9 and *V. cyclitrophicus* V13, respectively. Hence, both of V9 and V13 were regarded as non- or low virulent to *Artemia* nauplii.

So far, some models of vibriophage therapy using aquaculture animals have been successfully set up, and examples include: a *V. harveyi* phage improving shrimp (*Penaeus monodon*) larval survival (Vinod *et al*., 2006); a *V. parahaemolyticus* phage preventing vibriosis in *Artemia franciscana* (Martínez-Díaz & Hipólito-Morales, 2013); and a *V. anguillarum* phage increasing survival of Atlantic salmon with vibriosis (Higuera *et al*., 2013). Here, two *Vibrio* isolates with their phages were successfully isolated. Unfortunately, both of the bacterial hosts showed non- or low virulence to the animals tested in this study. Thus, an *in vivo* phage therapy model could not be set up using these *Vibrio* isolates and their phages. However, according to previous studies (Angulo *et al*. 1994; Gatesoupe *et al*., 1999; Jensen *et al*., 2003; Beaz-Hidalgo *et al*., 2008a; Reid *et al*., 2009), turbot and cod larvae, and corkwing wrasse may be useful as future challenge models for determining the pathogenicity of *V. splendidus* V9. Bivalve models may also be worth testing for the pathogenicity of *V. cyclitrophicus* V13. Using virulent bacterial strains whose pathogenicity has been well studied to isolate phages may provide a more direct approach to set up phage therapy models and so this was pursued in Chapter 3.

Chapter 3

Phage Therapy against *Aeromonas salmonicida* subsp. *salmonicida* Infections in a Rainbow Trout (*Oncorhynchus mykiss*) Model

3.1 Introduction

Aeromonas salmonicida subsp. *salmonicida* generally referred to as typical *A. salmonicida* is the causative agent of furunculosis which is a systemic disease of salmonid fish and has caused significant economic losses in worldwide aquaculture (Bernoth, 1997; Burr & Frey, 2007; Dallaire-Dufresne *et al*., 2014). The use of vaccines and antibiotics has been generally successful in preventing the disease and stopping heavy mortalities during outbreaks of furunculosis, respectively. However, the applications of vaccines have also shown a number of adverse effects (Midtlyng, 1996; Mutoloki *et al*., 2004; Sørum & Damsgård, 2004; Berg *et al*., 2006; Haugarvoll *et al*., 2010; Satoh *et al*., 2011), and using antibiotics leads to the rapid emergence of antibiotic resistant bacteria and the potential retention of the compounds in fish products destined for human consumption (Reith *et al*., 2008; Kim *et al*., 2011; Austin & Austin, 2012; Vega-Sánchez *et al*., 2014; Coscelli *et al*., 2015).

Using phages as bioagents is an alternative approach for the treatment or prophylaxis of bacterial infectious diseases in aquaculture (Nakai & Park, 2002). Since Nakai and Park with their colleagues initiated the exploration of the potential of using phage therapy in aquaculture (Park *et al.*, 1997; 1998; Nakai *et al.*, 1999; Park *et al.*, 2000; Park & Nakai, 2003), more and more bacterial pathogens and aquaculture animals have been involved in this practice worldwide. However, so far, only a few attempts have been made on the experimental applications of phage therapy to control *A. salmonicida* infections (Imbeault *et al*., 2006; Verner-Jeffreys *et al*., 2007; Kim *et al*., 2015).

In this study, two phages of a pathogenic *A. salmonicida* strain were isolated and characterised. Their abilities to inhibit *A. salmonicida* growth *in vitro*, as well as the efficiency of phage therapy against *A. salmonicida* infections in a rainbow trout model were investigated.

3.2 Materials and methods

All the media and chemicals were purchased from Oxoid (Basingstoke, UK), and Sigma-Aldrich (Basingstoke, UK), respectively, unless otherwise stated.

3.2.1 Sample collection

A water sample (100 mL) was collected using a 250 mL sterile laboratory capped bottle (Fisherbrand, UK) from the outlet pond of a Scottish fresh water fish farm in July 2012. The water temperature was 13 °C.

3.2.2 Culture of bacteria

The *A. salmonicida* strains used in this study were cultured on TSA plates at 22 °C or in TSB at 22 °C with orbital vibration at 140 rpm, except the cultures used for challenge experiments which were incubated at 15 °C. The methods used for preparing bacterial suspension for challenges were the same as Section **2.2.5.2.**

3.2.3 Enrichment and isolation of *A. salmonicida* phages

Pathogenic *A. salmonicida* subsp. *salmonicida* Hooke (Vivas *et al*., 2004), was obtained from the collection of Bacteriology Laboratory, University of Stirling, and used as the host in the phage enrichment process. This strain was isolated from rainbow trout in England in 1991 by Professor Brian Austin. The method used was similar to Section **2.2.3**, except 1 mL of *A. salmonicida* Hooke fresh culture was added into the water sample and TSB mixture, which then was incubated at 22 °C, 140 rpm for 24 hours.

The double agar overlay plaque assay as Section **2.2.3** was used for phage isolation, purification and enumeration, except the media used were TSA and TSB, and the temperature for incubation was 22 °C. *Aeromonas salmonicida* Hooke was used as the indicator bacterial strain.

As the *A. salmonicida* phages in this study replicated poorly in liquid broth (data not shown), a soft-agar method adapted from Fortier & Moineau (2009) was used for phage propagation, and also the preparations of phage for stocking and *in vivo*

injections. Firstly, the double agar overlay TSA plates were made following the same method for phage isolation, but with a greater number of phage aimed for complete lysis. The plates were incubated at 22 ℃ for 18-24 hour, and then the top agar was scraped and transferred into a centrifuge tube by a sterile spreader. The bottom plates were rinsed with 0.5 mL of phage SM buffer to recover residual phages and transferred into the same centrifuge tube. The supernatant was removed following one centrifugation at 4,000 x *g* for 10 min. Finally, the phage suspension was harvested by another centrifugation at 4,000 x *g* for 10 min and filtration through sterile 0. 20 µm filters. The methods for phage preservation were the same as described in Section **2.2.3**.

3.2.4 Phage morphology observation by TEM

The filtered propagated phage suspensions were used for morphology observations by TEM following the negative staining method as described in Section **2.2.6.1**.

3.2.5 Host range test

In the phage host range test, 15 *A. salmonicida* subsp. *salmonicida* strains and 2 *A. hydrophila* strains, which were either from the laboratory collection or purchased from the National Collection of Industrial, Marine and Food Bacteria (NCIMB), were included (Table 3.1). The spot test method of Section 2.2.6.2 was applied here, and *Aeromonas salmonicida* subsp. *salmonicida* strain Hooke was used as the positive control.

Table 3.1 Bacterial strains used for phage host range test.

Identification	Collection Number	A-layer	Source
Aeromonas salmonicida subsp. *salmonicida*	Hooke	+	Laboratory collection, England, 1991
	NCIMB 1102^{T}	-	Purchased from NCIMB
	MT 004	-	Laboratory collection, Ellis *et al.*, 1988;
	MT 423	+	Laboratory collection, Hirst & Ellis, 1994
	Fy	undetermined	Laboratory collection Scotland
	N5	undetermined	Laboratory collection Scotland
	76	undetermined	Laboratory collection Atlantic salmon, England 1976
	LL	undetermined	Laboratory collection, salmon farm, Scotland
	B01004	undetermined	Laboratory collection rainbow trout, Farm code 1 Scotland, 2001
	B99106	undetermined	Laboratory collection rainbow trout, Farm code 2 Scotland, 1999
	B99109	undetermined	Laboratory collection Atlantic salmon, Farm code 3, Scotland, 1999
	B05050	undetermined	Laboratory collection rainbow trout, Farm code 4 Scotland, 2005
	B02085(1)	undetermined	Laboratory collection Atlantic salmon, Farm code 5, Scotland, 2002
	B05059	undetermined	Laboratory collection, cod Farm code 6 Scotland, 2005

	B04061	undetermined	Laboratory collection, cod Farm code 7 Scotland,2004
	B99059	undetermined	Laboratory collection brown trout, Farm code 1 Scotland, 1999
A. hydrophila	NCIMB9240^T	n/a	Purchased from NCIMB
	NCIM1134	n/a	Purchased from NCIMB

+: positive; -: negative; n/a: not applicable.

NCIMB = National Collection of Industrial, Marine and Food Bacteria, Aberdeen.

Farm code, the location of the farms is confidential but randomly allocated numerical codes were provided per isolate to indicate that these had been recovered from different clinical outbreaks in different farm locations.

3.2.6 Determination of phage genome size by pulsed field gel electrophoresis (PFGE)

Phage genome sizes were determined using PFGE, because it saves the steps of phage DNA extraction and endonuclease digestion, when compared with the restriction endonuclease analysis. The methods were modified from Sambrook & Russell (2001) and Bartie *et al*. (2012). Briefly, 1 µL of DNase I (1 µg mL^{-1}) was added to 10 µL of phage suspension and the mixture was incubated at 37 °C for 30 min. Then 4 µL of 2.5 x SDS-EDTA Dye Mix (0.4 % (v/v) of 20 % (w/v) SDS solution, 30 mM EDTA, 0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF, 20 % (w/v) sucrose) was added, and the mixture was incubated at 65 °C for 5 min. Samples were stored at 4 °C until use.

Samples were loaded into separate wells of 1% agarose gel (Gibco, UK), which was run using a Rotaphor (R) Type V system (Biometra, UK) in 0.25 x TBE at 14 °C, 6 V cm^{-1} and a 120° angle for 20 hours with a 2-10 s switch time and rotor speed of 9. Lambda PFG Ladder (NEB, UK) and Lamada DNA/*Hin*d III Marker (Thermo Scientific) were used as the molecular size markers. The gels were stained with 0.5 ug/mL ethidium bromide solution for 30 min and de-stained in distilled water for 10 min. Finally, the gel was visualised using UV light.

3.2.7 *In vitro* test of phage inhibition on *A. salmonicida*

Aeromonas salmonicida Hooke, and *A. salmonicida* phages pAS-3 and pAS-6 were involved in this experiment. Phage preparation and fresh bacterial broth culture were co-inoculated in 250 µL of TSB at MOI = 1,000. The cultures were incubated with shaking at 140 rpm and 22 °C. In the bacterial growth control group, containing only sterile phage SM buffer was inoculated with bacteria. TSB without any inoculum was used as the blank control to monitor for any contaminations. Each treatment had four replicates. The experiment was carried out using 96-well flat bottom microplates (SARSTEDT, UK), which were read at OD_{600} every hour using a microplate reader (Synergy HT, BioTek, UK) from 0-18 hour.

3.2.8 *In vivo* trials in rainbow trout

All the juvenile rainbow trout used in the following challenge trials were from a same fish farm in Scotland. The conditions for keeping the fish, the methods for preparing bacterial inoculums and the passages of *A. salmonicida* Hooke, were the same as described in Section **2.2.5.1**, except that for *A. salmonicida* Hooke OD_{600} = 0.2 was equal to approximately 1 x 10^8 CFU mL^{-1}. All the injections in these trials were intraperitoneal. Phage preparations were tested for sterility by plating onto sterile TSA plates and incubating at 22 °C for 48 hours, and then checking for bacterial growth.

3.2.8.1 Dose response of *A. salmonicida* Hooke in rainbow trout

To estimate the relationship between the concentrations of *A. salmonicida* Hooke injected into rainbow trout and the cumulative moralities of the fish population, bacterial concentrations of 1 x 10^2, 10^3 and 10^4 CFU $fish^{-1}$ were injected into three groups of 6 fish per group. Another group of fish were injected with sterile saline as controls. The fish were kept for 14 days, and checked four times daily for morbidity/mortality. Freshly dead, moribund fish and survivors were observed and sampled for external and internal clinical signs of disease and bacterial recovery. Recovered bacteria were presumptively identified as the challenge strain by the production of a brown diffusible pigment when inoculated onto TSA plates, and identification was confirmed using a species specific PCR with the primer set Fer-3 (5’-

CGGTTTTGGCGCAGTGACG-3')/Fer-4 (5'-AGGCGCTCGGGTTGGCTATCT-3'), flanking a fragment of the *fstA* gene (coding for the ferric-siderophore receptor) (Beaz-Hidalgo *et al*., 2008b). The PCR reactions were performed in the following conditions: an initial denaturation at 95 °C for 2 min followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min. Then, the reaction was completed by extension at 72 °C for additional 5 min.

3.2.8.2 *In vivo* safety test of phage preparations

As the raw phage preparations to be used for phage therapy contained bacterial components, a safety test was carried out before the trial, to assess whether or not the phage preparations of pAS-3 and pAS-6 would cause any mortality or clinical signs of diseases in rainbow trout. The safety test used three groups of 5 fish per group. In two of the groups, 100 µL of 10^8 PFU mL^{-1} pAS-3 or pAS-6 were injected into each group, respectively. Sterile phage SM buffer was injected into the control group. The fish were kept for 14 days, and checked four times daily for any gross clinical signs of disease, and morbidity/mortality which would be further investigated by post-mortem, phage and bacterial recovery from kidney, and histopathology. All fish that survived were observed and sampled at the end of the experiment for any external or internal clinical signs of disease.

3.2.8.3 Phage therapy against the infection caused by *A. salmonicida*

The design of the phage therapy experiment against *A. salmonicida* Hooke infections in rainbow trout is shown in Table 3.2. *Aeromonas salmonicida* Hooke was injected into all the fish using a concentration of 1 x 10^2 CFU $fish^{-1}$ which should cause approximately 40 % mortality from the previous challenge study (Section **3.2.8.1**). Phage preparations of pAS-3 or pAS-6 were then immediately injected into two treatment groups of fish, respectively, using a MOI=10,000. The reasons for choosing MOI=10,000 were: first, using a lower MOI = 1,000 gave a good result of inhibition on bacterial growth *in vitro*, so using MOI=10,000 would be expected to give a better inhibition *in vivo*; and second, using MOI=10,000 meant 1 x 10^6 PFU $fish^{-1}$ of phage preparation would be injected, this concentration was lower than the one used in the

phage preparation safety test, which meant this concentration is supposed to be safe for fish. Sterile phage SM buffer was injected into the remaining treatment group. All the treatments had three replicates, and 10 fish were included in each replicate. Five fish were injected with sterile saline and phage SM buffer only and these were negative control. The fish were kept for 14 days, and checked four times daily for morbidity/mortality. Freshly dead, moribund fish and 50 % of the survivors from each group were sampled for bacterial recovery. Two of the *A. salmonicida* colonies recoved from each fish were selected randomly to test for phage sensitivity using phages pAS-3 and pAS-6.

Table 3.2 Experimental design of the phage therapy trial in rainbow trout (concentration of *Aeromonas salmonicida* Hooke injected = 10^2 CFU fish^{-1}, multiplicity of infection (MOI) = 10,000).

Treatment group	Treatment	No. Fish per replicate	No. replicates
1	phage SM buffer	10	3
2	treated with pAS-3	10	3
3	treated with pAS-6	10	3

3.2.9 Statistical analysis

Minitab software version 16 (Minitab Inc., Pennsylvania) was used in this study to perform basic descriptive statistics and comparisons using a significance level of 5% (P = 0.05). Prior to analysis, datasets were checked for normality using the Anderson-Darling test. Post-hoc analyses were carried out using Tukey's multiple comparison tests with values considered significantly different at P-values <0.05. Analysis of variance (ANOVA) manipulated by general linear models (GLMs) considering treatment as a fixed factor was used to analyse cumulative mortalities.

3.3 Results

3.3.1 *Aeromonas salmonicida* phages

Using the water sample collected from the outlet pond of a Scottish fresh water fish farm and *A. salmonicida* Hooke as the indicator, two phages were isolated according to different plaque morphologies. The phage forming clear plaques was named pAS-3, and the phage forming turbid plaques was named pAS-6 (Figure 3.1).

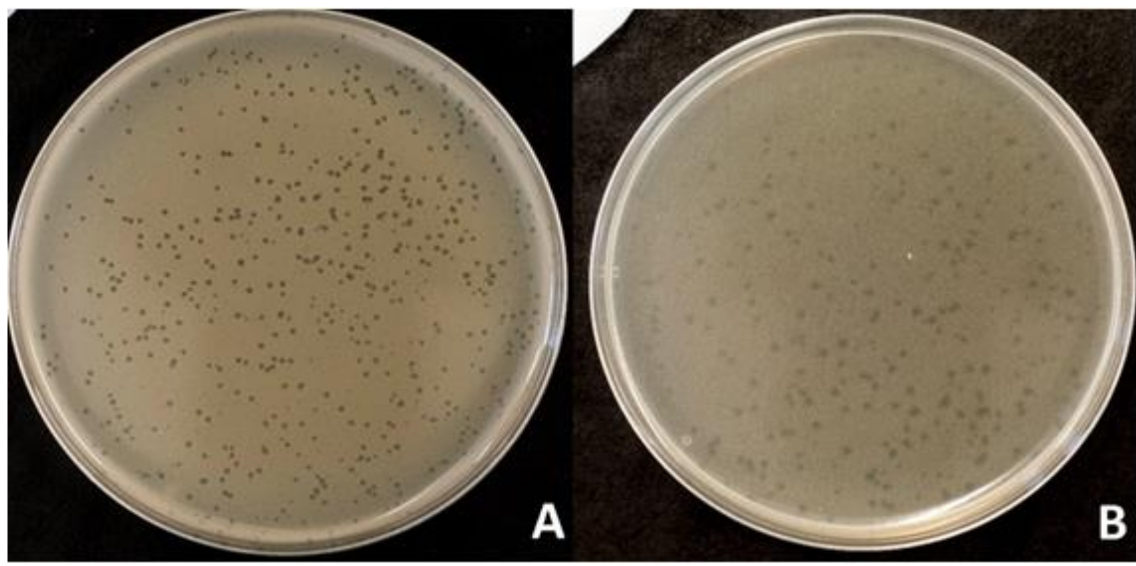

Figure 3.1 Plaque morphology of *Aeromonas salmonicida* phages using *A. salmonicida* strain Hooke as the indicator. (A) *A. salmonicida* phage pAS-3 formed clear plaques; (B) *A. salmonicida* phage pAS-6 formed turbid plaques.

3.3.2 Phage morphology

Both pAS-3 and pAS-6 were tailed phages and belonged to the order of *Caudovirales*. Phage pAS-3 showed the morphological characteristics of family *Siphoviridae* (Ackermann, 2009b): a hexagonal head (diameter approximately 50 nm) with a noncontractile and rigid tail (approximately 100 nm long). Phage pAS-6 showed the morphological characteristics of family *Podoviridae* (Ackermann, 2009b): an icosahedral head (diameter approximately 55 nm) with a short tail (Figure 3.2).

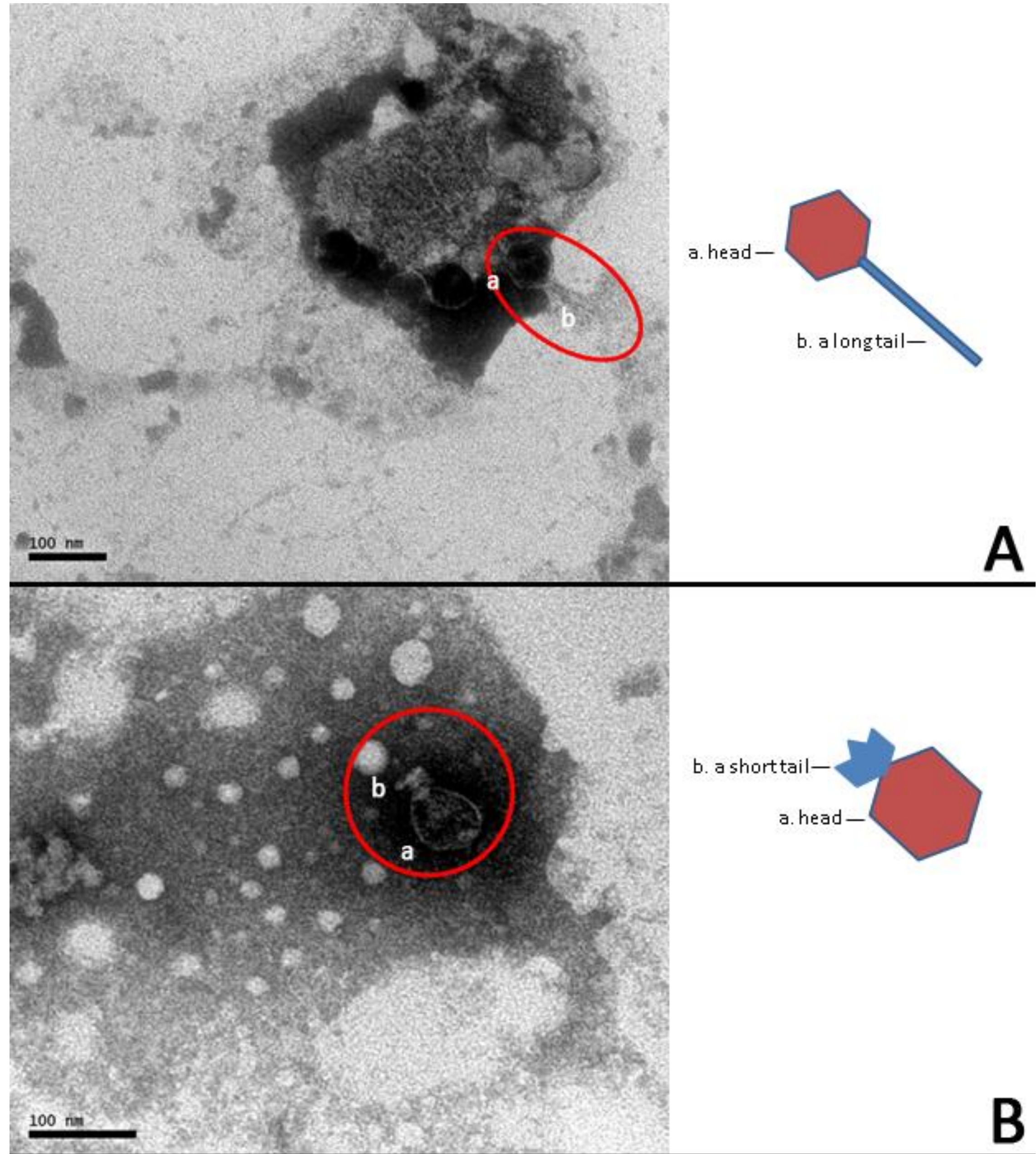

Figure 3.2 Phage morphology under transmission electron microscope and descriptions in schematic. (A) *Aeromonas salmonicida* phage pAS-3 with the morphological characteristics of family *Siphoviridae*; (B) *A. salmonicida* phage pAS-6 with the morphological characteristics of family *Podoviridae*. Red circles show phage positions.

3.3.3 Phage host range

In the host range test, phages pAS-3 and pAS-6 lysed the *A. salmonicida* strains B01004, B99059, B99106 and 76 only. These isolates were all recovered from clinical cases of furunculosis in farmed fish in the UK.

3.3.4 Phage genome size

The genome DNA of pAS-3 and pAS-6 showed a very similar position which was just a little higher than the band of 48.5 kb from Lambda PFG Ladder on the electrophoretogram. Therefore, the genome sizes of both *A. salmonicida* phages pAS-3 and pAS-6 were determined as approximately 50 kb (Figure 3.3).

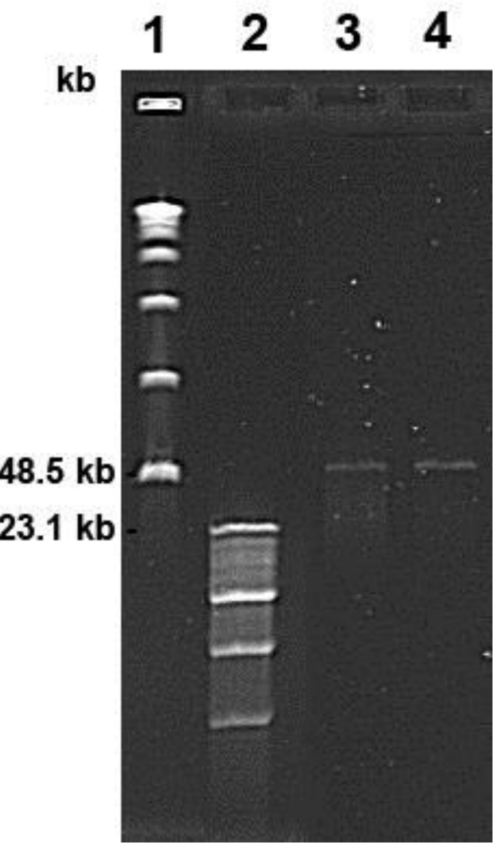

Figure 3.3 Electrophoretogram showing the genome DNA sizes of *Aeromonas salmonicida* phages determined by pulsed field gel electrophoresis: 1. Lambda PFG Ladder; 2. Lamda DNA/*Hin*d III Marker; 3. genome DNA of pAS-3; 4. genome DNA of pAS-6.

3.3.5 Inhibition of the growth of *A. salmonicida* Hooke *in vitro*

As shown in Figure 3.4, control inoculations of *A. salmonicida* Hooke with phage SM buffer reached logarithmic phase at 7 hour, and grew continuously to the stationary phase with a final OD_{600} value ≈ 0.6. The inoculations of *A. salmonicida* Hooke with phage pAS-6 showed a similar growth trend to that of the control, but there was some inhibition of growth and the culture reached a lower final OD_{600} value ≈ 0.52. In contrast, phage pAS-3 totally inhibited the growth of *A. salmonicida* Hooke. No contamination was observed from the blank control.

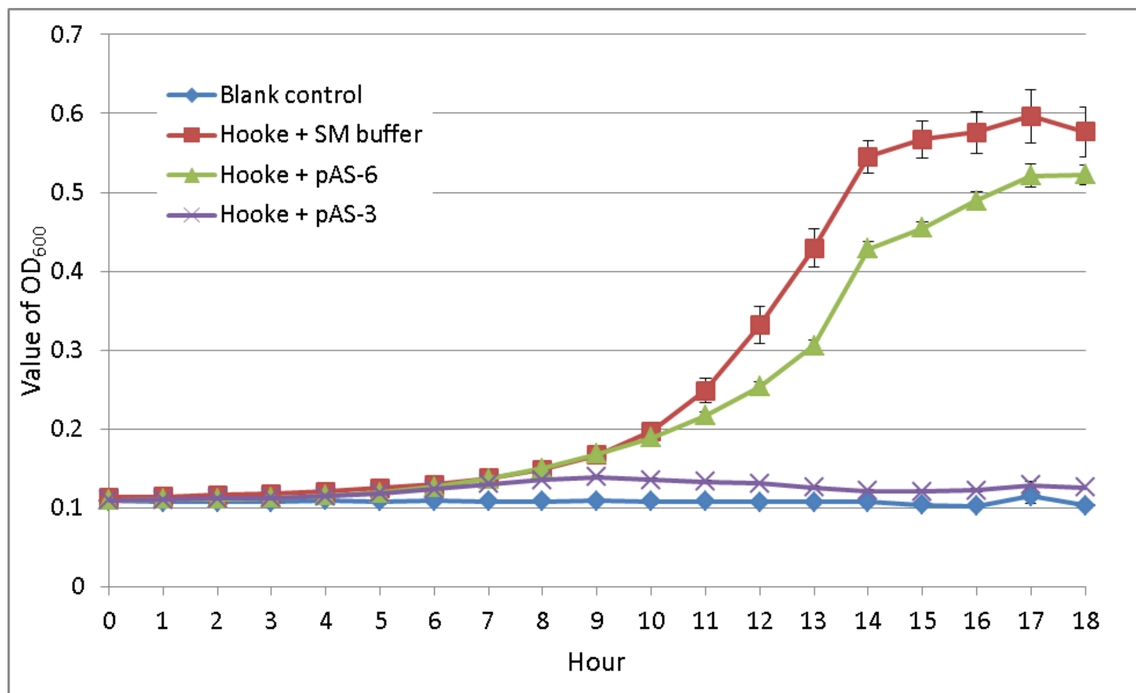

Figure 3.4 *In vitro* test of phage inhibition on the growth of *Aeromonas salmonicida* Hooke. Multiplicity of infection (MOI) = 1,000. The results were shown as mean ± standard deviation from four replicates (not all error bars visable).

3.3.6 Dose response challenge

At the end of this 14 days *A. salmonicida* challenge, 2, 5 and 5 fish were dead or moribund in the groups that had been injected with concentrations of 1 x 10^2, 10^3, 10^4 CFU $fish^{-1}$ generating final mortalities of 33 %, 83 % and 83 %, respectively, with no mortalities observed in the uninfected control group (Figure 3.5). Across all the challenge groups, mortality commenced at day 3 and ceased by day 8. All the dead or

moribund fish showed internal clinical signs of haemorrhaging over the abdominal walls, viscera and intestinal tract, and bacteria were recovered from the kidney. No clinical signs were observed nor were any bacteria recovered from the surviving fish, including the control group. All the recovered bacteria produced brown pigment on TSA plates and their identity was confirmed as *A. salmonicida* using specific PCR.

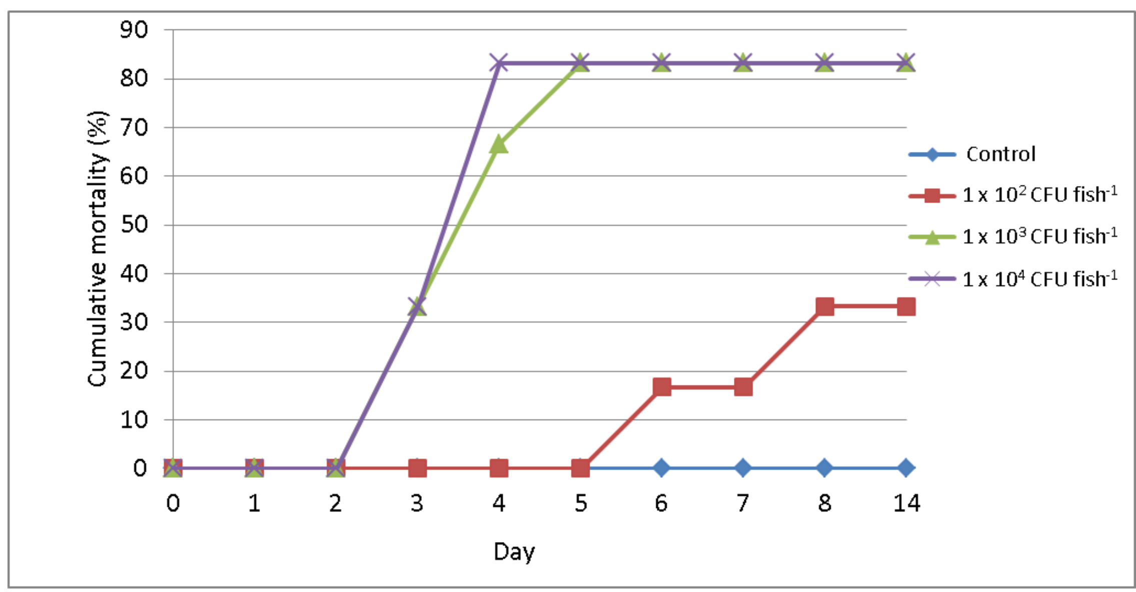

Figure 3.5 Mortality responding in rainbow trout to different injection concentrations of *Aeromonas salmonicida* Hooke. No death happened from day 8-14.

3.3.7 Safety test of phage preparations

None of the injected fish died or were moribund during the 14-day safety test of *A. salmonicida* phage pAS-3 and pAS-6 preparations (data not shown). At the end of the experiment, no external or internal clinical signs of disease were observed from the sampled fish including the control group.

3.3.8 Phage therapy in rainbow trout model

At the end of the 14-day phage therapy trial, the cumulative mortalities of the treatments with phage SM buffer, phage pAS-3 and phage pAS-6 were 37 %, 17 % and 27 %, respectively. Mortalities occurred rapidly in the control group from day 3 to day 6, while this was slower and delayed in the phage treated groups. Mortalities occurred from day 4 to day 8, and day 3 to day 10, respectively, in the phage pAS-3 and pAS-6

treatment groups (Figure 3.6). There were, however, no significant differences between the final cumulative mortalities and time to death of the three treatment groups (p>0.05). No fish died in the control group. Bacteria with brown pigmentation on TSA were recovered from all the freshly dead or moribund fish, but no bacteria were recovered from the survivors. In the phage sensitivity test, all of the selected colonies from all the dead or moribund fish were still sensitive to *A. salmonicida* phages pAS-3 and pAS-6.

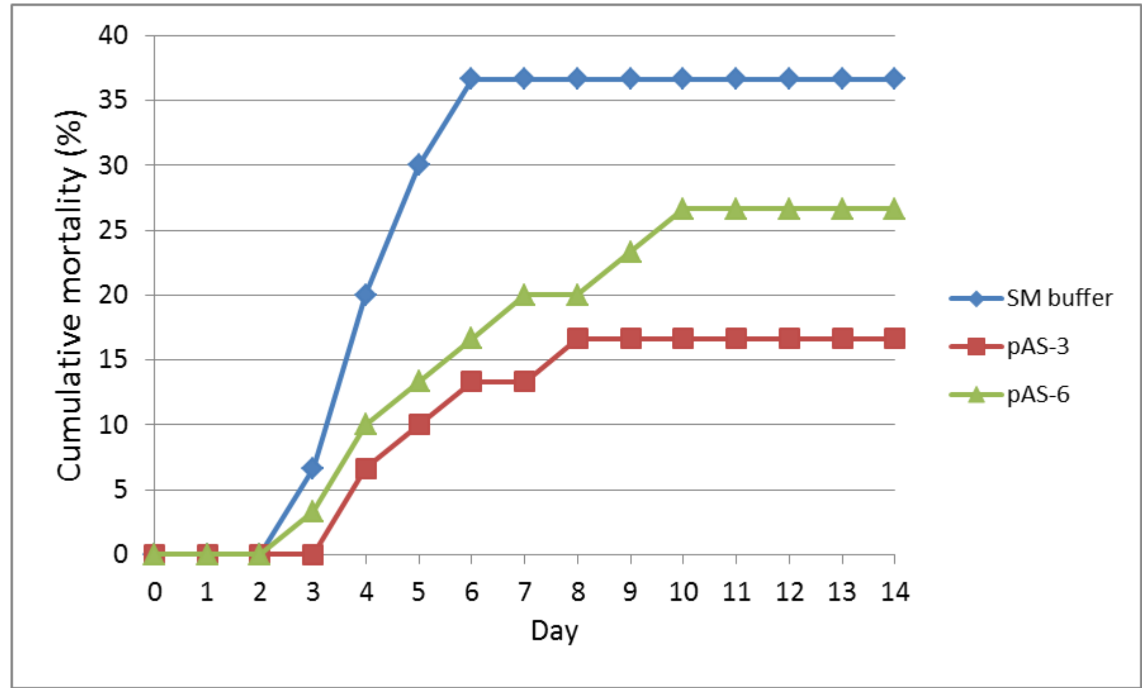

Figure 3.6 Cumulative mortalities of three treatment groups (phage SM buffer, phage pAS-3, phage pAS-6) in the phage therapy trial.

3.4 Discussion

An *in vivo* phage therapy model would not be set up in Chapter 2 due to a lack of pathogenicity of the bacterial strains in tested animals. Therefore, a pathogenic *A. salmonicida* subsp. *salmonicida* Hooke was used as the host to isolate phages. Considering the bacterial host was isolated from diseased fish, and any phages isolated would be applied in an aquaculture environment, a water sample collected from the outlet pond of a Scottish fresh water fish farm was used for phage isolation.

A number of *A. salmonicida* phages have been reported (Paterson *et al*., 1969; Kim *et al*., 2012a; b; c; d). Based on morphological characteristics, most of them are myophages, and only a few are podophages, whereas, to the best of our knowledge, no siphophages have been reported. In this study, two *A. salmonicida* phages were isolated. According to their morphological characteristics, pAS-3 was identified as a siphophage, and pAS-6 was identified as a podophage. The isolation of these two phages enriched the biodiversity of *A. salmonicida* phages.

In the host range test, both *A. salmonicida* phages, pAS-3 and pAS-6, were able to lyse four out of 15 strains of the *A. salmonicida* subsp. *salmonicida* strains tested. These four strains were isolated from diseased salmonid fish in the UK, but in different years. From these results, it is speculated that both *A. salmonicida* phage pAS-3 and pAS-6 have a relatively narrow host range. According to the studies of Kim *et al*. (2012a; b; d), *A. salmonicida* phages can have a broad host range, or be extremely host specific: *A. salmonicida* myophage PAS-1 showed broad infectivity in all the tested *A. salmonicida* strains (17), and another myophage phiAS5 was able to infect all the tested *A. salmonicida* subsp. *salmonicida* and subsp. *masoucida* strains (16), as well as seven *A. hydrophila* strains and one *A. sobria* strain, whereas *A. salmonicida* podophage phiAS7 was only able to infect one of the tested *A. salmonicida* subsp. *salmonicida* strains tested.

PFGE has been employed to resolve fragments of genomic DNA or whole viral genomic DNAs with the range of 0.2 kb to 12 Mb in many research work (Lingohr *et al*., 2009). Thus, in this study, PFGE was applied to detect the genomic DNA sizes of *A. salmonicida* phage pAS-3 and pAS-6. According to the results from PFGE, pAS-3 and pAS-6 had genome sizes of approximately 50 kb, which is close to the genome sizes of *A. salmonicida* podophage phiAS7 and myophage PAS-1 (42kb and 48 kb, respectively), but much smaller than that of *A. salmonicida* myophage phiAS4 and phiAS5 (164 kb and 225 kb, respectively) (Kim et a., 2012a; b; c; d).

In the *in vitro* growth inhibition experiment, with the MOI=1,000, pAS-3 successfully inhibited the growth of *A. salmonicida* Hooke during the whole 18 hours experiment,

whereas pAS-6 showed a weak ability of inhibiting Hooke growth. To investigate whether or not the ability of inhibiting bacterial growth *in vitro* can reflect the efficiency of phage therapy *in vivo*, both pAS-3 and pAS-6 were tested in a rainbow trout *in vivo* model.

Raw phage preparations of pAS-3 and pAS-6 were used for *in vivo* phage therapy. The reasons for not highly purifying the phages using CsCl density gradient centrifugation were: firstly, the particle production of pAS-3 and pAS-6 were relatively low, so it will be very hard to have a visible band of phage after the centrifugation; secondly, the steps of carrying out CsCl density gradient centrifugation are complicated and there is a danger of chemical poisoning from CsCl. Considering that raw phage preparations may contain bacterial components which may be harmful to fish and cause clinical signs or even mortality, *in vivo* safety tests of the raw phage preparations were conducted using a small number of rainbow trout before starting the phage therapy trial. One hundred microlitres of raw phage preparation was injected into fish by i.p. at a concentration of 10^7 PFU fish^{-1}. No mortality or any clinical signs of diseases were observed in the injected fish during 14 days. This showed that it was safe to administer the raw phage preparations to fish by i.p., which is consistent with Verner-Jeffreys *et al*. (2007): even though bacterial endotoxins were present at 10^6-10^7 Endotoxin Units per milliliter (EU mL^{-1}) in the raw preparations of *A. salmonicida* phages, no adverse effects were observed in the Atlantic salmon injected by i.p. with 0.1 mL of the raw phage preparations containing a phage concentration of 10^8 PUF mL^{-1} up to 42 days post-injection.

The optimum growth temperature of *A. salmonicida* is between 22 and 25 °C. However, some studies have reported that even growing *A. salmonicida* in this optimum temperature range may lead to the loss or inactivation of some virulence genes (Ishiguro *et al*., 1981; Stuber *et al*., 2003; Daher *et al*., 2011). To maintain the virulence of *A. salmonicida* Hooke, cultures were incubated at 15 °C, and this was also the same temperature for keeping fish in the challenge trials. In the does response trial, a bacterial concentration of 1 x 10^3 CFU fish^{-1} led to mortalities in 5 out of 6 fish, which illustrated that *A. salmonicida* Hooke was very virulent to rainbow trout with an

LC_{50} of < 1 x 10^3 CFU $fish^{-1}$. As *A. salmonicida* Hooke was so virulent, a concentration of ≥ 1 x 10^3 CFU $fish^{-1}$ may lead to a very high mortality, and this would overwhelm the effectiveness of phage therapy. Therefore, a lower concentration of 1 x 10^2 CFU $fish^{-1}$ was chosen to use in the therapy trial, aiming to acquire a total mortality of approximately 40 % in the control fish group (without phage treatment).

Considering the results from *in vitro* inhibition where pAS-3 completely inhibited Hooke growth in 18 hours with an MOI=1,000, a higher MOI=10,000 was used in the phage therapy trial to try to achieve better inhibition *in vivo*. This MOI value was also used by Kim *et al*. (2015) in their study of using phage against *A. salmonicida* infections in rainbow trout showing notable protective effects. Studies of Nakai *et al*. (1999) and Verner-Jeffreys *et al*. (2007) also showed that the early receiving phage treatment post bacterial challenge, the better protective effects could be had. Therefore, in this work, phage preparation was injected immediately after the bacterial challenge.

According to the results, the groups treated with phages pAS-3 and pAS-6 showed low of mortality and postponed time of death compared with the SM buffer treated control group. However, there were no statistically significant differences between the cumulative mortalities and time to death of the phage treated and untreated control groups. The results may be attributed to the following reasons. First, the bacterial concentration used in this study may be too low to show effective phage protection. Most of the phage therapy studies in fish by i.p. used bacterial concentrations which caused 100 % or nearly 100% mortalities in control fish group (Nakai *et al*., 1999; Verner-Jeffreys *et al*., 2007; Mahmoud & Nakai, 2012; Kim *et al*, 2015), so that even if a small number of fish survived in the phage treatment groups, the protection may be significant. However, in the work of Jun *et al*. (2013), phage treatment gave a 100 % protection on cyprinid loach when the bacterial concentration used caused 40 % mortality in the non-treatment control group. Second, the bacterial components in the raw phage preparations may reduce the activity of phages in the therapy. Verner-Jeffreys *et al*. (2007) also used raw phage preparations in their phage therapy trial, where phage treatment and non-treatment control groups had the same mortalities at the end of the experiment (MOI = 190,000). In contrast, Kim *et al.* (2015), using highly

purified phage preparations, led to 25 % survival rate in the phage treatment group whereas 0 % survival rate incurred in non-treatment control group (MOI = 10,000). Third, the fish group size used in this study was relatively small, so that it would be very hard to obtain statistically significant differences between the cumulative mortalities of the phage treated and untreated groups. The latter may be the most likely explanation and therefore the phage therapy experiment should be repeated with larger groups of fish.

Two of *A. salmonicida* colonies recovered from each died or moribund fish during the phage therapy trial were randomly selected for phage sensitivity test. All of them were still sensitive to the phages, i.e. no phage resistance was detected. Beside the possibility that no phage resistance happened during the experiment, the number of colonies selected for phage sensitivity test may not big enough to detect the phage-resistant mutants, as the resistance may just happen in a small portion of the recovered bacteria. Furthermore, the resistant mutants may remain the same phenotype with the sensitive colonies, which results the difficulty of identifying the mutants from a mix culture of both resistant and sensitive colonies. The mechanisms that confer protection against bacteriophages are still unclear. In the study of Verner-Jeffreys *et al*. (2007), although a phage cocktail of three phages were used in the phage therapy experiment, a 'light', more translucent, phenotype of *A. salmonicida* was recovered and showed resistance to all the three phages. Noteworthily, the resistant isolates were not only isolated from the phage treatment groups, but also the untreated challenge control fish, which indicated that the presence of bacteriophage was not necessary to initiate resistance.

From the results of this present study, there seems to be a correlation between the plaque morphology, the ability of inhibiting bacterial growth *in vitro*, and the efficiency of controlling bacterial infections *in vivo,* although the latter could not be proven statistically. *Aeromonas salmonicida* phage pAS-6 formed turbid plaques on bacterial lawn, weakly inhibited the growth of *A. salmonicida* Hooke *in vitro*, and showed a low efficiency of controlling *A. salmonicida* infections *in vivo*, whereas phage pAS-3 formed clear plaques, inhibited *A. salmonicida* Hooke completely *in vitro*, and showed a better

efficiency of phage therapy *in vivo*. Thus, it is speculated that phages forming clear plaques and showing high ability to inhibit bacterial growth *in vitro*, may also give greater efficiency for controlling bacterial infections *in vivo*, and influence the choice of phages for phage therapy in future.

To date, only a few attempts have been made to demonstrate the experimental applications of phage therapy to control *A. salmonicida* subsp. *salmonicida* infections, and effectiveness is still controversial: in a rainbow trout (i.m. injection) model (Kim *et al*., 2015), significant protective efficacy of phages was observed, whereas in a brook trout (water-borne) model (Imbeault *et al*., 2006) and an Atlantic salmon (i.p. injection) model (Verner-Jeffreys *et al*., 2007), only a delay in the onset of infection was observed. Therefore, in this study, an *in vivo* model was set up to estimate the effectiveness of phage therapy to against *A. salmonicida* subsp. *salmonicida* infections in rainbow trout. Under our experimental conditions, the phage treated and non-treated groups did not show significant differences in the final cumulative mortalities and the time to death. Future studies to improve this work should focus on choosing a more appropriate bacterial concentration for challenges, enhancing the purity of phage preparations, and using a larger number of fish per experimental group.

Chapter 4

Characteristics of the Phage-resistant Mutant Derived from a Phage-sensitive Pathogenic *Aeromonas salmonicida* Strain

4.1 Introduction

The increasing incidence of infections that are due to antibiotic resistant bacteria (Levy & Marshall, 2004; Bush *et al*., 2011; Bragg *et al*., 2014) has led to renewed interest in bacteriophages and bacteriophage therapy. Phages have the advantage of existing in abundance in nature and being easily isolated. They replicate and decline along with bacterial growth, kill specific bacteria, and co-evolve with their bacterial hosts (Loc-Carrillo & Abedon, 2011; Oliveira, *et al*., 2012). Precisely because of these advantages, a number of phage therapy trials have already been successfully carried out to control bacterial diseases in plants (Balogh *et al.,* 2010), poultry (Huff, *et al*., 2002, 2003), livestock (Johnson *et al*., 2008), humans (Abedon, *et al*., 2011; Chanishvili, 2012), and animals in aquaculture (Nakai & Park, 2002; Oliveira, *et al*., 2012; Higuera, *et al*., 2013; Richards, 2014; Rao & Lalitha, 2015).

However, phage therapy also has its limitations (Skurnik & Strauch, 2006; Loc-Carrillo & Abedon, 2011; Oliveira, *et al*., 2012), including major concerns about the emergence of phage-resistant mutants generated during therapy (Gill & Hyman, 2010; Labrie, *et al*., 2010). These phage-resistance mechanisms include preventing phage adsorption, preventing phage DNA entry, cutting phage nucleic acids, and the abortive infection systems i.e. interference can be made at different steps required for a successful infection of a phage (Hyman & Abedon, 2010; Labrie, *et al*., 2010). These mechanisms, however, are still not well understood. Among then, one system which acts by specifically cleaving the incoming phage DNA as it enters the host cells, namely the CRISPR-Cas systems (Clustered Regularly Interspaced Short Palindromic Repeats loci, coupled to CRISPR-associated genes) is considered to provide adaptive immunity to prokaryotes using sequence memory to target invading DNA (Dupuis *et al*., 2013; Attar, 2015). Due to the different reasons leading to phage-resistance, changes related with characteristics and virulence may occur in a bacterium (Guglielmotti *et al*., 2006; Stoddard *et al*., 2007; Capparelli *et al*., 2010b). Therefore, better understanding of the generation and characteristics of phage-resistant bacterial mutants, and estimating the change of virulence compared with the parental wild-type strains, is important and critical for the success of phage therapy.

Nowadays, insects are widely used as feasible and convenient model systems to evaluate virulence of numerous human pathogens. The greater wax moth (*Galleria mellonella*) larvae have the advantages of low costs, convenient injection, they require little space or specialist infrastructure, they are an ethically acceptable animal model, and can be reared over a wide temperature range (Mukherjee *et al*., 2011; Desbois & Coote, 2012). Numerous studies have successfully used the greater wax moth larvae model for various purposes including testing of antimicrobial agents (Desbois & Coote, 2011; Hill *et al*., 2014), estimating virulence of pathogenic bacteria (Jander *et al*., 2000; Silver *et al*., 2011; Evans & Rozen, 2012), testing of immune response testing (Schuhmann *et al*., 2003), and even phage therapy (Hall *et al*., 2012; Abbasifar *et al*., 2014). Here, we applied this model to assess virulence of *A. salmonicida* strains, as a previous study has shown that fish pathogen virulence can be reliably assessed in the greater wax moth larvae (McMillan *et al*., 2015).

In Chapter 3, two *Aeromonas salmonicida* phages, pAS-3 and pAS-6 were isolated and used in an *in vivo* phage therapy model against *A. salmonicida* infections in rainbow trout. No phage-resistant mutants were isolated either from the dead or moribund fish, or from the surviving fish at the end of the experiment. In this chapter, phage-resistant mutants of *A. salmonicida* Hooke were induced by repeating challenges with phage pAS-3, and then the characteristics and virulence of a selected mutant was studied and compared with the wild-type *A. salmonicida* strain in order to (i) understand the generation and characteristics of the pAS-3 induced phage-resistant mutants of *A. salmonicida*; and (ii) estimate the consequence of using *A. salmonicida* phage pAS-3 in phage therapy when phage-resistant mutant cells emerge.

4.2 Materials and methods

All the media and chemicals were purchased from Oxoid (Basingstoke, UK), and Sigma-Aldrich (Basingstoke, UK), respectively, unless otherwise stated.

4.2.1 Induction of phage-resistant mutants *in vitro*

To induce phage-resistant mutants, 10 µL of fresh overnight culture of *A. salmonicida* wild-type strain Hooke was added to 1 mL pAS-3 preparation (~ 10^7pfu mL^{-1}), then incubated overnight in a Kuhner shaker incubator (ISF-1-W, Switzerland) at 15 ℃, 140 rpm. After 24 hours, 50 µL of the mixture was aseptically spread onto a TSA plate, which was incubated at 15 ℃ for 2 days. A loopful of the mixture was picked and streaked onto a fresh TSA plate to allow growth of single colonies, which then were purified and marked for a phage-sensitivity test using the spot testing method (Kutter, 2009) with *A. salmonicida* phages pAS-3 and pAS-6. If all the generated bacterial strains were still sensitive to the phages, the above procedure would be repeated for another round, until at least one of the generated strains showed negative results (no plaques formed) in the phage-sensitivity test, which meant that phage-resistant mutants had been acquired. The mutants were stored on preservation beads (Technical Service Consultant Ltd, UK) at -70 ℃. One of the phage-resistant mutants, termed HM, was chosen for the subsequent analysis.

4.2.2 Identification of the phage-resistant mutant

The conventional tests, excluding O/129 test for bacterial identification were performed following the methods described in Chapter 2 (Section **2.2.4.1**).

The *A. salmonicida* specific primer set Fer-3/Fer-4 (Beaz-Hidalgo *et al*., 2008b) was used to confirm the identity of the phage-resistant mutant. The PCR conditions consisted of an initial denaturation step at 95 ℃ for 10 min followed by 30 cycles of amplification at 95 ℃ for 1 min, 60 ℃ for 1 min and 72 ℃ for 50 sec, then the reaction was completed by a final elongation step of 72 ℃ for 5 min. The PCR products were analysed using 1.2 % (w/v) agarose gel (containing 0.5 µg mL^{-1} ethidium bromide) electrophoresis at 80 V for 40 min and visualised under UV light using a transilluminator (Syngene, BioImaging).

Aeromonas salmonicida wild-type strain Hooke was used as the control in all the above tests.

4.2.3 Growth temperature range and colony morphology

Single colonies of Hooke and HM were aseptically streaked onto TSA plates, which were then incubated at 4 ℃, 15 ℃, 22 ℃ and 30 ℃, and observed for colony morphology every 24 hours for 7 days.

4.2.4 A-layer detection

To detect the A-layer protein, HM was streaked onto plates of Coomassie Brilliant Blue agar (TSA supplemented with 0.01 % (w/v) Coomassie Brilliant Blue R250) (Cipriano & Bertolini, 1988) and Congo Red agar (TSA supplemented with 0.02 % w/v congo red and 0.15 % bile salts) (Ishiguro *et al*., 1985; Bashar, *et al*., 2011), which two methods have been used in many studies for A-layer detection, but never been compared in a same study. The plates were incubated at 15 ℃ for 3-5 days. A-layer positive strains produce dark blue colonies on Coomassie Brilliant Blue agar, and red-coloured colonies on Congo Red agar, whereas the A-layer negative strains are not stained. The Hooke strain was used as the positive control in the tests.

4.2.5 Autoagglutination test

A single colony of HM was inoculated into a sterile round bottom test tube containing 5 mL of TSB. After overnight incubation with shaking at 15 ℃, 140 rpm, the tube was incubated for 3-6 hours at 15 ℃. Bacterial autoagglutination was indicated by the presence of visible cell clumps at the bottom of the tube, as observed by the naked eye. *Aeromonas salmonicida* Hooke was used as a positive control in the test.

4.2.6 Observation of bacterial ultra-thin sections by transmission electron microscopy (TEM)

Overnight cultures of HM cells in TSB were harvested by centrifugation (1-14 Microfuge, Sigma) at 5,000 *x g* for 5 min, and fixed in 1.5 mL of 2.5 % (v/v) glutaraldehyde overnight at 4 °C, followed by washing twice with 0.1 M sodium cacodylate buffer (pH 7.2). The fixed cell pellets were stored at 4 °C until they were post-fixed in 1 % (w/v) osmium buffer in closed vials for 1 hour at room temperature. The following steps of preparing the bacterial ultr-thin sections were conducted by the

technician Mr. Linton Brown. The pellets were then washed for 3 x 5 min with distilled water. 'En-bloc' staining was undertaken with 2 % (w/v) uranyl acetate in 30 % acetone in dark for 1 hour, after which the pellets were dehydrated through an acetone series of ascending concentrations (60 %, 90 % and 100 %) at room temperature. Pellets were then infiltrated with agar low viscosity resin (ALVR) on a rotator (Taab, UK), following which the pellets were finally embedded in green block moulds and polymerised in oven at 60 °C overnight. Ultra-thin sections at 100 µm thickness were prepared from the resin blocks using a microtome (Reichert Ultracut E, Leica, UK) and placed on 200 square mesh Formvar-coated copper grids. The sections were observed under a Philips (FEI) CM120 Biotwin Transmission Electron Microscope (TEM). Hooke was processed in the same way and used as comparison.

4.2.7 Antibiotic sensitivity assay

The procedure was modified from the Kirby-Bauer agar disc diffusion method (Bauer *et al.*, 1996). The overnight broth culture of HM was adjusted to OD_{600} of 0.5 using a spectrophotometer (2041, Cecil, UK), of which 100 µL was aseptically spread onto a TSA plate. Antibiotic discs (Oxoid) of amoxicillin (AML, 10 µg), enrofloxacin (ENR, 5 µg), florfenicol (FFC, 30 µg), oxolinic acid (OA, 2 µg), oxytetracycline (OT, 30 µg) and sulphamethoxazole (SXT, 25 µg) were applied, and the diameters of the inhibition zones were measured after 48 hours of incubation at 15 °C. The cut-off points for the antibiotic sensitivity profiles were: 0-10 mm in diameter = resistant, 11-15 mm in diameter = partial sensitivity, >16 mm in diameter = sensitive. *Aeromonas salmonicida* Hooke was used as comparison, and this experiment was repeated twice.

4.2.8 Virulence assessment using the greater wax moth larvae model

Freshly purchased greater wax moth larvae (UK Waxworms Limited) were used as the model to assess the virulence of the parental *A. salmonicida* wild-type strain Hooke and the phage-resistant mutant HM. Two hundred and twenty larvae were randomly divided into 11 groups, each comprising 20 larvae. The groups were injected in the last left proleg using a 50 µL Hamilton syringe with 10 µL of the *A. salmonicida* wild-type strain Hooke cells (10^3, 10^4, 10^5, 10^6 and 10^7 CFU mL^{-1}), the phage-resistant mutant HM

cells (10^3, 10^4, 10^5, 10^6 and 10^7 CFU mL^{-1}) or sterile saline, respectively (the wild and mutant cells were resuspended with sterile saline). The infected larvae were then kept in sterile petri dishes for 48 hours at 15 °C. Dead larvae were removed from the petri dishes, and the mortality of each group was recorded every 24 hours. The concentrations of the injected bacterial preparations were calculated using the Miles & Misra viable bacterial colony count method (Miles *et al.*, 1938). The LC_{50} values were calculated using the Probit analysis method (Sakuma, 1998) with IBM SPSS V19. Bacteria were recovered from the haemolymph of dead larvae using Aeromonas Medium Base with Ampicillin Selective Supplement following the manufacturer's instruction to avoid the contamination from the original microflora of the moth larvae. Both *A. salmonicida* Hooke and HM grew on the Aeromonas Medium agar plates before the challenge. The identification of Hooke and HM recovered from the larvae was carried out by PCR amplification using the specific primer set (Fer-3/Fer-4) for *A. salmonicida* and phage-sensitivity test using phages pAS-3 and pAS-6.

4.3 Results

4.3.1 Acquisition of phage-resistant mutants *in vitro*

Aeromonas salmonicida wild-type strain Hooke was challenged by *A. salmonicida* phage pAS-3 to induce *A. salmonicida* phage-resistant mutants. In the second round of induction, three single colonies were picked randomly and purified on TSA plates. All of these three acquired strains showed resistance to both *A. salmonicida* phages pAS-3 and pAS-6 in the phage-sensitivity test, whereas the parental wild-type strain Hooke showed sensitivity (Figure 4.1). The three phage-resistant cultures were regarded as phage-resistant mutants. One of the three mutants, named HM, was chosen to be used in subsequent experiments.

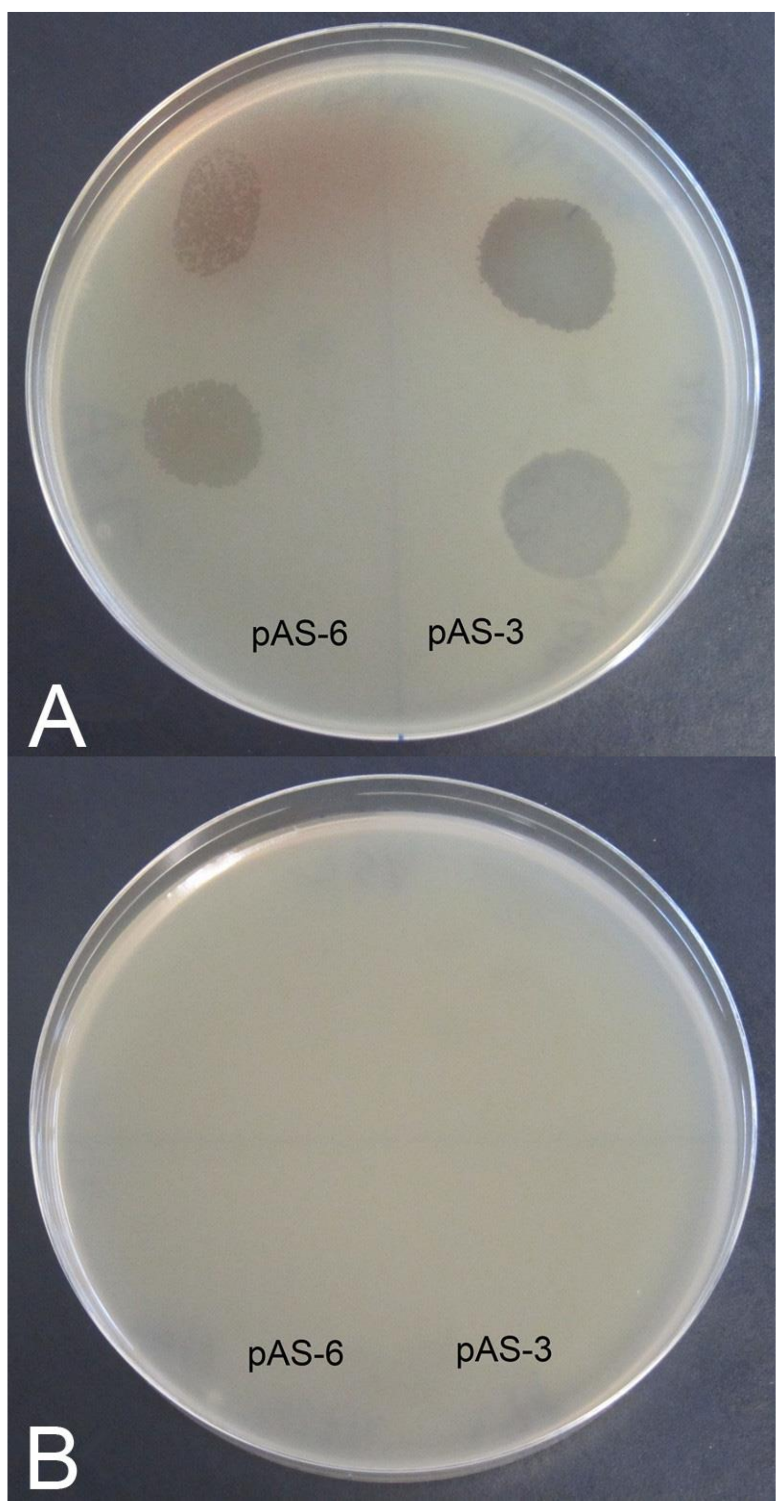

Figure 4.1 Phage-sensitivity tests of *Aeromonas salmonicida* strains HM and Hooke using *A. salmonicida* phages pAS-3 and pAS-6. Visible plaques formed on the bacterial lawn of *A. salmonicida* strain Hooke (A), whereas no plaques formed on that of HM (B).

4.3.2 Identification of HM as a *A. salmonicida* Hooke phage-resistant mutant

In the identification tests, HM gave a series of the same results with *A. salmonicida* Hooke: Gram negative short rods, non-motile, oxidase positive, facultative anaerobic and API 20E profile of 6206104 (ADH, LDC, CIT, GEL, GLU, MAN and OX were positive, the rest of the tests were negative).

In the molecular identification tests, the expected 422 bp PCR amplification products of *A. salmonicida* specific primer set (Fer-3/Fer-4) were visualised on the gel from both HM and Hooke genomic DNA (Figure 4.2).

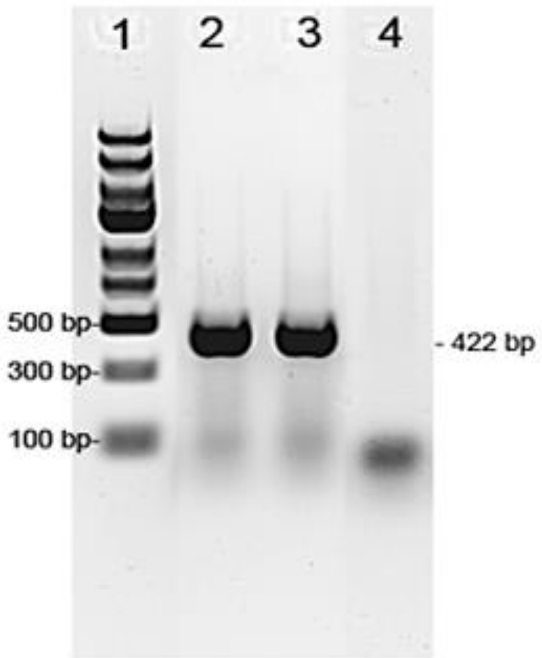

Figure 4.2 Identification of the *Aeromonas salmonicida* mutant HM by PCR using *A. salmonicida* specific primer set (Fer-3/Fer-4): 1. DNA Ladder; 2. Amplicon from the genomic DNA of phage-resistant mutant HM; 3. Amplsicon from the genomic DNA of wild-type strain Hooke; 4. Blank control no DNA template.

4.3.3 Growth temperature range

In the growth temperature range experiment, both *A. salmonicida* HM and Hooke grew at all the test temperatures (4 ℃, 15 ℃, 22 ℃ and 30 ℃). As observed by morphological differences in colony presentation, when incubated at the lower temperatures (4 ℃ and 15 ℃), HM and Hooke had similar sizes of colonies; whereas

when incubated at the higher temperatures (22 ℃ and 30 ℃), HM formed larger colonies than Hooke within the same incubation time (Figure 4.3), especially at 30 ℃, at which Hooke barely grew, but HM grew well. In this test, 22 ℃ was regarded as the optimum growth temperature for both Hooke and HM.

Both Hooke and HM *A. salmonicida* strains produced diffusible brown pigment on TSA plates at 15 and 22 ℃. However, the colonies from Hooke were regarded as 'rough', while colonies from HM were regarded as 'smooth' (Figure 4.3).

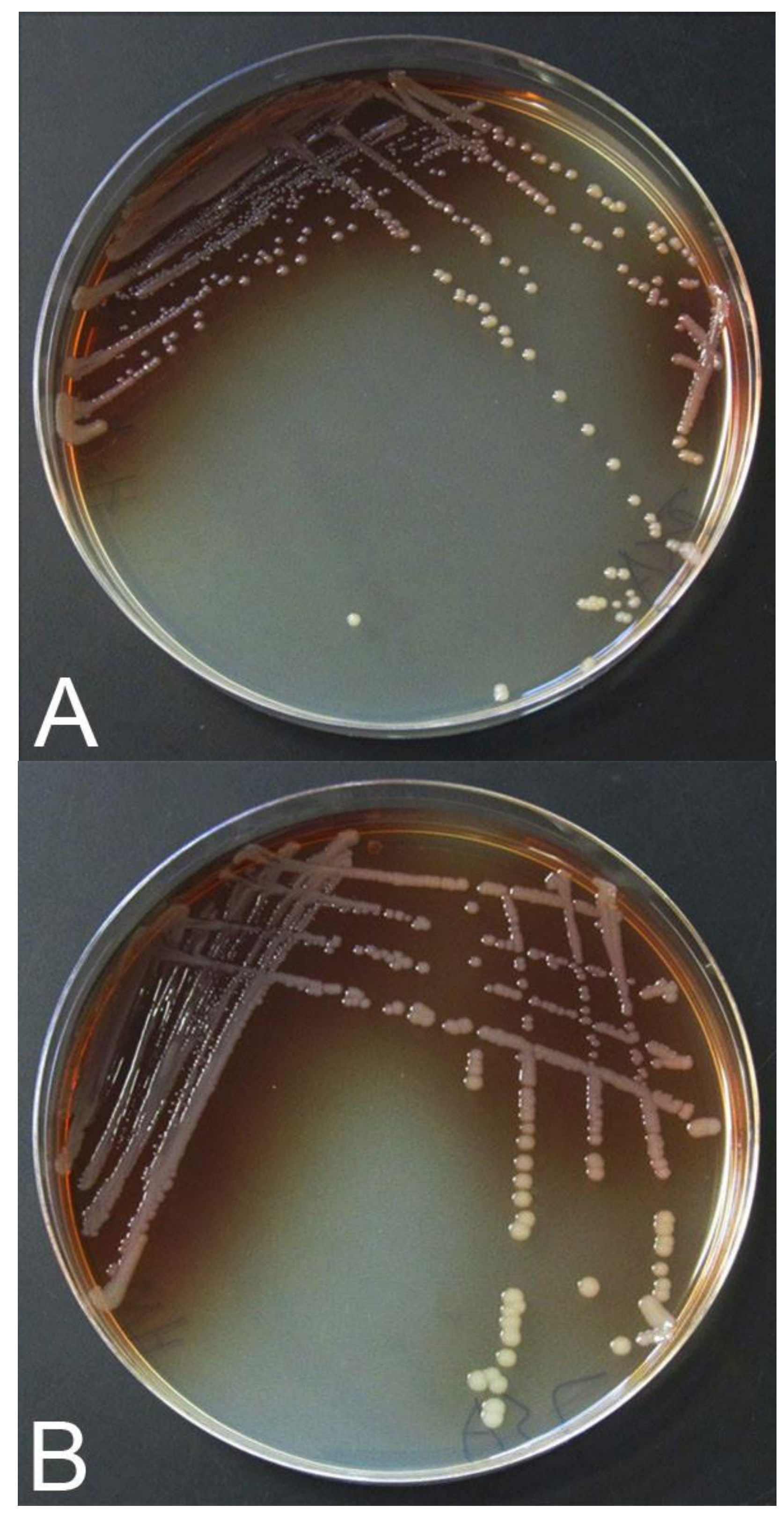

Figure 4.3 Colony morphology of *Aeromonas salmonicida* wild-type strain Hooke (A) and phage-resistant mutant HM (B) on TSA plates incubated at 22 °C for 3 days.

4.3.4 Detection of A-layer

Aeromonas salmonicida strains Hooke and HM were inoculated onto Coomassie Brilliant Blue agar and Congo Red agar plates to detect the presence of A-layer protein. *Aeromonas salmonicida* Hooke produced dark blue colonies on Coomassie Brilliant Blue agar, whereas HM produced creamy colonies (Figure 4.4A), which indicated that the latter was A-layer negative. Both Hooke and HM formed red-coloured colonies on Congo Red agar, although the colour of HM colonies was slightly lighter than that of Hooke colonies, which indicated A-layer positive results for both of the wild-type strain and the mutant (Figure 4.4B).

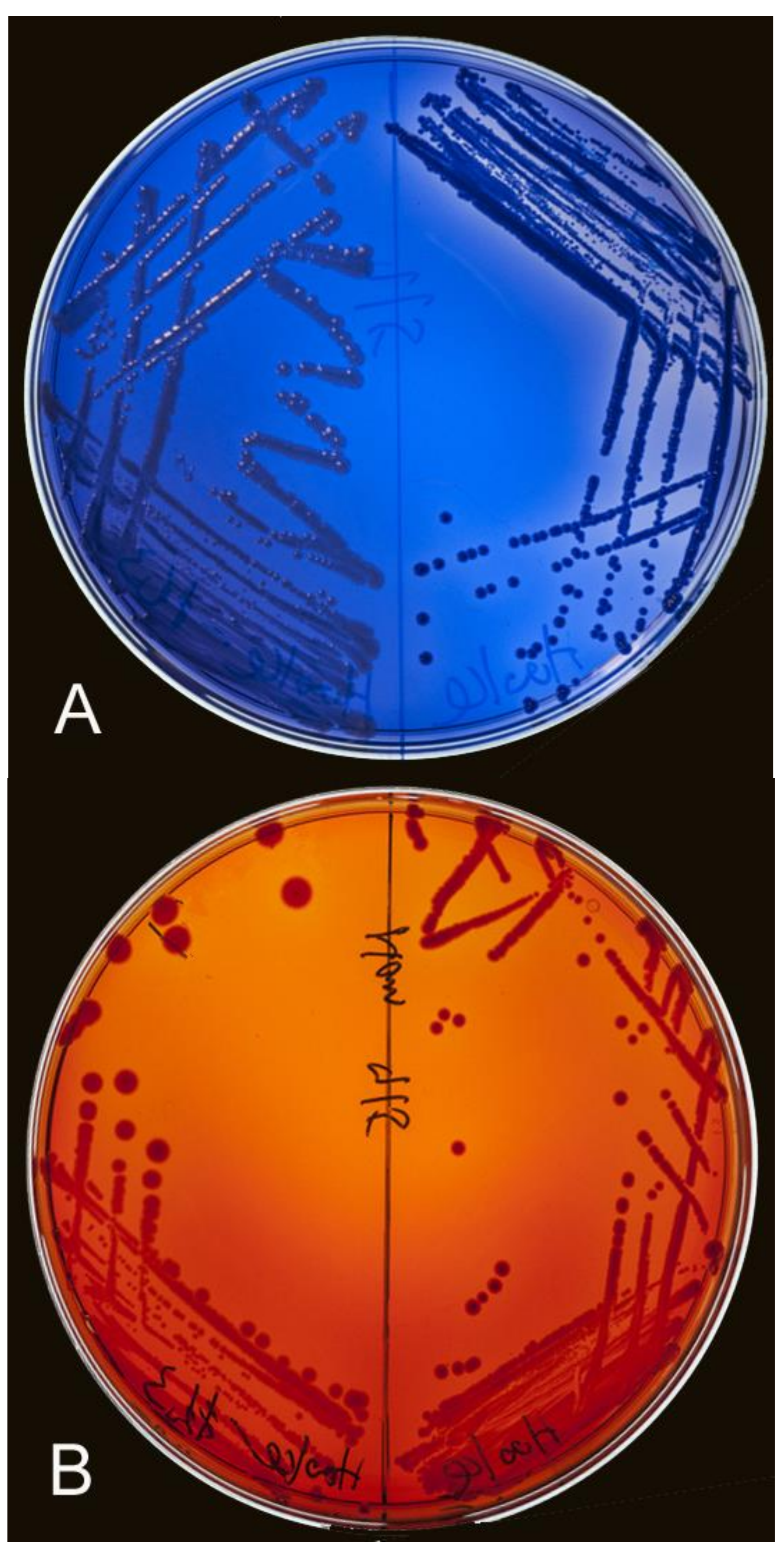

Figure 4.4 Detection of *Aeromonas salmonicida* A-layer: (A) phage-resistant mutant HM (left) and wild-type strain Hooke (right) on Coomassie Brilliant Blue agar; (B) HM (left) and Hooke (right) on Congo Red agar.

4.3.5 Autoagglutination ability

After standing in the 15 °C incubator for 3-6 hours, the wild-type strain *A. salmonicida* Hooke formed visible cell clumps at the bottom of the test tube, while the phage-resistant mutant HM formed a homogeneous cell suspension in TSB medium, indicating that the autoagglutination ability of HM decreased dramatically compared with the wild-type strain Hooke (Figure 4.5). Autoagglutination is associated with the presence of A-layer (Ishiguro *et al*., 1981; Evenberg *et al*., 1982).

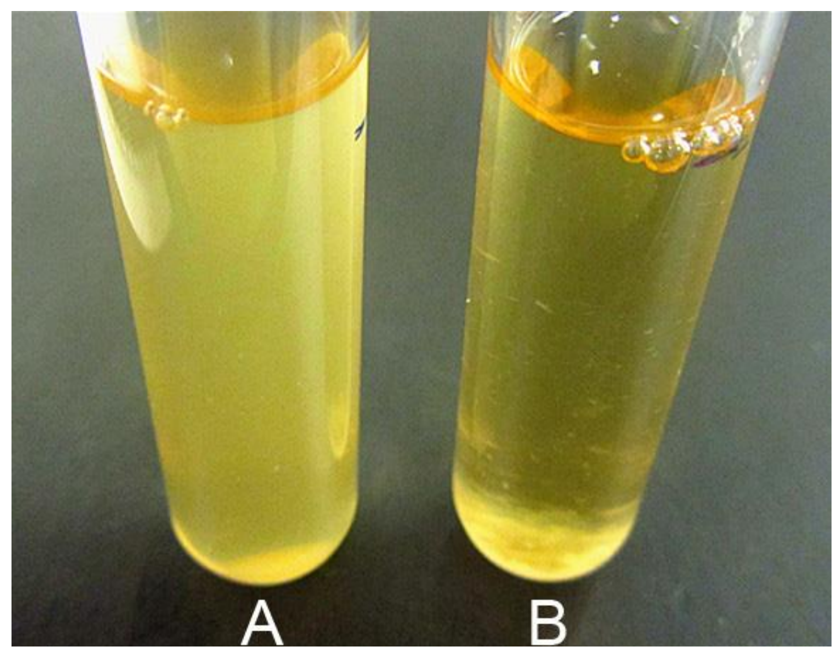

Figure 4.5 The autoagglutination phenotype: (A) *Aeromonas salmonicida* phage-resistant mutant HM did not autoagglutinate, forming a homogeneous cell suspension and (B) *A. salmonicida* wild-type strain Hooke autoagglutinated, forming visible cell clumps on the bottom of the test tube.

4.3.6 Bacterial cell morphological comparison of *A. salmonicida* Hooke and HM under TEM

The ultra-thin sections of both *A. salmonicida* Hooke and HM were observed by transmission electron microscopy (TEM) at a magnification of X 15,000. No obvious difference was observed from the inner cell areas. However, a layer of thick and furry structure (considered as the A-layer) was observed on the surface of *A. salmonicida* wild-type strain (Hooke) cells, whereas this structure appeared to be very thin or absent at some parts of the phage-resistant mutant (HM) cells, indicating a

degradation of A-layer on the *A. salmonicida* phage-resistant mutant (HM) cells. Moreover, gaps between the outer membrane and plasma membrane were observed from the HM cells, i.e. expansions of the periplasmic space were observed, where the furry outer membrane structure completely disappeared (Figure 4.6).

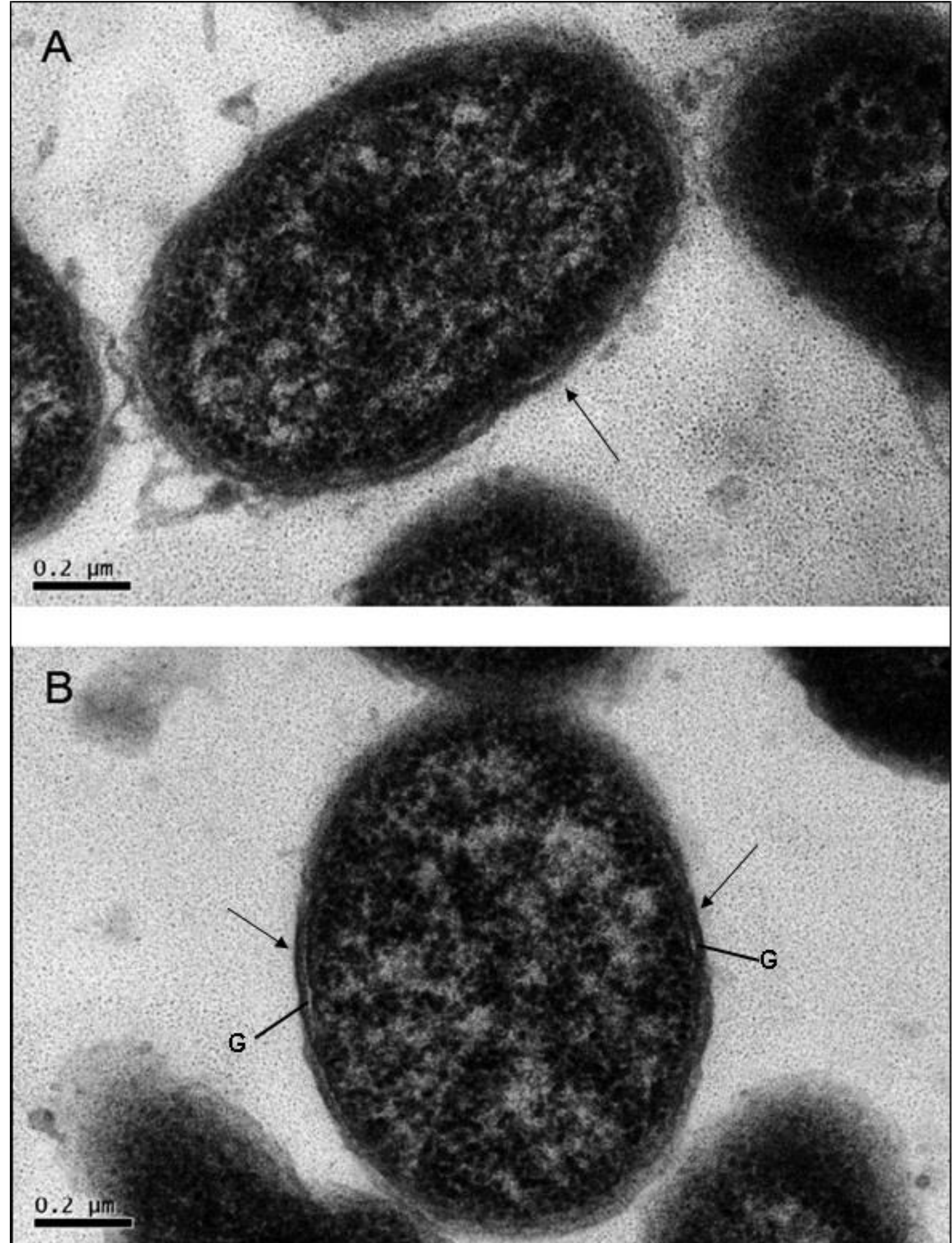

Figure 4.6 Bacterial cell morphology of *Aeromonas salmonicida* wild-type strain Hooke and phage-resistant mutant HM under transmission electron microscope. (A) wild-type strain Hooke, arrow indicates the outer membrane structure (A-layer); (B) phage-resistant mutant HM, arrows indicate the missing of A-layer, 'G' indicates the enlarged periplasmic space.

4.3.7 Antibiotic sensitivity

In the antibiotic sensitivity assay, *A. salmonicida* HM and Hooke showed the same sensitivities to all six antibiotics tested. Both were resistant to oxytetracycline (30 µg), but sensitive to amoxicillin (10 µg), enrofloxacin (5 µg), florfenicol (30 µg), oxolinic acid (2 µg) and sulphamethoxazole (25 µg) (Table 4.1).

Table 4.1 Antibiotic sensitivity results of *Aeromonas salmonicida* wild-type strain Hooke and phage-resistant mutant HM.

Antibiotics (µg)	Hooke	HM
Oxytetracycline (30)	R	R
Amoxicillin (10)	S	S
Enrofloxacin (5)	S	S
Florfenicol (30)	S	S
Oxolinic acid (2)	S	S
Sulphamethoxazole (25)	S	S

R: resistant; S: sensitive.

µg = microgram; information in brackets is the concentration of antibiotic per disc.

4.3.8 Virulence of *A. salmonicida* strains

A serial of dilutions of *A. salmonicida* Hooke and HM were injected into the greater wax moth larvae to assess the virulence. At the end point, i.e. 48 hours post-injection, the LC_{50} of Hooke and HM were 6.46×10^4 and 2.66×10^4 CFU $larva^{-1}$, respectively (Table 4.2). The dead larvae turned dark brown or black, whereas the larvae that survived were a light creamy colour. *Aeromonas salmonicida* Hooke and HM were both isolated from the haemolymph of dead larvae in pure culture on the Aeromonas selective medium plates. The identities of recovered *A. salmonicida* Hooke and HM were confirmed by the specific PCR products of 422 bp, and phage-sensitivity characters (recovered Hooke and HM were still phage-sensitive and phage-resistant, respectively). The group injected with sterile saline remained alive throughout the experiment.

Table 4.2 The LC_{50} of *Aeromonas salmonicida* wild-type strain Hooke and phage-resistant mutant HM to the greater wax moth larvae at 48 hours post-injection (group size per concentration=20).

Group	Concentration (CFU larva^{-1})	Mortality (%)	LC_{50} (CFU larva^{-1})
	8.0 x 10^0	0	
	8.0 x 10^1	0	
Hooke	8.0 x 10^2	5	6.46 x 10^4
	8.0 x 10^3	15	
	8.0 x 10^4	65	
	2.0 x 10^1	5	
	2.0 x 10^2	10	
HM	2.0 x 10^3	35	2.66 x 10^4
	2.0 x 10^4	100	
	2.0 x 10^5	100	
Sterile saline	10 µL	0	-

LC_{50}: lethal dose 50%; % = percentage.

4.4 Discussion

In this study, phage-resistant mutants derived from the pathogenic *A. salmonicida* strain Hooke were induced by repeating challenges using *A. salmonicida* lytic phage pAS-3. To imitate the aquatic environment, induction in liquid medium was chosen and considered to be simpler to acquire phage-resistant mutants than using solid agar. Other similar liquid induction methods, including one termed secondary culture technique, have been used in many studies to obtain phage-resistant mutants (Quiberoni *et al*., 1998; Guglielmotti *et al*., 2006; Stoddard *et al*., 2007).

In this study, the characteristics of *A. salmonicida* phage-resistant mutant HM and the wild-type strain Hooke were compared. Although the strains showed the same sensitivity to 6 antibiotics tested, they differed for other parameters. Differences were

shown in that the phage resistant mutant formed 'smooth' colonies on TSA, did not autoagglutinate, formed creamy colonies on Coomassie Brilliant Blue agar and showed the degradation of a thick/furry layer structure on the cell surface under TEM, all indicating a deficiency of the A-layer (Ishiguro *et al*., 1981; Cipriano & Bertolini, 1988; Bernoth, 1990; Austin & Austin, 2012). However, the phage-resistant mutant HM formed red colonies on Congo Red agar as did the wild-type strain Hooke. It indicates that Congo Red agar may not be as sensitive as Coomassie Brilliant Blue agar for detecting a deficiency of the A-layer of *A. salmonicida*. Expansion of the periplasmic space was observed in the phage-resistant mutant HM cells, where the A-layer structure had disappeared completely. It is speculated that the components related with the assembly of the A-layer were blocked in the periplasmic space, and could not be transferred to the surface of the outer membrane, which led to the deficiency of the A-layer (Belland & Truse, 1985).

An earlier study (Ishiguro *et al*., 1984) reported that the A-layer may act as a phage receptor, since phage TP446 adsorbed to all of the tested *A. salmonicida* strains that possessed the A-layer, but failed to adsorb to mutants lacking the A-layer. On the other hand, studies by Rodgers *et al*. (1981) and Ishiguro *et al*. (1983) reported that cell wall lipopolysaccharide (LPS) is a phage receptor, or at least related with *A. salmonicida* phage adsorption, as extracted LPS from *A. salmonicida* could inactivate phages, and mutants shown to be defective in LPS structure, were also resistant to the phages. Dooley *et al*. (1989) believed that the assembly of the A-layer required the presence of O-polysaccharide chains from LPS, indicating that changes of LPS can also lead to the deficiency of the A-layer on the cell surface. Therefore, it is deduced in this study that the A-layer either directly acted as the receptor of *A. salmonicida* phage pAS-3, or was affected indirectly by the change of an unknown phage receptor.

In this study, a phenomenon of cross-resistance was observed: the *A. salmonicida* phage-resistant mutant HM induced by challenging with the phage pAS-3, also acquired the resistance to phage pAS-6. It is speculated that phages pAS-3 and pAS-6 may share a same receptor on their *A. salmonicida* host, or at least the changing or absence of the receptor used by pAS-3 has a negative effect on the receptor used by

pAS-6. According to Chapter 3, pAS-3 and pAS-6 were isolated from a same environmental sample; pAS-3 was identified as a *Siphoviridae* phage, whereas pAS-6 was identified as a *Podoviridae* phage. Therefore, hypotheses are made that tailed phages from different families can use a same receptor on the bacterial host surface; moreover pAS-3 and pAS-6 may be in a competitive relationship in the natural environment.

Based on the functional similarity in the innate immune responses to invasive infection between insects and fish, McMillan *et al*. (2015) performed work with 11 wild-type isolates of *V. anguillarum* an important fish pathogen, and found the virulence of these isolates correlated positively and significantly in the greater wax moth larvae and Atlantic salmon infection models. Their work showed that the greater wax moth larva could be a suitable alternative host for studying virulence of fish pathogens. Thus, in this study, the virulence of *A. salmonicida* strains was assessed using the greater wax moth larvae model. This model could be considered as a reliable reflection of the virulence of these *A. salmonicida* strains in fish (rainbow trout), but future work is needed to confirm this. Moreover, this experiment of virulence assessment in the greater wax moth larvae model was only carried out once with no replicates. Therefore, the results should be confirmed by repeating the experiment with additional replicates.

Here, the *A. salmonicida* phage-resistant mutant HM showed a deficiency in the A-layer, and no decline of virulence in the greater wax moth larvae model when compared with its parental wild-type strain Hooke. The A-layer is one of the important virulence factors of *A. salmonicida*, and some early studies have reported an association between the possession of the A-layer and virulence (Udey & Fryer, 1978; Ishiguro *et al*., 1981; Kay *et al*., 1981). However, other work carried out by Adams *et al*. (1988), Ellis *et al*. (1988) and Bernoth (1990) indicated that A-layer negative stains can also cause mortality in rainbow trout. Therefore, presence of the A-layer cannot be considered as a reliable indicator of virulence of *A. salmonicida*.

The mechanisms of phage-resistance are complicated (Hyman & Abedon, 2010; Labrie, *et al*., 2010). In this study, it is likely that the generation of the phage-resistant mutants was due to the absence of the phage receptor on the cell surface. However, this hypothesis needs to be proven by further work, e.g. testing phage sensitivity on spontaneous or engineered A-layer negative *A. salmonicida* Hooke mutant, or testing whether or not phages pAS-3 and pAS-6 can be inactivate by purified A-layer protein. Moreover, the fact that the mutant HM remained resistant after single-colony purification and in the absence of phage pAS-3 suggests that the phage resistance was inheritable, i.e. a genetic change, but not a physiological response (Stoddard *et al*., 2007).

Although in Chapter 3 phage-resistant mutants were not isolated in the phage therapy experiment against *A. salmonicida* infections in rainbow trout, there is still the possibility that phage-resistant mutants may be found in further *in vivo* applications. In the study of Verner-Jeffreys *et al*. (2007), phage-resistant mutants had a more translucent phenotype when recovered from fish that died during the phage therapy trial, and still appeared to be A-layer positive. Thus, the *A. salmonicida* phage-resistant mutant HM was induced by challenging with phage pAS-3, and the characteristics were compared with that of the parental wild-type strain Hooke. The mutant showed a better ability than the parental strain to grow at higher temperatures (22 ℃ and 30 ℃), but no obvious difference at 15 ℃ or below, which is the more suitable water temperature for rearing rainbow trout compared with 22 ℃. Therefore, at the lower end of the fish rearing temperature, the conditions will not favour the growth of the phage-resistant mutant cells from this study. Moreover, the phage-resistant mutant HM showed A-layer deficiency, but its virulence did not decline when tested using the wax moth larvae model. Although, some studies have reported that phage-resistant bacteria frequently have reduced or lost virulence (Smith & Huggins, 1983; Park *et al*., 2000; Capparelli *et al*., 2010a; Laanto *et al*., 2012), no declind in virulence of the phage-resistant mutant HM from this study indicates the importance of fully testing the virulence of phage-resistant mutants before carrying out large scale field trials of phage therapy.

Chapter 5

General Discussion and Conclusions

5.1 Overview of this study

Bacteriophages are a subgroup of prokaryotic viruses that specifically invade bacterial cells. Not long after their discovery in 1917, they were applied as antibacterial agents against bacterial infections in animals and humans. However, due to the discovery and widespread application of antibiotics, many institutes stopped their research on phage therapies (Sulakvelidze *et al.*, 2001; Summers, 2001). The increasing incidence of infections due to antibiotic resistant bacteria has led to renewed interest in phages and phage therapy. Although phage therapy has been applied to control bacterial diseases in plants, poultry, livestock and humans, applications in aquaculture are still relatively limited.

Some *Vibrio* spp., especially *V. anguilarum*, *V. salmonicida*, *V. harveyi* and *V. tapetis*, are known bacterial pathogens that cause diseases in aquatic animals (e.g. fish, crustaceans and molluscs) (Daniels & Shafaie, 2000; Austin, 2010; Austin & Austin, 2012; Romalde *et al*., 2013). These diseases caused by the genus *Vibrio*, named vibriosis, have brought enormous economic loss to the worldwide aquaculture industry (Liu *et al*., 2013a). As phages occupy all the habitats where their bacterial hosts thrive (Skurnik & Strauch, 2006; Wittebole *et al*., 2014), in Chapter 2, the work started with isolating lytic vibriophages and their bacterial hosts from a same aquaculture environmental sample. Two vibriophages (PVS9 and PVC13) and their *Vibrio* hosts (V9 and V13) were successfully isolated, identified and characterised. However, these two *Vibrio* strains showed non- or low virulence to rainbow trout, goldsinny wrasse and *Artemia* in the experiments of pathogenicity determination. An *in vivo* phage therapy model could not be set up using these *Vibrio* isolates and their phages.

Instead in Chapter 3, *Aeromonas salmonicida* subsp. *salmonicida* Hooke, whose pathogenicity in salmonids had been proven in a number of published and laboratory works (Robertson *et al*., 2000; Vivas *et al*., 2004; Kim & Austin, 2006; Brunt *et al*., 2007; Scott *et al*., 2013), was used to isolate phages for phage therapy. Two phages pAS-3 and pAS-6 were successfully isolated. Rather than doing many studies on the

constituent traits, e.g. adsorption rate, burst size, or lysis time, the work in Chapter 3 focused on the traits i.e. plaque morphology, ability of inhibiting bacterial growth *in vitro*, and the safety of phage preparation, which may be directly related with the efficiency of phage therapy. Using *A. salmonicida* strain Hooke and its phages pAS-3 and pAS-6, an *in vivo* rainbow trout intraperitoneal injection model was developed. To determine the protection afforded by the phages, indicators which included lower cumulative mortalities and a delay of time to death in the fish groups provided with the phage treatment were used and compared with the control animals. However, in this study no significant differences were observed in either of these two indicators between the phage treated and untreated control fish groups. Whilst initially disappointing it was speculated that the low bacterial concentration used, the low purity of phage preparations, and the small number of fish used in the study may have compromised the results. Further work is required to refine these challenge factors and to fully determine the potential of these phages for use in aquaculture.

Although in Chapter 3, no phage-resistant mutants were isolated from the dead fish, the generation of the mutants is still a major concern for phage therapy. For this reason, phage-resistant mutants of *A. salmonicida* Hooke were induced *in vitro* by repeating challenges with phage pAS-3, and then the characteristics and virulence of a selected mutant HM were studied and compared with the wild-type *A. salmonicida* strain Hooke. The phage-resistant mutant HM showed A-layer deficiency, but grew better at higher temperatures (22 ℃ and 30 ℃), and its virulence did not decline in the greater wax moth larvae model. These results indicated the importance of fully testing the virulence of phage-resistant mutants before carrying out large scale field trials of phage therapy.

5.2 Future work

Besides the future work that has been highlighted based on the results from each chapter, further research is also required.

5.2.1 Further characterisation of phages

As mentioned above, further studies are required on characterisation of phages. Understanding the characteristics, such as adsorption rate, burst size, lysis time, and stability may help with improving the efficiency of phage therapy. Moreover, knowing a complete genome sequence is very important and especially useful for classifying phages and checking for virulence genes (which maybe passed to the bacterial hosts to cause diseases in animals); thus whole genome sequencing should be conducted.

5.2.2 Different administration methods of phages in phage therapy

Although intraperitoneal injection has been used in many phage therapy studies in fish for delivery of phages (Table 1.1), for application in aquaculture immersion (water-borne) or oral (adding phages in the feed) administration may prove more convenient and cost effective. Several studies have been conducted comparing the efficiency of these different administration methods (Nakai *et al*, 1999; Verner-Jeffreys *et al*., 2007; Prasad *et al*., 2011; Jun *et al*., 2013). However, there is still a lack of experimental data, which is needed for a better understanding of these delivery methods.

5.2.3 Controlling infections caused by atypical *Aeromonas salmonicida*

The diseases caused by atypical *A. salmonicida* in ballan wrasse (*Labrus bergylta*), one of the cleaner fish species being used as a biological control against sealice infections in farmed salmon, is currently a major concern in aquaculture in Scotland. One study reported that a phage could lyse an atypical *A. salmonicida* strain (Kim *et al*., 2012c), so using phage therapy in controlling infections caused by atypical *A. salmonicida* would be worth while exploring.

5.3 Recommendations for improvements in fundamental phage research

During this study, it has been observed that the field of fundamental phage research requires some improvements. Therefore, in this section the following recommendations are proposed for improvements which would benefit all researchers in the field, especially the ones new to working with phages.

5.3.1 Enriching the public resource of phages

In microbiology, establishing, maintaining and enriching collections of microorganisms are essential and important for many reasons including the conservation of genetic resources, exploring biodiversity, and providing the foundation for scientific research and biotechnological processes in industries (Fortier & Moineau, 2009). The same holds true for phages. Public phage collections should collect, preserve and distribute reference phages for education and research purposes. However, there are only a few public collections, including the American Type Culture Collection (www.atcc.org) and the German Collection of Microorganisms and Cell Cultures (www.dsmz.de), that have phages resource. So far, the only public collection devoted entirely to phages is the Félix d'Herelle Reference Center for Bacterial Viruses (www.phage.ulaval.ca) located at the Laval University, Quebec, Canada (Fortier & Moineau, 2009). Moreover, the amount of phages that have been isolated and studied is very low. The total number of phages in the biosphere has been estimated at over 10^{30} particles (Ackermann, 2011), however, from 1959 to 2012, only 6196 phages were reportedly examined by electron microscopy (Ackermann & Prangishvili, 2012). Even though, novel phages are continually reported at a rate of 100 per year (Ackermann, 2007), this is still not sufficient for the increasing demand from various phage research projects and applications. Moreover, for many bacteria their phages have not been isolated (Ackermann & Prangishvili, 2012).

The Science Education Alliance Phage Hunters Advancing Genomics and Evolutionary Science (SEA-PHAGES) project led by Professor Graham Hatfull from the Howard Hughes Medical Institute (HHMI), is an inspiring example for the expansion of public phage collections. This project began in the autumn of 2008, and has involved over 4,800 first-year undergraduate students at 73 institutions across the United States. The students are trained to isolate and name mycobacteriophages from their own soil samples which were collected across and outside the United States, and then the phage genomes were sequenced and analysed. Since the start of the programme, 3,000 new phages have been isolated, and more than 450 mycobacteriophage genomes have been sequenced and annotated, and deposited in GenBank and

PhagesDB to share with the scientific community (Jorda *et al.*, 2014). This project successfully involved introducing undergraduate students in authentic research to enhance their interest in biology, and their findings really contribute to the phage diversity and phage genomics data base.

Thus, a rich public phage collection with detailed information e.g. sample collection, host range, genome sequence, will provide a good resource for selecting candidates for phage therapy and other phage works that will directly or indirectly support phage therapy research. Researchers can select the phages with the characteristics that they need from the collection directly, so that it will save time and materials on phage isolation and characterisation. Therefore, new projects should build and enrich the phage collections. People who are interested in phage study should be involved in these projects, and samples collected from different environments should be used to isolate phages. Moreover, sharing phage collections and other phage resources with the scientific community would benefit the whole field of phage research.

5.3.2 Publishing a phage atlas

According to Ackermann (2011), over 96 % of the phages that have been isolated are tailed. It may be that the tailed phages are more numerous than other phages in nature, so that they are easier to isolate. However, there is another reason that should be considered: the tailed phages have notable morphological characteristics, i.e. icosahedral heads and tails, because of this, they are much easier to recognise than the phages without notable and unique morphological characteristics. When using a phage sample that is not highly purified (as in this study where phage lysates were used) for morphology observation, apart from phages, many objects from the bacterial cells can also be observed under TEM. For a researcher with insufficient experience with phage morphology, it is very hard to identify a non-tailed phage from other distractions such as debris from bacterial cells, so that some phages might be omitted due to having no typical shape characteristics. Good quality photographs of tailed phages observed using TEM can be found relatively easily from the internet, whereas photographs of non-tailed phages can be hardly found. Similarly, it is also not easy for new researchers to identify phage plaque morphology when isolating phages from

environmental samples. Small plaques can be misjudged as air bubbles, oil bubbles, or small cracks of agar on the plates. Therefore, an atlas containing TEM photographs of different phage morphologies, especially the non-tailed phage ones, and phage plaque morphologies would be very helpful for researchers to use for phage isolation and identification. However, to the best of my knowledge, there is no such an atlas or online repository available.

5.3.3 Cross-contamination control

Working with different phages at same time incurs a high risk of cross-contamination between these phages, especially for the phages that grow poorly in liquid broth and have to be propagated using soft-agar method (such as the *A. salmonicida* phages in Chapter 3). The manipulation steps (e.g. collecting the top agar and transferring to centrifuge tubes, and filtration) of preparing phage suspensions are complicated and have to be carried out in open spaces rather than closed containers. In Chapter 3, two phages having different plaque morphologies (pAS-3 formed clear plaques, pAS-6 formed turbid plaques) were studied together and therefore any cross-contamination was easily detected. However, using morphological characteristics of plaques to differentiate phages cannot be effective when phages form similar plaques. Low-cost, fast and effective methods have been developed for detecting bacterial cross-contamination, such as colony morphology, Gram staining, specific PCR, and biochemical characteristics. However, there is a general lack of such methods for phages. Developing new methods and searching for new characteristics to differentiate phages is crucial for detecting new phages and any cross-contamination. Meanwhile, careful manipulation, phage-specific laboratory systems, and effective disinfection procedure are important for preventing phage cross-contamination.

5.4 Prospects for phage therapy in aquaculture

According to the existing research data (Table 1.1), it has been shown that phage therapy can be considered as an alternative approach to control bacterial infections in aquaculture. However, considering these mixed results, where some bacteria appear to be more easily controlled than others, and the total number of bacterial pathogens

and the wide range of cultured species in aquaculture, it is clear that this research area is still in its infancy, and further studies are needed to be carried out to complement the database.

It is likely that phage therapy will not totally replace conventional antibiotic treatment, but provide an additional option for treating infections, especially these caused by antibiotic-resistant bacteria. Phage therapy provides different routes for phage administration to aquaculture animals, which can be chosen according to the realistic situations. On-spot treatment is suitable for treating ulcerative skin lesions (Khairnar *et al*., 2013); for deep, systemic infections, injection has been commonly employed; however, for larvae, tiny fish, shrimp and shellfish, injection is not feasible, whereas immersion, water-borne, or oral administration are more practical. It has been shown that higher protection was observed in fish that were first injected with phages and then 1 h later injected with the pathogen, than that in the fish were first injected with the pathogen and then the phages (Mahmoud & Nakai, 2012). This suggests that phages may play a better role in prophylaxis than treatment. However, it is not easy for phages to remain *in vivo* without the presence of the bacterial host, as phages are normally cleared by the fish immune system in a few days (Verner-Jeffreys *et al*., 2007). Regularly adding phages in feed and/or farming water to keep a high number of phages in the intestinal and water environments may provide a way of prophylaxis. Using phage cocktails is widely viewed as a more practical approach than using a single phage, which may overcome the limitations of phage resistance and 'too specific' killing (Richards, 2014). Therefore, phage cocktails are more suitable to be applied in the cases of treating multiple infections and preventing secondary infections.

Although, a range of companies specialising in bacteriophages have appeared or become established in recent years, demonstrating a growing commercial interest in phage therapy, there are still intellectual property (IP) challenges and regulatory hurdles which deter investments in phage therapy (Henein, 2013; Verbeken *et al*., 2014). In practice, patents on bacteriophage products are important tools for attracting investors to new companies keen on developing therapeutic bacteriophages. Investing in a product without full IP and particularly patent protection is unlikely to be

financially viable (Henein, 2013). Patenting of phages that have been isolated from the environment is currently possible, but it provides limited IP protection, as unique characters have to be provided to differentiate the patented phage from other phages. In addition competitors may easily isolate a same phage from the commercialised phage preparation or from the environment where the phage product has been applied. Different pathways of patenting in the US and EU also adds to this challenge (Pirnay *et al*., 2012).

The lack of a regulatory framework for developing phage therapy products or using phage therapy in real cases is another hurdle in the commercialisation of phage therapy. It has raised attention and debates on the uses of phage therapy for humans, since it is hard to fit phages into any of the existing category of human medicinal products (Pirnay *et al*., 2012; Henein, 2013; Huys *et al*., 2013; Verbeken *et al*., 2014). This problem should be the same for phage therapy in aquaculture; however, to the best of my knowledge, no literature has discussed it. Different countries may set different guidelines for phage therapy in aquaculture, according to their national veterinary medicines legislation. However, all the phage therapy products should meet the three scientific criteria: quality, safety, and efficacy (Henein, 2013; Verbeken *et al*., 2014). Moreover, as the 'arms race' between bacteria and phages is ongoing, legislation should allow new phages to be quickly used in phage therapy activities and products.

5.5 Other possible applications of phages in aquaculture

Besides phage therapy, there are other feasible and potential applications of phages, which can be applied alone, or in combination with phage therapy in aquaculture.

5.5.1 Phage and antibiotic synergy

Both phage therapy and antibiotic treatment have advantages and disadvantages in combating bacterial infections. Phages with antibiotic synergy could lead to a better treatment effect than when just applyied them independently, as phages and antibiotics can give help to each other during the treatment. Comeau *et al*., (2007)

reported that sub-lethal concentrations of certain antibiotics which inhibit bacterial cell division and trigger the SOS system can substantially stimulate the host bacterial cells to release more virulent phage particles, which could be useful for phage therapy. The study of Hagens *et al*. (2006) revealed that upon infection with filamentous phage Pf1, the host *Pseudomonas aeruginosa* strain showed a significant increase in sensitivity to the antibiotics including gentamicin, chloramphenicol and carbenicillin, and a *P. aeruginosa* strain harbouring a plasmid-borne gentamicin resistance gene could be resensitised to the antibiotic. Moreover, this study also showed that mice were rescued from lethal infections with *P. aeruginosa* K strain by concomitant treatment with phage Pf1 and low concentrations of gentamicin, however, neither therapy was able to rescue the mice when applied alone. This study presented a good exploration of how phage and antibiotic synergy could be used to combat pathogenic bacteria.

5.5.2 Using phages as biocontrol agents for food safety applications

Consumption of raw or undercooked seafood is known as a health risk to consumers, and bacterial contamination is the main cause of sea-food borne diseases (Iwamoto *et al*., 2010). Fish and fishery products are vulnerable to bacterial contamination, especially filter feeders such as mussels and oysters (Popovic *et al*., 2010). In addition, aquatic products may be contaminatedby the marine environment, or during handling, processing and preparation (Iwamoto *et al*., 2010). Therefore, using antimicrobial agents to eliminate the contaminations of fishery and aquaculture products is very important. The interest in natural antimicrobial compounds has increased due to alterations in consumer opinions towards the use of chemical preservatives in foodstuff and food processing surfaces (Sillankorva *et al*., 2012). Phages are naturally antimicrobial and their effectiveness in controlling bacterial pathogens in food industry has led to the development of different phage products already approved by the U.S. Food and Drug Administration (USFDA) (Sillankorva *et al*., 2012). One of these products, bacteriophage Listex P100, has been used to control *Listeria monocytogenes* contaminations of raw salmon and fresh channel catfish fillets (Soni & Nannapaneni, 2010; Soni *et al*., 2010). Moreover, the studies of Rong *et al*. (2014) and Jun *et al*. (2014) have applied phages to reduce the number of *V. parahaemolyticus* in oyster

processing. Hence, phages can be considered as effective antimicrobial agents to control bacterial contaminations in many aquatic products.

5.5.3 Using phages to manipulate intestinal microbiome of fish

The human gut microbiome plays crucial nutrition and health roles in metabolic functions, influencing the development of the immune system, and protecting from colonisation by pathogens (Hooper *et al*., 2002; Dethlefsen *et al*., 2007). Although, based on the current studies, little is known about the bacterial communities and their establishment, or their diversity in fish, it is speculated that the intestinal microbiota in fish play similar roles in nutrition and health functions including digestion of algal cells, production of amino acids, secretion of inhibitory compounds against colonisation of bacterial pathogens, metabolism of dietary substrates, immune system modulation, and reproduction and overall population dynamics (Nayak, 2010; Ghanbari *et al*., 2015). Therefore, manipulation of the intestinal microbiome can possibly improve the overall health status of the host fish, including nutrient digestion, synthesis, absorption, pathogen resistance, growth, sexual maturation, morphogenesis, and survivorship in stocked fish (Llewellyn et al., 2014).

Phages are known to significantly affect microbial community composition in many ecological niches, including the mammalian gut. For example, *Enterococcus faecalis* strain V583 produces a phage øV1/7 which confers an advantage to *E. faecalis* V583 during competition with other *E. faecalis* strains *in vitro* and *in vivo* (Duerkop *et al*., 2012). This work proposed that *E. faecalis* V583 uses phage particles to establish and maintain dominance of its intestinal niche in the presence of closely related competing strains, and therefore, phages can impact the dynamics of bacterial colonisation in the mammalian intestinal ecosystem. Another study of Stern *et al*. (2012) addressed that phage-bacterial attack-resistance interactions occur within the human gut microbiome and could possibly affect microbiota composition and human health. Although studies on phages in the intestinal tract of fish and other aquatic animals have not been reported, phage-bacterial interaction may yield new applications of phages in aquaculture to manipulate the structure and functions of intestinal microbiomes of aquatic animals to improve their health status.

5.5.4 Using phage-resistant mutants as vaccines

The generation of phage-resistant mutants derived from pathogenic bacteria is not always bad. In the studies of Capparelli *et al*. (2010a; b), a phage-resistant attenuated mutant A172 of *Staphylococcus aureus* could confer broad immunity against *Staphylococcal* infection in mice: live A172 cells, its heat-killed vaccine, anti-A172 mouse antibodies, and the capsular polysaccharide of A172 all afforded significant protection from lethal infections of the *S. aureus* parental strain A170, and antibiotic-resistant pathogenic *S. aureus* strains. The results suggested that selection for phage-resistant mutants can facilitate bacterial vaccine preparations.

5.5.5 Using phages as vaccine delivery vehicles

There are two different ways of using phages as potential vaccine delivery vehicles: by vaccinating with phages carrying vaccine antigens on their surface or by using phage particles to deliver a DNA vaccine expression cassette (Clark & March, 2004a). In the former, vaccine antigens can be displayed to a phage surface either by generating a transcriptional fusion to a coat protein or by being artificially conjugated (Clark & March, 2006). Screening phage-display libraries can provide novel or potential vaccine antigents, and conjugating specific antigenic peptides or proteins to the surface of phages increases the range of antigens that can be displayed (Clark & March, 2006). In the latter, the vaccine gene controlled by a eukaryotic expression cassette is cloned into a phage genome, and purified whole phage particles are injected into the host. The phage coat protects the vaccine DNA from degradation, and target the vaccine to the antigen-presenting cells (Clark & March, 2006). It has been shown that when compared with standard DNA vaccines, phage-delivered DNA vaccines are more efficient, and superior antibody responses have been shown in animals (Clark & March, 2004b; March *et al*., 2004; Clark *et al*., 2011). The possibility of producing a 'hybrid phage vaccine' has been proposed, where a DNA vaccine is contained within the same phage particle that displaying the variant of the same antigen on the surface. It is expected that such a vaccine will efficiently target both the humoral and cellular arms of the immune system (Clark & March, 2004a).

Phage vaccination has the advantages of being cheap to manufacture, little technical expertise is needed, large numbers of doses can be quickly produced, and multiple vaccines can be delivered in one phage (Clark & March, 2004a,b; March *et al*., 2004; Clark *et al*., 2011). Moreover, because phages (lambda) are very stable at a range of temperatures and pH, oral administration of phage-delivered DNA vaccines via drinking water may be possible (Jepson & March, 2004), which also means it will be suitable for the aqueous environment in aquaculture.

5.6 Final conclusions

Different approaches were used to isolate and characterise bacteriophages from two aquaculture environments for the studies of phage therapy. A rainbow trout intraperitoneal injection model was set up using a pathogenic *A. salmonicida* strain and its two phages. Fish treated with phages showed protection reflected by lower cumulative mortalities and a delayed time to death, however, significant differences between the treated and control groups could not be demonstrated. Futher work is required to determine if these phages have potential in phage therapy. Although the culture temperature of some fish species may not favour the emergence of phage-resistant bacterial mutants during phage therapy, their characteristics, especially virulence, still need to be well studied before carrying out large scale field trials. Combining the study results from other researchers, it appears feasible to use phage therapy as an alternative approach to control bacterial infections in aquaculture, but further studies are required to focus on improving effectiveness, and to overcome the concrete limitations and hurdles in application and commercialisation. Moreover, a broader range of applications of phages in aquaculture should be explored.

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