Study of *Staphylococcus pseudintermedius* phages: towards the development of phage therapy

by

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Declarations

The work presented in this document is original. All work, except where stated in the text, was carried out by the author of this thesis. The thesis has not been submitted for a degree at another university.

Abstract

The extensive use of antibiotics has led to the emergence of methicillin-resistant *Staphylococcus pseudintermedius*, a bacterium causing difficult-to-treat canine skin infection (pyoderma). The administration of bacteriophages (phage therapy) can be an alternative to antibiotic therapy. Lytic phages, which lyse their host, are considered the only appropriate type of phages for phage therapy as opposed to temperate phages, which can survive within their host (lysogeny). However, it is possible to mutate temperate phages so that they cannot establish lysogeny anymore. Phage λ virulent (Vir) mutants have lost the operator to which the CI repressor binds to inhibit the expression of lytic genes. As a result, Vir mutants are strictly lytic.

The work presented in this thesis was undertaken to isolate *S. pseudintermedius* phages and gain knowledge about their biology with the aim to develop phage therapy to treat pyoderma. The work was novel; very few data were available on *S. pseudintermedius* phages and no data have been published on phage therapy to treat canine skin infection.

Four temperate phage candidates were selected after phenotypic and genotypic characterisation. No lytic phages were found. Random mutagenesis approaches were unsuccessful for the isolation of Vir mutants. An operator and three point mutations leading to the absence of CI repressor binding to this operator were identified through gel shift assay. These mutations should lead to a virulent phenotype if introduced in the relevant phage genome through site-directed mutagenesis. A PCR-based assay was performed to explore how widespread lysogeny was in *S. pseudintermedius*: 11 out of 45 tested strains were positive for the presence of prophage genes. Bioinformatic analyses revealed some of the genetic characteristics of *S. pseudintermedius* phages: genomic circular permutation and the presence of a genetic switch similar to that of phage λ.

The work reported in this thesis represents a first step towards understanding the biology of *S. pseudintermedius* phages and developing phage therapy.

List of abbreviations

BAM (file)	Binary version of a SAM file
BHI	Brain heart infusion
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
bp	Base pairs
CDS	Coding DNA sequence
CFU	Colony-forming unit
dsDNA	Double-stranded DNA
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
EM	Electron microscopy
EOP	Efficiency of plating
HTH (motif)	Helix-Turn-Helix
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria broth
MOI	Multiplicity of infection
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRSP	Methicillin-resistant *Staphylococcus pseudintermedius*
MSSP	Methicillin-sensitive *Staphylococcus pseudintermedius*
NCBI	National Center for Biotechnology Information
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerisation chain reaction
PFGE	Pulsed-field gel electrophoresis
PFU	Plaque-forming unit
Poly(dI-dC)	Poly(deoxyinosinic-deoxycytidylic) acid
qPCR	Quantitative polymerisation chain reaction
RNAP	RNA polymerase
RFLP	Restriction-fragment length polymorphism
RT	Room temperature

SAM (file)	Sequence alignment/map
SDS	Sodium dodecyl sulphate
SGS	Sequence-generation sequencing (methods)
SIG	*Staphylococcus intermedius* group
ssDNA	Single-stranded DNA
ST	Sequence type
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TSB	Tryptic soya broth
UV (light)	Ultraviolet

Chapter 1 Introduction

1.1. Antibiotics for the treatment of bacterial infections

1.1.1. Brief history of the discovery and development of antibiotics

The very first antimicrobial agent to be used in human medicine was discovered by Ehrlich in 1909 and was commercialised for the treatment of syphilis under the name Salvarsan. In 1928, Fleming discovered that penicillin was responsible for the inhibition of growth of *Staphylococcus aureus* around the colony of a fungus belonging to the *Penicillium* genus (Saga and Yamaguchi, 2009). Penicillin came into clinical use in 1940, saving the lives of thousands of soldiers during World War II, following the development by Florey and Chain of a protocol for the purification of penicillin on a large scale (Chain *et al.*, 2005). By 1945 penicillin was being mass-produced for widespread use in human medicine. At the same time the first synthetic antibiotics, the sulfonamides, were developed (Aminov, 2010), and the first effective anti-tuberculosis drug, streptomycin, was discovered by Schatz and Waksman (Zumla *et al.*, 2013). The next twenty years saw the discovery of numerous new classes of antibiotics (Fernandes, 2006), a lot of which were isolated from soil bacteria.

1.1.2. The classes of antibiotics

Antibiotics are usually classified based on their chemical structure and their mechanism of action (Table 1.1). Antibiotics can be bactericidal, e.g. antibiotics inhibiting cell wall synthesis, or only bacteriostatic. They can also target specifically Gram-positive or Gram-negative bacteria, or they can have a broader spectrum and target both (Coates *et al.*, 2011).

Table 1.1: Characteristics of the different classes of antibiotics. Broad-spectrum drugs target both Gram-negative and Gram-positive bacteria.

Mechanism of action	Class of antibiotics		Examples	Bactericidal (BC)	Spectrum of activity
Inhibition of cell wall synthesis	β-lactams	Penicillins	Penicillin, amoxicillin, methicillin	BC	Broad spectrum
		Cephalosporins	Cephalothin, cefalexin	BC	Broad spectrum
		Carbapenems	Imipenem, doripenem	BC	Broad spectrum
	β-lactamase inhibitors		Clavulanic acid, tazobactam		Used with β-lactams
	Glycopeptide		Vancomycin	BC	Gram-positive bacteria
	Polymyxins		Polymyxin B, polymyxin E	BC	Gram-negative bacteria
	Bacitracin		Bacitracin	BC	Gram-positive bacteria
Inhibition of protein synthesis	Aminoglycosides		Streptomycin, kanamycin	BC	Broad spectrum
	Tetracyclines		Tetracycline, doxycycline		Broad spectrum
	Macrolides		Erythromycin, clarithromycin		Broad spectrum
	Chloramphenicol		Chloramphenicol		Broad spectrum
	Lincosamides		Lincomycin, clindamycin		Anaerobe + Gram-positive bacteria
	Oxazolidinone		Linezolid		Gram-positive bacteria
	Streptogramins		Quinupristin, dalfopristin		Gram-positive bacteria
Inhibition of DNA synthesis	Fluoroquinolones		Nalidixic acid, ciprofloxacin	BC	Broad spectrum
Inhibition of RNA synthesis	Rifamycins		Rifampicin, rifaximin	BC	Gram-positive bacteria
Inhibition of mycolic acid synthesis	Isoniazid		Isoniazid	BC	*Mycobacterium*
Inhibition of folic acid synthesis	Sulfonamides		Sulfadiazine, sulfisoxazole		Gram-positive bacteria
Depolarization of cell membrane	Lipopeptide		Daptomycin	BC	Gram-positive bacteria
Inhibition of ATPase	Diarylquinoline		Bedaquiline	BC	*Mycobacterium*

1.1.3. Antibiotic resistance

1.1.3.a. The emergence of resistant pathogens

The rise of antibiotic resistance started almost immediately after antibiotics were used for the first time. The first report of bacterial resistance to penicillin was published in 1942 (Peacock and Paterson, 2015). Genes coding for resistance mechanisms are themselves not new (D'Costa *et al.*, 2011) but the strong selective pressure resulting from the large-scale use of antibiotics in human medicine led to the selection of resistant microorganisms and the spread of resistance genes to clinically important bacterial species (Davies and Davies, 2010). In the 1960s to 1970s, while antibiotic resistance was increasing, the discovery rate of antibiotics was declining. The approach used since then to tackle emerging resistance of pathogens has been the modification of existing antibiotics (e.g. 1st, 2nd, 3rd and 4th generations of cephalosporins). Very few new classes of antibiotics have been discovered, the only truly novel agents that have been launched in the past 40 years are linezolid, daptomycin and bedaquiline (Overbye and Barrett, 2005, Bassetti and Righi, 2013).

1.1.3.b. The emergence of "superbugs"

The combination of many factors including high usage of antibiotics in human medicine but also in agriculture, improper use of the drugs (e.g. in the case of a viral infection or poor compliance in treatment), the diminished investment in antibiotic discovery by the pharmaceutical industry and the spread of resistance genes in the environment has led to a critical situation nowadays (Aminov, 2010). The human population is now faced with so-called superbugs resistant to almost every, if not all, antibiotics currently available (Rossolini *et al.*, 2014). A report published in 2009 estimated that each year about 25,000 patients in the European Union die from an infection with multidrug-resistant bacteria (Norrby *et al.*, 2009). The same report estimates that the economic costs due to these infections result in extra healthcare costs and productivity losses of at least 1.5 billion euros each year.

1.1.3.c. Multidrug-resistant *Staphylococcus aureus*, one of the most notorious superbugs

S. aureus is an opportunistic pathogen that causes multiple types of infections including skin, osteoarticular and urinary tract infections (Tong *et al.*, 2015).

Resistant strains were rapidly selected after the introduction of penicillin in human medicine. This antibiotic targets enzymes involved in cell-wall synthesis called the penicillin-binding proteins (PBPs). The β-lactam ring present in the penicillin's structure plays a major role in its inhibitory effect (Figure 1.1). The mechanism of resistance is the production of a β-lactamase, an enzyme that hydrolyses the β-lactam ring and inactivates the drug (Peacock and Paterson, 2015).

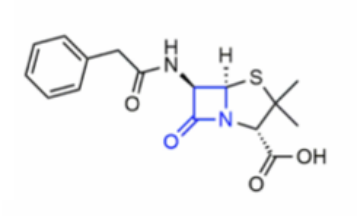


Figure 1.1: Penicillin contains a β-lactam ring (in blue) that plays a major role in its inhibitory effect on cell wall synthesis (picture from Fair and Tor, 2014).

A β-lactamase-resistant penicillin derivative, called methicillin (or meticillin), was commercialised in 1959. Methicillin-resistant *S. aureus* (MRSA) isolates were reported for the first time the following year. Resistance to methicillin is not due to β-lactamase production and results from the acquisition of the *mecA* gene from another bacterial species. This gene is located on a large mobile genetic element called Staphylococcal Chromosomal Cassette (SCC) *mec* and encodes a new PBP, termed PBP2a (Stapleton and Taylor, 2002). This enzyme has reduced affinity for β-lactams making this class of antibiotics ineffective at treating MRSA infections. Although methicillin is not used clinically anymore, the term MRSA has persisted, it now refers to multidrug-resistant *S. aureus,* and the term methicillin resistance denotes resistance to virtually all β-lactams.

1.2. Phage therapy

1.2.1. Phage therapy in the pre-antibiotic era: from initial enthusiasm to downfall in western countries

Bacteriophages, which name literally means "eaters of bacteria", were discovered and described twice by Frederick Twort in 1915 and Félix D'Hérelle in 1917 (Duckworth, 1987). Following this initial discovery, exploiting the ability of phages to infect and kill bacteria (section 1.4.2) became the main focus of phage research with the aim to develop phage-based antibacterial agents. D'Hérelle and other

researchers obtained encouraging results when using phages to treat dysentery, cholera and staphylococcal skin diseases in the 1920s and 1930s (Sulakvelidze *et al.*, 2001, Kutter *et al.*, 2010). Enthusiasm for this new cure grew fast in an era where treatment options for bacterial diseases were extremely limited. In the 1930s, companies such as Eli Lilly were commercialising phage products for human use (Housby and Mann, 2009).

Despite the initial enthusiasm, controversy arose in the scientific community over the validity of phage as a therapeutic agent because of the inconsistent successes and failures of phage therapy. Possible reasons for this are that standardized, controlled trials did not exist at the time and that the biology of phages was poorly understood (Tsonos *et al.*, 2013). Some scientists thought phages were viruses while others believed they were lytic enzymes (Wittebole *et al.*, 2014). General overenthusiasm and exaggerated claims about commercial phage preparations meant that phages were sometimes used in situations where they were not adapted, with disastrous consequences (Barrow and Soothill, 1997).

The popularity of phage therapy did not improve after the introduction of antibiotics in human medicine in 1940 (section 1.1.1). Antibiotics offered significant advantages compared to phage therapy. Their broad spectrum of activity meant that they could be used without the need to know which pathogen caused the infection (Villa and Veiga-Crespo, 2010). This was not the case with phages (section 1.2.4). The pharmacology of antibiotics was also easier to characterise due to their chemical nature. Their defined chemical structure meant they were easy to patent to secure return on investments for pharmaceutical companies. Overall, antibiotics were quite uniformly and dramatically effective against important diseases including tuberculosis, they were relatively cheap to produce and were quickly made widely available.

Politics also played a role in the downfall of phage therapy. Indeed, it had been employed in the Soviet Union and the German army during World War II and was regarded with increasing suspicion by the former Allies as a consequence of the Cold War. Eventually phage therapy was abandoned in the West (Summers, 2012).

1.2.2. Phage therapy in the Soviet Union

On the contrary to western countries, the study and use of phages as therapeutic agents never stopped in the Soviet Union. The Eliava Institute, founded in Georgia in 1923, and the Hirszfeld Institute, founded in Poland in 1952, became major centres for the development of phage therapy benefitting from important governmental support. Numerous studies were carried out to test phage therapy for the treatment and prophylaxis of enteric diseases, lung and skin infections through topical or oral administration with high reported success rates (Alisky *et al.*, 1998). In 1963-1964, an extensive study involving more than 30,000 children was conducted to evaluate the utility of phages to prevent dysentery. The reported incidence of dysentery was 2.6-fold higher in the placebo group than in the phage-treated group (Kutter *et al.*, 2010). Between 1981 and 1986, 550 patients suffering from skin infections were treated with phages. The authors reported positive results in 92% of cases (Slopek *et al.*, 1987).

Most of the literature reporting these successes was in Russian or Polish. It was later made available to the wider scientific community by translating it into English. Unfortunately, the controls and methods used in a lot of these studies do not conform to current practise in the West (Sulakvelidze *et al.*, 2001). Despite this, phage therapy has remained, at least in Georgia, part of the standard healthcare system even after antibiotics were introduced (later than in the West) and the collapse of the Soviet Union in 1991 (Kutateladze and Adamia, 2008). More recently, the successful use of PhagoBioDerm, a biodegradable matrix impregnated with phages and antibiotics (Markoishvili *et al.*, 2002), was reported for the healing of infected radiation burns (Jikia *et al.*, 2005).

1.2.3. Uses of bacteriophages in the West

1.2.3.a. Non-therapeutic uses of phages

Phage therapy may have been abandoned in western countries but bacteriophages themselves were not forgotten. In the 1940s, bacteriophages were identified as model organisms for pioneering experiments leading the way to molecular biology. Studies on phages helped establish that DNA is the genetic material. Temperate phages, of which the phage λ that infects *Escherichia coli*, were used to explore how genes are regulated (Keen, 2015). Phage-encoded enzymes such as DNA/RNA polymerases,

ligases, and exo- and endonucleases are nowadays used in molecular cloning. The study of bacterial resistance mechanisms to phage infection led to the identification of restriction enzymes and the CRISPR-Cas system providing more tools for molecular biologists (Rohwer and Segall, 2015). In the food industry, phages are nowadays used for decontamination or treatment purposes. EcoShield™ and ListShield™, both commercialised by Intralytix, are used for the decontamination of red meat and food-processing facilities respectively (Figure 1.2) (Sillankorva *et al.*, 2012). Omnilytics developed AgriPhage™, a phage preparation for the treatment of bacterial diseases on tomato and pepper plants (Zaczek *et al.*, 2015).

Figure 1.2: Commercial packaging of ListShield™, targeting *Listeria monocytogenes* in food-processing plants (picture from Sulakvelidze, 2013).

Phages are also used in laboratories and commercially for the identification and detection of pathogens such as *Listeria monocytogenes*, *S. aureus*, *Salmonella* and *Mycobacterium* (Loessner *et al.*, 1996, Monk *et al.*, 2010, Lienemann *et al.*, 2015, Rees, 2006).

1.2.3.b. Renewed interest in phage therapy

As a result of the antibiotic resistance crisis, phages are being reconsidered as alternative therapeutics to treat and prevent bacterial infections. In the 1980s Smith and Huggins carried out a significant series of carefully controlled studies in the UK. They reported the successful use of phages to protect mice, calves, piglets and lambs from *E. coli* infections (Smith and Huggins, 1982, 1983). Interestingly, they found that in some cases phage therapy was superior to antibiotic treatment. In the 1990s further encouraging results were obtained by Soothill in animal studies (Soothill, 1992, 1994).

In 2000, the US government described antibiotic resistance as "a growing menace to all people" (Thiel, 2004) and started encouraging phage research. In 2014, the European Parliament proposed a motion for the resolution of antibiotic resistance asking member states to prioritise the development of phage therapy (Blondin *et al.*, 2014). In 2015, the US government released a National Action Plan for Combating Antibiotic-Resistant Bacteria (Carter *et al.*, 2015) supporting among other options the development of alternative therapeutics including phage. At the same time a few phase I/II clinical trials were carried out in humans, demonstrating the general safety and suggesting the efficacy of phage treatment through oral, topical and intravenous applications (Bruttin and Brüssow, 2005, Sarker *et al.*, 2012, Wright *et al.*, 2009, Speck and Smithyman, 2016).

In parallel to clinical trials, which aim is to produce reliable data on a medium to large scale, phage therapy is being used sporadically in Europe on a very small scale as a last resort treatment, under "compassionate use" regulation, for human patients with otherwise untreatable infections (Verbeken *et al.*, 2012, Rose *et al.*, 2014). Such experimental treatments have not been described in animal health but one clinical trial was performed to evaluate the topical treatment of otitis of dogs with a phage mixture (Hawkins *et al.*, 2010). The authors reported the absence of toxicity and the possible role of the phage mixture in the treatment of otitis.

1.2.4. Pros and cons of phage therapy

There are advantages to phage treatment as well as drawbacks. Many ways to overcoming the latter have been proposed (Table 1.2). One major advantage is the specificity of phages for their host that leads to minimal disruption of the normal flora, on the contrary to some broad-spectrum antibiotics. Another advantage is that phage therapy is effective against antibiotic-resistant bacteria because the mechanism of action is different from all antibiotics (Hanlon, 2007). Phages are also able to amplify *in situ* as long as the host is present, which offers the possibility for single-dose treatment and potentially reduced costs (Loc-Carrillo and Abedon, 2011).

Table 1.2: Advantages and drawbacks of phage therapy, and possible solutions to overcoming the disadvantages.

Advantages	Drawbacks	Possible solutions
Narrow host range = minimal disruption of normal flora	Narrow host range = doctors need to know what pathogen causes the infection	➔ Use phage cocktails to broaden the spectrum
Phages can multiply *in situ* = potential for single-dose treatment	Phages are quickly cleared from the bloodstream by the immune system	➔ Prefer topical applications or select long-circulating phages
Low toxicity	Phages may contain harmful genes (e.g. virulence genes)	➔ Perform whole-genome sequencing to ensure absence of harmful genes
Ability to destroy biofilms	Phage lysis causes the release of bacterial components and/or toxins	➔ Engineer phages that kill but do not lyse their host
Cheap to produce	Difficult to patent	➔ Patent method of isolation/production or engineer therapeutic phages
Effective against antibiotic-resistant bacteria	Regulatory hurdles	➔ Develop a new regulatory framework to accommodate phage therapy
Resistance to phages occurs at a lower frequency compared to resistance to antibiotics (Kutateladze and Adamia, 2010)	Development of resistance to phages	➔ Use phage cocktails or isolate new phages that overcome the resistance.

The development of resistance against phages is a concern, however phages can quickly adapt and overcome the resistance (Samson *et al.*, 2013). It is possible to regularly select new phages able to infect the resistant bacteria. It has been proposed to select (and re-select if needed) specific phages through a patient-specific approach in hospitals for life-threatening situations (Huys *et al.*, 2013). For other situations patients could use commercial phage cocktails that would follow the normal phases of drug development but would need regular updating (similarly to vaccines). Current regulations do not facilitate either of these approaches and a new regulatory framework accommodating phage therapy may be needed (Verbeken *et al.*, 2014b). In some cases, the development of resistance to phages may have a more positive outcome: the reduction of the bacterial host's fitness. Some structures on the bacterial cell surface that phages use as receptors to recognise their host (section 1.4.2.a.i) can be virulence factors, e.g. lipopolysaccharide in *Salmonella* or the antigen K in *E. coli*. The loss or modification of these structures leads to resistance against phages but also reduces the virulence (Leon and Bastias, 2015).

An issue associated with the commercial development of phage therapy is the difficulty for private companies to secure intellectual property (IP) because the idea of using phages therapeutically is not new and phages are ubiquitous. Patenting a method of isolation/production of phages or engineering phages may be ways to create IP and ensure return on investments (Pirnay *et al.*, 2012). Phage engineering is becoming more and more accepted within the phage community and by regulatory authorities such as the Food and Drug Administration (communication of Scott Sticbitz, FDA representative at the Evergreen Phage Meeting 2015) as one of the ways forward to develop phage therapy. A few companies are now taking on the challenge to bring commercial phage preparations to the market (Henein, 2013).

Phage therapy offers an interesting alternative to antibiotic treatment with numerous advantages, and its disadvantages can be overcome. Given the emergency of the situation for patients infected with multi-drug resistant bacteria, some even argue that all relevant academic laboratories, authorities and industries, have a moral duty to investigate or facilitate the investigation of phage therapy with the aim to alleviate suffering (Verbeken *et al.*, 2014a).

1.2.5. Choosing a model disease to investigate the effectiveness of phage therapy

As mentioned in the previous sections, phage therapy has been tested for the treatment of various types of infections and it is possible to argue that some diseases are better models than others to investigate the effectiveness of phage therapy. Enteric diseases are not good models. Most are acute, self-limiting infections and by the time symptoms show, i.e. diarrhoea, antibacterial treatment is not much use for ameliorating the condition of the patient. Rehydration therapy is the cornerstone of treatment for diarrhoea (Casburn-Jones, 2004). Moreover, the use of antibiotics for treating infectious diarrhoea is likely to select resistant bacteria and should be avoided except in severe cases of cholera or typhoid fever (Diniz-Santos *et al.*, 2006). It is not expected that phages would bring any more benefit than antibiotics in cases of diarrhoea.

Surface infections such as skin or ear infections are better models because topical application is possible. When applied topically, phages are less likely to be rapidly cleared by the immune system, compared to phages applied intravenously, even though interaction with the immune system is still possible (Clark and March, 2006). Through topical application phages are not exposed to inactivating gastric acid as are phages administered orally. Surface infections can also be chronic and resistant to antibiotic treatment (Abedon *et al.*, 2011), offering the possibility to show the effectiveness and usefulness of phage therapy as an alternative to antibiotic treatment.

1.3. The bacterium *Staphylococcus pseudintermedius*

1.3.1. A commensal, part of the normal canine cutaneous flora

Staphylococcus pseudintermedius is a coagulase-positive *Staphyloccocus* species that colonises dogs and sometimes other animals such as cats (Bardiau *et al.*, 2013) and rats (Himsworth *et al.*, 2013). In healthy dogs, *S. pseudintermedius* is part of the normal cutaneous flora and colonizes the skin, hair (Allaker *et al.*, 1992a) and mucocutaneous sites such as the mouth, nose and anus (Devriese and De Pelsmaecker, 1987, Allaker *et al.*, 1992b).

The carriage rates reported by several cross-sectional studies conducted on large populations of healthy dogs (n > 100) are rather variable. For example two studies showed that *S. pseudintermedius* was present on 46.2% and 87.4% (Hanselman *et al.*, 2009, Rubin and Chirino-Trejo, 2011) of healthy dogs. This variability probably reflects diversity in the numbers and types of body sites that were sampled and in the methods used for sampling (Bannoehr and Guardabassi, 2012). The health status of the dogs may also influence *S. pseudintermedius* carriage. A study showed that the carriage rate was higher in dogs suffering from atopic dermatitis (eczema) compared to healthy dogs (Fazakerley *et al.*, 2009).

1.3.2. An opportunistic pathogen

S. pseudintermedius is also an opportunistic pathogen that has been identified as the main causative agent of ear and skin infections (pyoderma) (van Duijkeren *et al.*, 2011b). Abnormal host factors such as hypersensitivities, eczema and ectoparasites are the primary causes of pyoderma (Bloom, 2014). *S. pseudintermedius* is thought to act as a secondary infectious agent or lesion contaminant. Bacterial pyoderma can be classified into surface, superficial and deep pyoderma depending on the depth of the lesion (Ihrke, 1987). Superficial pyoderma is the most common form and manifests as hair loss (alopecia) areas spread throughout the body, epidermal collarettes, and papules or pustules (Hillier *et al.*, 2014) (Figure 1.3).

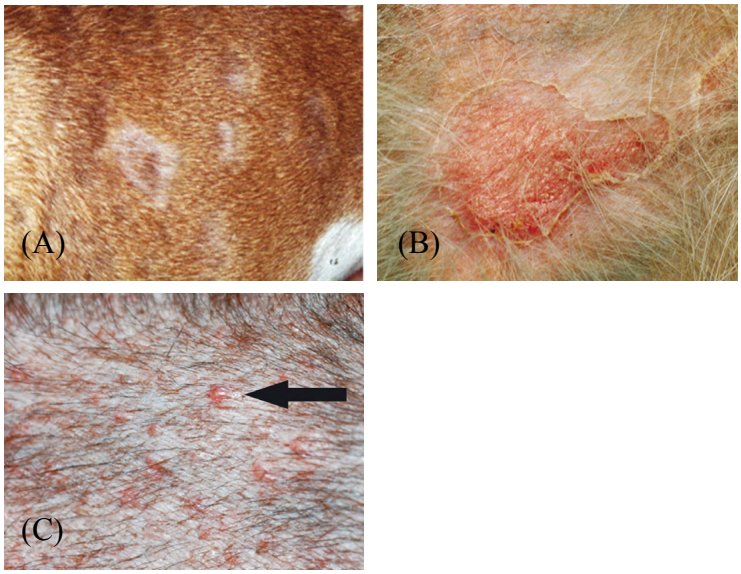


Figure 1.3: Superficial pyoderma is the most common form of the disease and is associated with (A) hair loss (alopecia) areas spread throughout the body, (B) epidermal collarettes, and (C) pustules (arrow) and papules (pictures from Hillier *et al.*, 2014).

Until 2005, *Staphylococcus intermedius* was thought to be the major causing agent of pyoderma in dogs. The description of *S. pseudintermedius* (Devriese *et al.*, 2005) has led to the re-classification of the *S. intermedius* group (SIG). It is now divided into four species: *S. intermedius*, *S. pseudintermedius* and *S. delphini* groups A and B (Sasaki *et al.*, 2007). *S. pseudintermedius* is nowadays identified through a series of biochemical tests and PCR amplification of the thermonuclease (*nuc*) gene following an initial cultivation step and selection of colonies with a typical *Staphylococcus* morphology.

1.3.3. *S. pseudintermedius* and antibiotic resistance

1.3.3.a. The emergence of methicillin-resistant *S. pseudintermedius* and pyoderma treatment options

S. pseudintermedius is one example of an animal pathogen that has acquired resistance, showing that antibiotic resistance has become a problem not only in human health but also in animal health. *S. pseudintermedius* has historically been considered slow to acquire resistance to antibiotics (Ravens *et al.*, 2014). However, the past fifteen years have seen the emergence of methicillin-resistant *S. pseudintermedius* (MRSP), which acquired its resistance in a similar way to MRSA (section 1.1.3.c). MRSP is associated with postoperative infections and urinary tract

infections in addition to skin infections (Kadlec and Schwarz, 2012) and studies showed that MRSP carriage is much lower than methicillin-susceptible *S. pseudintermedius* (MSSP) carriage (between 1% and 7% of healthy dogs) (Nienhoff *et al.*, 2011, Davis *et al.*, 2014, Kjellman *et al.*, 2015). The treatment of pyoderma used to be empirical and involved systemically administered antibiotics such as amoxicillin-clavulanic acid, clindamycin or cefalexin (Table 1.1). Systemic treatments were, and still are, coupled with the topical application of antibacterial agents like hydroxyl acids, benzoyl peroxide or ethyl lactate. Typically, three to six weeks of therapy are required and recurrent infections may occur, especially in dogs suffering from atopic dermatitis (Dowling, 1996, Summers *et al.*, 2014).

MRSP isolates are considered resistant to all β-lactams, which means that amoxicillin cannot be used to treat MRSP infections. Moreover, additional antibiotic resistance genes can insert into the Staphylococcal Chromosomal Cassette (section 1.1.3.c) (Holden *et al.*, 2004, Descloux *et al.*, 2008) and this explains why MRSP is often resistant to several non-β-lactam antibiotics (Garbacz *et al.*, 2013, Onuma *et al.*, 2012, Murayama *et al.*, 2013). The treatment of pyoderma therefore becomes much more difficult and recurrent infections are more likely to occur due to treatment failure.

1.3.3.b. Molecular characterisation and genetic aspects of MRSP isolates

Studies carried out over the past five years showed that MRSP strains constitute a rather clonal population with predominant lineages identified through multi-locus typing (MLST). MLST characterises isolates by sequencing internal fragments of multiple housekeeping genes (loci). The different sequences present within a bacterial species are assigned as distinct alleles. The combination of alleles at each chosen locus defines the allelic profile or Sequence Type (ST). Seven loci are used to discriminate MRSP strains: *tuf* (elongation factor Tu), *cpn60* or *hsp60* (heat-shock protein 60), *pta* (phosphotransacetylase), *purA* (adenylsuccinate synthetase), *fdh* (formate dehydrogenase), *ack* (acetate kinase) and *sar* (sodium sulphate symporter) (Solyman *et al.*, 2013). ST71 and ST68 are predominant in Europe and North America respectively (Kadlec *et al.*, 2010).

Two other molecular methods are used to precisely type *S. pseudintermedius* clones:

- *Spa* typing, that involves the amplification, sequencing and analysis of the polymorphic repeat X region of the protein A, a cell wall constituent of *Staphylococci* (Tang *et al.*, 2000).
- SCC*mec* typing based on the type of recombinase genes, the class of *mec* gene and its associated regulatory sequences (IWG-SCC, 2009).

The geographic restriction described above probably reflects the rather recent emergence of methicillin-resistant strains but the situation is evolving rapidly. One particular clone, ST71-t02(*spa*)-II-III(SCC*mec*), has been isolated since 2007 from European countries such as Germany, Spain, Sweden, Norway, Finland and the UK (Bannoehr *et al.*, 2007, Ruscher *et al.*, 2010, Gómez-Sanz *et al.*, 2011, Grönthal *et al.*, 2014) and later from other parts of the world such as Japan, Hong-Kong and South America (Bardiau *et al.*, 2013, Boost *et al.*, 2011, Quitoco *et al.*, 2013).

A study published in 2015, comparing twelve *S. pseudintermedius* genomes, suggested that the success of this clone and other isolates from ST71 and ST68 lineages depends on their ability to acquire novel mobile genetic elements through horizontal gene transfer (HGT) (McCarthy *et al.*, 2015). According to this study MRSP evolved through a stepwise accumulation of SCC*mec*, transposon-like elements and also core genome mutations conferring fluoroquinolone resistance. Interestingly, this study also suggested that, in contrast to *S. aureus*, HGT in *S. pseudintermedius* occurs predominantly through bacteriophage transduction rather than plasmid conjugation. The role of phages in the transfer of genetic material between *S. pseudintermedius* strains was further suggested in a study published in 2016 (Couto *et al.*, 2016). This study also found that some prophage genes were upregulated in MRSP compared to MSSP, suggesting that prophages may have a role in bacterial fitness in *S. pseudintermedius*.

1.3.4. *S. pseudintermedius* in humans

S. pseudintermedius has high host specificity and humans do not naturally carry this bacterium. However, transient carriage of *S. pseudintermedius* in humans is possible especially for people from the veterinary profession (Bond and Loeffler, 2012). A study involving 128 veterinarians showed a relatively high MRSP carriage rate (3.9 %) persistent over a period of one month (Paul *et al.*, 2011). Another survey of 171 veterinary dermatology staff in North America showed that nine individuals (5.3%) were colonised by MRSP. Interestingly, concordant strains of MRSP were isolated from the pets of three of these individuals (Morris *et al.*, 2010). Indeed, pet owners are likely to be colonised by MRSP whose origin can be attributed to their pet animal (Guardabassi *et al.*, 2004, van Duijkeren *et al.*, 2011a). These results also suggest that, although MRSP has not been shown to be more virulent than MSSP, it seems to have better ability to colonize humans.

The zoonotic transmission of MRSP is a concern because the bacterium can cause infections in humans. The first case of *S. pseudintermedius* infection in a human was described in 2006 (Van Hoovels *et al.*, 2006). Two more recent cases were due to ST71 strains (Stegmann *et al.*, 2010, Starlander *et al.*, 2014). It has been suggested that ST71 strains have a particular ability to invade human hosts because they adhere equally well to canine and human corneocytes. The adherence to corneocytes is an important step in skin colonisation and pathogenesis, and *S. pseudintermedius* usually adheres significantly better to canine corneocytes compared to human ones (Latronico *et al.*, 2014). Another concern associated with the transmission of MRSP from animals to humans is that MRSP may transfer its mobile SCC*mec* to methicillin-susceptible *S. aureus* present on humans, converting it into MRSA (Cohn and Middleton, 2010).

1.3.5. Phage therapy for the treatment of canine pyoderma

The development of phage therapy to treat canine pyoderma was chosen for several reasons:

- Pyoderma is becoming a serious issue in animal health because of antibiotic-resistant *S. pseudintermedius* and phage therapy is an interesting alternative.
- Canine skin infection constitutes a good model disease for testing the effectiveness of phage therapy (section 1.2.5): it is sometimes chronic and

offers the possibility to use a topical treatment, avoiding part of the immune response and its inhibitory effects.

- Developing phage therapy in animals would constitute a proof-of-concept facilitating its introduction in human health.

A phage-based commercial product, Staphage Lysate (SPL)®, Delmont Laboratories, is already available to help with the treatment of staphylococcal infections in the dog but it is said to only stimulate the immune response rather than directly kill the bacteria responsible for the infection. The aim in this project was to develop a treatment relying on the lytic activity of intact phages.

1.4. The biology of bacteriophages

1.4.1. The classification of bacteriophages

Bacteriophages are the most abundant organisms on Earth. It is estimated that there are more than 10^{31} phage particles on the planet, with approximately ten phages for every bacterial cell (Karam, 2005). They exist in various forms and sizes that can be classified. Several classification schemes were proposed during the first half of the 20th century. The International Committee on Taxonomy of Viruses (ICTV), created in 1971, currently recognises one order, thirteen families and 80 genera of phages (Krupovic *et al.*, 2016). While families are defined by the nature of the nucleic acid and particle morphology (Figure 1.4), there are no universal criteria for genus and species determination (Ackermann, 2006). The ICTV has adopted the "polythetic species concept" where a species of viruses is defined by a set of properties and no single property is absolutely essential. The advantage of this concept is that it accommodates the inherent variability of viruses (Fauquet, 2008).

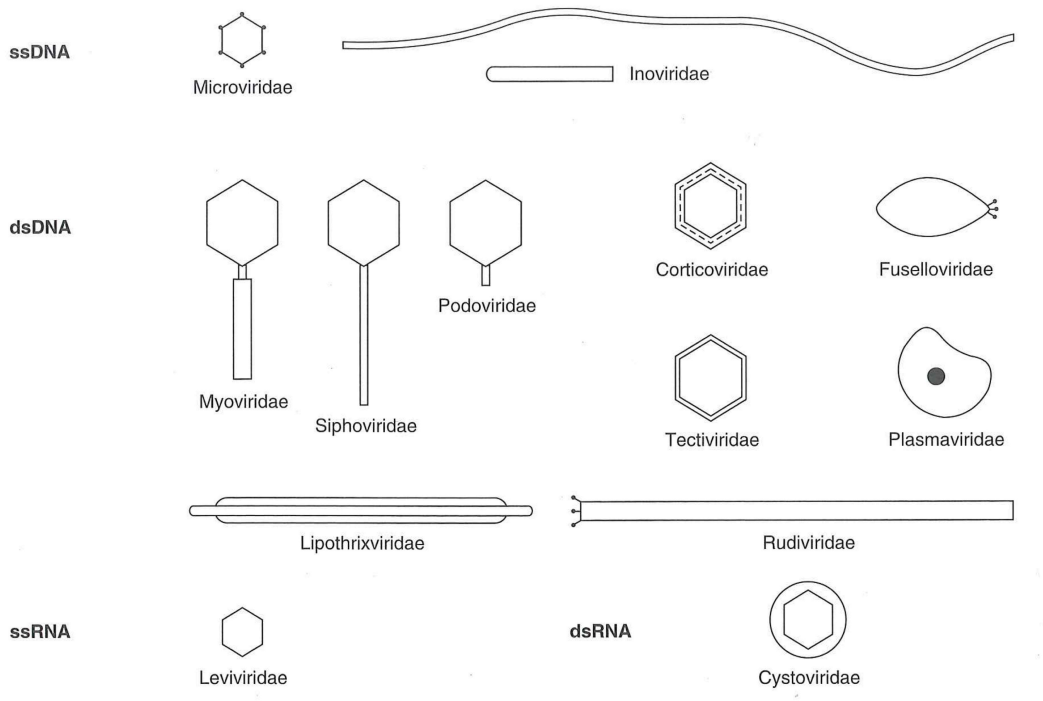


Figure 1.4: the ICTV currently recognises 13 families of phages defined by the nature of the nucleic acid and particle morphology (picture from Ackermann, 2006).

Classification schemes based on comparative genomics or proteomics, which are not taken into account in the ICTV classification, have been proposed. However, the

current knowledge of phage genomics and proteomics is limited to a few phage groups and it may be too early to establish a universal system based on these approaches (Nelson, 2004). The ICTV classification therefore remains relevant.

1.4.2. The phage life cycles

When considering the *Caudovirales*, the tailed double-stranded (ds) DNA phages that constitute 96% of all known phages (Fokine and Rossmann, 2014), one can distinguish two types: lytic phages, which lyse their host after infection and release new viruses, and temperate phages, which have the ability to survive within their host (lysogeny) and occasionally enter the lytic cycle (Kropinski, 2006) (Figure 1.5).

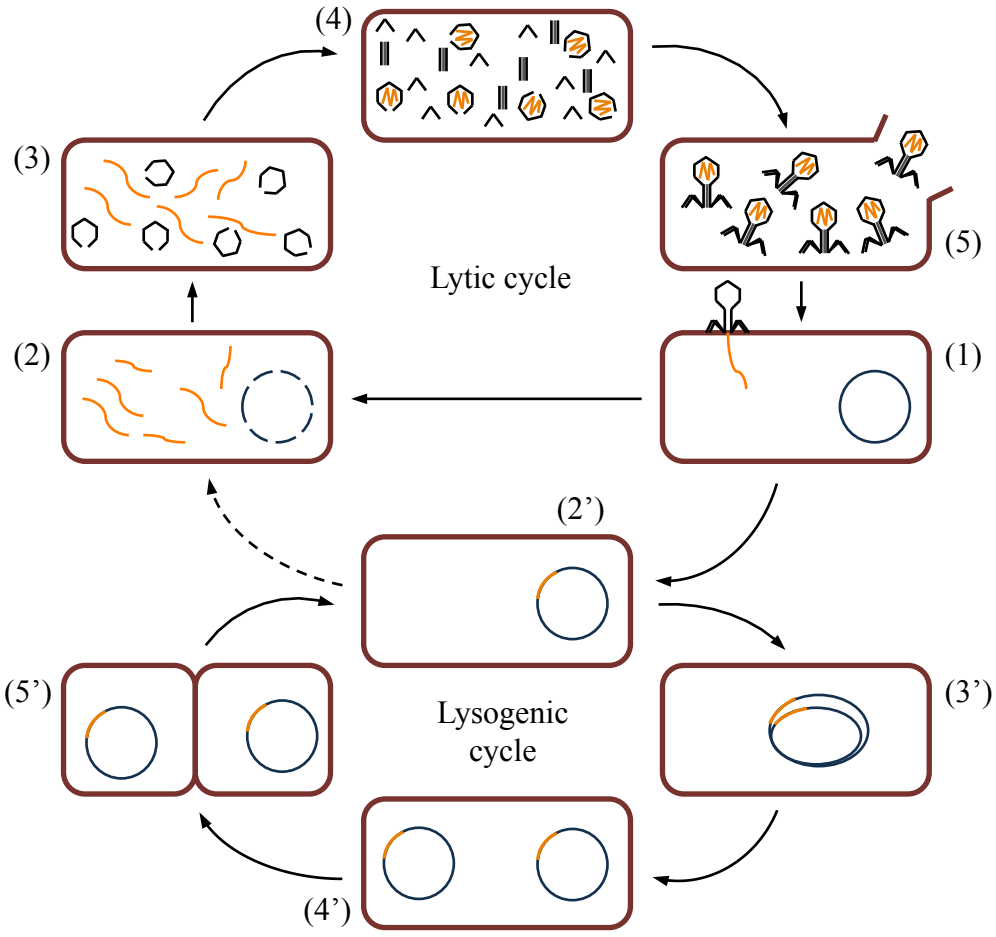


Figure 1.5: The lytic cycle involves (1) attachment to the host and DNA injection, (2) DNA replication, (3) DNA transcription, (4) virion assembly and (5) host lysis resulting in the release of new viruses. During the lysogenic cycle, (2') the phage DNA integrates into the host's chromosome and (3', 4', 5') is transmitted to daughter cells. The prophage can occasionally re-enter the lytic cycle.

Some temperate phages integrate into the host's chromosome (as depicted in Figure 1.5) while others, such as P1, are maintained as a separate episome that replicates independently from the chromosome (Sternberg and Austin, 1981).

1.4.2.a. The lytic cycle

1.4.2.a.i. Phage adsorption to the host cell surface

The first step of a phage's lytic cycle is the adsorption of a phage particle to a host cell. This occurs through interaction of the distal end of the phage tail, varying from a thin tail tip (with or without tail fibres) to a much larger baseplate (Chaturongakul and Ounjai, 2014), with cell-surface components (Lindberg, 1973). In phage λ, LamB and OmpC, both outer membrane proteins, are recognised by the tail fibres (Hendrix and Casjens, 2006). The siphophage SPP1 infecting *Bacillus subtilis* recognises YueB, a membrane anchored-protein, with its tail tip after initial interaction with cell wall teichoic acids (Baptista *et al.*, 2008). Wall teichoic acids were also shown to be adsorption receptors for *S. aureus* phages (Xia *et al.*, 2011, Uchiyama *et al.*, 2014). Once a phage finds its receptor at the surface of its host cell, the binding interaction becomes irreversible and the proteins at the tip of the tail undergo conformational changes to allow the ejection of the DNA from the capsid into the cell (Rakhuba *et al.*, 2010).

1.4.2.a.ii. DNA translocation into host cells

The mechanism of DNA translocation through the cell wall and membrane is not fully understood. In phages with contractile tails, like the myovirus T4, the internal tail tube may be pushed through the cell wall and membranes, and thus act as a DNA conduit directly into the host cell (Mosig and Eiserling, 2006). For siphophages, which tail is non-contractile, a channel may form from host proteins and/or phage proteins (Letellier *et al.*, 1999). DNA ejection relies in some phages at least partially on the pressure inside the capsid resulting from the tight packaging of genomic DNA (Inamdar *et al.*, 2006). In other phages, DNA ejection is enzyme-driven (Grayson and Molineux, 2007). The process is often facilitated by the action of tail-associated enzymes such as endopeptidase and cell wall hydrolase that target the integrity of the host cell wall (Rashel *et al.*, 2008, Rodríguez-Rubio *et al.*, 2013).

1.4.2.a.iii. DNA transcription and replication

Once it has entered the cell the linear genome of tailed dsDNA phages, is circularised through the action of the bacterial ligase on its complementary ends (see section 1.4.2.a.iv). The transcription of phage genes, a temporally regulated process, then starts. Genes can be classified into early, middle and late genes (Pero *et al.*, 1979, Kassavetis and Geiduschek, 1984, Madsen and Hammer, 1998). Transcription is usually mediated by the host RNA polymerase (RNAP) but some phages, like T7 and T3, use their own RNAP to transcribe middle and late genes (Krüger and Schroeder, 1981).

Early genes code for transcriptional regulators (Hendrix and Casjens, 2006) and proteins involved in DNA replication such as single-stranded (ss) DNA binding protein (Shokri *et al.*, 2009), helicase (Lee and Richardson, 2011), Holliday junction resolvase (Zecchi *et al.*, 2012), dUTPase (Wheeler *et al.*, 1996) and DNA polymerase (Mueser *et al.*, 2010). Some phages encode only a subset of DNA replication proteins, including an origin-specific replication initiation protein, and recruit the host DNA replication machinery (Seco *et al.*, 2013).

DNA replication happens following two modes. The first mode involves bidirectional replication of the DNA circle, also called circle-to-circle replication (Taylor and Wegrzyn, 1995) (Figure 1.6). After a few rounds, DNA replication switches to a second mode and produces DNA concatemers constituted of mature phage genomes joined together in a head-to-tail manner (Fujisawa and Morita, 1997). The concatemers arise by rolling circle, i.e. unidirectional replication of a circular genome from an origin of replication, which is used just once (Novick, 1998).

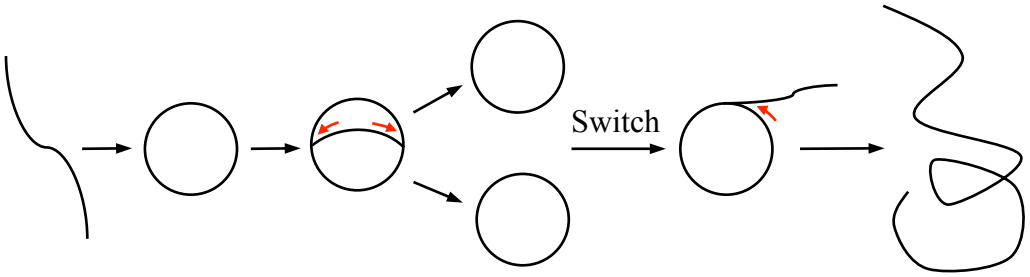


Figure 1.6: The linear phage genome is circularised following translocation into the host. The DNA circle is first replicated through bidirectional replication. After a few rounds, replication switches to unidirectional or rolling circle replication. This produces DNA concatemers constituted of mature phage genomes all joined together. Red arrows indicate the direction(s) of replication (figure based on Taylor and Wegrzyn, 1995 and modified).

The products of middle genes include regulatory proteins that control the transcription of late genes (Lobocka *et al.*, 2012). These code for virion components (capsid and tail proteins), proteins involved in DNA packaging into the phage heads and proteins necessary for host lysis (Mosig and Eiserling, 2006, Gupta, 2008).

1.4.2.a.iv. Virion assembly and DNA packaging

Virion assembly occurs as a result of ordered interactions among the structural proteins (Mosig and Eiserling, 2006, Hendrix and Casjens, 2006). Head assembly is initiated from the portal protein, or portal vertex (Bazinet and King, 1985, Olia *et al.*, 2011), and involves the polymerization of capsid proteins into an icosahedral structure around scaffolding proteins (White *et al.*, 2012, Black and Rao, 2012). These are degraded by a protease when the assembled proheads undergo maturation (Ray *et al.*, 2009, Medina *et al.*, 2010).

Individual genome units are then cut and packaged by the terminase into the proheads from the concatemers produced during DNA replication, using the hole in the portal vertex as an entrance point (Black, 1989). The large subunit of the terminase interacts with the procapsid portal and has ATPase and endonuclease activities. It therefore provides the energy necessary for DNA translocation and is responsible for generating individual genome units by cutting both strands of DNA at the ends of each genome unit (Black, 2015). The small subunit of the terminase has a DNA-binding activity and stimulates packaging (Koti *et al.*, 2008).

There are two DNA packaging strategies that lead to either genomes with cohesive ends or circularly permuted genomes. In phages with cohesive ends such as phage λ (Catalano *et al.*, 1995), the DNA concatemer is first cut by the terminase in a sequence-dependent manner at the *cos* site. DNA is then translocated into the capsid. The second cut occurs at a second specific DNA site (Oram and Lindsay, 2011). This leads to phage particles containing genomes with distinct ends at identical locations in the sequence (Figure 1.7, A and B). The ends are "cohesive" because the terminase generates protruding single strands that are complementary to each other.

In phages with circularly permuted genomes such as T4 or SPP1 (Jardine and Anderson, 2006), the terminase makes a sequence-specific cleavage at the *pac* site and the subsequent cuts (blunt cuts) are made non-specifically in the DNA substrate once the capsid is filled to capacity. These phages are therefore called "headful packaging" phages. The packaged DNA is usually slightly longer than a complete genome. It contains a full set of genes plus an extra copy of a varying subset of genes. There are two consequences associated with this:

- The ends of the genome "move" along the sequence (Casjens and Gilcrease, 2009). They are not conserved within phage particles (Figure 1.7, C and D) and that is the reason why genomes are called circularly permuted.
- A terminal repeat, consisting of a few hundred bases identical to the beginning of the genome sequence and coming from the next genome unit in the concatemer, is present at the end of each packaged genome unit.

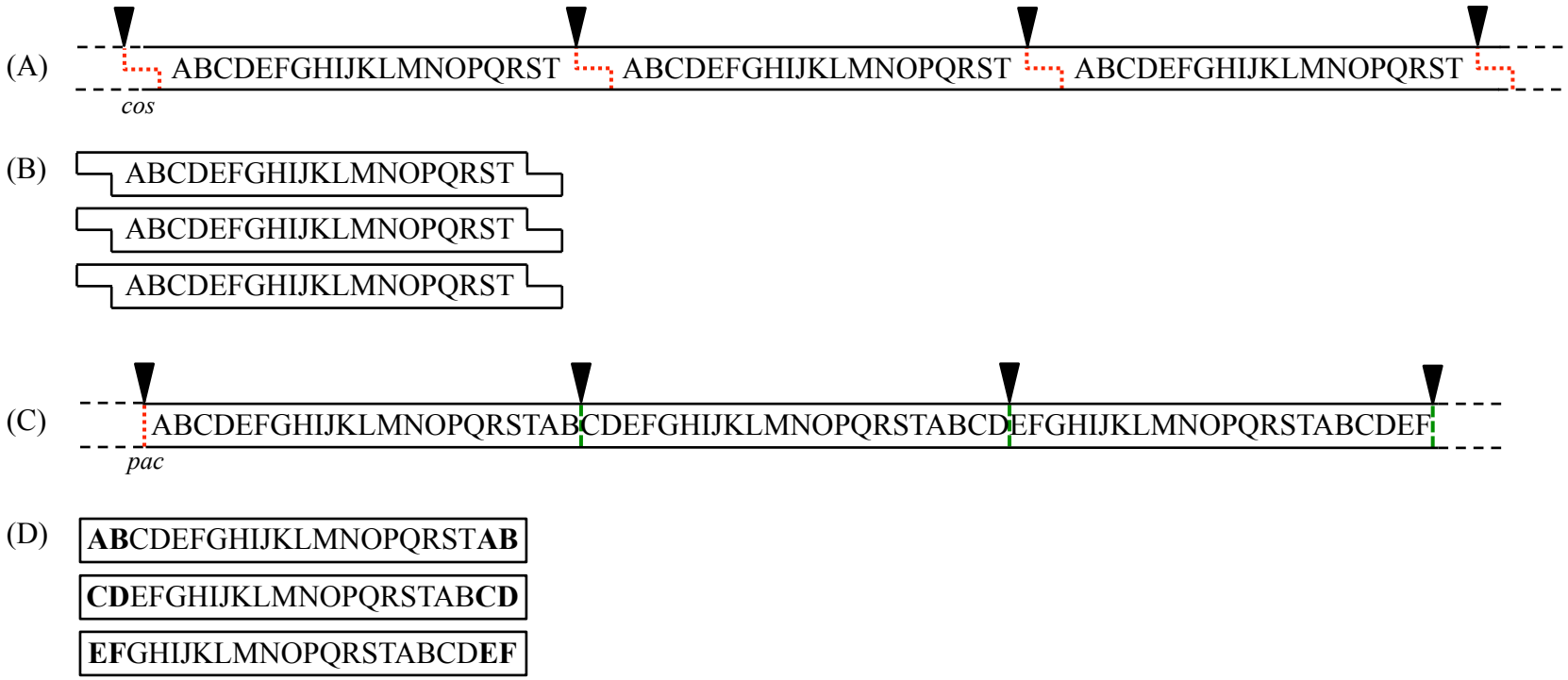


Figure 1.7: (A) In phages with cohesive ends, the terminase (black triangles) cuts DNA concatemers in a sequence-dependent manner (red dotted lines). The first cut occurs at the *cos* site and is followed by DNA translocation into the capsid. (B) The resulting DNA fragments have distinct ends at identical locations in the sequence. (C) In phages with circularly permuted genomes, the terminase first cuts at the *pac* site. Subsequent cuts are non-specific (green dotted lines) and occur once the capsid is full. The packaged DNA is usually slightly longer than a complete genome and this causes the ends to "move" along the sequence. (D) The resulting phage particles contain a full set of genes plus some duplicated genes leading to the presence of terminal repeats (in bold), and the genome ends are not identical.

Following DNA packaging, the terminase complex is replaced by neck proteins, which serve as a plug to prevent DNA leakage from the capsid (Cardarelli *et al.*, 2010). Neck proteins are also called head-tail connector proteins because they form an interface between head and tail that allow binding of the two virion components (Rishvod *et al.*, 1998, Auzat *et al.*, 2014).

The tail is assembled separately and later attached to the capsid. The process starts at the distal end of the tail, from the initiator complex that corresponds to the future tail tip or baseplate (Xu *et al.*, 2014, Maxwell and Davidson, 2014) (Figure 1.8). The tail proteins polymerize around a tape-measure protein (TMP) that determines the length of the tail (Katsura, 1990, Abuladze *et al.*, 1994, Aksyuk and Rossmann, 2011). When the tail reaches the length of the TMP, a terminator protein stops polymerization by binding at the top of the tail tube (Pell *et al.*, 2009). The terminator and the head-tail connector proteins interact to connect the head and tail. Side tail fibres, when present, are assembled at the same time (Leiman *et al.*, 2010, Aksyuk and Rossmann, 2011).

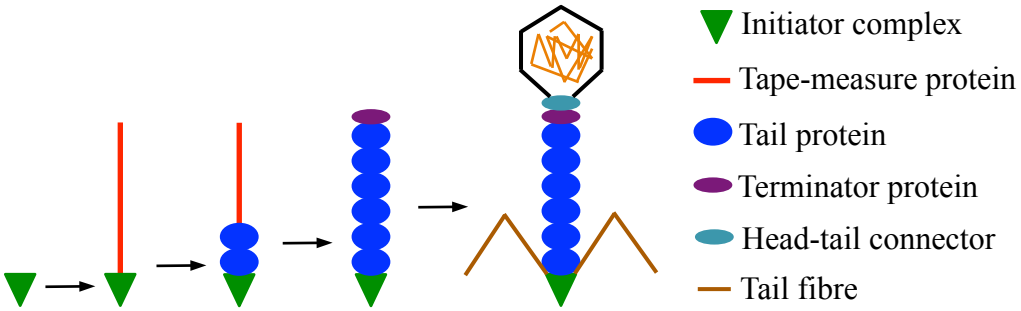


Figure 1.8: Phage assembly starts from the initiator complex. The tail proteins polymerize around a tape-measure protein (TMP). When the tail reaches the length of the TMP, a terminator protein stops polymerization by binding at the top of the tail tube. The terminator and the head-tail connector proteins interact to connect the head and tail. Side tail fibres are assembled at the same time (figure based on Pell *et al.*, 2009 and modified).

1.4.2.a.v. Host cell lysis

The last stage of a phage's lytic cycle is cell lysis that results in the release of progeny ready to infect new host cells. In dsDNA phages, cell lysis requires the holin and the endolysin (Young, 1992). Endolysins are cell-wall degrading enzymes and holins form holes in the cell membrane leading to its permeabilisation (Grundling *et*

al., 2001, Young and Bläsi, 1995). During the virion assembly period, endolysins accumulate in the cytosol while holins accumulate in the membrane (Young and Wang, 2006). Then suddenly, at a precise scheduled time, the holins form micrometer-scale lesions in the membrane, releasing the endolysins that rapidly degrade the peptidoglycan (To and Young, 2014). The holin-endolysin lysis system has been studied extensively in the coliphages λ and T4 (Wang *et al.*, 2000, Moussa *et al.*, 2014) and similar systems were identified in phages infecting other bacteria such as *S. aureus* (Mishra *et al.*, 2013).

1.4.2.b. The lysogenic cycle

1.4.2.b.i. Decision between lysis and lysogeny in phage λ

When it infects its host a temperate phage has to "choose" between entering the lytic cycle and staying inside the host as a prophage. The regulatory pathways behind the decision between lysis and lysogeny have been extensively studied in phage λ and are described below.

The lysis-lysogeny decision is made about ten to fifteen minutes after infection and involves the expression of two phage proteins: CII, a transcriptional activator, and CIII (Little, 2006). Both proteins interact with host factors in a way that is believed to allow sensing of the physiological state of the host cell and influences the lysis-lysogeny decision (Cheng *et al.*, 1988) The intracellular concentration of CII is the key determinant for lysogeny establishment (Kihara *et al.*, 1997). When CII accumulates inside the host above a threshold level it promotes lysogenization by:

- Activating the expression of the CI repressor from the CII-dependent promoter P_{RE} (Figure 1.9). This leads to very high levels of CI in the cell and repression of the expression of lytic genes (Belfort and Wulff, 1974).
- Activating the expression of the integrase from the CII-dependent promoter P_{I}, which start site is within the *xis* gene. Thus integrase and no excisionase is produced during the establishment of lysogeny (Campbell, 2006).
- Stimulating the production of an antisense transcript from a third CII-dependent promoter P_{AQ}. This antisense RNA opposes expression of the *Q* gene, which product activates the expression of late genes (Ho and Rosenberg, 1985, Wegrzyn and Wegrzyn, 2005).

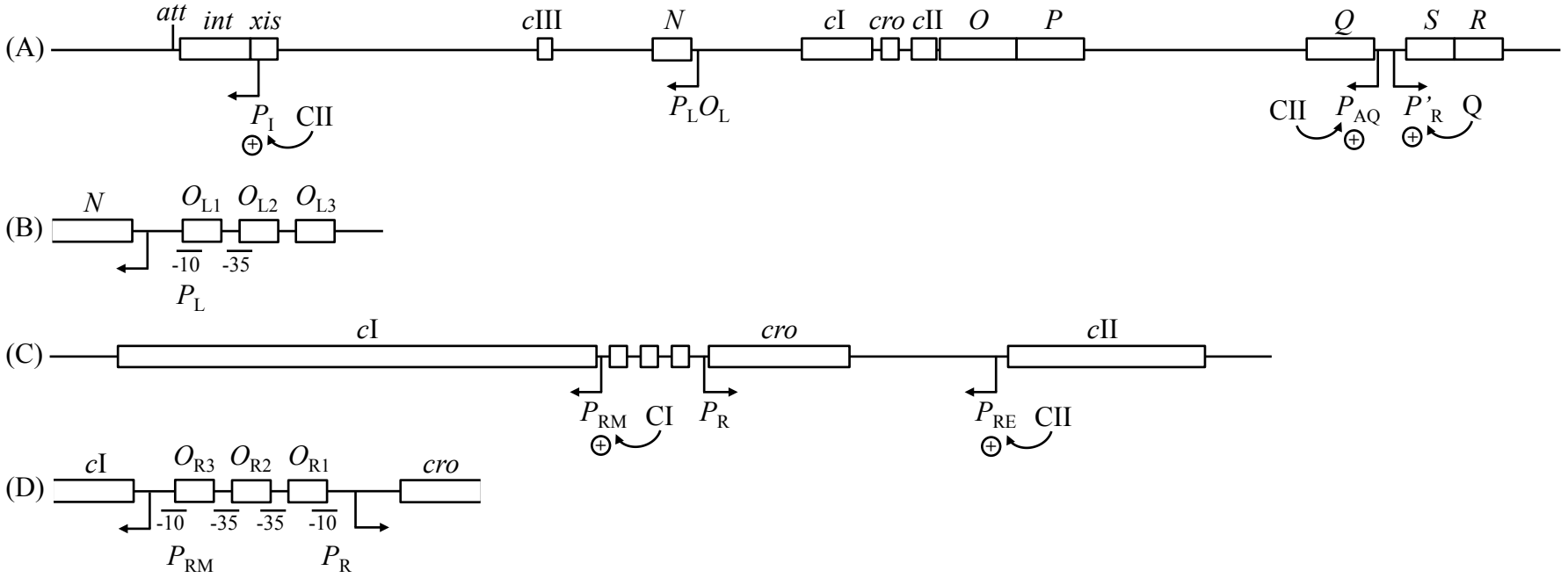


Figure 1.9: (A) Map of part of the λ genome. *att*: site of reciprocal recombination for integrating and excising phage DNA, *int*: integrase, *xis*: excisionase, P_I: CII-dependent promoter controlling the expression of *int* during lysogenization, *c*I, *c*II and *c*III: genes involved in the establishment and/or maintenance of lysogeny (see text), *N*: N controls the expression of early lytic genes, P_LO_L: promoter region controlling the expression of *N*, *cro*: gene involved in the genetic switch from lysogeny to lysis, *O* and *P*: genes involved in DNA replication, *Q*: Q activates the expression of late genes, such as *S* and *R*, from the P'_R promoter, P_{AQ}: CII-dependent promoter coding for an antisense RNA that opposes the expression of *Q*. (B) Close-up of the P_LO_L region. There are three operator sequences, O_{L1}, O_{L2} and O_{L3}, overlapping the promoter. (C) Close-up of the *c*I/*cro* region. *c*I is transcribed from the CII-dependent promoter P_{RE} during lysogenization and from the P_{RM} promoter after integration into the host's genome. *cro* is transcribed from the P_R promoter. (D) Close-up of the *c*I/*cro* intergenic region. There are three operator sequences, O_{R1}, O_{R2} and O_{R3}, overlapping both promoters (figure based on Little, 2006 and modified).

1.4.2.b.ii. Integration of the phage DNA into the host's chromosome

The phage λ DNA integrates into the host's genome through site-specific recombination between the *att*B (BOB') site on the bacterial chromosome and the *att*P (POP') site on the circularised phage genome (Hsu *et al.*, 1980) (Figure 1.10). The crossover event takes place within, or at the boundaries of, a fifteen-base pair core sequence, called 'O', that is identical on both the host and phage DNA. The arms of the *att* sites (P, P', B and B') are all different from each other (Landy and Wilma, 1977). The reaction requires a specialised phage-encoded integrase and the bacterial integration host factor (IHF) (Tal *et al.*, 2014). It generates *att*L (left prophage end) and *att*R (right prophage end) at the junctions between the integrated prophage and the bacterial chromosome (Mumm *et al.*, 2006).

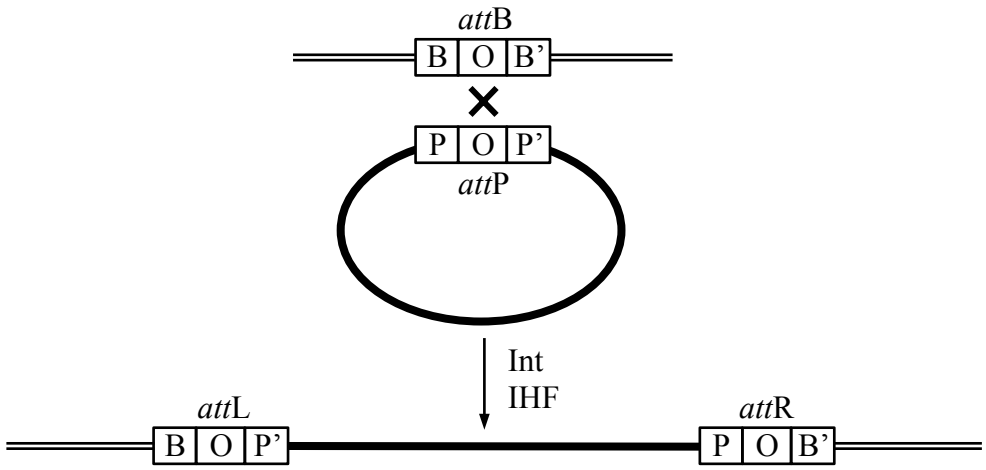


Figure 1.10: The phage λ DNA integrates into the host's genome through site-specific recombination between *att*B (BOB') on the host's chromosome (double line) and *att*P (POP') on the circularised phage genome (thick black line). The reaction is mediated by the phage integrase (Int) and the host integration factor (IHF) and generates *att*L (left prophage end) and *att*R (right prophage end) at the junctions between the integrated prophage and the bacterial chromosome.

1.4.2.b.iii. Maintenance of lysogeny

Lysogeny is a very stable state that, in phage λ, is maintained by the CI repressor. This protein prevents transcription from two promoters that are key in the lytic cycle: P_L and P_R (Figure 1.9). P_L controls the expression of the *N* gene, which product activates the expression of early lytic genes. P_R controls *Q* and *cro*, which products activate the expression of late lytic genes and promote lytic development, respectively (Oppenheim *et al.*, 2005). CI therefore maintains λ as a prophage by

preventing the transcriptional cascade that leads to cell lysis and release of progeny. Added to this, CI promotes its own expression by acting positively on the promoter P_{RM} (Friedman and Court, 2001).

The CI repressor binds to an operator region O_R situated between the *c*I and *cro* genes and overlapping both P_{RM} and P_R. O_R is composed of three binding sites O_{R1}, O_{R2}, O_{R3} (Figure 1.9, C and D) (Ptashne, 2004). When maintaining lysogeny, CI binds strongly to O_{R1} as a dimer and cooperatively to O_{R2}, forming a tetramer (Figure 1.11, A). In this situation, CI represses P_R by preventing RNAP binding through steric hindrance, but promotes transcription from P_{RM} (Bakk *et al.*, 2004). CI also binds to another operator region O_L situated upstream of the *N* gene. Similarly to O_R, O_L is composed of three binding sites O_{L1}, O_{L2} and O_{L3} overlapping P_L. CI binds cooperatively to O_{L1} and O_{L2} as a tetramer and this represses transcription from P_L (Hochschild, 2002). Another level of cooperativity exists where the CI tetramer bound at O_R interacts with the tetramer bound at O_L forming an octamer and bending the DNA (the two regions are separated by 2.4 kilobases) (Little and Michalowski, 2010) (Figure 1.11, B). This strengthens the interaction of CI with the DNA and improves the repression of P_R and P_L. It also facilitates the cooperative binding of CI dimers to O_{R3} and O_{L3} when the intracellular concentration of CI is high. This leads to repression of P_{RM} and serves as a feedback loop to maintain a stable level of CI inside the cell (Dodd *et al.*, 2005).

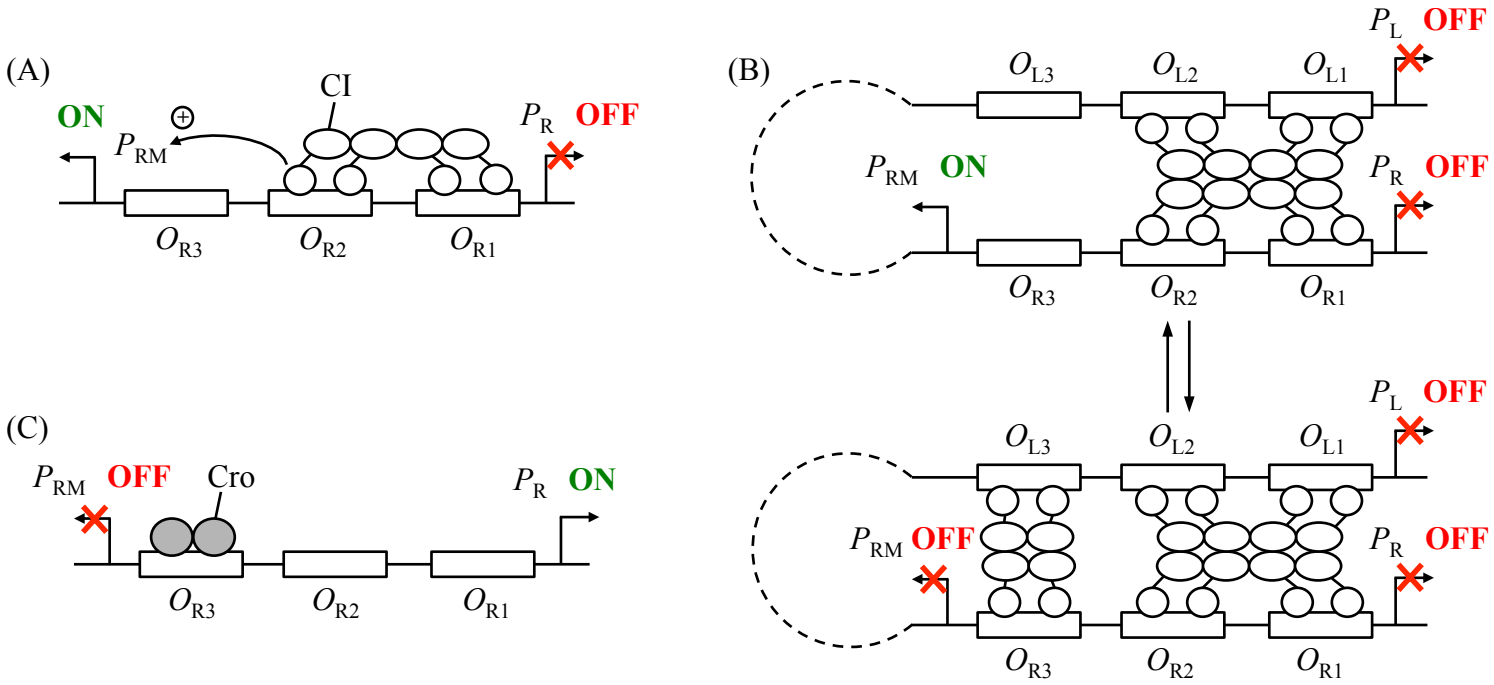


Figure 1.11: (A) At moderate levels of CI, two CI dimers bind to O_{R1} and O_{R2}, forming a tetramer. This represses P_R and activates P_{RM}. A CI monomer is constituted of two domains linked by a hinge region. (B) CI tetramers bound at O_R and O_L interact, forming an octamer. This improves the repression of P_R and P_L. At high levels of CI, CI dimers bind to O_{R3} and O_{L3}, turning P_{RM} off. (C) At moderate levels of Cro, a Cro dimer binds to O_{R3}, repressing the expression of CI from P_{RM} (figure based on Little, 2006 and modified).

1.4.2.b.iv. Switch from lysogenic state to lytic development

As mentioned in section 1.4.2, prophages occasionally switch to lytic development. This happens when the host SOS stress response, an error-prone DNA repair system (D'Ari, 1985), is induced. The two main components of this system are the proteins RecA and LexA. RecA is a protein able to recognise short stretches of ssDNA and LexA is a transcription repressor that binds upstream of the SOS genes inhibiting their expression in absence of DNA damage. When DNA damage occurs leading to accumulation of ssDNA at replication sites, RecA is activated. It then binds to the ssDNA and acquires a co-protease activity, which facilitates the self-cleavage of the LexA repressor. This results in the derepression of the SOS genes and production of proteins involved in DNA repair (Janion, 2008).

The CI repressor is a structural homolog of LexA and under SOS activation RecA catalyses the self-cleavage of CI. This leads to a drop in the intracellular concentration of CI and derepression of P_R and P_L. As a result the Cro repressor is expressed. This protein can bind to the same operator sites as CI but with opposite affinities. The strong binding of Cro to O_{R3} represses the expression of CI from P_{RM}, making the switch to lytic development irreversible (Schubert *et al.*, 2007) (Figure 1.11, C). In absence of the CI repressor, the *N* gene is expressed, starting the transcriptional cascade leading to lysis. The phage integrase and excisionase are also expressed and mediate the excision of the prophage from the host's genome (Casjens and Hendrix, 2015). The phage DNA is then ready for completion of the lytic cycle. The whole system is effectively a way for λ prophages to detect DNA damages that are likely to kill their host and lead to their own destruction if they do not escape (Galkin *et al.*, 2009).

1.4.2.b.v. Immunity associated with lysogeny

The presence of the CI repressor inside a lysogenic cell (or lysogen) means that if it is infected by another phage similar to the λ prophage, CI has the ability to bind to the operator sites of the entering phage and stop its lytic development. The presence of a prophage in a cell therefore confers immunity to its host against further infection with a similar phage (Fogg *et al.*, 2010). This is called homoimmunity (Dimmock, 2016). For this reason, the region covering *c*I, *cro*, O_R and O_L is often referred to as the λ immunity region (Wilgus *et al.*, 1973).

Heteroimmunity, also called superinfection exclusion, exists as well. It refers to the resistance of a lysogen against infection by a phage different from that carried in the bacterial genome (Ali *et al.*, 2014). While homoimmunity occurs after nucleic acid uptake, superinfection exclusion confers resistance against phage infection by means of preventing the uptake of nucleic acid, such as altering the host cell surface to prevent phage adsorption or blocking DNA translocation (Mahony *et al.*, 2008).

1.4.2.b.vi. Lysogeny in other temperate phages

Similar genes and genetic organisation are found in temperate phages other than coliphages (Neve *et al.*, 1998, Madsen and Hammer, 1998). Operator sites, *c*I and *cro* repressors genes, and an *att* site resembling those of phage λ are found in the genome of the *S. aureus* phage ϕ11 (Lee and Iandolo, 1988, Das *et al.*, 2007, Biswas *et al.*, 2014). This suggests that similar regulatory pathways and genetic switches are present in temperate phages other than λ to establish, maintain and exit lysogeny.

1.4.3. Lytic phages are required for phage therapy

Lytic phages are regarded as the only type of phages appropriate for phage therapy (Ghanna and Mohammadi, 2012). Temperate phages are expected to be less effective at clearing a bacterial infection because after infecting their host there is a chance that they will enter the lysogenic cycle and not kill the host. They are associated with lysogenic conversion, the modification of the bacterial phenotype, sometimes in ways that result in increased virulence. They may also transfer genes, e.g. antibiotic resistance genes and pathogenicity determinants, from one host to another when they become prophages (Abedon *et al.*, 2011). It is however possible to isolate mutants of temperate phages that are no longer able to establish lysogeny. Such mutants may have lost the *c*I repressor gene or other genes involved in lysogeny (integrase, excisionase, etc.). Other virulent (Vir) mutants have mutations in the CI repressor binding site leading to reduced affinity of the protein for its site, like λvir phages (Oppenheim and Salomon, 1970). Vir mutants have the ability to overcome homoimmunity (Figure 1.12).

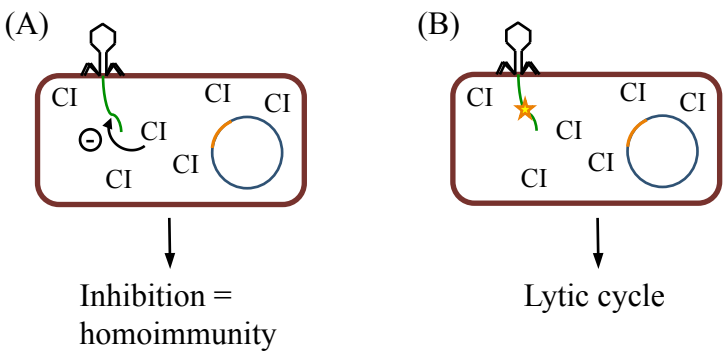


Figure 1.12: (A) When a lysogen is infected by a phage that is similar to the prophage present in its chromosome, the lytic cycle is prevented through homoimmunity. (B) A Vir mutant has one or several mutations (shown as a star) in the CI binding site and can therefore overcome homoimmunity.

1.5. Contents of this thesis

1.5.1. Aims and objectives of the work reported in this thesis

The work that was undertaken to gain knowledge about bacteriophages infecting *S. pseudintermedius* is presented in this thesis. This was done towards the general aim of this PhD project: developing phage therapy to treat canine skin infection. The work was divided into four objectives:

- The isolation and characterisation of *S. pseudintermedius* phages with the aim to select the best candidate(s) for phage therapy (Chapter 3).
- The isolation of Vir mutants of *S. pseudintermedius* phages because the ones that were found during this project were all temperate (Chapters 4 and 5).
- Bioinformatic analyses to learn about some of the characteristics of *S. pseudintermedius* phages on the protein and genome levels (Chapters 5 and 6).
- The study of the ecology of *S. pseudintermedius* and its phages to try and understand why the isolation of *S. pseudintermedius* phages proved difficult and show whether lysogeny is widespread in this pathogen (Chapter 7).

1.5.2. Specific introductions in the experimental chapters

More extensive introductions are given in the above-mentioned experimental chapters on matters specific to each chapter. Methods for isolating lytic and temperate phages are introduced in Chapter 3. Homoimmunity and the advantage of using Vir mutants are further discussed in Chapter 3 too. Methods for the

mutagenesis of phages to obtain Vir mutants are presented in Chapters 4 and 5. Details about the characteristics of operator sequences, a type of mutations leading to a Vir mutant phenotype and how to analyse the structure of proteins *in silico* are introduced in Chapter 5. DNA sequencing technologies and methods for the analysis of DNA sequencing data are presented in Chapter 6. The ecology of bacteria and their phages, and how it influences where the latter can be found are discussed in Chapter 7.

Chapter 2 Material and methods

2.1. Bacterial strains

MRSP and MSSP strains were obtained from laboratories in Europe and North America. Their characteristics are listed in Table 2.1, Table 2.2 and other publications (Descloux *et al.*, 2008, Perreten *et al.*, 2010, Ben Zakour *et al.*, 2012). Material transfer agreements were signed with the relevant institutions.

2.2. Culture and storage of bacterial strains

Bacterial strains were cultivated on Blood Agar plates (Blood Agar Base, Oxoid + 5% sheep blood). Liquid culture was performed by adding one colony into 5 mL Brain Heart Infusion (BHI) (Atlas, 1997) and incubating overnight at 37°C with shaking. In the next sections, an overnight culture refers to a liquid bacterial culture prepared as described above with an Optical Density at 600 nm (OD_{600nm}) = 0.3 following a 10^{-1} dilution. Strains were stored on Blood Agar plates in the fridge for short-term storage and at -80°C in cryotubes (1 mL overnight culture + glycerol at a final concentration of 16%) for long-term storage.

2.3. Bacterial growth curve

100 µL overnight culture was added to 50 mL BHI and cultivated at 37°C with shaking. 1 mL samples were taken at regular time intervals and OD_{600nm} was measured. Appropriate dilutions of each time point were plated to determine the number of Colony Forming Units per mL (CFU/mL) and the correspondence with OD_{600nm}. Bacterial growth curves and the determination of growth phases are presented in section 4.3.2.a. In the next sections, a bacterial culture at mid-exponential phase refers to a culture which OD_{600nm} = 0.3 after around three hours of cultivation.

Table 2.1: Characteristics of the *S. pseudintermedius* strains used in the project for the induction of prophages through mitomycin C exposure. Some strains are referred to with an alternative name in publications (shown in brackets). SCC*mec* = staphylococcal cassette chromosome *mec* element, Spa type = staphylococcal protein A type, n/t = not tested.

Name (Alternative name)	Methicillin resistance	Sequence Type	Spa type	SCC*mec* type	Source	Animal	Country of origin	Owner
E086 (V0703277)	MRSP	ST71	t02	II-III	synovia	dog	The Netherlands	J. Wagenaar
E123 (AS4)	MRSP	ST71	t02	II-III	skin	dog	USA	S. Weese
E025 (06-1065)	MRSP	ST68	t06	V	skin	dog	USA	D. Bemis
E122 (A42)	MRSP	ST68	t06	V	skin	dog	USA	S. Weese
E125 (129Ab)	MRSP	ST58	t06	VII	healthy animal	dog	USA	S. Weese
E069 (KM241)	MRSP	ST73	t24	VII	otitis externa	dog	Switzerland	V. Perreten
E126 (182Ab)	MRSP	ST113	t06	IV	nose	dog	Canada	S. Weese
E139 (DK639)	MRSP	ST258	t02	II-III	otitis externa	dog	Denmark	A. Moodley
AB178	MRSP	n/t	n/t	IV	wound	dog	Sweden	S. Borjesson
AB190	MRSP	n/t	t02	II-III	udder	horse	Sweden	S. Borjesson
AB252	MRSP	n/t	t06	II-III	wound	dog	Sweden	S. Borjesson
AB255	MRSP	n/t	n/t	n/t	wound	dog	Sweden	S. Borjesson
AB312	MRSP	n/t	t02	II-III	wound	dog	Sweden	S. Borjesson
AB316	MRSP	n/t	t35	n/t	ear	dog	Sweden	S. Borjesson
AB680	MRSP	n/t	t29	II-III	unknown	dog	Sweden	S. Borjesson
08BKT31634	MRSP	n/t	t10	II-III	post-operative wound	dog	Sweden	S. Borjesson
S56C3	MSSP	n/t	n/t	n/t	ear	dog	Denmark	A. Moodley

Name (Alternative name)	Methicillin resistance	Sequence Type	Spa type	SCC*mec* type	Source	Animal	Country of origin	Owner
S56D2	MSSP	n/t	n/t	n/t	ear	dog	Denmark	A. Moodley
S56H7	MSSP	n/t	n/t	n/t	ear	dog	Denmark	A. Moodley
S56F3	MSSP	n/t	n/t	n/t	ear	dog	Denmark	A. Moodley
S57E7	MSSP	n/t	n/t	n/t	ear	dog	Denmark	A. Moodley
S60C4	MSSP	n/t	n/t	n/t	ear	dog	Denmark	A. Moodley
S60C6	MSSP	n/t	n/t	n/t	ear	dog	Denmark	A. Moodley
S60D6	MSSP	n/t	n/t	n/t	ear	dog	Denmark	A. Moodley
S60D7	MSSP	n/t	n/t	n/t	ear	dog	Denmark	A. Moodley
S61A3	MSSP	n/t	n/t	n/t	wound	dog	Denmark	A. Moodley
S61A8	MSSP	n/t	n/t	n/t	wound	dog	Denmark	A. Moodley
S61B7	MSSP	n/t	n/t	n/t	vulva	dog	Denmark	A. Moodley
S61D1	MSSP	n/t	n/t	n/t	eye	dog	Denmark	A. Moodley
S61H5	MSSP	n/t	n/t	n/t	wound	dog	Denmark	A. Moodley
S61I9	MSSP	n/t	n/t	n/t	ear	dog	Denmark	A. Moodley
S62A2	MSSP	n/t	n/t	n/t	ear	dog	Denmark	A. Moodley
S63G7	MSSP	n/t	n/t	n/t	wound	dog	Denmark	A. Moodley
S76I4	MSSP	ST273	n/t	IV	skin	dog	Denmark	A. Moodley
JZ22	MSSP	n/t	n/t	n/t	dermatitis	dog	Sweden	S. Borjesson
JZ31	MSSP	n/t	t65	n/t	dermatitis	dog	Sweden	S. Borjesson
JZ56	MSSP	n/t	t30	n/t	dermatitis	dog	Sweden	S. Borjesson
JZ133	MSSP	n/t	n/t	n/t	dermatitis	dog	Sweden	S. Borjesson

Name (Alternative name)	Methicillin resistance	Sequence Type	Spa type	SCC*mec* type	Source	Animal	Country of origin	Owner
JZ146	MSSP	n/t	t63	n/t	dermatitis	dog	Sweden	S. Borjesson
JZ151	MSSP	n/t	t64	n/t	dermatitis	dog	Sweden	S. Borjesson
JZ152	MSSP	n/t	t01	n/t	dermatitis	dog	Sweden	S. Borjesson
JZ170	MSSP	n/t	t15	n/t	dermatitis	dog	Sweden	S. Borjesson
JZ208	MSSP	n/t	t05	n/t	dermatitis	dog	Sweden	S. Borjesson
JZ220	MSSP	n/t	t50	n/t	dermatitis	dog	Sweden	S. Borjesson
AB561	MSSP	n/t	n/t	n/t	dog bite	human	Sweden	S. Borjesson
AB564	MSSP	n/t	n/t	n/t	dog bite	human	Sweden	S. Borjesson

Table 2.2: Characteristics of the remaining *S. pseudintermedius* strains and other bacterial strains used in the project. Some strains are referred to with an alternative name in publications (shown in brackets). SCC*mec* = staphylococcal cassette chromosome *mec* element, Spa type = staphylococcal protein A type, n/t = not tested.

Name (Alternative name)	Methicillin resistance	Sequence Type	Spa type	SCC*mec* type	Source	Animal	Country of origin	Owner
E029 (6940)	MRSP	ST71	t02	II-III	skin	dog	Italy	A. Battisti
E045 (19698)	MRSP	ST71	t02	II-III	wound	dog	Sweden	U. Andersson
E046 (AB34)	MRSP	ST71	t02	II-III	surgical wound	dog	Sweden	U. Andersson
E047 (KM061849)	MRSP	ST71	t02	II-III	rhinitis	cat	Switzerland	V. Perreten
E052 (VPL07229)	MRSP	ST71	t02	II-III	otitis externa	dog	Switzerland	V. Perreten
E061 (KM08465)	MRSP	ST71	t02	II-III	inflammation	dog	Switzerland	V. Perreten

Name (Alternative name)	Methicillin resistance	Sequence Type	Spa type	SCC*mec* type	Source	Animal	Country of origin	Owner
E064 (KM1381)	MRSP	ST71	t02	II-III	fistula	dog	Switzerland	V. Perreten
E075 (IMD071045)	MRSP	ST71	t02	II-III	fistula	dog	Switzerland	V. Perreten
E133 (Sp73)	MRSP	ST71	t06	II-III	urine	dog	Canada	S. Weese
E134 (Sp74)	MRSP	ST71	t06	II-III	otitis externa	dog	USA	S. Weese
E140 (DK729)	MRSP	ST71	t02	V	bite wound	dog	Denmark	A. Moodley
E017 (06-255)	MRSP	ST68	t06	V	pyoderma	dog	USA	D. Bemis
E018 (06-1164)	MRSP	ST68	t06	V	skin	dog	USA	D. Bemis
E019 (06-1400)	MRSP	ST68	t06	V	pyoderma	dog	USA	D. Bemis
E020 (06-2584)	MRSP	ST68	t06	V	pyoderma	dog	USA	D. Bemis
E022 (06-632)	MRSP	ST68	t06	V	skin	dog	USA	D. Bemis
E023 (06-815)	MRSP	ST68	t06	V	pyoderma	dog	USA	D. Bemis
E026 (06-1460)	MRSP	ST68	t06	V	pyoderma	dog	USA	D. Bemis
E135 (Sp75)	MRSP	ST68	t06	V	urinary tract infection	dog	USA	S. Weese
E136 (Sp80)	MRSP	ST68	t06	V	surgical wound	dog	USA	S. Weese
HK2	MSSP	n/t	n/t	n/t	ear	dog	China	A. Moodley
HK14	MSSP	n/t	n/t	n/t	ear	dog	China	A. Moodley
Y1	MSSP	n/t	n/t	n/t	ear	dog	China	A. Moodley
S66E5	MSSP	ST269	n/t	IV	skin	dog	Denmark	A. Moodley
S76G8	MSSP	ST45	n/t	n/t	ear	dog	Denmark	A. Moodley
ED99	MSSP	n/t	n/t	n/t	pyoderma	dog	UK	J. Fitzgerald
S. delphini	n/t	n/t	n/t	n/t	unknown	unknown	Denmark	A. Moodley

Name (Alternative name)	Methicillin resistance	Sequence Type	Spa type	SCC*mec* type	Source	Animal	Country of origin	Owner
S. intermedius	n/t	n/t	n/t	n/t	unknown	unknown	Denmark	A. Moodley
MRSA2	MRSA	n/t	n/t	n/t	unknown	unknown	UK	G. Amos
MRSA6	MRSA	n/t	n/t	n/t	unknown	unknown	UK	G. Amos
MRSA8	MRSA	n/t	n/t	n/t	unknown	unknown	UK	G. Amos

2.4. Plate assay

Phages were isolated and amplified using plate assay and plate washes as previously described (Sambrook *et al.*, 1989b). Tryptic Soya Broth (TSB) (Atlas, 1997) molten agar (TSB broth + 0.4% agarose + 5 mM $CaCl_2$), TSB plates (TSB + 0.8% agarose + 5 mM $CaCl_2$), Ringers + Mg solution (0.15 M NaCl, 4.0 mM KCl, 4.0 mM $CaCl_2$ and 1 mM $MgSO_4$) and an overnight bacterial culture was used. To ensure the clonality of newly isolated phages, a single plaque was picked with a Pasteur pipette and re-plated twice as previously described (Sambrook *et al.*, 1989b). In the next sections, a top agar seeded with bacteria refers to 5 mL molten agar, prepared as described above, mixed with 100 µL overnight culture.

2.5. Isolation of phages from faeces and soil

5 grams of faecal material or soil were homogenised with 30 mL of buffer (0.25 M NaCl + 5 mM $CaCl_2$) (Nälgård, 2011) and incubated overnight at 37°C to allow diffusion of phages. Tubes were centrifuged twice 15 min at 2,000 rpm, filtered (0.45 µm filters) or unfiltered supernatants were spotted directly onto top agars seeded with bacteria. Enrichment was also attempted by mixing 5 mL of chloroform treated-supernatant + 100 µL overnight bacterial culture (strains E018, E133, E139 or E140 chosen according to their diverse Sequence Types, see Table 2.1 and Table 2.2) + 5 mM $CaCl_2$ + 5 mL BHI, incubating overnight at 37°C with shaking and spotting filtered supernatants onto top agar seeded with bacteria to see plaques. All environmental samples were handled in the containment suite level 2 for safety reasons.

2.6. Isolation of phages from water samples

Water samples were filtered (0.20 µm filters), 10 mL of each filtered sample were mixed with 10 mL BHI + 100 µL overnight bacterial culture (strains E018, E029, E139, AB178, JZ22 or ED99, chosen according to their diverse Sequence Types and methicillin resistance status, see Table 2.1 and Table 2.2) + 5mM $CaCl_2$ and incubated overnight at 37°C with shaking to enrich the sample. Tubes were centrifuged 10 min at 4,000 rpm, and filtered supernatants were spotted onto top agar seeded with bacteria to see plaques.

2.7. Isolation of phages from skin swabs

Swabs and their transport gels were soaked in 1 mL 0.5 M NaCl + 0.1 M $CaCl_2$ (Prof. Finn Vogensen, personal communication) for 2h for phages to diffuse. Supernatants were taken without disturbing the settled swab or gel and spotted onto top agar seeded with bacteria (strains E018, E069, E133, E139, S66E5 or S76G8, chosen according to their diverse Sequence Types and methicillin resistance status, see Table 2.1 and Table 2.2) to see plaques.

2.8. Isolation of temperate phages through co-culture

Bacterial strains were cultivated in BHI until they were at mid-exponential phase. 1 mL of the cultures were mixed together and incubated overnight at 37°C with shaking (Nälgård, 2011). Cells were centrifuged 10 min at 4,000 rpm and 10 µL of supernatant were spotted onto top agar seeded with bacteria to investigate the presence of phages. Alternatively, 100 µL of overnight cultures of cultivating strain and donor strain were added to 5 mL BHI. Cells were centrifuged after overnight incubation at 37°C with shaking. 500 µL of supernatant and 100 µL of cultivating strain were mixed and the presence of phages was investigated through plate assay.

2.9. Induction of temperate phages with mitomycin C

100 µL overnight culture of a potential lysogen was added to 5 mL of BHI and incubated 1h at 37°C with shaking (until $OD_{600nm} = 0.1$). 0.5 µg/mL mitomycin C (Mitomycin C from *Streptomyces caespitosus*, Sigma) was added to the culture and incubated for 3h at 37°C with shaking. The culture was centrifuged 10 min at 4,000 rpm, the supernatant was filtered with a 0.2 µm syringe filter and spotted onto top agar seeded with bacteria to see plaques.

2.10. Titration of phage lysates

Phage lysates were diluted and plate assay was performed with appropriate dilutions. Plaques were counted the next day to determine the titre in Plaque Forming Units per mL (PFU/mL).

2.11. Host range screening

Phage lysates were diluted and 10 µL of 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions were spotted on top agar seeded with bacteria. Results were observed after incubation overnight at 37°C. Plaques were counted at two or three dilutions. If results were not consistent at different dilutions, the experiment was repeated.

2.12. Genomic DNA extraction from phages

0.1 µg/mL proteinase K (Promega), 2.5 mM EDTA and 0.5% SDS (w/v) were added to 1 mL of high titre phage lysate (10^{9}-10^{10} PFU/mL) and incubated 1h at 56°C to digest the capsid. 1 vol. phenol-chloroform-IAA (Phenol:Chloroform:Isoamylalcohol, 25:24:1, pH 8.0, Sigma) was added and mixed well. After centrifugation at 12,000 rpm for 10 min, the upper layer was transferred to a new tube. This step was repeated twice. 1 vol. chloroform was added, mixed well and centrifuged at 12,000 rpm for 5 min. The upper layer was transferred to a new tube, 0.1 vol. 3 M sodium acetate and 1 vol. 100% ethanol were added and incubated overnight at -20°C. After centrifugation at 12,000 rpm for 5 min, the supernatant was discarded and the pellet was washed with 70% ethanol before being dried at 60°C for 2h and dissolved in TE Buffer + 0.05 mg/mL RNase (Amresco).

2.13. DNA concentration measurement

DNA concentration was measured with a NanoDrop spectrophotometer (Fisher Scientific). If a very accurate reading was required, DNA concentration was measured with the Qubit® dsDNA BR assay kit and a Qubit® 2.0 fluorometer following the manufacturer's instructions (Life Technologies).

2.14. Phage genomic DNA digestion

Phage genomic DNA was digested with *Eco*RI (Thermo Scientific) and *Sau*3AI (Fisher BioReagents) for 1h at 37°C following the manufacturer's instructions. 2 mM spermidine was added to facilitate digestion.

2.15. DNA gel electrophoresis

Gel electrophoresis was performed with 0.8 to 1% agarose gels containing 0.004% (v/v) ethidium bromide and 1x Tris-Acetate-EDTA (TAE) buffer. Gels were run 1h at 120 V. Gel pictures were taken with a Gene Flash transilluminator (Syngene Bio

Imaging). The GeneRuler 1kb DNA Ladder (Thermo Fisher Scientific), the 1kb DNA Ladder (Promega) and the 100bp DNA Ladder (Promega) were used as molecular weight markers. All sizes in the next chapters are shown in base pairs.

2.16. Pulsed-Field Gel Electrophoresis (PFGE)

50 µL of high titre phage lysate (10^9-10^{10} PFU/mL) were mixed with 50 µL of 2% Low Melting Point (LMP) agarose (Promega) in plug moulds. Plugs were left to set in the fridge for 5 min before being incubated overnight at 56°C with 1 mL lysis buffer (0.1 M EDTA, 0.1 M Tris-HCl pH 8.0, 1% SDS and 0.5 mg/mL proteinase K) to digest the phage capsid. Plugs were washed with 1 mL 0.5x Tris-Borate-EDTA (TBE) buffer for 20 min and slid into the wells of a 1% PFGE agarose gel. The gel was run for 20h (default settings on Chef Mapper machine, Biorad) and stained with 0.002% (v/v) ethidium bromide after migration was complete. The Molecular Weight Marker for DNA 0.1-200 kb from λ phage (Sigma) was used and sizes are shown in kilobase pairs in the next chapters.

2.17. Whole-genome sequencing and assembly

Phage genomes were sequenced through a single-plaque sequencing method as previously described (Kot *et al.*, 2014). Genome assembly was performed with the software CLC *de novo* Assembly Cell 4.0 (CLC Bio).

2.18. Whole-genome annotation and alignment

Phage genomes were annotated with the software Prokka, that uses the SwissProt protein database as a reference (Seemann, 2014). Genomes were aligned with the programme Mauve (Darling *et al.*, 2010) using progressiveMauve algorithm and its default settings. Genomes were also aligned with the programme Easyfig (Sullivan *et al.*, 2011) using the default settings and showing coding DNA sequences (CDSs) instead of genes. Colours codes for CDSs of known function were added to the CDS features in the GenBank files before performing alignment, as described in the Easyfig 2.1 user manual.

2.19. Electron microscopy of phage particles

10 µL of high titre lysate (10^9-10^{10} PFU/mL) were placed onto a formvar-carbon grid for 1 min. The surface liquid was blotted with a paper towel and the sample was

fixed and stained by adding 10 µL of 2% uranyl-acetate. The grid was blotted again and loaded onto a JEM-2010 electron microscope (JEOL). Samples were observed at 40,000x and 50,000x magnification.

2.20. Measuring phages on electron microscopy pictures

Electron microscopy pictures were printed out on paper and the size of the capsid, tail and baseplate of each phage were measured in centimeters (Figure 2.1). The sizes were converted into nanometers by using the scale on each picture. Measurements were taken on six different phage particles in total, originating from two lysates that were produced independently.

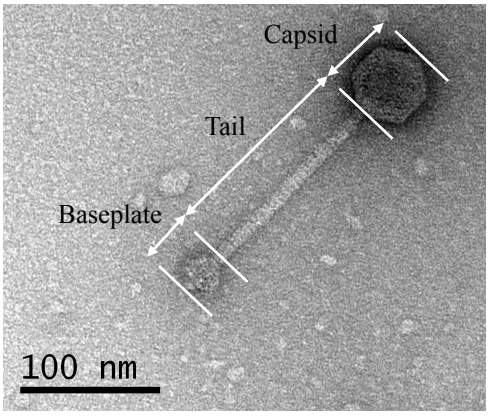


Figure 2.1: The size of the capsid, tail and baseplate of each phage were first measured in centimeters and then converted into nanometers by using the scale on each picture.

2.21. Phage growth curve

Bacterial cells from an overnight culture and phage particles were mixed together at a chosen MOI (Multiplicity Of Infection – relation between number of phages : number of bacterial cells) in 5 mL BHI and incubated at 37°C with shaking. To follow phage growth, 100 µL samples were taken at regular time intervals, centrifuged, filtered, and plate assay was performed with the supernatants to determine the titre in PFU/mL.

2.22. Phage propagation in liquid culture

Bacterial strains were cultivated in 250 mL BHI (in 1L flasks) at 37°C with shaking until mid-exponential phase. The culture was then split into up to ten times 25 mL (in 125 mL flasks), 5 mM $CaCl_2$ were added to each culture and phages were added at

an MOI of 1:1000. Cultures were incubated overnight at 37°C with shaking, the next day they were centrifuged and filtered to recover phage particles.

2.23. Phage concentration on Amicon filters

Following phage propagation, 15 mL of phage lysate were added to a 50-mL Amicon Ultra Centrifugal Filters Units (Millipore) and centrifuged at 2,200 rpm until the volume reached 500 µL or less. The concentrated lysate was pipetted into a 1.5 mL tube. The filter was washed with 1 mL Ringers + Mg that was added to the concentrated lysate.

2.24. Testing the pre-attachment phase

Bacterial cells at mid-exponential or stationary phase and phage particles were mixed together at an MOI of 1:1 in 1 mL BHI + 5mM $CaCl_2$. Samples were incubated for 0, 5, 10, 15, 20, 25 and 30 min at room temperature (RT). Tubes were centrifuged for 1 min at 5,000 rpm to separate free phages from cells. Appropriate dilutions of the supernatants were plated out to determine the titre in PFU/mL.

2.25. Phage mutagenesis through exposure to hydroxylamine

200 µL of high titre phage lysate (10^8 to 10^{12} PFU/mL) were exposed to hydroxylamine as previously described (Davis *et al.*, 1980) and incubated at 37°C until reaching 99.9% killing. The mutagenized phages were dialysed overnight at 4°C against 1L Ringers + Mg. After dialysis, the phages were appropriately diluted with BHI to reach an MOI of 1:1 when added to a bacterial culture at mid-exponential phase. 5 mM $CaCl_2$ were added to the culture and it was incubated overnight at 37°C with shaking. The next day the culture was centrifuged, the supernatant was filtered, concentrated on an Amicon filter and plated with the phage lysogen (300 µL of concentrated mutagenized phages on one plate). The protocol optimisation process is described in section 4.3.

2.26. Phage mutagenesis through exposure to ultraviolet (UV) light

500 µL of high titre phage lysate (10^9 to 10^{12} PFU/mL) were 10-fold diluted with Ringers + Mg, transferred to a small Petri dish and exposed to UV light (253.7 nm, 0.707 mW/cm^2) for 20s. UV-irradiated phages were mixed with bacterial cells at mid-exponential phase at an MOI of 1:1. For this, an appropriate volume of bacterial

culture at mid-exponential phase was centrifuged down and re-suspended in 3 mL BHI. The UV-irradiated phages were allowed to pre-attach to bacterial cells for 15 min at RT in the small volume of liquid thus obtained (5 mL phages + 3 mL bacteria). BHI was then added up to 25 mL and the culture was incubated overnight at 37°C with shaking. The next day the culture was centrifuged, the supernatant was filtered, concentrated on an Amicon filter and plated with the lysogen. All manipulations and overnight incubation with UV-irradiated phages were performed in the dark with a safe red light to avoid photoreactivation. The protocol optimisation is described in section 4.4.

2.27. Phage evolution through serial passaging on a permissive host

Phages were added to 10 mL of bacterial culture at mid-exponential phase at a range of MOIs (1:50, 1:100, 1:500 and 1:1000) and incubated overnight at 37°C with shaking. The next day, cultures were centrifuged, supernatants were filtered and titres were checked through plate assay. The process was repeated six times by using the lysate from the previous step with the highest titre. All the filtered lysates were kept at +4°C until the end of the experiment. They were then concentrated on Amicon filters and plated with the phage lysogen (see also section 4.5).

2.28. Production of cell lysate for Electrophoretic Mobility Shift Assay (EMSA)

400 µL of overnight bacterial culture were added to 5 mL BHI and incubated for 4h at 37°C with shaking. The culture was then centrifuged, re-suspended in 500 µL ice-cold washing buffer (50 mM Tris HCl, 5 mM EDTA, pH 8.0), centrifuged again and re-suspended in 700 µL ice-cold lysis buffer (50 mM Tris HCl, 0.15 M NaCl, 4.0 mM KCl, 4.0 mM $CaCl_2$ and 1 mM $MgSO_4$, 5 mM EDTA, 10% glycerol, 200 µg/mL phenylmethylsulfonyl fluoride, pH 8.0). The mixture was transferred to a ribolysing tube containing glass beads and was ribolysed at 6,000 rpm for 2 x 40s. The ribolysed cells were centrifuged for 10 min at 10,000 rpm and the supernatant was frozen at -80°C until further use. Protein concentration was measured using the Pierce™ BCA Protein Assay kit (Thermo Scientific).

2.29. Production of dsDNA probes for EMSA through PCR

The PCR mixture consisted of 10 pmol of ssDNA (ordered from Sigma) in a 25 µL final volume composed of 1x Q5® buffer (New England Biolabs), 0.2 mM dNTPs

(New England Biolabs), 0.5 µM forward and reverse primers (Table 2.3), 0.5 unit of Q5® DNA polymerase (New England Biolabs) and nuclease-free water. Reactions mixtures were thermally cycled according to Table 2.4. The length of the dsDNA probes (100 bp) was checked by gel electrophoresis before pooling five PCR reactions together and purifying the product using the QIAquick® PCR Purification kit (Qiagen).

2.30. Radiolabelling of dsDNA probes

The radiolabelling mixture consisted of 20 pmol of dsDNA probe in a 50 µL final volume composed of 1x T4 Kinase buffer (New England Biolabs), 20 units of T4 Polynucleotide Kinase (New England Biolabs), 1 nmol [γ-^{32}P]ATP (Perkin Elmer, 3,000 Ci/mmol, 10 mCi/mL) and nuclease-free water. The mixture was incubated for 30 min at 37°C then 20 min at 65°C to inactivate the kinase. The radiolabelled probe was purified using the QIAquick® PCR Purification kit (Qiagen) and stored at +4°C until further use. DNA concentration was measured in ng/µL using a UVette® and a Biophotometer (Eppendorf).

2.31. Protein binding to dsDNA probes and EMSA

The protein binding mixture consisted of 5 ng of radiolabelled DNA in a 40 µL final volume composed of 1x binding buffer (10x binding buffer: 1.5 M NaCl, 40 mM KCl, 40 mM $CaCl_2$ and 10 mM $MgSO_4$, 50 mM EDTA), 1 µg poly(dIdC), 20 µg proteins from cell lysate (section 2.28) and nuclease-free water. The mixture was incubated for 30 min at 37°C. 10 µL 4x non-denaturing loading buffer (0.2 M Tris HCl, pH 6.8, 40% glycerol, 0.04% bromophenol blue) were added and the binding reaction was loaded onto a native 4% polyacrylamide gel that was run for 2h at 250 V. After migration, the gel was dried and an X-ray film was exposed to the gel for 3h to see the result.

2.32. Cloning and expressing the phage *c*I repressor gene into *E. coli*

The SpT5 *c*I gene was amplified through PCR in a mixture consisting of 1 µL SpT5 genomic DNA in a 25 µL final volume composed of 1x Q5® buffer (New England Biolabs), 0.2 mM dNTPs (New England Biolabs), 0.5 µM forward primer and reverse primer (Table 2.3), 0.5 unit of Q5® DNA polymerase (New England Biolabs) and nuclease-free water. Reaction mixtures were thermally cycled according to Table

2.4. The PCR product was purified using the QIAquick® PCR Purification kit (Qiagen).

The purified product was A-tailed by mixing 30 µL of purified PCR product with 1x Taq Buffer A (KAPA Biosystems), 0.25 mM dATP (Invitrogen), 5 units of KAPA Taq polymerase (KAPA Biosystems) and nuclease-free water in a 40 µL final volume, and incubating at 95°C for 5 min, then 72°C for 20 min.

The A-tailed product was gel purified and subcloned into the pGEM-T Easy Vector and *E. coli* JM109 cells following the manufacturer's instructions (Promega). Positive (white) colonies were selected and plasmid miniprep was performed.

Both pGEM-T + insert and pET28a (Novagen) plasmids were digested with HindIII and NdeI using the MutiCORE buffer and following the manufacturer's instructions (Promega). The excised insert and the digested pET28a plasmid were gel purified and subcloned into *E. coli* JM109 cells following the manufacturer's instructions (Promega). Kanamycin-resistant colonies were selected and plasmid miniprep was performed.

BL21(DE3)pLysS Competent Cells were transformed with the purified pET28a plasmid + insert or the empty pET28a vector following the manufacturer's instructions (Promega). Kanamycin-resistant colonies were selected and cultivated overnight in LB (Luria Broth) + 25 µg/mL kanamycin. The next day, cultures were set up in triplicate: 3x pET28a + insert + isopropyl β-D-1-thiogalactopyranoside (IPTG), 3x pET28a + insert and 3x empty pET28a + IPTG, and for this 1 mL overnight culture was added to 50 mL LB + 50 µg/mL chloramphenicol + 25 µg/mL kanamycin and cultures were incubated at 37°C with shaking. When OD_{600nm} reached 0.6, 0.25 mM IPTG was added to the relevant cultures to induce gene expression. Cultures were incubated at 25°C overnight. The next day, cultures were centrifuged 5 min at 4,000 rpm and pellets were used to produce cell lysate as described in section 2.28 (see also Figure 5.9). Miniprep was performed with the GeneJET Plasmid Miniprep kit (Thermo Scientific). After each miniprep, plasmid sequences were checked through Sanger sequencing using the M13 or T7 primers for pGEM-T and pET28a respectively (Table 2.3). DNA gel purification was performed with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel).

2.33. Polyacrylamide gel electrophoresis (PAGE)

PAGE was performed with Mini-PROTEAN® Tris/Tricine Precast Gels (New England Biolabs) and 1x RunBlue Native Run Buffer (Expedeon). Gels were run for 45 min at 160 V and stained with the InstantBlue reagent (Expedeon) following the manufacturer's instructions. The Color Prestained Protein Standard, Broad Range (11 – 245 kDa) (NEB) was used as molecular weight marker. All sizes in the next chapters are shown in kiloDaltons.

2.34. Calculating the molecular weight of the CI repressor protein

The theoretical molecular weight of the CI repressor was calculated with the Protein Molecular Weight calculator (http://www.bioinformatics.org/sms/prot_mw.html) using the translated sequence of the protein (CDS: locus SpT5_016 in SpT5 genome, base range: complement (14,496..14,825), GenBank accession number: KX827368).

2.35. Visualising CI repressor structures

Visualisation and comparison of CI repressor structures were carried out with the Deep View – Swiss-PdbViewer software, using the Magic Fit > C-carbons function (Guex and Peitsch, 1997). The crystal structures of the N-terminal domain of the λ and TP901-1 CI repressors were used for comparison and are listed in the PDB (Protein Data Bank) as 1LMB and 3ZHM respectively.

2.36. Protein and short DNA sequence alignments

Protein and short DNA sequence alignments were performed with the online tools Clustal Omega (Sievers *et al.*, 2011), or protein blast using the blastp algorithm (NCBI) using the default settings.

2.37. End-point PCR primer design

Once the area to be targeted through PCR was selected, it was loaded into the NCBI online tool Primer-BLAST and specific primers were designed according to the desired settings (Table 2.3). The specificity of the primers was tested against the NCBI nucleotide (nr) database.

Table 2.3: Characteristics of the PCR primers and oligonucleotides used in the project. The SpT5 operator and its mirror image are in bold and underlined.

Primer/oligo name	Sequence (5'-3')	PCR target	GenBank accession no. or reference	Primer position (base range)	Amplicon's size (bp)
SpT5_F1	CGTCGTTGGTAATGAAGTGGC	Phage SpT5 DNA	KX827368	16,592 – 16,612	125
SpT5_R1	CTGTTCTTACCTGACCTGCGT			16,696 – 16,716	
SpT152_F1	CAGCGGCTTTTGACTGAAACA	Phage SpT152 DNA	KX827369	16,730 – 16,750	485
SpT152_R1	CGAGACAAGACGGAACGACA			16,266 – 16,285	
SpT252_F1	AGGGTGGGAATCTTTTTGTGGA	Phage SpT252 DNA	KX827370	20,942 – 20,963	261
SpT252_R1	ACGGCTCTCGCTAACAAACA			20,703 – 20,722	
SpT99F3_F1	ACGTGAATACGAAGAAGCTGTTGA	Phage SpT99/F3 DNA	KX827371	4,065 – 4,088	375
SpT99F3_R1	CTTGTTCGTAAGTACGCGCCC			4,419 – 4,439	
SpT5_op_F1	GACATGTGAGAACGATGGAT	EMSA ssDNA probes	N/A	3' end of EMSA probe	100
SpT5_op_R1	TTTCGGCTCGCAATCTTTTTA			5' end of EMSA probe	
SpT5_op (EMSA probe)	GACATGTGAGAACGATGGATGTTAAGGTATAAAC A**GTTCTC**AAAA**GAGAAC**GAAGGAGGTGACAAAA TGGTACTTGATCTAAAAAGATTGCGAGCCGAAA	N/A (= not applicable)	N/A	N/A	N/A
SpT5_mir (EMSA probe)	GACATGTGAGAACGATGGATGTTAAGGTATAAAC A**CAAGAG**AAAA**CTCTTG**GAAGGAGGTGACAAAA TGGTACTTGATCTAAAAAGATTGCGAGCCGAAA	N/A	N/A	N/A	N/A
SpT5_*c*I_F1	CAATCATATGAGAAGCAATGATGAAATAATCAC	SpT5 *c*I repressor gene	KX827368	14,800 – 14,825	346
SpT5_*c*I_R1	CAATAAGCTTTTATTTATCGCGTGATTTCC			14,496 – 14,515	
M13_forward	GTTTTCCCAGTCACGAC	Sequencing of pGEM-T Easy Vector	Promega	2,949 – 2,972	N/A
M13_reverse	CAGGAAACAGCTATGAC			176 - 197	

Primer/oligo name	Sequence (5'-3')	PCR target	GenBank accession no. or reference	Primer position (base range)	Amplicon's size (bp)
T7_forward	TAATACGACTCACTATAGGG	Sequencing of pET28a plasmid	Novagen	367 - 386	N/A
T7_reverse	GCTAGTTATTGCTCAGCGG			68 - 86	
SpT99F3_low_F1	TGGGGAAGAAAGGCTCGGA	Either side of change in coverage	KX827371	18,257 – 18,275	315
SpT99F3_high_F1	TACTGTGCAATTTGCGCTTG			18,552 – 18,571	
SpT5_F1_cir	TCGTTAATATACTAACGCAAT	SpT5 genome ends	KX827368	39,619 – 39,639	431
SpT5_R1_cir	AACATTTAGTACAACGCTCGCTA			223 - 245	
SpT152_F1_cir	GATGCAACCCCAATTAATTCCAT	SpT152 genome ends	KX827369	40,707 – 40,729	818
SpT152_R1_cir	TGGATTCCACCAAAAGCTGTC			417 - 437	
SpT252_F1_cir	TATTGAGTGCAGCAGGCCG	SpT252 genome ends	KX827370	39,875 – 39,893	447
SpT252_R1_cir	TCGCTATAGGTGCCATTGCT			209 - 228	
SpT99F3_F1_cir	TTTTTAAAACGAGGAACGTCTCGGG	SpT99/F3 genome ends	KX827371	40,611 – 40,635	415
SpT99F3_R1_cir	CTCTTATCTATTGCATGGCGGTG			252 - 274	
Int_F1	GGCCGAGGGTATATTGACCG	Phage integrase gene	KX827368	11,433– 11,452	578
Int_R1	GAGGCTAAAAGCGCAGAAGC			11,991 – 12,010	
Pse F2	TRGGCAGTAGGATTCGTTAA	*S. pseudintermedius nuc* gene	AB327164 (Sasaki *et al*. 2010)	44 - 63	926
Pse R5	CTTTTGTGCTYCMTTTTGG			951 - 969	
qPse F1	ATTTGGGAACGCTAAAACATT	*S. pseudintermedius nuc* gene (qPCR)	AB327164 (Sasaki *et al*. 2010)	982 - 1002	183
qPse R1	ATTCAAGCGCTCATTGATAC			1,145 – 1,164	
SpT5_im_F1	GATACTAAGAGCTTTTGCAAAATC	SpT5 immunity region	KX827368	14,646 – 14,669	462
SpT5_im_R1	GCCATTTCATCTTGTGTGATTC			15,066 – 15,087	

2.38. End-point PCR

The PCR mixture consisted of 1 µL of DNA extract or one colony in a 25 µL final volume composed of 1x PCR Master Mix (Promega), 0.4 µM forward and reverse primers (Table 2.3), 0.5 mg/mL Bovine Serum Albumin (BSA) and nuclease-free water. Reactions mixtures were thermally cycled according to Table 2.4.

Table 2.4: Description of the end-point PCR programmes that were used in the project. When performing colony PCR, the first step was extended to 5 min.

PCR target	PCR programme
Phage DNA (Warwick phage-specific PCR)	1 x 2min at 94°C
	15s at 94°C 30 x 15s at 64°C 45s at 72°C
	1 x 2 min at 72°C
EMSA ssDNA	1 x 30s at 98°C
	10s at 98°C 30 x 15s at 60°C 10s at 72°C
	1 x 2 min at 72°C
SpT5 *c*I repressor gene	1 x 30s at 98°C
	10s at 98°C 30 x 15s at 64°C 15s at 72°C
	1 x 2 min at 72°C
SpT5 immunity region	1 x 2min at 94°C
	15s at 94°C 30 x 15s at 60°C 45s at 72°C
	1 x 2 min at 72°C

PCR target	PCR programme
Phage DNA (genome ends)	1 x 2min at 94°C
	15s at 94°C 30 x 25s at 64°C 45s at 72°C
	1 x 2 min at 72°C
Phage integrase gene	1 x 2min at 94°C
	15s at 94°C 30 x 15s at 59°C 45s at 72°C
	1 x 2 min at 72°C
S. pseudintermedius nuc gene	1 x 2min at 95°C
	30s at 95°C 30 x 35s at 56°C 1 min at 72°C
	1 x 2 min at 72°C

2.39. Determining sequencing read coverage across phage genomes

The sequencing reads stored in FASTQ files were aligned, or mapped, against the assembled genome with the programme BWA-MEM (Li and Durbin, 2009). The SAM (Sequence Alignment/Map) file thus obtained was converted into a sorted BAM file (binary version of a SAM file) with the application Samtools. The sorted BAM file was read with the programme Qualimap (Garcia-Alcalde *et al.*, 2012). Qualimap produced a report containing information useful to evaluate the quality of

sequencing data, including read coverage across each phage genome. The plot data were exported into an Excel file to produce diagrams (detailed description of the steps in Appendix A).

2.40. Visualising read alignment across theoretical genome ends

Phage genomes were cut in half and both original ends were pasted together in a fasta file. Sequencing reads were aligned with the cut-and-pasted version of each genome following the method described above. The obtained sorted BAM file was read with the programme Artemis (Rutherford *et al.*, 2000) using the BAMView option (Carver *et al.*, 2010) that allows the visualisation of sequencing reads aligned against a reference genome.

2.41. Spiking faeces with *S. pseudintermedius* cells

100 µL BHI containing 10^8 down to 10^2 CFU of the strain E140 were added to 0.1 gram of faeces in a 2-mL tube. Liquid and faeces were mixed together with a thin metallic spatula and DNA was extracted for further tests (section 2.43).

2.42. Spiking swabs with *S. pseudintermedius* cells

100 µL BHI containing 10^8 down to 10^2 CFU of the strain E140 were pipetted onto Sterilin® flocked regular swabs (Appleton Woods) and left to dry for an hour. DNA was extracted for further tests (section 2.44).

2.43. DNA extraction from cells and faeces

DNA extraction from cells and faeces was performed using the FastDNA™ Spin Kit for Soil (MP Biomedicals) from 350 µL overnight culture or 0.1 gram of faeces following the manufacturer's instructions.

2.44. DNA extraction from skin swabs

DNA extraction from skin swabs was performed using the NucleoSpin® Tissue kit (Macherey-Nagel) following the manufacturer's instructions for DNA extraction from buccal swabs. The buffer used at step 1 was Phosphate-Buffered Saline (PBS).

2.45. Swab and faecal sample collection

Skin swabs and faecal samples were collected by veterinarians at various locations: Kenilworth Avonvale practice, Warwick Avonvale practice, Pride Veterinary Centre (Derby), Bristol veterinary school and Lene Boysen's veterinary dermatology practice (Copenhagen, Denmark). Sampling kits containing tubes, swabs and minimal sample collection requirements were provided. Veterinarians were asked to collect swabs from three places on the body of one dog (bare skin if healthy, or pyoderma lesions) and collect a faecal sample from the same animal when possible. Faecal samples were also obtained from dogs owned by colleagues in Denmark, from litterbins in Coventry and from dogs staying at the Castledean Boarding Kennel (Coventry). Samples were kept in the fridge until analysis.

2.46. Quantitative PCR (qPCR) primer design

Both qPCR reverse primers were designed to hybridise to regions specific to *S. pseudintermedius* based on an alignement of the *nuc* genes of several *Staphylococcus* species (*S. aureus*, *S. delphini* and *S. intermedius*) generated with the software MEGA (Tamura *et al.*, 2011). The amplicon size was 183 bp (size range for qPCR primers: 100 to 200 bp) (Table 2.3).

2.47. qPCR assay for the detection of *S. pseudintermedius*

The qPCR mixture consisted of 10 µL of DNA extract in a 25 µL final volume composed of 1x of Power SYBR® Green PCR Master Mix (Fisher Scientific), 0.9 µM forward and reverse primers (Table 2.3), 1.0 mg/mL BSA and nuclease-free water. qPCR reactions were performed in MicroAmp® Fast Optical 96-well Reaction plates (Fisher Scientific) using a 7500 Fast Real-Time PCR System and the 7500 Software (Applied Biosystems). Plates were sealed with MicroAmp® Optical Adhesive Films (Fisher Scientific) and reaction mixtures were thermally cycled once at 50°C for 2 min, once at 95°C for 10 min, and 40 times at 95°C for 15s and 60°C for 1 min. Genome copy standards were used to generate a standard curve and thus genomic DNA from *S. pseudintermedius* was used to obtain a dilution range from 1,000,000 to 1 genome(s). The theoretical genome copy numbers in the standards were calculated with an online tool (http://cels.uri.edu/gsc/cndna.html) based on the amount of DNA in the standard solution in nanograms and the length of the genome (2.8 Mbp for strain E140). The geometric mean of Ct values was determined with a

threshold of 0.03 and converted into absolute genomic quantities by plotting against the genome copies of the standard curve.

Chapter 3 Isolation and characterisation of *S. pseudintermedius* phages

3.1. Introduction

The initial stage of this PhD project was dedicated to the isolation and characterisation of *S. pseudintermedius* phages. This work was novel; no peer-reviewed published work was available regarding phages infecting this bacterium. However, techniques for the isolation of bacteriophages have been developed during the 100 years following their discovery and could be applied to *S. pseudintermedius* phages. These techniques are straightforward and usually involve very little processing of the samples. If the sample is liquid, it can be directly spotted onto a top agar seeded with the host to see plaques after overnight incubation. If the sample is not liquid, it can be homogenised with a buffer and then spotted onto an agar plate seeded with bacteria. With these simple techniques, phages were isolated from a variety of sources such as water (Karumidze *et al.*, 2013), faeces (Golomidova *et al.*, 2007, Owens *et al.*, 2013) and soil (Salifu *et al.*, 2013, Cross *et al.*, 2015).

Sometimes the concentration of phages in a sample is too low to be detected by spotting a few tens of microliters onto bacteria. To overcome this problem, the sample can be enriched through addition of bacteria and nutrients followed by overnight incubation (Kim *et al.*, 2012, Lee *et al.*, 2011). If the phages present in the sample can infect the host, phage amplification will occur bringing the concentration to a level detectable via plate assay. The isolation of *S. pseudintermedius* lytic phages was attempted by screening dog faeces, dog skin swabs, soil and water through similar methods, with or without enrichment. The choice of samples to screen was based on the assumption that the pathogen could be present in those samples and that phages would co-reside with their host. This was discussed in more detail in Chapter 7.

Another source of bacteriophages are the prophages present in lysogens. These can be captured through co-culture where two or more bacterial strains are cultivated together overnight. Prophages in the genome of the donor strain(s) may spontaneously enter the lytic cycle, lyse their host and infect the cultivating strain(s) present in the culture. Phages amplified this way can be recovered by spotting the culture supernatant onto the cultivating strain. This method was used for the isolation

of *S. intermedius* phages (before the re-classification of the SIG group, see section 1.3.2) with the aim to develop phage typing of *S. intermedius* strains (Overturf *et al.*, 1991).

As mentioned in section 1.4.2.b.iv, prophage induction can also occur when inducing the host SOS stress response. The antibiotic mitomycin C is a DNA replication inhibitor that was isolated from the microorganism *Streptomyces caespitosus*. It induces cross-links in the DNA that trigger the SOS response (Tomasz and Palom, 1997). Mitomycin C exposure of λ lysogens was shown to induce lytic growth of the prophages through activation of RecA and self-cleavage of the CI repressor (Roberts and Roberts, 1975). A RecA-LexA-dependent pathway similar to that of *E. coli* was described in *S. aureus* (Bisognano *et al.*, 2004) and temperate phages of *Staphylococus epidermidis* were isolated through mitomycin C exposure (Gutiérrez *et al.*, 2010). This indicated that temperate phages of *S. pseudintermedius* might be inducible through mitomycin C treatment. It was therefore attempted.

Once phages were isolated, they were characterised genotypically and phenotypically with a range of methods commonly found in the literature (Kulikov *et al.*, 2012, Shen *et al.*, 2012, Kesik-Szeloch *et al.*, 2013). These methods included:

- Restriction-Fragment Length Polymorphism (RFLP) where phage genomic DNA was digested with restriction enzymes and patterns of digestion were compared to each other to evaluate diversity between genomes.
- Pulsed-Field Gel Electrophoresis (PFGE) to determine the phages' genome size
- Host range testing
- Whole-genome sequencing and alignment to compare phage genomes
- Electron microscopy to study the phages' morphology. This would provide information about their taxonomy.

3.2. Isolation of *S. pseudintermedius* phages from dog samples

3.2.1. Phages isolated in Denmark prior to the PhD project

S. pseudintermedius phages were isolated from dog faeces at the University of Copenhagen prior to this PhD project (Nälgård, 2011). They were first thought to be lytic because of their environmental origin (dog faeces) and were named accordingly

SpLx (for *Staphylococcus pseudintermedius* Lytic phage, x = number). Following initial characterisation, ten phages were selected and provided by our collaborators in Copenhagen for further analysis in this PhD project.

3.2.2. Further attempts at isolating phages from dog faeces, skin swabs and soil

Over a period of four months, more than 100 dog faecal samples were collected from different sources in the United Kingdom (boarding kennels, veterinary practices, the Bristol veterinary school, litterbins) and screened for the presence of phages following a method similar to the one previously used in Denmark. A few soil samples from the Tocil Wood area situated on the University of Warwick campus where dogs were walked regularly were screened (section 2.5). Nine Thames water samples collected downstream of wastewater treatment plants during a different project were screened too because water is a well-known source of phages (section 2.6). In addition, skin swabs collected by veterinarians from healthy dogs or dogs with suspected pyoderma in the Coventry area were screened for the presence of phages (sections 2.7 and 2.45). All these attempts remained unsuccessful.

Three weeks were spent at the University of Copenhagen to screen faecal samples collected in Denmark for the presence of phages using the same equipment and the same method as previously used there. Skin swabs collected in Denmark from dogs with suspected pyoderma were screened too (sections 2.7 and 2.45), but no lytic phages were isolated.

For all of the above experiments, bacterial strains with different characteristics and Sequence Types (STs) were used to try and capture the diversity of phages hypothetically present in environmental samples. STs included ST68 and ST71, which are most common in the USA and in Europe respectively (section 1.3.3.b). Isolating phages against these strains was therefore the most relevant to this PhD project.

3.3. Isolation of temperate *S. pseudintermedius* phages

3.3.1. Co-culture with several bacterial strains

Temperate phages were isolated through co-culture in Denmark prior to this PhD project. They could not be re-amplified from the stock lysates stored at -80°C so the

experiment was repeated in Denmark following the same protocol (section 2.8). Four different mixes (A, B, C and D) composed of seven different *S. pseudintermedius* strains were tested (Table 3.1). The choice of bacterial strains to use in this experiment was based on the result of two previous experiments performed by collaborators in Denmark. In the first experiment, a plaque-like inhibition zone was observed when spotting the supernatant of an overnight culture of the MSSP S61A8 strain onto a lawn of the MRSP E140 strain. In the second experiment, the ability of MSSP cultures to form inhibition zones onto lawns of MRSP strains was further studied. The supernatants of 44 MSSP overnight cultures were spotted onto lawns of eleven MRSP strains (Nälgård, 2011). The selection of these strains was based on their sequence type (ST71 and ST68) and their availability at the time of the experiment. The three MSSP strains (S61A8, S61H5 and S63G7) that formed inhibition zones on one or several MRSP strains were kept to perform co-culture with combinations of the same eleven MRSP strains.

After overnight incubation, the supernatant of each mixed culture was spotted onto all fourteen bacterial strains used in the experiment. Plaques were seen on four MRSP strains when spotting the supernatant of mixes A, B and D. No plaques were seen when spotting mix C on any of the strains. This way twelve temperate phages were isolated (Table 3.2). These phages were considered independent from each other until further analysis and were named SpTx (*Staphylococcus pseudintermedius* Temperate phage, x = number) depending on the co-culture mix they came from and the bacterial strain on which they formed plaques.

Table 3.1: Eleven MRSP strains and three MSSP strains were used to isolate temperate phages through co-culture. The bacterial strains were combined in four different mixes (A, B, C and D) composed of seven different *S. pseudintermedius* strains (✔ = strain was present in mix).

S. pseudintermedius strains	Methicillin resistance	Sequence Type	Country of origin	Co-culture mixes			
				A	B	C	D
E029	MRSP	ST71	Italy	✔		✔	
E045	MRSP	ST71	Sweden	✔			✔
E086	MRSP	ST71	The Netherlands	✔		✔	
E123	MRSP	ST71	USA	✔		✔	
E133	MRSP	ST71	Canada	✔	✔		✔
E140	MRSP	ST71	Denmark	✔	✔		✔
E018	MRSP	ST68	USA		✔	✔	
E022	MRSP	ST68	USA		✔	✔	
E025	MRSP	ST68	USA		✔		✔
E122	MRSP	ST68	USA		✔		✔
E136	MRSP	ST68	USA				✔
S61A8	MSSP	n/t	Denmark	✔			✔
S61H5	MSSP	n/t	Denmark		✔	✔	
S63G7	MSSP	n/t	Denmark			✔	

Table 3.2: After co-culture, twelve temperate phages were isolated when spotting the supernatants of mixes A, B and D onto four MRSP strains. No phages were isolated from mix C. SpT = *Staphylococcus pseudintermedius* Temperate phage.

S. pseudintermedius strains	Methicillin resistance	Sequence Type	Country of origin	Co-culture mixes		
				A	B	D
E029	MRSP	ST71	Italy	SpT1	SpT2	SpT3
E045	MRSP	ST71	Sweden	SpT4	SpT5	SpT6
E086	MRSP	ST71	The Netherlands	SpT7	SpT8	SpT9
E133	MRSP	ST71	Canada	SpT10	SpT11	SpT12

3.3.2. Co-culture with two bacterial strains

When using multiple strains for co-culture it was subsequently difficult to determine where the isolated phage(s) came from. This might cause Intellectual Property issues since the strains used in this project were obtained from other researchers or institutes. To avoid problems in the event of patenting or commercialising a product derived from this project, it was decided to use only two strains at a time for subsequent co-culture experiments (section 2.8). This way the phage donor strain was easily identifiable.

A number of combinations were tested (Table 3.3). Four cultivating strains, E018, E136, E086 and E140, were selected because they exhibited the Sequence Types 68 and 71. A fifth cultivating strain, ED99, was chosen because it did not to contain any prophages in its genome (Ben Zakour *et al.*, 2012). Using this strain meant that homoimmunity would be avoided (section 1.4.2.b.v) and this might help capture phages unable to form plaques on the other cultivating strains. Donor strains were selected based on their sensitivity or resistance to methicillin, their geographical origin and their sequence type.

Only one combination led to the isolation of a phage: phage SpT99/F3 came from the strain S56F3 and was cultivated on the strain ED99. It was possible that homoimmunity and other phage resistance mechanisms (e.g. restriction-modification systems) prevented the isolation of phages on the other cultivating strains. The fact that only one phage was isolated on ED99 suggested that homoimmunity might not be the main phage resistance mechanism in *S. pseudintermedius*.

Table 3.3: Five cultivating strains were co-cultivated with 37 donor strains. The combination ED99 / S56F3 was the only one that led to the isolation of a phage. (-) = no phage was isolated, (+) = a phage was isolated, n/t = not tested.

Donor strains	Methicillin resistance	Sequence Type	Country of origin	Cultivating strains				
				E018	E136	E086	E140	ED99
E029	MRSP	ST71	Italy	n/t	-	n/t	n/t	-
E045	MRSP	ST71	Sweden	n/t	-	n/t	n/t	-
E086	MRSP	ST71	The Netherlands	n/t	-	n/t	n/t	-
E123	MRSP	ST71	USA	n/t	-	n/t	n/t	-
E133	MRSP	ST71	Canada	n/t	-	n/t	n/t	-
E140	MRSP	ST71	Denmark	n/t	-	n/t	n/t	-
E018	MRSP	ST68	USA	n/t	n/t	-	n/t	n/t
E022	MRSP	ST68	USA	n/t	n/t	-	n/t	n/t
E025	MRSP	ST68	USA	n/t	n/t	-	n/t	n/t
E122	MRSP	ST68	USA	n/t	n/t	-	n/t	n/t
E136	MRSP	ST68	USA	n/t	n/t	-	n/t	n/t
E139	MRSP	ST258	Denmark	n/t	-	-	n/t	-
AB190	MRSP	n/t	Sweden	-	n/t	n/t	-	n/t
AB312	MRSP	n/t	Sweden	-	n/t	n/t	-	n/t
AB316	MRSP	n/t	Sweden	-	n/t	n/t	-	n/t
AB680	MRSP	n/t	Sweden	-	n/t	n/t	-	n/t
HK2	MSSP	n/t	China	-	n/t	n/t	-	n/t
HK14	MSSP	n/t	China	-	n/t	n/t	-	n/t

Donor strains	Methicillin resistance	Sequence Type	Country of origin	Cultivating strains				
				E018	E136	E086	E140	ED99
Y1	MSSP	n/t	China	-	n/t	n/t	-	n/t
S56F3	MSSP	n/t	Denmark	-	n/t	n/t	-	+
S56H7	MSSP	n/t	Denmark	-	n/t	n/t	-	-
S57E7	MSSP	n/t	Denmark	-	n/t	n/t	-	-
S60C4	MSSP	n/t	Denmark	-	n/t	n/t	-	-
S60C6	MSSP	n/t	Denmark	-	n/t	n/t	-	-
S60D6	MSSP	n/t	Denmark	-	n/t	n/t	-	-
S60D7	MSSP	n/t	Denmark	-	n/t	n/t	-	-
S61A3	MSSP	n/t	Denmark	n/t	-	-	n/t	-
S61A8	MSSP	n/t	Denmark	n/t	-	-	n/t	-
S61B7	MSSP	n/t	Denmark	n/t	-	-	n/t	-
S61H5	MSSP	n/t	Denmark	n/t	-	-	n/t	-
S63G7	MSSP	n/t	Denmark	n/t	-	-	n/t	-
JZ31	MSSP	n/t	Sweden	-	n/t	n/t	-	n/t
JZ56	MSSP	n/t	Sweden	-	n/t	n/t	-	n/t
JZ133	MSSP	n/t	Sweden	-	n/t	n/t	-	n/t
JZ146	MSSP	n/t	Sweden	-	n/t	n/t	-	n/t
JZ151	MSSP	n/t	Sweden	-	n/t	n/t	-	n/t
JZ152	MSSP	n/t	Sweden	-	n/t	n/t	-	n/t

3.3.3. Induction of prophages through mitomycin C exposure

The induction of prophages through exposure to mitomycin C (section 2.9) was attempted on 46 bacterial strains (Table 2.1) and seven phages were isolated. They were called SpT08, SpT86, SpT123, SpT152, SpT178, SpT252 and SpT316 based on the bacterial strains they came from: 08BKT31634, E086, E123, JZ152, AB178, AB252 and AB316 respectively. SpT123 and SpT178 proved to be very difficult to amplify to a high titre so both were set aside.

At this stage, 30 phages in total had been isolated (eighteen phages at Warwick and ten phages in Denmark). A summary of their characteristics including their strain of origin (when applicable) and the strain on which they were propagated is given in Table 3.4. Most of these phages were isolated from and/or propagated on ST71 MRSP strains from different geographical origins. No phages were either isolated or propagated on ST68 strains despite such strains being used as donor or cultivating strains in all the experiments described above. This suggested that ST68 strains are particularly resistant to phage infection. Following isolation, the 28 selected phages (30 phages minus SpT123 and SpT178) were characterised to determine whether they were different genotypically and phenotypically.

Table 3.4: 30 phages in total were isolated either before or during this PhD project. Most of these phages were isolated from and/or propagated on ST71 MRSP strains from different geographical origins. No phages were isolated or propagated on ST68 strains. N/A: not applicable, n/t: not tested.

Bacteriophages	Method of isolation	Bacterial strain of origin / Propagation strain	Methicillin resistance	Sequence Type	Country of origin
SpL7	Isolation from faeces	N/A			
		E133	MRSP	ST71	Canada
SpL8	Isolation from faeces	N/A			
		E133	MRSP	ST71	Canada
SpL9	Isolation from faeces	N/A			
		E133	MRSP	ST71	Canada
SpL10:1	Isolation from faeces	N/A			
		E133	MRSP	ST71	Canada
SpL10:2	Isolation from faeces	N/A			
		E133	MRSP	ST71	Canada
SpL10:5	Isolation from faeces	N/A			
		E133	MRSP	ST71	Canada
SpL10:6	Isolation from faeces	N/A			
		E133	MRSP	ST71	Canada
SpL11	Isolation from faeces	N/A			
		E133	MRSP	ST71	Canada
SpL12	Isolation from faeces	N/A			
		E133	MRSP	ST71	Canada

Bacteriophages	Method of isolation	Bacterial strain of origin / Propagation strain	Methicillin resistance	Sequence Type	Country of origin
SpL13	Isolation from faeces	N/A			
		E133	MRSP	ST71	Canada
SpT1	Co-culture (mix A)	Unknown			
		E029	MRSP	ST71	Italy
SpT2	Co-culture (mix B)	Unknown			
		E029	MRSP	ST71	Italy
SpT3	Co-culture (mix D)	Unknown			
		E029	MRSP	ST71	Italy
SpT4	Co-culture (mix A)	Unknown			
		E045	MRSP	ST71	Sweden
SpT5	Co-culture (mix B)	Unknown			
		E045	MRSP	ST71	Sweden
SpT6	Co-culture (mix D)	Unknown			
		E045	MRSP	ST71	Sweden
SpT7	Co-culture (mix A)	Unknown			
		E086	MRSP	ST71	The Netherlands
SpT8	Co-culture (mix B)	Unknown			
		E086	MRSP	ST71	The Netherlands
SpT9	Co-culture (mix D)	Unknown			
		E086	MRSP	ST71	The Netherlands
SpT10	Co-culture (mix A)	Unknown			
		E133	MRSP	ST71	Canada

Bacteriophages	Method of isolation	Bacterial strain of origin / Propagation strain	Methicillin resistance	Sequence Type	Country of origin
SpT11	Co-culture (mix B)	Unknown			
		E133	MRSP	ST71	Canada
SpT12	Co-culture (mix D)	Unknown			
		E133	MRSP	ST71	Canada
SpT99/F3	Co-culture (two strains)	S56F3	MSSP	n/t	Denmark
		ED99	MSSP	n/t	UK
SpT08	Mitomycin C induction	08BKT31634	MRSP	n/t	Sweden
		E045	MRSP	ST71	Sweden
SpT86	Mitomycin C induction	E086	MRSP	ST71	The Netherlands
		S60D7	MSSP	n/t	Denmark
SpT123	Mitomycin C induction	E123	MRSP	ST71	USA
		S60D7	MSSP	n/t	Denmark
SpT152	Mitomycin C induction	JZ152	MSSP	n/t	Sweden
		E139	MRSP	ST258	Denmark
SpT178	Mitomycin C induction	AB178	MRSP	n/t	Sweden
		ED99	MSSP	n/t	UK
SpT252	Mitomycin C induction	AB252	MRSP	n/t	Sweden
		E045	MRSP	ST71	Sweden
SpT316	Mitomycin C induction	AB316	MRSP	n/t	Sweden
		E045	MRSP	ST71	Sweden

3.4. Genotypic and phenotypic characterisation of the isolated phages

3.4.1. RFLP analysis of the phages' genomes

To characterise the 28 phages, the first test that was performed was RFLP (Figure 3.1). It was carried out with two enzymes: *Eco*RI and *Sau*3AI. *Eco*RI recognises a six base-long digestion site (G^AATT_C) and *Sau*3AI recognises a shorter four base-long digestion site (^GATC_) (section 2.14). It was therefore likely that a higher number of *Sau*3AI sites compared to *Eco*RI sites were present in a given genome, meaning that the genome was more likely to be digested by *Sau*3AI than *Eco*RI. When using these two enzymes for RFLP it was hoped that digestion could be seen with at least *Sau*3AI. *Eco*RI digestion patterns, when digestion was possible, would bring additional information about diversity.

The genomic DNA from all the phages in this experiment was digested with *Eco*RI and *Sau*3AI, indicating that all phages contained dsDNA in their capsid. Digestion patterns were compared and according to RFLP analysis phages SpT1 to SpT12 were very similar. A preliminary host range study showed that all twelve phages exhibited the same host range except for phage SpT5 (Table 3.5). Phage SpT8 was kept alongside SpT5 as a representative of the eleven other phages. It later turned out that the discrepancy between SpT5 and the other phages was due to the fact that undiluted lysates were used at the time to test the host range. The inhibition zones seen on ST68 strains with all phages but SpT5 were due to lysis from without and not productive lysis. As a result, phage SpT5 alone was kept as representative of the twelve initial phages.

SpT08, SpT252 and SpT316 appeared very similar to each other based on RFLP results. SpT252 was kept for further work. SpT152 and SpT99/F3 showed unique patterns of digestion with both enzymes and were kept too. SpT86 proved to be very difficult to amplify to a high titre, hence the faint bands visible in Figure 3.1, E and F. Added to the fact that SpT86 was very unstable when stored at +4°C, this meant that this phage was not kept for further work.

Among the phages isolated in Denmark prior to this PhD project, three types of phages could be distinguished. One group included SpL8, SpL9 and SpL10:2. A second group included SpL7, SpL10:1, SpL10:6, SpL11, SpL12 and SpL13.

SpL10:5 exhibited a unique digestion pattern with both enzymes. One phage out of each group, SpL8, SpL11 and SpL10:5, were kept for further analysis.

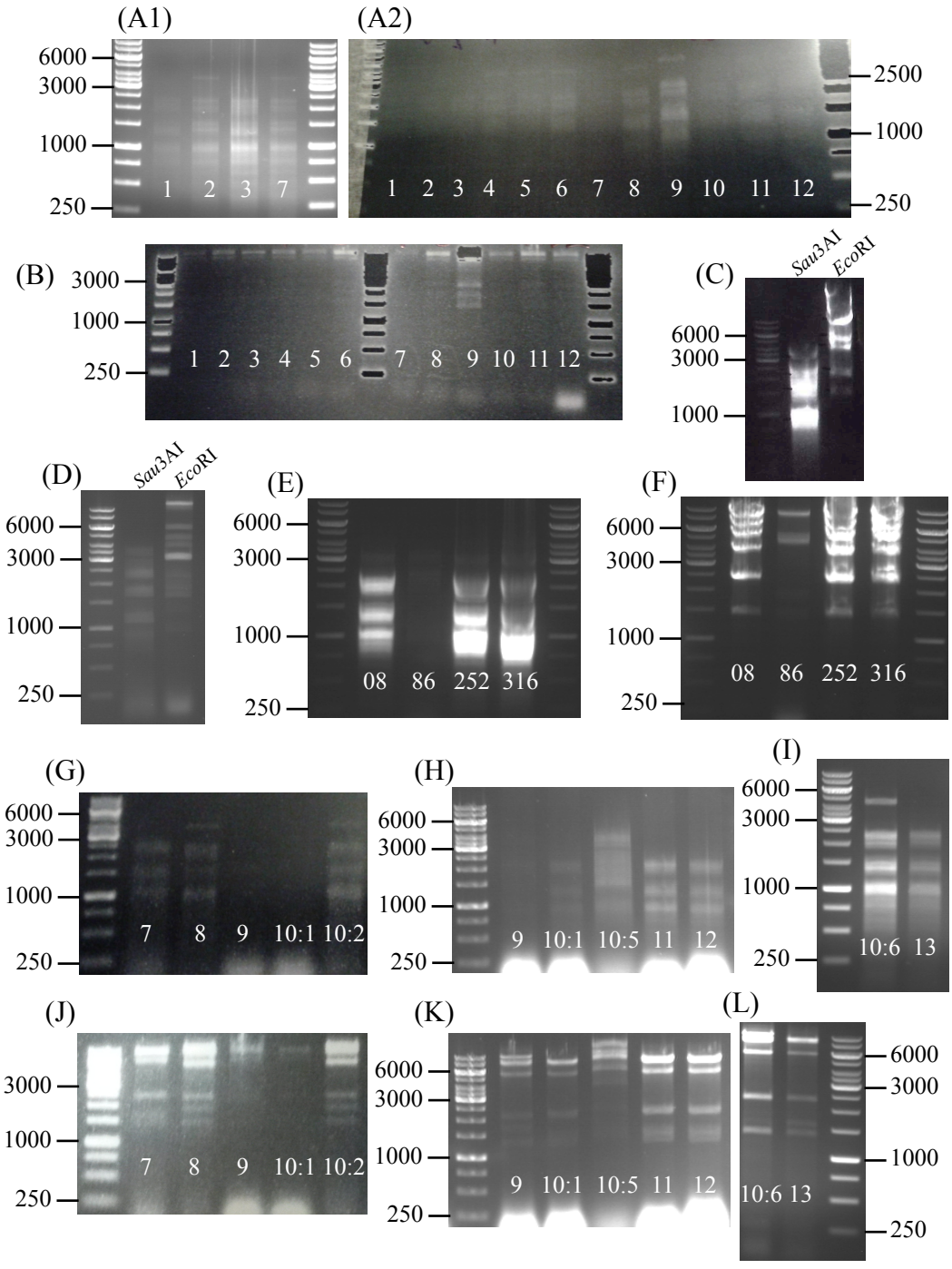


Figure 3.1: RFLP analysis of the *S. pseudintermedius* phages' genomic DNA revealed a variety of digestion patterns. (A1 and A2) SpT1 to SpT12 with *Sau*3AI and (B) *Eco*RI, (C) SpT152, (D) SpT99/F3, (E) SpT08, SpT86, SpT252 and SpT316 with *Sau*3AI and (F) *Eco*RI, (G, H, I) SpL7 to SpL13 with *Sau*3AI and (J, K, L) *Eco*RI.

Table 3.5: A preliminary host range study showed that phages SpT1 to SpT12 exhibited the same host range except for phage SpT5. In this small study, the host range was tested by spotting 10 µL of undiluted lysate onto a top agar seeded with bacteria. (+) = phage lysis was observed, (-) = no lysis.

S. pseudintermedius strains	Methicillin resistance	Sequence Type	Bacteriophages											
			SpT1	SpT2	SpT3	SpT4	SpT5	SpT6	SpT7	SpT8	SpT9	SpT10	SpT11	SpT12
E029	MRSP	ST71	+	+	+	+	+	+	+	+	+	+	+	+
E045	MRSP	ST71	+	+	+	+	+	+	+	+	+	+	+	+
E086	MRSP	ST71	+	+	+	+	+	+	+	+	+	+	+	+
E123	MRSP	ST71	+	+	+	+	+	+	+	+	+	+	+	+
E133	MRSP	ST71	+	+	+	+	+	+	+	+	+	+	+	+
E140	MRSP	ST71	+	+	+	+	-	+	+	+	+	+	+	+
E018	MRSP	ST68	+	+	+	+	-	+	+	+	+	+	+	+
E022	MRSP	ST68	+	+	+	+	-	+	+	+	+	+	+	+
E025	MRSP	ST68	+	+	+	+	-	+	+	+	+	+	+	+
E122	MRSP	ST68	+	+	+	+	-	+	+	+	+	+	+	+
E136	MRSP	ST68	+	+	+	+	-	+	+	+	+	+	+	+
S61A8	MSSP	n/t	+	+	+	+	-	+	+	+	+	+	+	+
S61H5	MSSP	n/t	+	+	+	+	-	+	+	+	+	+	+	+
S63G7	MSSP	n/t	+	+	+	+	-	+	+	+	+	+	+	+

In total seven phages were kept at this stage of the project for further work based on RFLP results (Figure 3.2).

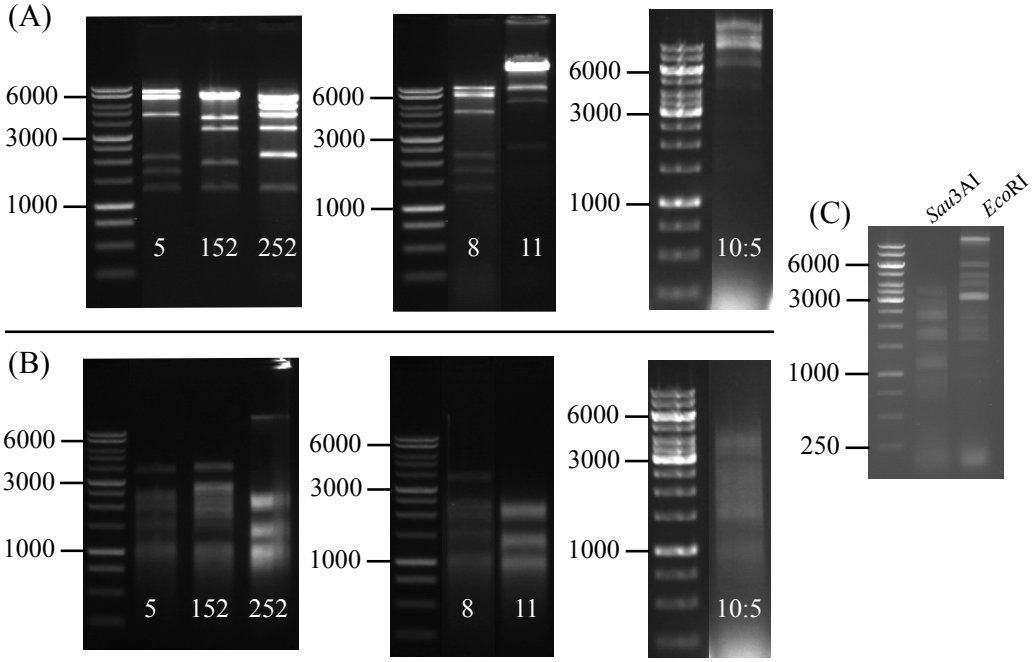


Figure 3.2: Seven phages were kept for further work after RFLP analysis. Digestion patterns of SpT5, SpT152, SpT252, SpL8, SpL11 and SpL10:5 with (A) *Eco*RI and (B) *Sau*3AI, and (C) digestion patterns of SpT99/F3.

Following RFLP analysis, PFGE was performed to determine the size of the selected phages' genomes. From now on in this chapter, phages isolated at Warwick (SpT5, SpT152, SpT252 and SpT99/F3) will be referred to as "the Warwick phages" and phages isolated in Denmark (SpL8, SpL10:5 and SpL11) will be referred to as "the Danish phages".

3.4.2. PFGE with the selected phages' genomes

After overnight digestion of the capsid, the genomic DNA of the selected phages was run on a PFGE gel for 20h (Figure 3.3 and section 2.16).

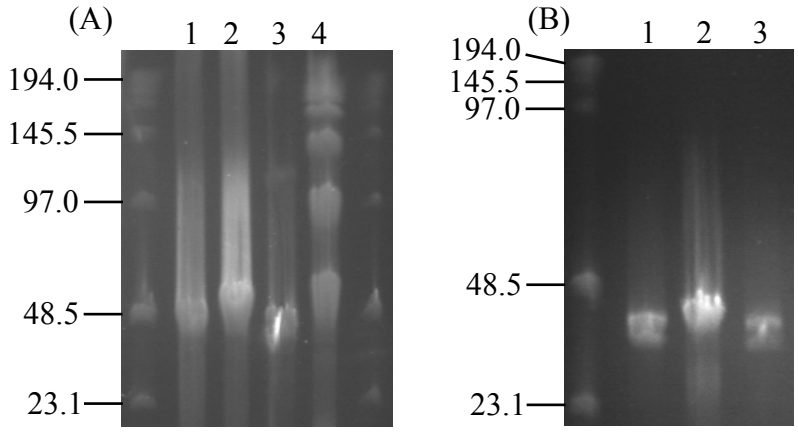


Figure 3.3: PFGE analysis revealed that the length of the genome of the seven analysed phages was between 40 and 50 kilobases long. (A) PFGE with the Warwick phages. 1: SpT5, 2: SpT252, 3: SpT152 and 4: SpT99/F3. (B) PFGE with the Danish phages. 1: SpL8, 2: SpL10:5 and 3: SpL11.

The length of the genome of the seven analysed phages was between 40 and 50 kilobases long. Temperate phages often have short genomes (around 40 kb or less) compared to lytic phages that can have much larger genomes up to 500 kb (Ackermann, 2006). The genome size of the Warwick phages was therefore consistent with the fact that they were temperate. The Danish phages exhibited the same genome size. This might indicate that they were temperate as well, even though they were considered lytic because they were isolated from the environment.

SpT99/F3 showed a unique PFGE pattern with bands around 50, 100, 150, 190 and 200 kb. A similar result was obtained when performing PFGE for the first time with SpT99/F3 genomic DNA (Figure 3.4). The gel presented in Figure 3.3 was performed later to confirm the result. For this, SpT99/F3 was re-amplified from the -80°C stock lysate to make sure fresh genomic DNA was used. The differences in migration distances between both gels were due to the fact that a different agarose was used for the second PFGE experiment (Figure 3.3).

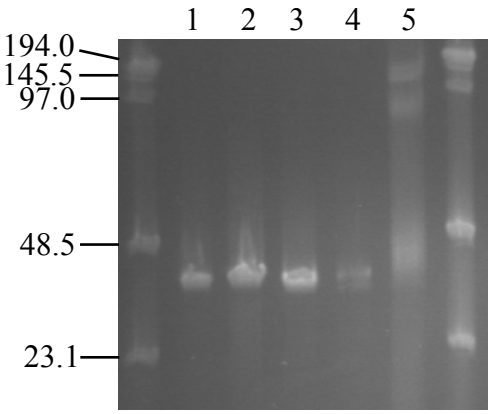


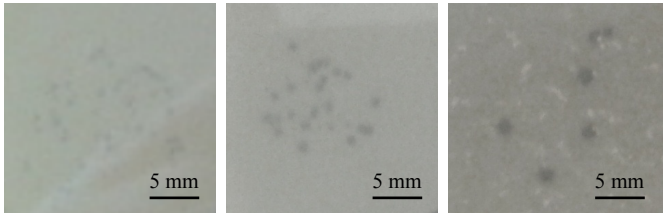
Figure 3.4: Similar migration patterns were observed when performing PFGE the first time with the Warwick phages. 1: SpT5, 2: SpT252, 3: SpT152, 4: SpT86 and 5: SpT99/F3.

The multiple bands corresponding to SpT99/F3 could have resulted from contamination of the phage's DNA with the host's DNA. If this was case, it was surprising that it did not happen with the other phages that were prepared for PFGE the same way as SpT99/F3. These bands could also correspond to concatemers of SpT99/F3 genomes. Another possibility was the presence of a second phage (or more) in the SpT99/F3 lysate, which genome was visible on the gel. In spite of this unusual PFGE pattern, SpT99/F3 was kept for further analysis.

3.4.3. Host range screening and Efficiency Of Plating (EOP)

To characterise the selected phages phenotypically, the host range and EOP (section 2.11) were determined on a library of 72 methicillin-sensitive or methicillin-resistant *S. pseudintermedius* strains obtained from different parts of the world (Sweden, Denmark, UK, USA, China) (Table 3.6). Each phage was tested once on each bacterial strain. The phage titre on the test strain was divided by the phage titre on the reference strains to calculate the EOP.

Table 3.6: The host range of the studied phages was narrow (growth on less than half of 72 *S. pseudintermedius* strains) and three different plaque sizes were observed. Each phage was tested once on each bacterial strain. EOP was calculated by dividing the phage titre on the test strain by the phage titre on the reference strain (in grey). n/t = not tested, (-) = no growth, S = small plaques, M = medium-sized plaques, L = large plaques (see pictures below – magnification is the same for all three pictures).



small medium large

S. pseudintermedius strains	Methicillin resistance	Sequence Type	Bacteriophages						
			SpT5	SpT152	SpT252	SpT99/F3	SpL8	SpL10:5	SpL11
E029	MRSP	ST71	$8.7x10^{-1}$ S	-	8.5 S	-	$4.1x10^{-1}$ S	2.5 M	$8.5x10^{-1}$ M
E045	MRSP	ST71	1.0 M	-	1.0 S	-	1.0 S	1.0 M	1.0 M
E046	MRSP	ST71	-	-	-	-	-	-	-
E047	MRSP	ST71	$7.0x10^{-1}$ M	-	$9.7x10^{-1}$ S	-	1.2 S	3.5 M	$8.2x10^{-1}$ M
E052	MRSP	ST71	-	-	-	-	-	-	-
E061	MRSP	ST71	$6.1x10^{-1}$ S	-	$5.7x10^{-1}$ S	-	$4.2x10^{-1}$ S	2.0 S	$8.2x10^{-1}$ M
E064	MRSP	ST71	$7.4x10^{-1}$ M	-	$4.5x10^{-1}$ S	-	$2.9x10^{-1}$ S	3.5 M	1.18 M
E075	MRSP	ST71	-	-	-	-	-	-	-
E086	MRSP	ST71	1.3 S	-	5.2 S	-	1.0 S	2.5 M	$9.3x10^{-1}$ M
E123	MRSP	ST71	1.3 S	-	2.2 S	-	$3.7x10^{-1}$ S	3.0 M	$6.6x10^{-1}$ M

S. pseudintermedius strains	Methicillin resistance	Sequence Type	Bacteriophages						
			SpT5	SpT152	SpT252	SpT99/F3	SpL8	SpL10:5	SpL11
E133	MRSP	ST71	1.8 M	-	4.7 M	-	$7.5x10^{-1}$ S	2.5 M	$9.3x10^{-1}$ M
E134	MRSP	ST71	1.1 M	-	5.0 M	-	$4.2x10^{-1}$ S	2.5 M	$8.8x10^{-1}$ M
E140	MRSP	ST71	-	-	-	-	-	-	-
E017	MRSP	ST68	-	-	-	-	-	-	-
E018	MRSP	ST68	-	-	-	-	-	-	-
E019	MRSP	ST68	-	-	-	-	-	-	-
E020	MRSP	ST68	-	-	-	-	-	-	-
E022	MRSP	ST68	-	-	-	-	-	-	-
E023	MRSP	ST68	-	-	-	-	-	-	-
E025	MRSP	ST68	-	-	-	-	-	-	-
E026	MRSP	ST68	-	-	-	-	-	-	-
E122	MRSP	ST68	$8.0x10^{-7}$ S	-	-	-	-	-	-
E135	MRSP	ST68	-	-	-	-	-	-	-
E136	MRSP	ST68	-	-	-	-	-	-	-
E069	MRSP	ST73	$6.5x10^{-3}$ S	-	-	-	$2.9x10^{-3}$ S	$2.5x10^{-4}$ S	$4.9x10^{-6}$ S
E125	MRSP	ST58	-	-	-	-	-	-	-
E126	MRSP	ST113	-	-	-	-	-	-	-
E139	MRSP	ST258	$2.0x10^{-1}$ M	1.0 S	$3.7x10^{-2}$ S	-	$8.3x10^{-2}$ S	$3.5x10^{-2}$ M	-
AB178	MRSP	n/t	-	-	-	-	-	-	-
AB190	MRSP	n/t	1.3 S	-	6.7 M	-	$8.3x10^{-1}$ S	3.0 M	1.0 M
AB252	MRSP	n/t	-	-	-	-	-	-	-

S. pseudintermedius strains	Methicillin resistance	Sequence Type	Bacteriophages						
			SpT5	SpT152	SpT252	SpT99/F3	SpL8	SpL10:5	SpL11
AB255	MRSP	n/t	-	-	$2.5x10^{-6}$ S	-	-	-	$1.6x10^{-5}$ M
AB312	MRSP	n/t	1.2 S	-	3.0 S	-	$3.3x10^{-1}$ S	1.5 M	1.0 M
AB316	MRSP	n/t	-	-	-	-	-	-	-
AB680	MRSP	n/t	$3.3x10^{-6}$ S	-	4.2 M	-	$3.7x10^{-1}$ S	$4.0x10^{-1}$ S	1.2 M
08BKT..	MRSP	n/t	$1.3x10^{-1}$ L	-	-	-	-	-	-
HK2	MSSP	n/t	-	$4.2x10^{-1}$ M	$8.7x10^{-3}$ S	$5.1x10^{-1}$ S	$1.9x10^{-5}$ S	$1.6x10^{-1}$ L	$7.1x10^{-2}$ L
HK14	MSSP	n/t	-	-	-	-	-	-	-
Y1	MSSP	n/t	-	$1.2x10^{-5}$ M	-	$4.3x10^{-4}$ S	-	-	-
S56C3	MSSP	n/t	-	-	-	-	-	-	-
S56D2	MSSP	n/t	-	-	-	-	$6.4x10^{-4}$ S	-	-
S56F3	MSSP	n/t	$2.0x10^{-4}$ L	$1.2x10^{-6}$ M	$1.2x10^{-4}$ M	-	$1.8x10^{-5}$ L	$1.4x10^{-4}$ M	$1.4x10^{-5}$ M
S56H7	MSSP	n/t	-	-	-	-	-	-	-
S57E7	MSSP	n/t	$4.3x10^{-5}$ M	$1.2x10^{-4}$ S	$2.5x10^{-4}$ M	-	$1.3x10^{-4}$ L	$6.2x10^{-4}$ M	$7.1x10^{-3}$ M
S60C4	MSSP	n/t	-	-	-	-	-	-	-
S60C6	MSSP	n/t	$6.0x10^{-2}$ S	1.54 M	-	-	$5.5x10^{-1}$ S	$3.7x10^{-3}$ M	$2.9x10^{-1}$ M
S60D6	MSSP	n/t	$2.7x10^{-6}$ S	-	-	-	-	$3.7x10^{-6}$ S	$2.9x10^{-6}$ S
S60D7	MSSP	n/t	-	-	-	-	-	-	-
S61A3	MSSP	n/t	$7.3x10^{-6}$ S	-	-	-	-	-	$2.1x10^{-6}$ S
S61A8	MSSP	n/t	-	-	-	-	-	-	-
S61B7	MSSP	n/t	$1.3x10^{-7}$ S	-	-	$2.7x10^{-5}$ S	-	-	$7.1x10^{-7}$ M
S61D1	MSSP	n/t	-	-	-	-	-	-	-

S. pseudintermedius strains	Methicillin resistance	Sequence Type	Bacteriophages						
			SpT5	SpT152	SpT252	SpT99/F3	SpL8	SpL10:5	SpL11
S61H5	MSSP	n/t	-	-	-	-	-	-	-
S61I9	MSSP	n/t	$4.3x10^{-5}$ S	-	$1.5x10^{-4}$ L	$5.5x10^{-5}$ S	$9.1x10^{-4}$ L	$1.2x10^{-4}$ M	$7.1x10^{-4}$ M
S62A2	MSSP	n/t	-	-	-	-	-	-	-
S63G7	MSSP	n/t	$2.0x10^{-6}$ S	-	-	-	-	-	-
S66E5	MSSP	n/t	$8.4x10^{-4}$ S	-	$8.6x10^{-2}$ S	-	$2.7x10^{-3}$ S	$7.5x10^{-3}$ S	$7.1x10^{-4}$ S
S76G8	MSSP	n/t	-	-	-	-	-	-	-
S76I4	MSSP	n/t	-	-	-	-	-	-	-
JZ22	MSSP	n/t	$1.3x10^{-4}$ S	-	-	-	-	-	-
JZ31	MSSP	n/t	-	-	-	-	-	-	-
JZ56	MSSP	n/t	$3.3x10^{-6}$ S	$2.5x10^{-6}$ S	-	$7.1x10^{-4}$ S	-	$8.7x10^{-5}$ M	$3.6x10^{-5}$ M
JZ133	MSSP	n/t	-	-	-	$6.4x10^{-4}$ L	-	-	-
JZ146	MSSP	n/t	$6.7x10^{-5}$ S	$4.2x10^{-1}$ M	$3.5x10^{-4}$ S	-	-	$2.5x10^{-5}$ L	$6.4x10^{-5}$ L
JZ151	MSSP	n/t	-	-	-	-	-	-	-
JZ152	MSSP	n/t	$2.0x10^{-5}$ L	-	$5.0x10^{-6}$ S	-	-	$1.2x10^{-6}$ M	$7.1x10^{-6}$ M
JZ170	MSSP	n/t	-	-	$1.5x10^{-5}$ M	-	-	$2.5x10^{-6}$ M	-
JZ208	MSSP	n/t	-	-	$6.7x10^{-4}$ M	-	-	$5.0x10^{-4}$ S	$2.1x10^{-5}$ M
JZ220	MSSP	n/t	-	$4.2x10^{-7}$ L	$2.5x10^{-6}$ M	-	-	$5.0x10^{-6}$ M	$7.1x10^{-7}$ M
AB561	MSSP	n/t	$1.5x10^{-2}$ S	$9.2x10^{-3}$ S	-	$7.5x10^{-2}$ S	$2.4x10^{-1}$ S	$5.0x10^{-3}$ S	$3.6x10^{-1}$ M
AB564	MSSP	n/t	$3.3x10^{-3}$ S	$1.2x10^{-2}$ S	-	-	$6.4x10^{-3}$ S	$7.5x10^{-4}$ S	$1.4x10^{-2}$ S
ED99	MSSP	n/t	-	-	-	1.0 M	-	-	-

The host range of the studied phages was narrow (growth on less than half of the library). On the contrary to broad-host range phages such as phage K that can grow on almost all *S. aureus* strains (O'Flaherty *et al.*, 2005), they could not form plaques on all *S. pseudintermedius* strains.

SpT5 had the largest host range and formed plaques on 31 out of 72 strains (43%). Phages SpT252, SpL8, SpL10:5 and SpL11 exhibited the next broadest host ranges and these were similar to each other This suggested that these phages were less diverse than originally thought based on RFLP results. These five phages could form plaques on several ST71 strains at high EOP ($1.0x10^{-1}$ and over) and around half of the MSSP strains at low EOP (less than $1.0x10^{-4}$). No phage growth was observed on any ST68 strains except for phage SpT5 at a very low EOP ($8.0x10^{-7}$) on E122. This further supported the idea that ST68 strains are particularly resistant to phage infection (see section 3.3.3). SpT152 and SpT99/F3 exhibited the narrowest host ranges with growth on 11 (15%) and 8 (11%) out of 72 strains respectively. Their EOPs were from medium ($1.0x10^{-2}$) to low ($1.0x10^{-4}$) value and they could not form plaques on any MRSP strains except for SpT152 on E139.

Three plaque sizes were distinguished and the seven phages exhibited all three of them depending on the host. Most plaques were small or medium-sized and turbid. This was consistent with the fact that the Warwick phages were temperate and suggested once again that the Danish phages might be temperate too.

It was possible that *S. pseudintermedius* contained prophages similar to the tested phages. In what case the narrow host ranges could be explained by the phenomenon of homoimmunity. The use of Vir mutants insensitive to homoimmunity could therefore broaden the spectrum of the selected phages by allowing them to successfully infect lysogens (section 1.4.3).

3.4.4. Whole-genome sequencing

3.4.4.a. Whole-genome sequencing of the Danish phages

While the work presented above was carried out at the University of Warwick, the genomes of SpL8, SpL10:5 and SpL11 were sequenced and annotated by collaborators in Denmark. These three phages were first thought to be lytic because they were isolated from faeces and not through induction of prophages. The annotation of the genomes revealed that all three contained genes associated with lysogeny (integrase gene, *c*I repressor gene, etc.). SpL8, SpL10:5 and SpL11 were therefore likely to be temperate and, were these phages to be selected for phage therapy development, the isolation of Vir mutants became necessary.

3.4.4.b. Whole-genome alignment after sequencing the Warwick phages

The genomes of SpT5, SpT152, SpT252 and SpT99/F3 were sequenced and assembled in Denmark by Dr Witold Kot (section 2.17). They were then annotated at the University of Warwick by Dr Andrew Millard (section 2.18). These temperate phages contained genes typically associated with lysogeny and similar to those of phage λ, such as genes coding for integrases and Cro/CI repressors (see Chapter 6). To compare the seven genomes to each other and study genetic diversity in greater detail, they were aligned using the programme Mauve (Figure 3.5).

Figure 3.5: Whole-genome alignment performed with Mauve. The three Danish phages (SpL8, SpL10:5 and SpL11) were extremely similar. Genomic rearrangements (coloured blocks in different order) and unique (white) regions were seen in the Warwick phages' genomes (SpT5, SpT252, SpT152 and SpT99/F3.). SpT99/F3 was very different from the other six phages.

Mauve outlined regions of the genomes that aligned with parts of other genomes. These regions, circled by coloured blocks, were presumably homologous and internally free of genomic rearrangements. Within these blocks, the programme drew a similarity profile of the genome sequence. The height of the similarity profile corresponded to the average level of conservation in that region of the genome. Some areas were completely white meaning that they were not aligned and were unique to a particular genome. Regions outside the outlined blocks lacked any detectable homology (Darling *et al.*, 2010).

Mauve outlined five coloured blocks (yellow, green, blue, purple and red). Based on this alignment, the Danish phages were genetically very similar to each other with no unique (white) regions visible in their genome. This suggested that genetic diversity of these phages was not as high as the RFLP analysis indicated. Genomic rearrangements could be seen in SpT5, SpT252 and SpT152 where the same coloured blocks were outlined but in a different order. SpT5 was also very similar to the Danish phage genomes apart from genomic rearrangements. No unique regions were seen to differentiate SpT5 from the Danish phages. At this point it is important to note that the presumption that genomic rearrangements took place was based on the assumption that the studied genomes were not circularly permuted (section 1.4.2.a.iv). Bioinformatics analyses were carried out to determine whether the genomes had distinct cohesive ends or were circularly permuted (section 0).

On the contrary to the SpT5 genome, unique regions were visible in the genome of SpT252 and SpT152. For SpT152, this could be expected since this phage was isolated on an MRSP strain different from those used for the isolation of SpT5 and the Danish phages. Regarding the SpT99/F3 genome, Mauve attempted to outline homologous blocks but white regions were numerous within these blocks. A quarter of SpT99/F3 genome lacked any homology with the other genomes (regions outside blocks). This confirmed that this genome was unique among the *S. pseudintermedius* phages isolated in this project. The observation was consistent with the RFLP, PFGE and host range results.

The genome sizes determined through sequencing for SpT5, SpT152, SpT252, SpL8, SpL10:5 and SpL11 were around 40 kb (Table 3.7). They were consistent with the PFGE results. Similarly to the other phages, a single 40-kb long genome was detected and assembled for SpT99/F3. However, PFGE results for this phage suggested that more than one phage might be present in its lysate. Further analysis of the sequencing data was carried out to explore this possibility (section 6.4).

Table 3.7: Whole-genome sequencing revealed that the seven phage genomes were around 40 kilobases long. This was consistent with the PFGE results.

	Bacteriophages						
	SpT5	SpT152	SpT252	SpT99/F3	SpL8	SpL10:5	SpL11
Genome size (kb)	39.8	41.1	40.1	40.7	40.5	39.8	40.1

In conclusion, whole-genome alignment revealed that the Danish phages were virtually all the same phage. They were also very similar to SpT5 and this was consistent with host range results. For these reasons, SpT5 was kept and the Danish phages were set aside. SpT252 was very similar to SpT5 in terms of host range and genome sequence except for a unique region around 21,000 bp so it was kept too. SpT152 and SpT99/F3 were shown to be different from SpT5 and SpT252 both genotypically and phenotypically. They were kept for further work as well.

3.4.5. Determination of the phages' morphology through electron microscopy

The four selected phages were observed in electron microscopy (EM) to determine their morphology at the University of Warwick with Ian Hands-Portman's help (Figure 3.6 and section 2.19).

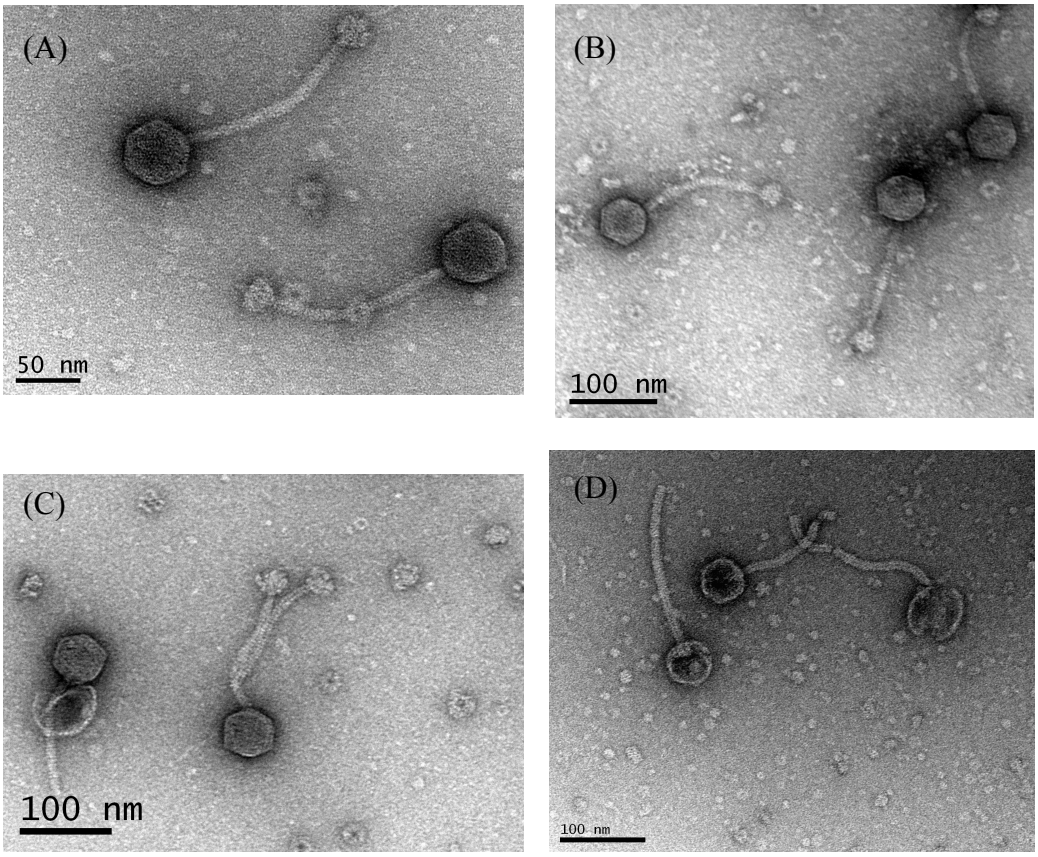


Figure 3.6: the Warwick phages exhibited morphologies similar to that of the *Siphoviridae* family with icosahedral capsids and long tails. (A) SpT5, (B) SpT152, (C) SpT252, (D) SpT99/F3

The four Warwick phages had icosahedral capsids and long tails (more pictures in Appendix B), and they measured between 225 and 275 nm in total (Table 3.8 and section 2.20). This type of morphology was similar to that of the *Siphoviridae* family, a family of bacteriophages characterised by linear genomic DNA, long non-contractile tails, and a total size of around 200 nm (Ackermann, 2006). Importantly all the SpT99/F3 phage particles that were observed in EM appeared to have empty heads (dark centre + white lining along the edge of the capsid) and no baseplate was seen at the tip of their tails. This strongly suggested that the genomic DNA was ejected during sample preparation, displacing the baseplate in the process. SpT99/F3 particles may also be naturally unstable. Indeed, it was noticed that the titre of

SpT99/F3 lysates decreased at a higher rate than lysates of the other three Warwick phages when stored at +4°C for several months. Measurements taken on SpT99/F3 phage particles during this project were therefore not fully representative of the size of viable SpT99/F3 particles.

Table 3.8: The capsid, tail and baseplate of phages SpT5, SpT152 and SpT252 were of similar sizes (in nm). For SpT99/F3, measurements were taken on particles with empty heads and were therefore not fully representative of the size of viable SpT99/F3 particles.

		Bacteriophages			
		SpT5	SpT152	SpT252	SpT99/F3
Size (nm)	Capsid	54.0	60.6	56.9	55.0
	Tail	144.4	143.9	139.3	199.8
	Baseplate	28.4	27.3	31.9	?
	Total length	226.9	231.8	228.1	271.7

SpT5, SpT152 and SpT252 exhibited similar capsid, tail and baseplate sizes. The structure of the baseplate was very different from the thin conical tail tip surrounded by tail fibres of phage λ (Casjens and Hendrix, 2015) (Figure 3.7, A). The comparatively large baseplate of SpT5, SpT152 and SpT252 was similar to that of the lactococcal phage TP901-1, another siphovirus (Spinelli *et al.*, 2014) (Figure 3.7, B). Tail fibres, either side tail fibres like that of λ or a central tail fibre like that of TP901-1, were not seen on the three Warwick phages. The absence of tail fibres has been observed in other *Siphoviridae*, such as the lactococcal phage p2 (Spinelli *et al.*, 2006) (Figure 3.7, C). It was, however, possible that tail fibres were not visible on the three phages because they have fallen off during sample preparation or because the appropriate staining technique was not used. One way to confirm whether this might be the case would be to take pictures of a phage known to have fibres using the same technique and check whether fibres can be seen.

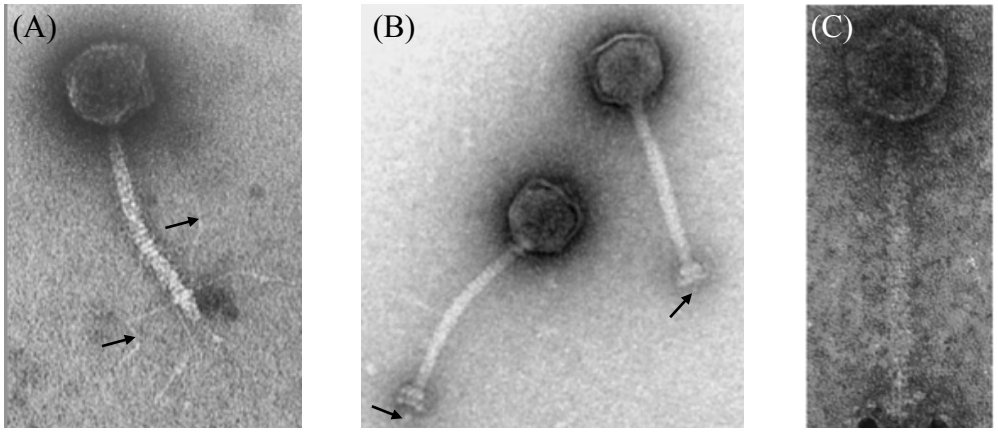


Figure 3.7: (A) Phage λ has a conical tail tip surrounded by side tail fibres, shown with black arrows (picture from Casjens and Hendrix, 2015). (B) The lactococcal phage TP901-1 has a larger baseplate with a short central tail fibre, shown with black arrows (picture from Spinelli *et al.*, 2014). (C) The lactococcal phage p2 has a larger baseplate without tail fibres (picture from Spinelli *et al.*, 2006).

Interestingly, the tail end of *Siphoviridae* phages, which recognises the receptor on the host cell surface, appears to display different morphologies depending on the nature of the receptor, i.e. a protein or carbohydrate. Phages that interact with protein moieties usually possess a thin, pointed end while those that interact with carbohydrate moieties display a much larger baseplate (Mahony and van Sinderen, 2012). This suggested that SpT5, SpT152 and SpT252 recognise carbohydrate moieties on the surface of their host.

It can be shown whether phages have contractile tails by observing them after having incubated them with their host or bacterial membranes. Phages and host/membranes should be incubated long enough so that attachment and DNA injection occurs but not so long that phages lyse their host. Contractile tails will appear shorter once DNA has been injected. Filtered lysates of the Warwick phages were used to generate the above pictures, no bacterial membranes were present. It was therefore not confirmed whether they had non-contractile tails.

3.5. Conclusions

The isolation of lytic *S. pseudintermedius* phages was attempted through screening of dog samples (faeces and skin swabs) and other samples (soil and water). Faecal samples were thought to be a particularly good source because ten phages infecting

this pathogen were successfully isolated in Denmark from this type of samples prior to this PhD project. After many attempts using different types of samples, from different sources, with different versions of protocols, no other phages were found and experiments were discontinued. One possible reason why phages were not found was simply that they were not present in the screened samples. These were chosen on the principle of co-residence between host and phages, but there was no evidence of the presence of *S. pseudintermedius* in the samples. A study of the ecology of *S. pseudintermedius* and its phages was designed to try and understand where the pathogen and its phages might be (Chapter 7).

The isolation of temperate phages through co-culture and mitomycin C exposure was more successful. In total, 28 phages were genotypically and phenotypically characterised. Following characterisation, four temperate phages isolated at the University of Warwick were selected. One of them, phage SpT5, was isolated through co-culture of seven *S. pseudintermedius* strains together and this meant the strain of origin of this phage was not known. Complementary experiments were carried out to identify the SpT5 lysogen (section 7.3.2.c). The ten Danish phages were shown to be probably temperate because they harboured genes typically associated with lysogeny (integrase gene, *c*I repressor gene, etc.).

The fact that all phages isolated in this project were temperate suggested that lysogeny was their preferred lifestyle. This is not unusual for phages infecting pathogens. Numerous phages infecting *S. aureus*, an opportunistic pathogen of the skin, are temperate (Deghorain and Van Melderen, 2012) and no lytic phages of *Clostridium difficile*, a pathogen responsible for nosocomial infections, have been isolated to this day (Hargreaves and Clokie, 2014). Lytic phages are more common with pathogens able to survive and thrive in the environment such as *Vibrio cholera* (Faruque *et al.*, 2005). *S. pseudintermedius* may occupy a small ecological niche restricted to the skin of dogs. If a bacterial pathogen is limited to replication associated with its animal host, the chance of a pathogen and a lytic phage encountering the animal host at the same time is limited. Lysogeny may be the best strategy for *S. pseudintermedius* phages to closely follow their bacterial host.

The presence of *S. pseudintermedius* temperate phages in the faecal samples screened in Denmark suggested that the host was present in these particular samples. This may have resulted from contamination with bacteria from the perineum, one of the carriage sites of *S. pseudintermedius* on dogs (Bannoehr and Guardabassi, 2012). It is important to note that the faecal samples from which the Danish phages were isolated were collected from a veterinary school where a number of pyoderma cases were handled every day. It is therefore possible that the prevalence of *S. pseudintermedius* was higher in that context and that contamination of faeces was likely to occur. For practical reasons most of the faecal samples analysed at the University of Warwick came from dogs not known to have been in contact with *S. pseudintermedius*. This may explain why no phages were found in these samples. However, the screening of faecal samples collected from the dermatology service of the Bristol veterinary school where pyoderma cases were handled did not lead to the isolation of lytic or temperate phages either. The presence of *S. pseudintermedius* and its phages in faeces may therefore be only occasional, even in an environment where the bacterium is likely to be more prevalent.

Whole-genome sequencing revealed that the Danish and Warwick phages (apart from SpT99/F3) were strikingly similar on the genetic level. For this reason the ten Danish phages were not kept for further work. It was interesting to note that, similarly to the Warwick phages, the Danish phages belonged to the family of *Siphoviridae* (Nälgård, 2011). This type might be the most common *S. pseudintermedius* phages. Other types may exist. A study showed that in *Clostridium difficile* different antibiotics (mitomycin C and norfloxacin) induced different families of phages (*Siphoviridae* or *Myoviridae*) (Nale *et al.*, 2012). This principle could be applied to *S. pseudintermedius* to isolate other families of phages.

The four Warwick temperate phages that were selected for further work after characterisation exhibited similar characteristics (Table 3.9).

Table 3.9: The four Warwick temperate phages exhibited similar characteristics: they belonged to the same phage family, and had similar phage particle and genomes sizes. For SpT99/F3, measurements were taken on particles with empty heads and were therefore not fully representative of the size of viable SpT99/F3 particles.

		Bacteriophages			
		SpT5	SpT152	SpT252	SpT99/F3
Phage family		*Siphoviridae*	*Siphoviridae*	*Siphoviridae*	*Siphoviridae*
Size (nm)	Capsid	54.0	60.6	56.9	55.0
	Tail	144.4	143.9	139.3	199.8
	Baseplate	28.4	27.3	31.9	?
	Total length	226.9	231.8	228.1	271.7
Genome size (kb)		39.8	41.1	40.1	40.7

The host range of these phages was narrow and none of them could infect ST68 *S. pseudintermedius* strains, one of the most common clones found in the USA. Added to the fact that they were temperate, these phages could not be considered ideal for phage therapy. As mentioned in section 1.2.4, it is becoming more and more accepted within the phage community and by regulatory authorities such as the Food and Drug Administration (FDA) that phage engineering is one of the ways forward to develop phage therapy because the perfect phage for one application may not exist in nature (Hodyra and Dabrowska, 2015). Based on this principle, mutagenesis approaches were used to isolate lytic mutants of the Warwick phages suitable for phage therapy (Chapters 4 and 5).

Acknowledgements

The Danish phages were isolated by Sofia Nälgård under the supervision of Prof. Luca Guardabassi (University of Copenhagen). Their genomes were sequenced and annotated by Dr Witold Kot and Dr Arshnee Moodley (University of Copenhagen). Samples (faeces, skin swabs and wastewater) used in this work were collected thanks to Sophie Bailey (Kenilworth Avonvale veterinary practice), Simon Davies (Warwick Avonvale veterinary practice), Paul Sands (Pride veterinary centre, Derby), Tristan Cogan (Bristol veterinary school), Lene Boysen (dermatology practice, Copenhagen), Dr Gregory Amos (University of Warwick), Dan Powell (Coventry City Council) and the Castledean boarding kennel's staff.

Chapter 4 Phage random mutagenesis for the isolation of Vir mutants

4.1. Introduction

Following isolation and initial characterisation, four phage candidates SpT5, SpT152, SpT252 and SpT99/F3 were kept for the development of phage therapy against *S. pseudintermedius*. These phages could not be used directly because they were temperate and only lytic phages are considered appropriate for phage therapy. To overcome this problem, it was decided to modify the selected phages through mutagenesis approaches. The objective was to isolate Vir mutants because of the advantages associated with the use of such mutants, e.g. the ability to overcome homoimmunity (sections 1.4.3 and 3.4.3). This property translates into the ability to form plaques on a lysogen that contains a similar prophage. The screening process for Vir mutants was based on this property and consisted in cultivating mutagenized phages on their respective lysogen. Having the lysogen of each phage was therefore necessary for these experiments. In the case of SpT5 the strain of origin (or lysogen) was not known because it was thought to have originated from a mix of bacterial strains (section 3.3.1). A new lysogen of SpT5 was isolated during the PhD project.

Two types of mutagenesis approaches were considered: random or site-directed mutagenesis. Random mutagenesis was attempted first because even though precise phage engineering is becoming more and more accepted, the commercialisation of GMOs may still be difficult particularly in Europe. Random mutagenesis methods were expected to produce mutants that should not qualify as GMOs because they could be isolated in nature and do not require the introduction of foreign DNA in their genome.

Two types of random mutagenesis methods were tested:

- The exposure of phage particles to hydroxylamine, a chemical that induces random mutations in the DNA (Davis *et al.*, 1980). It can usually penetrate the capsid of phages and induce mutations within 24 to 48 hours.
- The exposure of phage particles to ultraviolet (UV) light. It can induce mutations in the DNA such as pyrimidine dimers (Goodsell, 2001, Tropp, 2012) and has been used to mutagenize phage particles (Krwawicz *et al.*,

2003, Drake, 1966). Mutations are usually induced within a few tens of seconds.

In both cases the time necessary to reach 99.9% killing of phage particles, corresponding to a drop in titre by a factor of 10^3 PFU/mL, was determined. The idea that reaching 99.9% killing ensures optimal mutagenesis was discussed by Hopwood (Hopwood *et al.*, 1985) in the context of his work on *Streptomyces* and since became a general consensus when performing mutagenesis.

After exposure to the mutagen, mutations, that were likely to have occurred only on one strand, were expressed by cultivating mutagenized phages on a permissive host (the bacterial strain used to cultivate a given phage). This way, mutations would be stably integrated into the phage genome through DNA replication in the host (Griffiths *et al.*, 1999). Amplifying phages on plates at this step was too labour-intensive (too many plates needed) and could also be limiting in terms of phage titre. Amplification in liquid culture was preferred for this step because it might yield higher phage titres and increase the chances of finding a mutant. Optimisation was required to determine the optimal conditions for phage cultivation in liquid medium. Indeed, the efficiency of phage amplification depends on characteristics unique to a phage or group of similar phages. The adsorption rate (how quickly phages find and attach to host cells), the burst size (the number of phage particles produced during lysis) and the MOI are examples of such characteristics that can influence how efficiently phages amplify (Weld *et al.*, 2004). It may also happen that amplification in liquid culture remains impossible with some phages (Prof. David Hodgson, personal communication).

A third approach based on the ability of phages to evolve quickly was tested. This method called serial or multiple passaging was performed in the hope that a Vir mutant may arise spontaneously when sequentially amplifying phages on the same host. This principle has been used previously to isolate mutants with characteristics, e.g. host range, different from the original phage (Turner and Chao, 1998, Betts *et al.*, 2013). Once again, optimisation was needed to determine the best conditions for successful sequential amplification.

4.2. Isolating and confirming lysogens for use in screening for Vir mutants

As mentioned in section 4.1, lysogens were necessary for screening for Vir mutants after random mutagenesis. A lysogen of SpT5 was not directly available and a new one was isolated. This was done by scratching the centre of several SpT5 plaques cultivated on E045 (its cultivating strain) with a thin needle and re-streaking each obtained colony three times to ensure clonality. The lysogen of SpT5 was identified according to two criteria: resistance to lysogenised phage and production of phages when cultivated in liquid culture (Figure 4.1). One out of three putative lysogens could grow in presence of SpT5 indicating that it was likely to be a lysogen. This was further confirmed by the fact that the supernatant obtained after overnight culture of this strain could form plaques on a permissive host showing the production of phages in liquid culture. The identified lysogen was called E045 lys SpT5 ('lys' for 'lysogen').

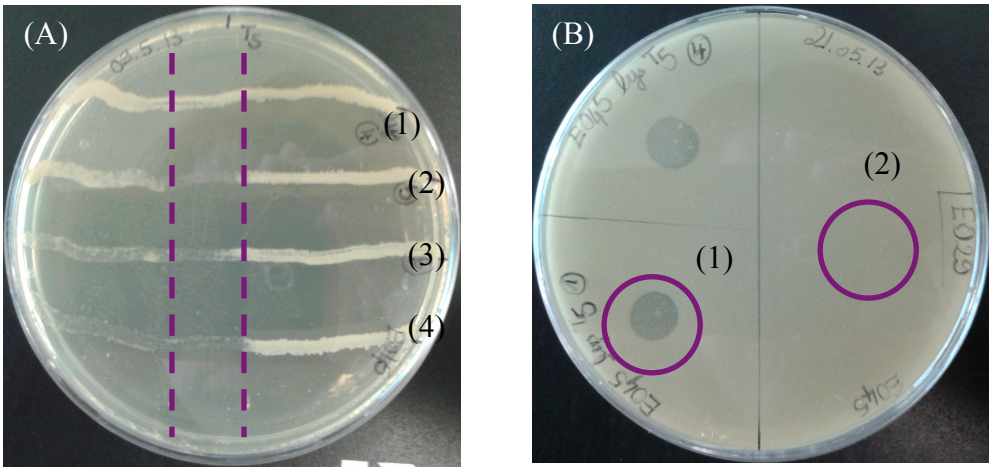


Figure 4.1: (A) Strain (1) was likely to be a lysogen of SpT5 because it could grow in presence of the phage (streaked vertically between the two dashed lines). Strains (2) and (3) could not grow in presence of the phage and were not lysogens. The non-lysogenized host (4) was used as a control. (B) The supernatant of an overnight culture of the lysogen (1) formed plaques when spotted on a sensitive host (E029). This illustrated the production of phages during overnight incubation. The non-lysogenized host (2) did not form plaques.

In the case of SpT152, SpT252 and SpT99/F3 the strain of origin was known. Primers specific to each phage were designed and used to check that the phages' strains of origin were real lysogens (Figure 4.2). Amplification could be seen on phage DNA and their corresponding lysogens confirming the lysogenic state of the bacterial strains. Once this was confirmed, phage mutagenesis for the isolation of Vir mutants was attempted.

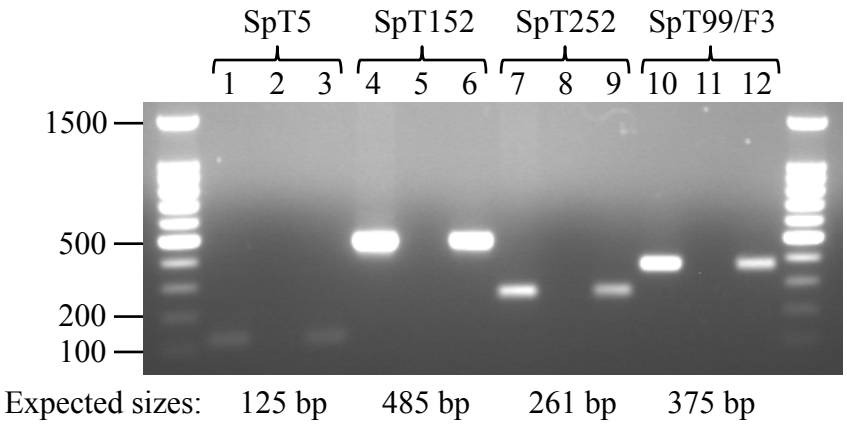


Figure 4.2: PCR amplification was seen on each phage DNA (first lane) and their corresponding lysogens (third lane) when using primers specific to each phage genome. This confirmed the lysogenic state of the bacterial strains. 1: SpT5, 2: E045, 3: E045 lys SpT5, 4: SpT152, 5: E139, 6: JZ152, 7: SpT252, 8: E045, 9: AB252, 10: SpT99/F3, 11: ED99, 12: S56F3.

4.3. Phage mutagenesis through exposure to hydroxylamine

4.3.1. Killing curve

The time necessary to reach the 99.9% killing point (drop in titre by a factor of 10^3 PFU/mL) when exposing phage particles to hydroxylamine was determined (section 2.25). This parameter was measured for the four phages (Figure 4.3).

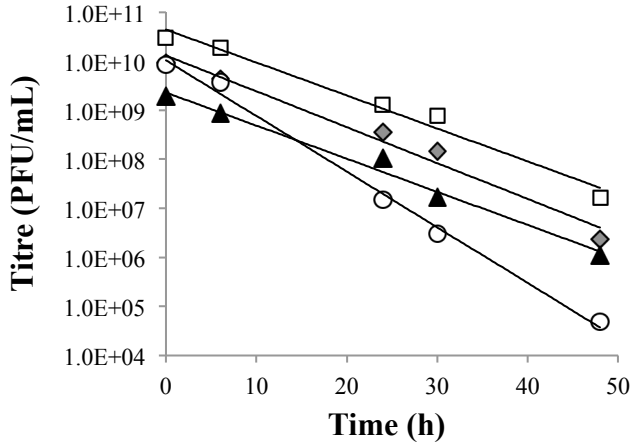


Figure 4.3: 26 hours for SpT99/F3 (circles) and 38 hours for SpT5 (squares), SpT252 (diamonds) and SpT152 (triangles) were necessary to reach 99.9% killing when exposing phage particles to hydroxylamine (Y axis in log10 scale).

It was determined that a 26-hour exposure for SpT99/F3 and a 38-hour exposure for the other phages were necessary to reach 99.9% killing. After exposure to the mutagen, the induced mutations were expressed by cultivating phage particles on a permissive host (the bacterial strain used to cultivate a given phage). A protocol was optimised to achieve successful phage amplification in liquid medium.

4.3.2. Phage amplification after mutagenesis for expression of mutations

4.3.2.a. Bacterial hosts' growth curves

Growth curves of the relevant bacterial hosts were determined (section 2.3) as supporting information for the design of phage amplification protocols in liquid medium. To be able to show bacterial growth in log(number of cells) as a function of time (instead of OD_{600nm} as a function of time), the correspondence between OD_{600nm} and CFU/mL was determined (Figure 4.4).

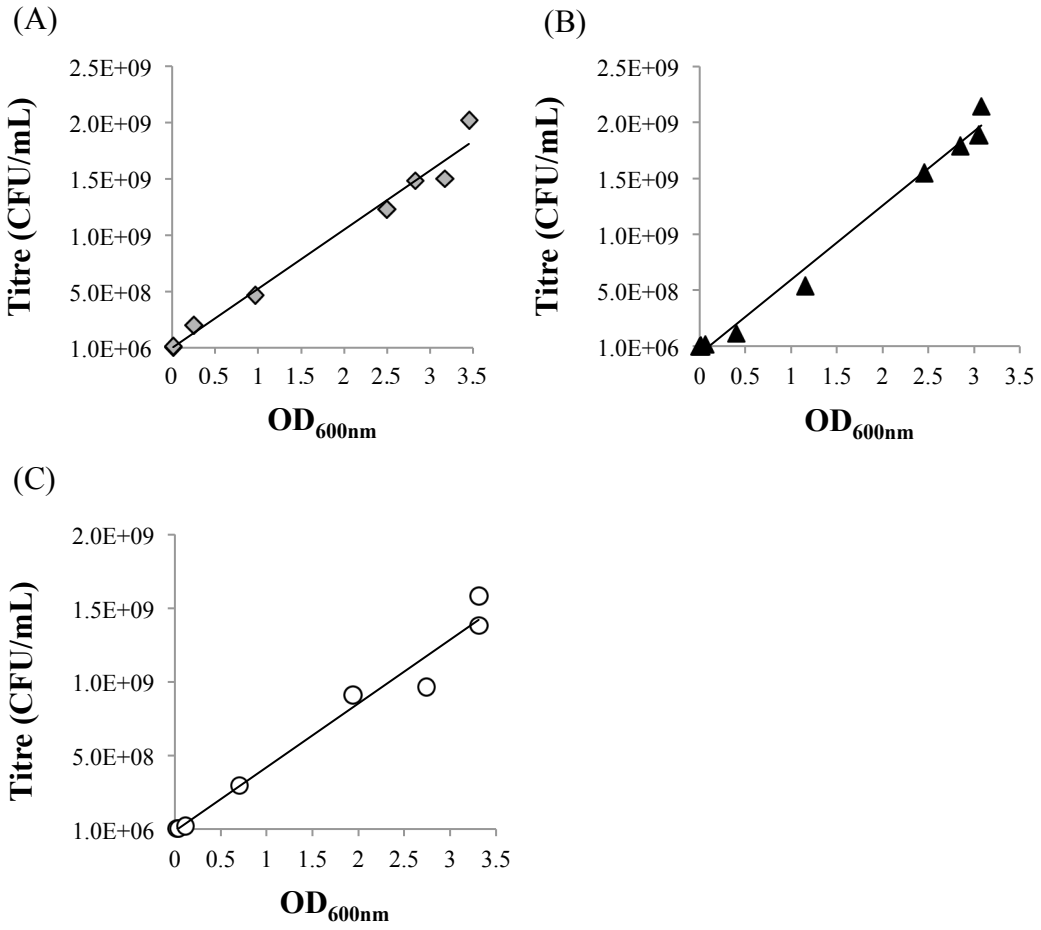


Figure 4.4: The correspondence between OD_{600nm} and CFU/mL was determined for the bacterial strains (A) E045, (B) E139 and (C) ED99. A linear relation was observed between both parameters.

A linear relation was observed between the number of CFU/mL and OD_{600nm} for values up to 3.5. However, to keep measured OD_{600nm} values within the linearity range defined by the Beer-Lambert law (from 0.1 to 1.0), samples were diluted when necessary before performing OD measurement. The obtained curves provided a rapid way to calculate the number of cells in a given volume of culture after measuring OD_{600nm}. This was useful when determining the growth curves of the relevant bacterial hosts (Figure 4.5) and when testing different ratios of phage particles and bacterial cells during optimisation of the phage amplification protocol.

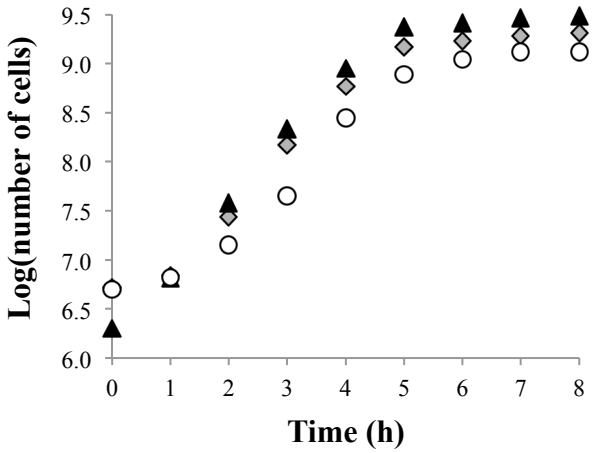


Figure 4.5: Growth curves of *S. pseudintermedius* strains E045 (diamonds), E139 (triangles) and ED99 (circles) at 37°C. Cells were at mid-exponential phase around three hours of cultivation, which corresponded to an OD_{600nm} value of 0.3.

For the three tested bacterial hosts, cells were found to be at mid-exponential phase around three hours of cultivation, which corresponded to an OD_{600nm} value of 0.3.

4.3.2.b. Pre-attachment phase and first attempts at phage amplification

To develop the phage amplification protocol, SpT5 was used as a model before applying the optimised protocol to other phages. These were similar to SpT5 (except SpT99/F3) so their optimal culture conditions should be similar. First, it was decided to introduce a pre-attachment phase in the protocol (section 2.24) where phages and cells were mixed together at an MOI of 1:1 in a small volume of medium (1 mL), allowing phages to attach to the cells before amplification. It was hoped that this would maximise phage infection and help make phage amplification successful. Two types of cells were tested: cells at mid-exponential phase and stationary phase, because the receptor used by phages to attach to the cells might be expressed at a specific time during cell growth (Figure 4.6).

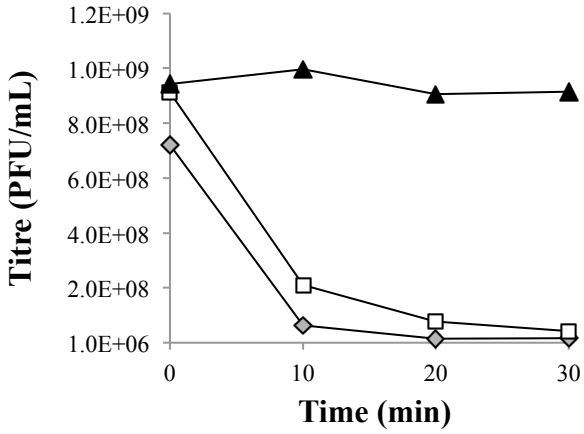


Figure 4.6: Kinetics of pre-attachment of SpT5 phage particles to bacterial cells (E045): no cells (triangles), with cells at mid-exponential phase (diamonds), with cells in stationary phase (squares). No difference was observed when using cells in exponential or stationary phase.

No difference was observed when using cells at mid-exponential or stationary phase, so it was decided to use cells at mid-exponential phase from then on. Ten to fifteen minutes of pre-attachment were enough for the initial phage titre to drop to less than 10% free phage particles. It was also determined that six hours were necessary for the phages to be amplified to a high titre (from 10^4-10^5 to 10^8 PFU/mL) on a permissive host after pre-attachment (Figure 4.7).

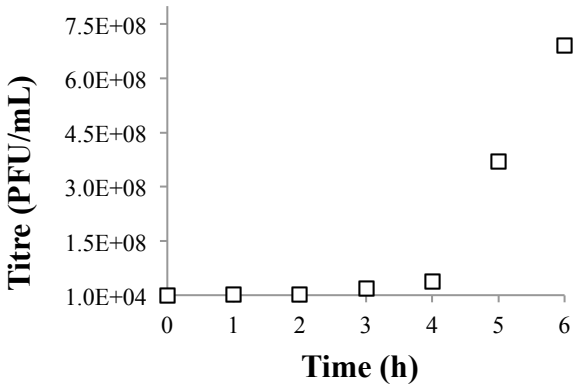


Figure 4.7: Successful amplification of phage SpT5 (from 10^4-10^5 to 10^8 PFU/mL) was achieved within six hours of incubation at 37°C with shaking, following a pre-attachment step at an MOI of 1:1 and the addition of BHI up to 5 mL final volume.

Based on the data presented above, the following protocol was used to mutagenize SpT5 phages: 10^8-10^9 PFU/mL were exposed to hydroxylamine (final volume: 1 mL,

see section 2.25). After a 38-hour exposure, the mutagenized phages, whose number had dropped to 10^5-10^6 PFU/mL, were dialysed to remove the remaining hydroxylamine. Pre-attachment was performed as described above (cells at mid-exponential phase + phages, at MOI 1:1, in 1 mL BHI) and phages were amplified over six hours at 37°C with shaking following the addition of BHI up to 5 mL final volume. Following amplification, the culture's supernatant was concentrated on an Amicon filter (section 2.23) and plated with the lysogen (300 µL phages on one plate – despite the high number of phages on one plate, bacterial growth was uniform indicating that lysis from without did not occur). After three attempts with this version of the protocol, no Vir mutants were isolated and the protocol further optimised.

4.3.2.c. Further optimisation of the protocol

It was hypothesised that the initial number of phage particles exposed to hydroxylamine (10^8-10^9 PFU) was too low and was reducing chances to obtain the right mutation leading to a virulent phenotype. To obtain lysates with very high titre, a protocol for the propagation of SpT5 in liquid culture involving overnight amplification in 25 mL BHI followed by 10x concentration on Amicon filters was developed (sections 2.22 and 2.23). MOI from 1:10 to 1:1000 were tested and it was found that when using an MOI of 1:1000 followed by 10x concentration, a titre of up to 10^{12} PFU/mL was obtained.

Mutagenesis with hydroxylamine was repeated using concentrated lysates: 10^{10}-10^{11} PFU/mL were exposed to hydroxylamine. After a 38-hour exposure, the titre of mutagenized phages had dropped to 10^7-10^8 PFU/mL and dialysis was performed. Phages were then amplified overnight by adding them to bacterial cells at mid-exponential phase at an MOI of 1:1 in BHI (final volume: 25 mL). A pre-attachment step was not performed in this version of the protocol because phages were left in contact with bacteria overnight. Following amplification, the culture's supernatant was concentrated and plated with the lysogen (300 µL concentrated phages on one plate). After three attempts, no Vir mutant of SpT5 was isolated. The same results were obtained with SpT252 and SpT152.

In the case of SpT99/F3, the amplification step was performed on plates as no amplification was obtained in liquid culture. For this, following hydroxylamine exposure 1 mL of mutagenized phages was divided into four times 250 µL that were plated with the SpT99/F3 bacterial host (ED99, four plates in total). Amplified phages were recovered after overnight incubation through plate wash. After three attempts, no Vir mutant of SpT99/F3 was isolated. It was decided to test other random mutagenesis approaches for the isolation of Vir mutants.

4.4. Phage mutagenesis through exposure to UV light

4.4.1. Killing curve

Similarly to mutagenesis with hydroxylamine, the time it takes to reach the 99.9% killing point was determined for each phage (section 2.26). It was found that exposure to UV light (253.7 nm, 0.707 mW/cm^2) for fifteen seconds for SpT99/F3 and for twenty seconds for the other phages was necessary to reach 99.9% killing (Figure 4.8).

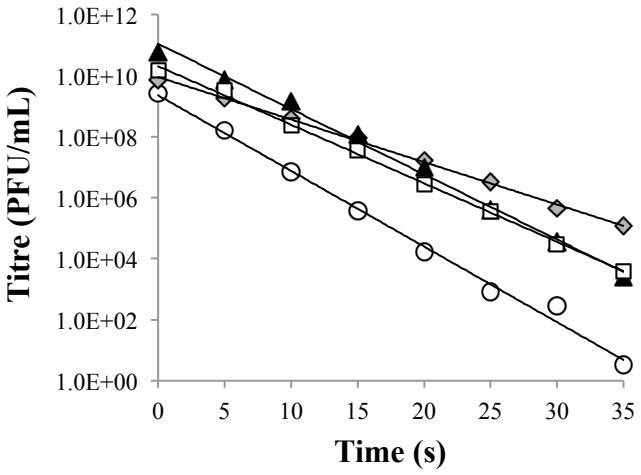


Figure 4.8: Exposure to UV light for fifteen seconds for SpT99/F3 (circles) and for twenty seconds for SpT5 (squares), SpT252 (diamonds) and SpT152 (triangles) was necessary to reach 99.9% killing (Y axis in log10 scale).

4.4.2. Phage amplification after mutagenesis for expression of mutations

Similarly to the protocol for hydroxylamine mutagenesis, mutations were expressed by cultivating mutagenized phages on a permissive host and the protocol was optimised using phage SpT5 as a model. Amplification after UV light exposure was

first attempted by mixing UV-irradiated phages (initial number: 10^{10}-10^{11} PFU/mL, final volume: 5 mL, see section 2.26) with bacterial cells at mid-exponential phase at an MOI of 1:1 in BHI (final volume: 25 mL) and incubating overnight at 37°C with shaking straight away. This resulted in poor phage amplification (titre remained around 10^5 PFU/mL following exposure to UV light and amplification).

The protocol was repeated with a pre-attachment step in a smaller volume of media: 5 mL of UV-irradiated phages (10^{10}-10^{11} PFU/mL) were mixed at an MOI of 1:1 with bacterial cells at mid-exponential phase that had been re-suspended in 3 mL BHI. The mixture was left at room temperature for fifteen minutes, then it was diluted with BHI (final volume: 25 mL) and incubated overnight at 37°C with shaking. With this methiod phage amplification was much better (from 10^5 to 10^{10} PFU/mL). Following amplification, the culture's supernatant was concentrated and plated with the lysogen (300 μL concentrated phages on one plate). The optimised protocol was tested three times and no Vir mutant of SpT5 was isolated. The same results were obtained with SpT252 and SpT152.

In the case of SpT99/F3, the amplification step was performed on plates. For this, following UV-light exposure 5 mL of UV-irradiated phages were divided into twenty times 250 μL that were plated with the SpT99/F3 bacterial host (ED99, twenty plates in total). Amplified phages were recovered after overnight incubation through plate wash. After three attempts, no Vir mutant of SpT99/F3 was isolated.

4.5. Phage evolution through serial passaging on a permissive host

The isolation of virulent mutants was attempted through natural phage evolution by cultivating phages multiple times on a permissive host (Figure 4.9 and section 2.27).

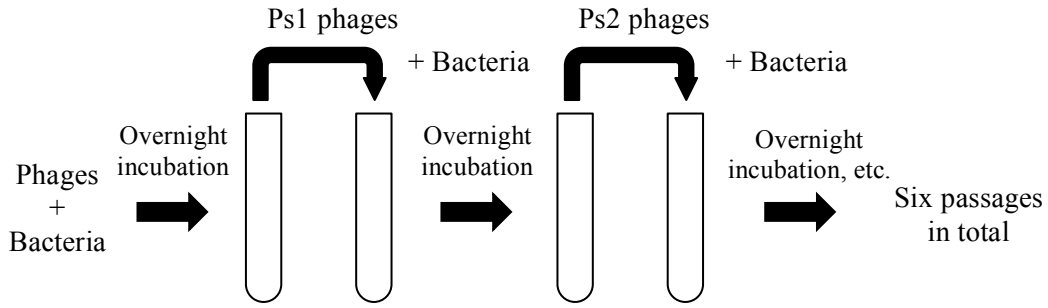


Figure 4.9: During the serial passaging experiment, the ancestral phages were mixed with bacteria and incubated overnight. Phages from this culture (Ps1 phages = passage 1 phages) were mixed with bacteria and incubated overnight. The process was repeated six times. At each step, the titre was checked and phages were added at an adapted MOI to naïve bacteria (never exposed to phages). The lysate resulting from each step was plated onto the lysogen to look for virulent mutants.

To ensure that satisfactory phage amplification occurred at each passage (from 10^6 to 10^{10} PFU/mL), phages (10^6 PFU/mL) were added to bacterial cells at mid-exponential phase at a range of MOIs (1:50, 1:100, 1:500 and 1:1000). The phage titre of each lysate was checked before performing the next passage. The lysate with the highest titre was used for the next step. Interestingly, phage SpT152 was found to generally better propagate at an MOI of 1:100 while SpT5 and SpT252 were successfully amplified at an MOI of 1:1000. After serial amplification the lysate resulting from each step and with the highest titre (six lysates for each phage) was concentrated and plated with the lysogen to look for Vir mutants (300 µL concentrated phages on one plate). The protocol was tested with SpT5, SpT152 and SpT252 but the isolation of Vir mutants remained unsuccessful. The protocol was not tested with SpT99/F3 as it involved phage amplification in liquid culture.

4.6. Conclusions

The isolation and characterisation of *S. pseudintermedius* phages led to the selection of four temperate phage candidates for the development therapy. The modification of these phages through random mutagenesis was attempted with the objective to isolate Vir mutants suitable for phage therapy. First, phage particles were exposed to

hydroxylamine, a chemical mutagen. This random mutagenesis method had been used successfully before to isolate Vir mutants of *S. aureus* phages (Rapson, 2002). In this project and in spite of extensive optimisation, it did not lead to the production of virulent mutants. Exposure to UV light did not lead to the isolation of Vir mutants either. The last method to be tested was multiple passaging of phages on a naïve host. This approach was based on the natural ability of phages to evolve quickly but once again the isolation of Vir mutants remained unsuccessful.

When exposing phage particles to hydroxylamine or UV light, killing was observed indicating that mutagenesis did occur and some of the induced mutations were lethal. It is possible that mutations that would theoretically lead to a virulent phenotype were also lethal, e.g. they led to the modification of an essential gene. The region that is mutated in lambdoid Vir mutants, the operator, is often situated between the promoters of the *c*I and *cro* genes (Ptashne *et al.*, 1980). For this reason, it was not expected that mutations in the operator in *S. pseudintermedius* temperate phages would be lethal. However, the location of the operator sequence(s) might be different in *S. pseudintermedius* phages. This possibility was further explored in Chapter 5.

Random mutagenesis could be attempted with other mutagens. The illumination of T4 phages in presence of dyes such as acridine orange and proflavine was used to produce mutants with different plaque morphologies (Ritchie, 1965, Spikes, 1968). The exposure of phage particles to 5-bromouracil during prophage replication or lytic growth was shown to cause DNA-base sequence changes in phage λ (Skopek and Hutchinson, 1982). It would also be possible to select deletion mutants using a heat-EDTA treatment step (Graham *et al.*, 1979, Sternberg *et al.*, 1979, Yamamoto *et al.*, 1968). In that case, the obtained mutants would not result from the introduction of point mutations in the genome but the deletion of whole regions of the DNA. The experiment is therefore more likely to produce mutants like clear-plaque mutants in which the *c*I gene is deleted. Mutants also lacking the operator might occur and would correspond to the type of virulent phages suitable for phage therapy.

Chapter 5 Study of the SpT5 operator region and CI repressor: towards site-directed mutagenesis for the isolation of Vir mutants

5.1. Introduction

Following the attempts at isolating lytic mutants of the four temperate phages through random mutagenesis approaches and the absence of success, it was decided to try site-directed mutagenesis instead. The advantage of this method was that it was possible to precisely choose the location of the mutations to introduce in the temperate phages to produce Vir mutants. It was also expected that using this strategy would generate interesting data about the general characteristics of the CI repressor and its binding site(s) in the phage genomes.

The first step towards site-directed mutagenesis was to find the CI repressor binding site(s), also called operator, within the phage genomes. As described in section 1.4.2.b.iii, phage λ has two operators composed of three binding sites O_{R1}, O_{R2} and O_{R3}. All three have elements of two-fold rotational symmetry (Maniatis *et al.*, 1975). These elements of symmetry, appearing as palindromes (or inverted repeats), have been identified in the operator sites of other temperate phages. Three palindromic operator sites O_L, O_R and O_D recognised by the CI repressor were found in the genome of the lactococcal temperate phage TP901-1 (Johansen *et al.*, 2003). Two fifteen-base inverted repeats with partial two-fold symmetry and the ability to bind the CI repressor were also identified in the *S. aureus* temperate phage ϕ11 (Ganguly *et al.*, 2009). In both cases and similarly to phage λ the operator sites were situated in the *c*I-*cro* intergenic region. Based on the assumption that operator sites would exhibit similar characteristics in *S. pseudintermedius* phages, sequence analysis of the *c*I-*cro* intergenic region was performed to find potential CI repressor binding sites. A single palindromic sequence was identified in the genome of phage SpT5.

This putative operator region was further studied through Electrophoretic Mobility Shift Assays (EMSA) to confirm whether it was an actual operator. EMSA are based on the ability of DNA-binding proteins such as the CI repressor to specifically recognise and bind to their target DNA sequence. In these assays, a piece of DNA containing the target sequence undergoes migration on a native PolyAcrylamide Gel Electrophoresis (PAGE) in presence or absence of the binding protein. When the

protein binds to the DNA, the molecular complex is much bigger than DNA alone. This slows down the migration and the migration pattern appears shifted upwards compared to free DNA (Brunelle *et al.*, 1985) (Figure 5.1).

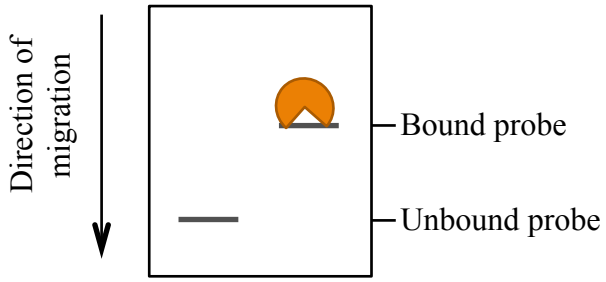


Figure 5.1: When a protein (orange)-DNA (grey) complex migrates on a native PAGE, its migration pattern appears shifted upwards compared to free DNA. This property is used in EMSA to study the ability of proteins to bind to DNA.

Purified proteins (Hendrickson and Schleif, 1985) or crude cell lysates (Ken and Hackett, 1991) can be used. To see the result, different DNA labelling methods are available: with fluorescence (Ruscher *et al.*, 2000), with biotin (Funabashi *et al.*, 2007), or with radioactive phosphate (^{32}P) which remains the most sensitive approach to this day. Radioactive labelling was chosen for the work described in this chapter. EMSA was first performed to test for specific binding between a DNA probe that contained the non-modified SpT5 putative operator sequence and lysogen cell lysate that hypothetically contained the SpT5 CI repressor. If specific binding occurred, it would indicate that the identified palindrome was an operator. Point mutations were then introduced in the potential operator and EMSA was carried out to determine whether binding occurred with the mutated probes. If binding was abolished, the corresponding mutation was considered a good candidate for site-directed mutagenesis.

Cell lysates were used in the EMSA experiments. It was therefore not possible to be certain that the protein responsible for the interaction was the SpT5 CI repressor. To confirm this, the SpT5 *c*I repressor gene was cloned and expressed in *Escherichia coli*. The gene was amplified through PCR and cloned into a plasmid vector. One important characteristic of a plasmid vector is the number of copies produced through replication in one host cell. It depends on the type of ORI (replication origin)

and leads to the classification of plasmid vectors into two categories: high copy number and low copy number plasmids (del Solar *et al.*, 1998). If the objective is to make lots of copies of a vector with insert, usually without expressing the gene (i.e. subcloning), a high copy number plasmid such as the pGEM series (500-700 copies per cell) can be used. When the objective is to express a recombinant protein, using a high copy number plasmid may lead to toxically high levels of recombinant protein in the cell. A low copy number plasmid such as the pET series (15-60 copies per cell) is more adapted (Rosano and Ceccarelli, 2014).

Once the DNA fragment was inserted into the vector, it was introduced into competent *E. coli* cells through heat-shock transformation (Froger and Hall, 2007). Marker genes such as antibiotic resistance genes (Padmanabhan *et al.*, 2011) or blue-white selection (Sambrook *et al.*, 1989a) were used to select for cells that were successfully transformed.

To express the cloned gene a pET vector containing the T7 promoter system was used. In this system, the gene of interest is cloned behind a promoter recognised by the phage T7 RNA polymerase (RNAP). The T7 RNAP gene is usually carried in the bacterial genome in a prophage (λDE3), under the control of the *lac*UV5 promoter that is induced by isopropyl β-D-1-thiogalactopyranoside (IPTG). Leaky expression of the T7 RNAP without IPTG induction may occur. Expressing the T7 lysozyme, provided by a plasmid (pLysS), helps reduce this. In absence of IPTG, the lysozyme binds to the RNAP and inhibits its activity. After IPTG induction, the level of T7 RNAP produced is too high to be inhibited (Rosano and Ceccarelli, 2014).

After cloning the SpT5 *c*I repressor gene, cell lysates of cultures expressing or not the repressor were used in EMSA to test for the ability of the recombinant protein to bind to the SpT5 operator sequence.

Finally, the tertiary structure of the SpT5 CI repressor protein was studied in more details to see how its DNA-binding ability translated on a structural level. The examination of protein structure is performed through Nuclear Magnetic Resonance (NMR) (Wüthrich, 2001) or crystallography (Smyth and Martin, 2000). Resolved and published protein structures are deposited in the Protein Data Bank (PDB). It is

sometimes not possible to observe a protein directly; it may for example not be possible to produce suitable crystals for crystallography or producing crystals may be too time-consuming. An alternative to direct examination of a protein is computational structure prediction. Currently, the most reliable way to predict protein structure is to use template-based modelling in which the tertiary structure of amino acid sequences is determined by using the similarity between the protein sequence of interest and other proteins whose tertiary structure is known. Several online tools such as those available on the Phyre2 web portal use this approach (Kelley *et al.*, 2015).

The 3D structure of proteins is directly linked to their function and structure comparison of two or more proteins might help highlight conserved domains with similar functions. This is particularly useful for the classification of new structures with unknown functions (Jones and Thornton, 2004). Structure comparison is even possible between proteins that have little amino acid sequence similarity because evolution conserves protein structure more than protein sequence (Marti-Renom *et al.*, 2009).

The tertiary structure of the SpT5 CI repressor protein was predicted from its amino acid sequence with the Phyre2 web portal. The predicted structure was compared with the crystal structures of the phage λ and phage TP901-1 CI repressors to look for conserved domains characteristic of this type of proteins.

5.2. Identification of a potential operator region in the genome of phage SpT5

Whole-genome sequencing and genome annotation of phage SpT5 revealed the presence of a *c*I-*cro* intergenic region similar to that of phage λ with *c*I and *cro* repressor genes (Figure 5.2). The presence of Shine-Dalgarno sequences from six to eight nucleotides upstream of the predicted start codons confirmed that these were likely to be real start codons. A palindrome (also called inverted repeat) was identified through sequence analysis within the SpT5 *cro* repressor gene. A spacer constituted of four adenosines was present between the two halves of the palindrome.

Phages often have more than one operator sites but in the case of SpT5 only the one palindrome was found. It was interesting to see that the putative operator was situated within the *cro* repressor gene because it might explain the lack of success of random mutagenesis approaches. The *cro* repressor gene is essential for entering the lytic cycle and only a few precise mutations could change the sequence of the operator without disrupting the coding sequence of the gene. This probably lowered the chances of obtaining the right mutation though random mutagenesis.

5.3. Testing protein binding to the putative operator sequence

To test whether the identified palindrome was an operator, EMSA were carried out (sections 2.28, 2.29, 2.30 and 2.31) using two 100-bp DNA probes: SpT5_op contained the putative operator sequence and SpT5_mir contained a mirror image of the same operator (Figure 5.3 and Table 2.3). In SpT5_mir, the sequence of the operator remained the same because of its symmetrical characteristics but it was reverted relative to the rest of the DNA sequence. This changed the nucleotides present on either side of the palindrome and may have an impact on protein binding if nucleotides other than those in the operator were involved in the protein-DNA interaction.

*c*I repressor gene SD

TTTCATCATTGCTTCTCATTTTAAGTATCTCCTGTGTATAAAATATTTGTTCTTATTTGTGAACAATTAGATTATAACATCGTTCCCAAAT

SD Met Leu Arg Tyr

GAATACAATATATCAGAAGAAAAAAACTTTATGATTTTTTTAGAGTAAAAAATGTTGACATGTGAGAACGATGG ATG TTA AGG TAT

Lys **Gln** **Phe** **Ser** Lys **Glu** **Asn** Glu *cro* repressor gene

AAA CAG TTC TCA AAA GAG AAC GAAGGAGGTGACAAAATGGTACTTGATCTAAAAAGATTGCGAGCCGAAAGAATTGCTTGTG

palindromic sequence

GAATACACAAGATGAAATGGCTAATTTAATGGGATGGAA

Figure 5.2: A *c*I-*cro* intergenic region similar to that of the coliphage λ and other phages was found in the genome of SpT5. *c*I and *cro* repressor genes (codons in green and red respectively) and a potential operator region in the form of a palindrome (blue) were identified. The first twelve codons of the *cro* repressor gene were translated. Amino acids which codons are covered by the palindrome are shown in bold. The sequence covered by the SpT5_op EMSA probe is underlined. SD = Shine Dalgarno sequence.

SpT5_op

5′GACATGTGAGAACGATGGATGTTAAGGTATAAACA**GTTCTC**AAAA**GAGAAC**GAAGGAGGTGACAAAATGGTACTTGATCTAAAAAGATTGCGAGCCGAAA3′

3′CTGTACACTCTTGCTACCTACAATTCCATATTTGT**CAAGAG**TTTT**CTCTTG**CTTCCTCCACTGTTTTACCATGAACTAGATTTTTCTAACGCTCGGCTTT5′

SpT5_mir

5′GACATGTGAGAACGATGGATGTTAAGGTATAAACA**CAAGAG**AAAA**CTCTTG**GAAGGAGGTGACAAAATGGTACTTGATCTAAAAAGATTGCGAGCCGAAA3′

3′CTGTACACTCTTGCTACCTACAATTCCATATTTGT**GTTCTC**TTTT**GAGAAC**CTTCCTCCACTGTTTTACCATGAACTAGATTTTTCTAACGCTCGGCTTT5′

Figure 5.3: The SpT5_op and SpT5_mir EMSA dsDNA probes contained the putative operator sequence (in bold) and a mirror image of the same operator respectively. In SpT5_mir, the putative operator was reverted relative to the rest of the DNA sequence.

The SpT5_op probe was radiolabelled and exposed to lysogen (E045 lys SpT5) and non-lysogen (E045) cell lysates. In the presence of lysogen cell lysate, that hypothetically contained the CI repressor, a shift was observed (Figure 5.4, A). Exposing SpT5_op to non-lysogen cell lysate did not result in any shift. When performing the same test with SpT5_mir, no shift was observed. This showed that binding occurred between the putative operator and a lysogen-specific factor, likely to be the CI repressor. The fact that no binding occurred with SpT5_mir suggested that nucleotides other than those in the operator were involved in the interaction. To further test the specificity of the interaction, a competition assay was performed where radiolabelled SpT5_op was exposed to lysogen cell lysate and increasing concentration of unlabelled probe (Figure 5.4, B). At high concentrations of cold probe the shift decreased significantly confirming the competition between hot and cold probes for binding to the hypothetical CI repressor. These data strongly supported the hypothesis that the identified palindrome was an operator sequence and the lysogen cell lysate contained a CI repressor.

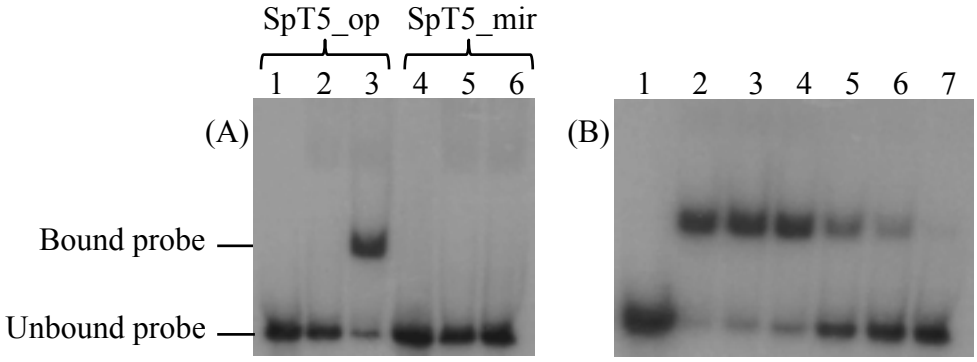


Figure 5.4: (A) A shift was observed when exposing a probe containing the operator sequence (SpT5_op) to lysogen cell lysate. No shift was seen when performing the same test with a probe containing a mirror image of the operator (SpT5_mir). 1 and 4 = free DNA, 2 and 5 = DNA + non-lysogen (E045), 3 and 6 = DNA + lysogen (E045 lys SpT5). (B) Competition assay: at high concentrations of cold SpT5_op the shift decreased significantly confirming the specificity of the binding interaction. 1 = free SpT5_op, 2 to 7 = hot SpT5_op + lysogen + 0, 2.5, 5, 25, 50 and 250 ng of cold SpT5_op.

Interestingly, similar results were obtained when using cell lysate of phage SpT252 lysogen. SpT252 was shown to be very similar to SpT5 (section 3.4.4.b) and the

same *c*I-*cro* intergenic region as SpT5 was found in the genome of SpT252 (Figure 5.5 and section 2.36).

```
SpT5        TTTCATCATTGCTTCTCATTTTAAGTATCTCCTGTGTATAAAATATTTGTTCTTATTTGT
SpT252      TTTCATCATTGCTTCTCATTTTAAGTATCTCCTGTGTATAAAATATTTGTTCTTATTTGT
            ************************************************************

SpT5        GAACAATTAGATTATAACATCGTTCCCAAATGAATACAATATATCAGAAGAAAAAAACTT
SpT252      GAACAATTAGATTATAACATCGTTCCCAAATGAATACAATATATCAGAAGAAAAAAACTT
            ************************************************************

SpT5        TATGATTTTTTTAGAGTAAAAAATGTTGACATGTGAGAACGATGGATGTTAAGGTATAAA
SpT252      TATGATTTTTTTAGAGTAAAAAATGTTGACATGTGAGAACGATGGATGTTAAGGTATAAA
            ************************************************************

SpT5        CAGTTCTCAAAAGAGAACGAAGGAGGTGACAAAATGGTACTTGATCTAAAAAGATTGCGA
SpT252      CAGTTCTCAAAAGAGAACGAAGGAGGTGACAAAATGGTACTTGATCTAAAAAGATTGCGA
            ************************************************************

SpT5        GCCGAAAGAATTGCTTGTGGAATACACAAGATGAAATGGCTAATTTAATGGGATGGAA
SpT252      GCCGAAAGAATTGCTTGTGGAATACACAAGATGAAATGGCTAATTTAATGGGATGGAA
            ************************************************************
```

Figure 5.5: Identical *c*I-*cro* intergenic regions were identified in the SpT5 and SpT252 genomes. The first codons of the *c*I repressor gene and the *cro* repressor gene are underlined in green and red respectively. The operator is boxed in blue.

When exposing the probe SpT5_op to cell lysate of the lysogen AB252, a shift was observed (Figure 5.6). When using SpT5_mir to perform the same test, no shift was observed. The non-lysogen strain (E045) was the same for SpT5 and SpT252 and the same results were obtained as before.

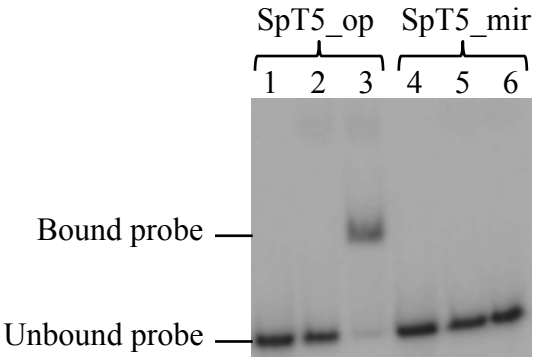


Figure 5.6: When exposing the probe SpT5_op to cell lysate of the lysogen AB252, a shift was observed. No shift was seen when using SpT5_mir. 1 and 4 = free DNA, 2 and 5 = DNA + non-lysogen (E045), 3 and 6 = DNA + lysogen (AB252).

5.4. Introduction of mutations in the operator sequence

The positions where to introduce mutations in the operator were carefully chosen. Indeed, the operator was found within the *cro* repressor gene and maintaining the integrity of this gene was crucial for the mutated phage to be able to enter the lytic cycle. To achieve this, point mutations were introduced by modifying the third nucleotide of each codon covered by both halves of the palindrome (Table 5.1). This way, the operator sequence was changed while the coding sequence was maintained.

The codon usage of staphylococcal phages was taken into account to introduce appropriate mutations. According to a study looking at codon usage in 40 staphylococcal phages, the Gln, Phe, Glu and Asn codons covered by the operator were minor codons (Bishal *et al.*, 2012). Minor codons correspond to low-abundance tRNA species and, as such, influence gene expression rate. In *Saccharomyces cerevisiae* they are frequently encountered in genes expressed at low levels (Raué *et al.*, 1990). In *E. coli*, rare codons are found within the first 25 codons of genes associated with essential cellular functions and were shown to have an inhibitory effect on gene expression (Chen and Inouye, 1994). Codon selection and optimisation have been used to improve protein expression in *E. coli* (Burgess-Brown *et al.*, 2008) and zebrafish (Horstick *et al.*, 2015). Here, the introduction of mutations in the operator changed minor codons into major codons. This would theoretically lead to a higher expression level of the *cro* repressor gene once one or several of these mutations were introduced in a phage. Since the objective of this work was to produce virulent mutants that systematically enter the lytic cycle, it was not considered a problem. Changing major codons into minor codons are likely to have more deleterious effects. The Ser codon present in the sequence was already a major codon and was replaced by another major codon.

Table 5.1: Five single-base mutations (orange) were introduced in both halves of the operator sequence (blue). Each DNA probe contained one mutation and was called after the amino acid which codon was modified (orange).

Probe name	Sequence
SpT5_op	Gln Phe Ser Lys Glu Asn (…) CA**G TTC TC**A AAA **GAG AAC** GA (…)
SpT5_Gln	Gln Phe Ser Lys Glu Asn (…) CA**A TTC TC**A AAA **GAG AAC** GA (…)
SpT5_Phe	Gln Phe Ser Lys Glu Asn (…) CA**G TT**T **TC**A AAA **GAG AAC** GA (…)
SpT5_Ser	Gln Phe Ser Lys Glu Asn (…) CA**G TTC TC**T AAA **GAG AAC** GA (…)
SpT5_Glu	Gln Phe Ser Lys Glu Asn (…) CA**G TTC TC**A AAA **GA**A **AAC** GA (…)
SpT5_Asn	Gln Phe Ser Lys Glu Asn (…) CA**G TTC TC**A AAA **GAG AA**T GA (…)

The five mutations were tested and three were found to prevent protein binding: Phe, Glu and Asn mutations (Figure 5.7). Interestingly, the Ser mutation that changed a base situated in the spacer had little effect on binding. This indicated that the spacer was not as critically involved (if at all) as the palindrome itself in the binding reaction.

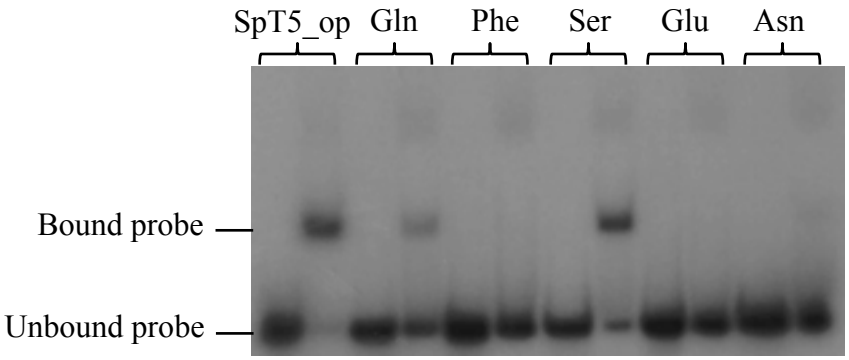


Figure 5.7: Three mutations, Phe, Glu and Asn, were found to prevent protein binding. The Gln mutation led to partial inhibition of protein binding. For each probe: left lane = no lysogen cell lysate and right lane = presence of lysogen cell lysate.

The Gln mutation led to partial inhibition of protein binding. Out of interest, the interaction between the SpT5_Gln probe and lysogen cell lysate was further studied.

5.5. Further tests with the SpT5_Gln probe

Competition assays were performed with radiolabelled wild-type (SpT5_op) and mutated (SpT5_Gln) probes. Both were exposed to lysogen cell lysate and challenged with increasing concentrations of unlabelled probes (Figure 5.8). It was shown that cold SpT5_Gln had little effect on protein binding to hot SpT5_op: a shift was still visible when adding 250 ng of cold mutated probe. On the other hand 25 ng of cold SpT5_op probe were enough to completely inhibit binding to the mutated probe. These results indicated that the affinity of the binding protein was higher for the SpT5_op probe than for the mutated probe.

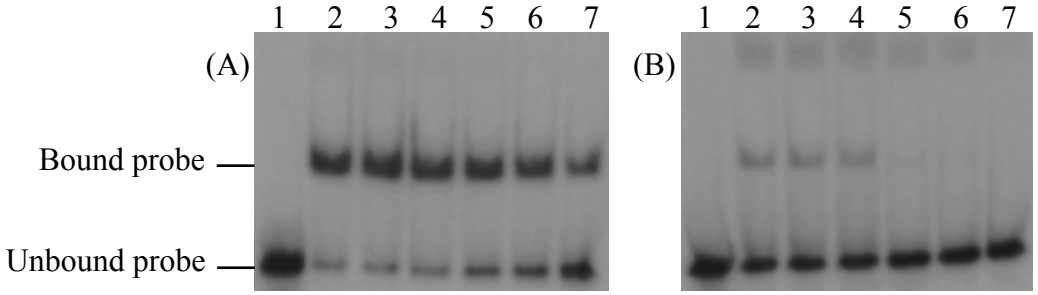
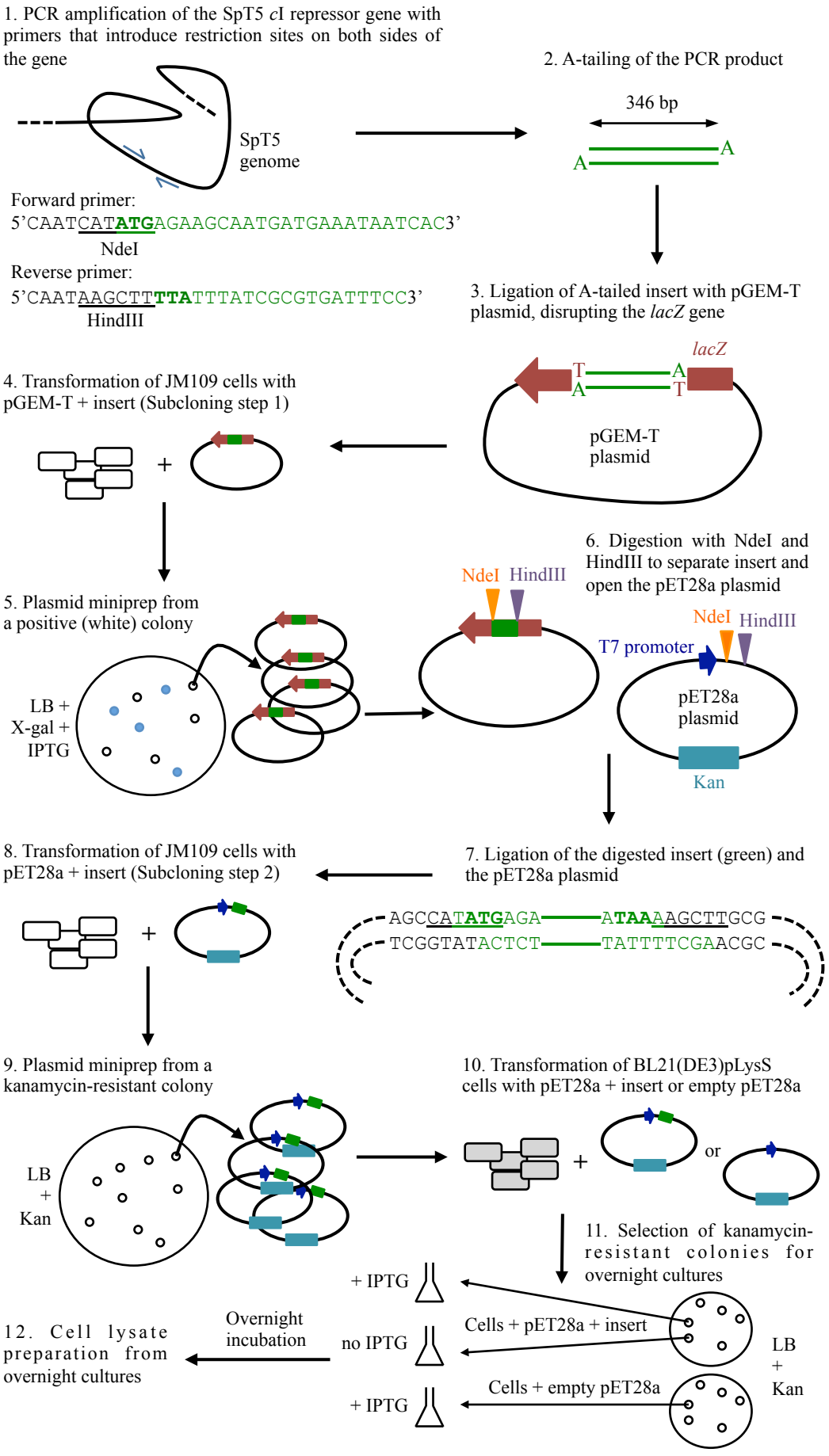


Figure 5.8: (A) SpT5_Gln had little effect on protein binding to hot SpT5_op: a shift was still visible when adding 250 ng of cold mutated probe. 1 = free SpT5_op, 2 to 7 = SpT5_op + lysogen + 0, 2.5, 5, 25, 50 and 250 ng of cold SpT5_Gln. (B) 25 ng of cold SpT5_op probe were enough to completely inhibit binding to the mutated probe. 1 = free SpT5_Gln, 2 to 7 = SpT5_Gln + lysogen + 0, 2.5, 5, 25, 50 and 250 ng of cold SpT5_op.

5.6. Cloning and expressing the SpT5 *c*I repressor gene in *E. coli*

All the experiments described above were performed using cell lysate and not purified protein. It was not possible to be sure that the protein responsible for the interaction was the bacteriophage CI repressor. To unequivocally confirm that the CI repressor was binding to the operator sequence, the *c*I repressor gene was cloned and expressed in *E. coli* (Figure 5.9 and section 2.32).

Figure 5.9 (next page): The SpT5 *c*I repressor gene was cloned and expressed in the *E. coli* BL21(DE3)pLysS strain following two subcloning steps using the *E. coli* JM109 strain. At steps 1 and 7 bases corresponding to the *c*I repressor gene are green, and the initiation and stop codons are in bold.


1. PCR amplification of the SpT5 cI repressor gene with primers that introduce restriction sites on both sides of the gene
SpT5 genome
Forward primer:
5'CAATCATATGAGAAGCAATGATGAAATAATCAC3'
NdeI
Reverse primer:
5'CAATAAGCTTTTATTTATCGCGTGATTTCC3'
HindIII
2. A-tailing of the PCR product
346 bp
A
A
3. Ligation of A-tailed insert with pGEM-T plasmid, disrupting the lacZ gene
lacZ
T
A
A
T
pGEM-T plasmid
4. Transformation of JM109 cells with pGEM-T + insert (Subcloning step 1)
+
5. Plasmid miniprep from a positive (white) colony
LB + X-gal + IPTG
6. Digestion with NdeI and HindIII to separate insert and open the pET28a plasmid
NdeI HindIII
NdeI HindIII
T7 promoter
pET28a plasmid
Kan
7. Ligation of the digested insert (green) and the pET28a plasmid
AGCCATATGAGA ATAAAAGCTTGCG
TCGGTATACTCT TATTTTCGAACGC
8. Transformation of JM109 cells with pET28a + insert (Subcloning step 2)
+
9. Plasmid miniprep from a kanamycin-resistant colony
LB + Kan
10. Transformation of BL21(DE3)pLysS cells with pET28a + insert or empty pET28a
+
or
11. Selection of kanamycin-resistant colonies for overnight cultures
+ IPTG
no IPTG
+ IPTG
Cells + pET28a + insert
Cells + empty pET28a
LB + Kan
Overnight incubation
12. Cell lysate preparation from overnight cultures

Cell lysates of IPTG-induced cultures, non-induced cultures and cells containing the empty pET28A plasmid were run on a polyacrylamide gel to confirm that the recombinant protein was effectively expressed (Figure 5.10). An additional band around 15 kDa, the theoretical size of the cloned phage repressor (section 2.34), was seen in the lysates of IPTG-induced cultures. This confirmed that the cloned *c*I repressor gene was expressed following IPTG induction.

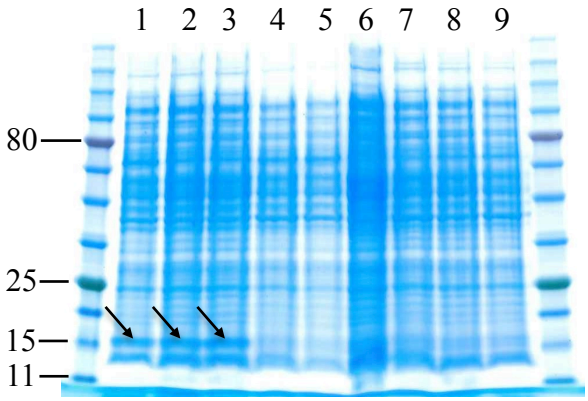


Figure 5.10: Cell lysates of IPTG-induced cultures (1, 2 and 3), non-induced cultures (4, 5 and 6) and cells containing the empty pET28A plasmid (7, 8 and 9) were run on a polyacrylamide gel. An additional band (shown with arrows) was seen in the lysates of IPTG-induced cultures. Its size (15 kDa) corresponded to the theoretical size of the phage repressor. This confirmed that the repressor was effectively expressed following IPTG induction.

5.7. Testing the binding ability of the recombinant CI repressor expressed in *E. coli*

After cloning the SpT5 *c*I repressor gene into *E. coli* and expressing the recombinant protein through IPTG induction, the DNA probes SpT5_op and SpT5_mir were exposed to cell lysates from IPTG-induced culture, non-induced culture and cells containing the empty cloning vector (Figure 5.11, A). A shift was observed in the presence of induced and non-induced cell lysates. After IPTG induction, the phage repressor was highly expressed and a high quantity of SpT5_op probe was bound by the recombinant protein. Consequently the quantity of emitted radiation was high and this made the corresponding shift (Figure 5.11, A, well 4) appear larger than the shift obtained with non-induced cell lysate (Figure 5.11, A, well 5). The fact that binding was observed with cell lysate from non-induced culture indicated that leaky gene expression occurred. Exposing SpT5_op to cell lysate from cells with the empty

cloning vector did not result in any shift and no shift was obtained when using SpT5_mir. These results confirmed that the DNA-binding protein responsible for the observed interaction was the CI phage repressor.

To check the specificity of the interaction between the operator and the recombinant repressor, a competition assay was carried out using radiolabelled SpT5_op, cold SpT5_op and non IPTG-induced cell lysate (Figure 5.11, B). The phage repressor was too highly expressed in the IPTG-induced cell lysate for a competition assay to be performed successfully (Figure 5.11, C). Similarly to Figure 5.4, the shift decreased significantly at high concentrations of cold probe (250 ng) confirming the specificity of the interaction between the recombinant repressor and the operator sequence.

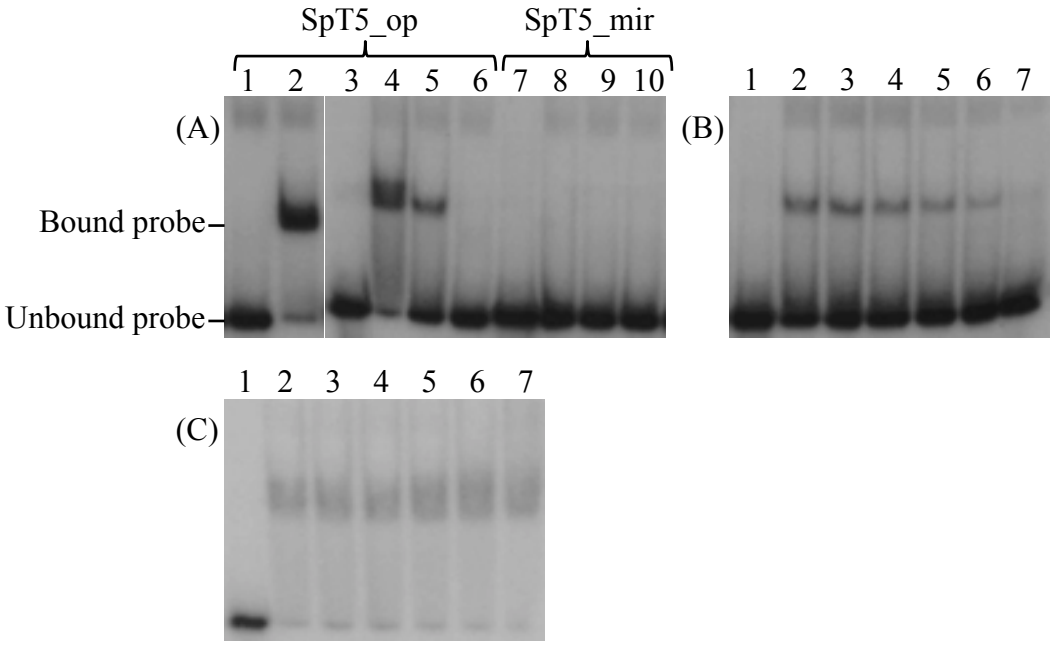


Figure 5.11: (A) A shift was observed when exposing SpT5_op to cell lysates of *E. coli* cultures expressing or not the SpT5 CI repressor. No shift was seen with SpT5_mir. 1 = DNA + non-lysogen (E045), 2 = DNA + lysogen (E045 lys SpT5), 3 and 7 = free DNA, 4 and 8: DNA + IPTG-induced culture, 5 and 9 = DNA + non-induced culture, 6 and 10 = DNA + *E. coli* with empty cloning vector. (B) Competition assay: the shift decreased significantly at high concentrations of cold SpT5_op confirming the specificity of the interaction. 1 = free SpT5_op, 2 to 7 = SpT5_op + non-induced lysate + 0, 2.5, 5, 25, 50 and 250 ng of cold SpT5_op. (C) Competition assay with IPTG-induced cell lysate: the shift did not decrease at high concentrations of cold SpT5_op. 1 = free SpT5_op, 2 to 7 = SpT5_op + induced lysate + 0, 2.5, 5, 25, 50 and 250 ng of cold SpT5_op.

5.8. Studying the structure of the SpT5 CI repressor

The secondary structure of the SpT5 CI repressor was predicted with the Phyre2 online tool based on the *c*I repressor gene's translated DNA sequence. The obtained structure was compared with crystal structures of the N-terminal domain of the CI repressor from the lactococcal phage TP901-1 (Frandsen *et al.*, 2013) (Figure 5.12) and the N-terminal domain of the CI repressor from phage λ (Figure 5.13), both found in the Protein Data Bank (PDB) (section 2.35). Comparison with CI repressors from staphylococcal phages was not possible because crystal structures were not available in the PDB.

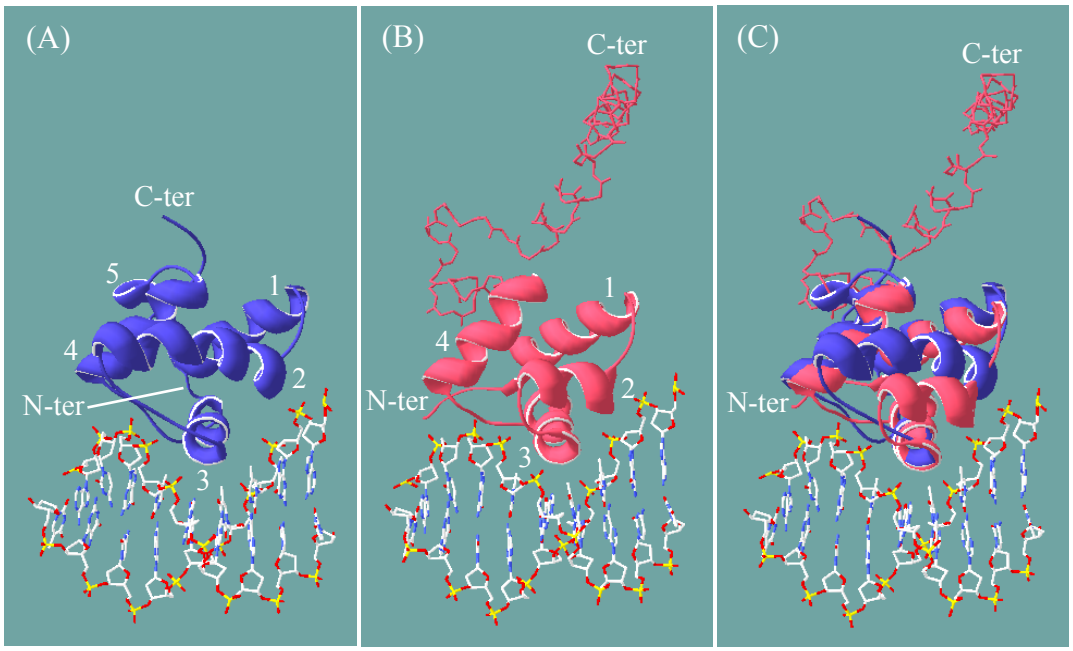


Figure 5.12: (A) The N-terminal domain of the CI repressor from the lactococcal phage TP901-1 is shown in complex with its operator half-site. N-ter: N-terminal end, C-ter: C-terminal end. α-helices were numbered from N-ter to C-ter. (B) The predicted structure of the SpT5 CI repressor is shown in the same position relative to the TP901-1 operator half-site. The four α-helices that were similar to the TP901-1 repressor are shown in ribbons. (C) When both structures were superimposed, helices 1, 3 and 4 aligned almost perfectly with each other. The N-terminal and C-terminal ends of only the SpT5 CI repressor are indicated.

The N-terminal domain of the TP901-1 phage CI repressor constitutes the protein's DNA binding domain and is essential for site-specific recognition. The domain is composed of five α-helices that form a compact globular domain. Helices 2 and 3 form a Helix-Turn-Helix (HTH) unit, a highly conserved structural motif found in

DNA-binding proteins (Pedersen *et al.*, 2010). The structure chosen to illustrate this was crystallised in complex with its operator half-site. Helix 3 fitted into the major groove of the DNA highlighting its role in the protein-DNA interaction (Figure 5.12, A). The SpT5 CI repressor exhibited a similar structure at its N-terminal end. It was composed of four tightly folded α-helices and helix 3 seemed to fit into the major groove of the DNA similarly to helix 3 in the TP901-1 repressor (Figure 5.12, B). This was consistent with the identification through blastp search of an HTH-like motif near the N-terminal end of the SpT5 repressor. It was noted that helix 2 was much shorter in the SpT5 repressor than in the TP901-1 repressor. When both structures were superimposed, helices 1, 3 and 4 aligned almost perfectly with each other (Figure 5.12, C). This strongly suggested that the SpT5 repressor had one DNA binding domain at its N-terminal end.

The C-terminal end of the SpT5 repressor could not be compared with the C-terminal domain of the TP901-1 repressor because no crystal structure was available in the PDB. However, it is known that the TP901-1 CI repressor binds operators as a dimer (Johansen *et al.*, 2003) and that the C-terminal part is required for oligomerization of the protein. It is also known that the C-terminal part of the protein is likely to be partially unfolded and more flexible because it was shown to be much more sensitive to proteolysis than the N-terminal domain (Pedersen *et al.*, 2010). One can hypothesise that, similarly to the TP901-1 repressor, the SpT5 repressor binds as a dimer to its operator and that its C-terminal end is involved in protein oligomerization. One monomer can interact with only one operator half-site and, as shown earlier in this chapter, mutations in either half of the SpT5 operator led to disruption of repressor binding (Gln and Phe in one half, Glu and Asn in the other half). This suggested that the SpT5 repressor does indeed form dimers. Added to this, the possibility that the C-terminal end may be partially unfolded might have made structure prediction difficult. This would explain the disorganised shape of the SpT5 repressor C-terminal end.

Following comparison with the TP901-1 repressor, the predicted structure of the SpT5 repressor was compared with the N-terminal domain of the phage λ CI repressor. Similarly to the TP901-1 repressor, the N-terminal domain mediates operator binding and is composed of five α-helices, two of which (helices 2 and 3)

form a HTH unit (Beamer and Pabo, 1992). The structure chosen in the PDB was crystallised as a dimer in complex with its full operator site. Helices 3 and 3' fitted into the major grooves of the DNA where the bases of the operator were situated, and helices 5 and 5' extended out from the globular domain to form part of the dimer interface (Figure 5.13, A).

Once again, the SpT5 CI repressor exhibited a similar structure at its N-terminal end with helix 3 seemingly fitting into the major groove of the DNA (Figure 5.13, B). When both repressors were superimposed, helices 1, 2, 3 and 4 aligned almost perfectly with minor differences in length and orientation (Figure 5.13, C). The C-terminal domain of the λ CI repressor mediates dimerization as well as interaction between two dimers bound to adjacent operator sites (cooperative binding, see section 1.4.2.b.iii). It is formed by a highly twisted seven-stranded β-sheet (Bell and Lewis, 2001). This structure is very different from the predicted structure of the SpT5 repressor's C-terminal end. Again, it is possible that the prediction is wrong or it is possible that the C-terminal end folds in a completely different way. It may even remain partially unfolded in the absence of a second monomer.

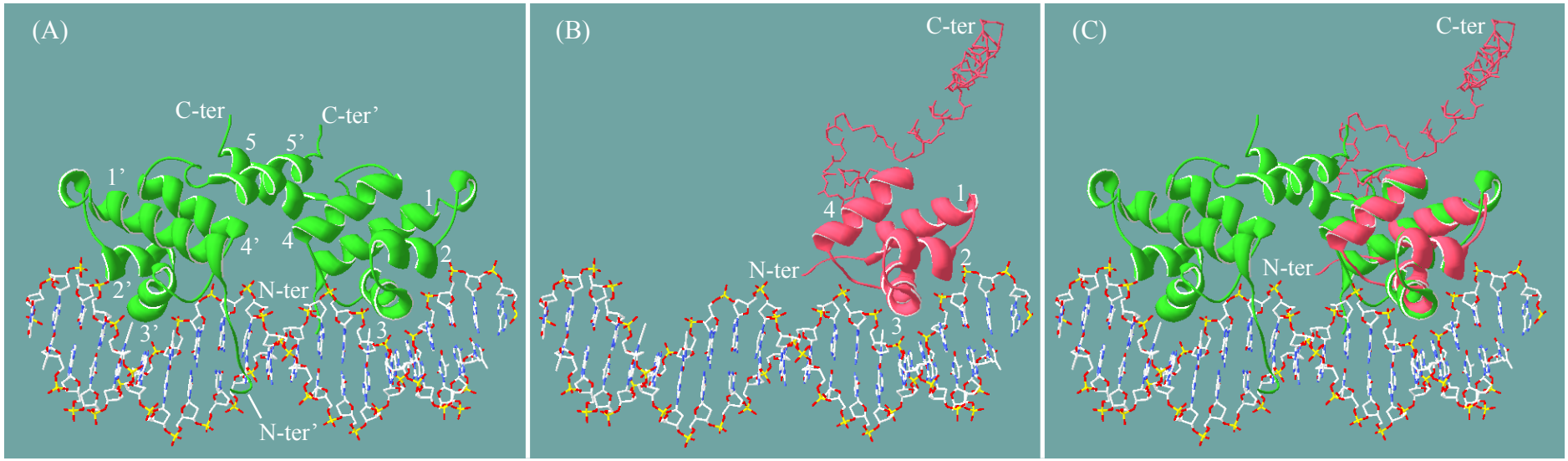


Figure 5.13: (A) A dimer of N-terminal domains of the λ CI repressor is shown in complex with its full operator site. N-ter, N-ter': N-terminal ends, C-ter, C-ter': C-terminal ends. α-helices were numbered from N-ter to C-ter. (B) The predicted structure of the SpT5 CI repressor is shown in the same position relative to the λ operator site. The four α-helices that were similar to the λ repressor are shown in ribbons. (C) When both repressors were superimposed, helices 1, 2, 3 and 4 aligned almost perfectly with minor differences in length and orientation. The N-terminal and C-terminal ends of only the SpT5 CI repressor are indicated.

In spite of structural similarities, the amino-acid sequences of the three CI repressors were very different from each other (Figure 5.14 and section 2.36). A few conserved amino acids (aa) were identified in the N-terminal domain of all three CI repressors where the HTH motif was situated. A RecA-dependent auto-cleavage site is situated in the linker region between the N-terminal and C-terminal domains of the λ CI repressor, more precisely between residues 111 and 112 (Pabo *et al.*, 1979). A similar auto-cleavage site might be present in a similar region in the other two proteins but the lack of sequence similarity did not allow any conclusive prediction.

The C-terminal domains of the λ and TP901-1 CI repressors were aligned and some similarities could be seen. No residues from the C-terminal end of the SpT5 CI repressor were aligned to these regions because the SpT5 protein was much shorter than the other two: 109 aa, 180 aa and 237 aa for the SpT5 repressor, the TP901-1 repressor and the λ repressor respectively. Other staphylococcal phage CI repressors identified through blastp search were of similar short sizes (109-110 aa). This difference in size may suggest that the C-terminal end of the SpT5 repressor and staphylococcal phage repressors fold differently in order to fulfil the same role as in other CI repressors, i.e. dimerization and multimerization.

Figure 5.14: The amino acid sequences of the λ, TP901-1 and SpT5 CI repressors were very different from each other. Residues corresponding to helix-turn-helix motifs were underlined. The N-terminal domains of the λ (1-92 aa) and TP901-1 (1-74 aa) CI repressors are circled in red. The C-terminal domains, involved in oligomerization, of the λ (136-237 aa) and TP901-1 (138-180 aa) CI repressors are boxed in blue. A RecA-dependent auto-cleavage site is situated in the linker region of the λ CI repressor between residues 111 and 112 (black triangle). (*) = single, fully conserved residue, (:) = residues with strong similar properties, (.) = residues with weak similar properties.

5.9. Conclusions

When random mutagenesis approaches were unsuccessful for the isolation of virulent mutants of the phages, it was decided to attempt site-directed mutagenesis of the operator region, a DNA sequence involved in the genetic switch between lysis and lysogeny and recognised by the CI phage repressor. A putative operator was found in the form of a palindromic sequence in the genome of phage SpT5. EMSA showed that specific binding occurred between a DNA probe containing the putative operator sequence and lysogen cell lysate. This confirmed that the identified sequence was an operator and that a lysogen-specific factor, hypothetically the SpT5 CI repressor, was responsible for the observed binding.

The characteristics of the SpT5 operator sequence were slightly different from other phage operators. Firstly, it was found within the *cro* repressor gene; operators involved in the control of the lysogeny-lysis genetic switch like in phage λ are often situated between the *cro* and *c*I promoters (Ptashne *et al.*, 1980). Secondly, only one

single palindrome was identified in the SpT5 genome when other temperate phages have up to three operators in the *cro*-*c*I intergenic region (Hochschild and Ptashne, 1986). It is possible that evolution selected for operators located within coding sequences in *S. pseudintermedius* phages, in what case such an operator would be expected to be present within the *c*I repressor gene in SpT5. This operator would be the Cro repressor-binding site. It was proposed that this operator sequence would be similar to the one identified within the *cro* repressor gene, based on the fact that a consensus sequence can often be derived from all the operators present in one phage genome (Citron *et al.*, 1989). However, a similar palindromic sequence or any sequences with elements of symmetry were not found when examining the *c*I repressor gene by eye. Further analyses of the SpT5 genome to look for palindromes or similar sequences with the help of various programmes were not successful either.

It is possible that operators in *S. pseudintermedius* phages are not all similar and some may not have elements of symmetry. Asymmetric operators were identified in the genome of the temperate mycobacteriophage L1 (Ganguly *et al.*, 2007). DNase I footprinting analysis could be performed to identify more operator sites in the SpT5 genome (Das *et al.*, 2009). In this test, DNA regions suspected to contain operator sites are subjected to DNase I digestion in presence of saturating amounts of DNA-binding proteins, i.e. CI or Cro repressors in this case. Regions recognised by the repressors are protected from digestion. The digestion patterns of DNA with and without the repressors are then compared to find where the proteins bind.

Once the operator was identified, point mutations were carefully chosen in a way that changed the operator sequence without disrupting the coding sequence of the *cro* repressor gene. Three mutations led to the absence of binding between dsDNA probes and the hypothetical CI repressor. If these mutations were introduced in the genome of phage SpT5, they should lead to a virulent phenotype. Site-directed mutagenesis itself was not performed because the PhD project was coming to an end and developing a protocol for modifying phage SpT5 might not be trivial. Two options were considered: the introduction of single-stranded (ss) pieces of DNA containing the desired mutation into cells lysogenized with SpT5 or the transfection (transformation with viral DNA) of non-lysogenised cells phage genomic DNA along with the same mutated ssDNA. In both cases, the successful modification of

the phage genome would rely on strand invasion. In this situation the ssDNA is expected to anneal with its complementary region in the phage genome (i.e. the operator sequence) forming a D-loop (D for displacement) (Noirot and Kolodner, 1998). The formation of such D-loops is known to generate active replication forks where the invading strand acts as a primer for DNA synthesis (Kogoma, 1996). This would result in the incorporation of the ssDNA in the genome and gene conversion. Any mismatches created by the presence of mutated bases in the ssDNA should be resolved with a 50% chance for the mutated base to be either kept or lost (Figure 5.15). The use of a large molar excess of ssDNA should increase the mutation rate.

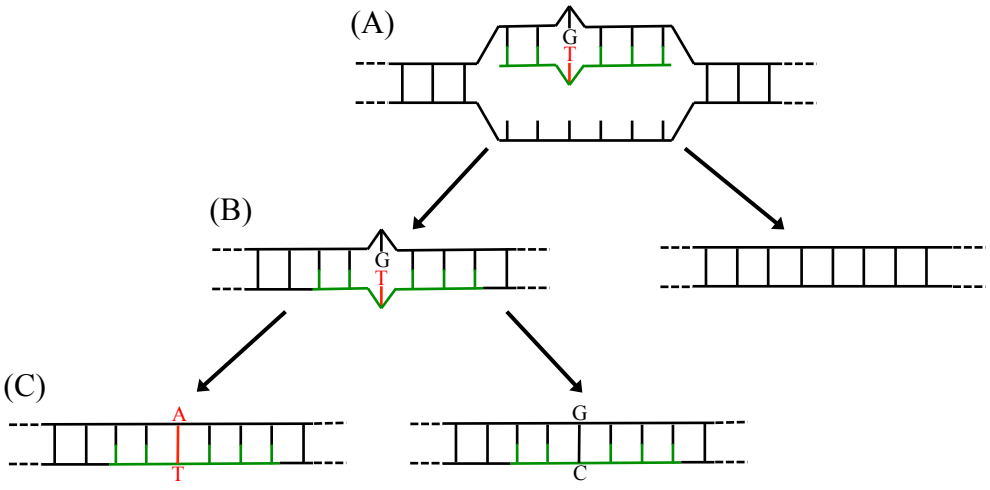


Figure 5.15: In site-directed mutagenesis through strand invasion and replication, (A) ssDNA hybridises with its complementary region forming a D-loop (D for displacement). (B) Both strands are replicated, resulting in the incorporation of the ssDNA. (C) Mismatches created by the presence of mutated bases (in red) in the ssDNA are resolved with a 50% chance for the mutated base to be either kept or lost.

The transformation of SpT5 lysogens with ssDNA alone could be performed through classic electroporation. Gram-positive bacteria, like *S. pseudintermedius*, are in general more resistant to DNA transformation than Gram-negative bacteria. The exterior peptidoglycan layer acts as a physical barrier for the DNA, making transformation of Gram-positive bacteria more challenging. Despite this, protocols for electroporation-mediated transformation of *Staphylococcus* species were successfully developed (Löfblom *et al.*, 2007, McNamara, 2008). Another challenge for the transformation of *Staphylococcus* species is the presence of restriction-

modification systems that digest foreign DNA. Specific lineages of *S. aureus* were found to possess such a system (Sau1) that blocks horizontal gene transfer (Waldron and Lindsay, 2006). It was hypothesised that inhibiting Sau1 may allow genetic manipulation of the relevant *S. aureus* strains. Similarly to *S. aureus*, *S. pseudintermedius* may have restriction-modification systems that make transformation more difficult.

For the introduction of ssDNA + phage genomic DNA it may be better to use protoplasts, where the cell wall was digested. It may help overcome some of the problems encountered in electroporation-mediated transformation especially when trying to introduce a piece of DNA as big as a phage genome. However, the possible presence of restriction-modification systems within the cells would still be an issue.

The advantage with both approaches (transformation of normal cells or protoplasts) compared to usual bacterial transformation is that there is no need to regenerate bacterial cells. The expected result is a modified phage and not a modified cell. Transformed cells would simply be cultivated in presence of the phage's lysogen. Modified phages (i.e. lytic mutants) would be expected to burst out of the transformed host and infect the neighbouring cells thanks to their ability to overcome homoimmunity. A low level of gene conversion would therefore not be a problem because obtaining a single plaque from a single mutant, derived from a single successful conversion event, would be enough to amplify it through further cultivation.

The protocols available in the literature for both methods were developed for *Staphylococcus* species that were not *S. pseudintermedius*. Developing a protocol for this particular species might have required extensive optimisation and it was estimated that this would not fit within the timeframe of the PhD project.

The SpT5 CI repressor was further studied. First, its gene was cloned and expressed in *E. coli*, and EMSA confirmed that it was the protein binding to the identified operator. Secondly, its tertiary structure was predicted and compared with the crystal structures of the λ and TP901-1 CI repressors. This study showed that the SpT5 repressor had a DNA-binding domain at its N-terminal end characterised by a helix-

turn-helix unit, a structural motif typical of DNA-binding proteins. This observation was consistent with the EMSA results. The secondary structure of the C-terminal end remained uncertain. It might be partially unfolded like the C-terminal domain of the TP901-1 phage repressor and it possibly mediates dimerization of two CI repressor monomers similarly to the C-terminal domain of the TP901-1 and λ repressors. It would be interesting to perform crystallography on the SpT5 CI repressor with or without DNA to determine how it interacts with its operator site. It would also be good to crystallize the protein as a monomer or a dimer to visualise the conformations of the C-terminal end when it is free and when it interacts with another monomer.

Chapter 6 Bioinformatics analyses of the four selected *S. pseudintermedius* phage genomes (SpT5, SpT152, SpT252 and SpT99/F3)

6.1. Introduction

Over the past decade, the development and improvement of sequencing technologies has enabled the fast, cheap and reliable sequencing of entire genomes. Sequencing methods are currently classified into three categories, or three "generations". First-generation sequencing, also called the chain-termination method, was develop by Sanger in 1975 (Sanger and Coulson, 1975). The Sanger method is still used nowadays for the sequencing of short pieces of DNA such as PCR products or plasmids. The length of a DNA fragment that is sequenced in one go, also called a read, is 800 bases on average. The main limitation of the Sanger method is the small amount of DNA that can be processed per unit of time (Schadt *et al.*, 2010).

To overcome the low throughput of the first-generation method, second-generation sequencing (SGS) methods were developed and were made commercially available for the first time in 2005. Much higher throughput is achieved with SGS by sequencing a large number of DNA molecules in parallel (van Dijk *et al.*, 2014). The first step consists of fragmenting the DNA template to build a library of nucleic acids (Figure 6.1). The fragmentation can be physical (e.g. sonication) or biochemical (enzyme-based treatments). During a tagging step, adapters (synthetic oligonucleotides of a known sequence) are ligated to the extremities of each DNA fragment, also called insert (Figure 6.2), and the library is clonally amplified through PCR performed with primers recognising the adapter sequences.

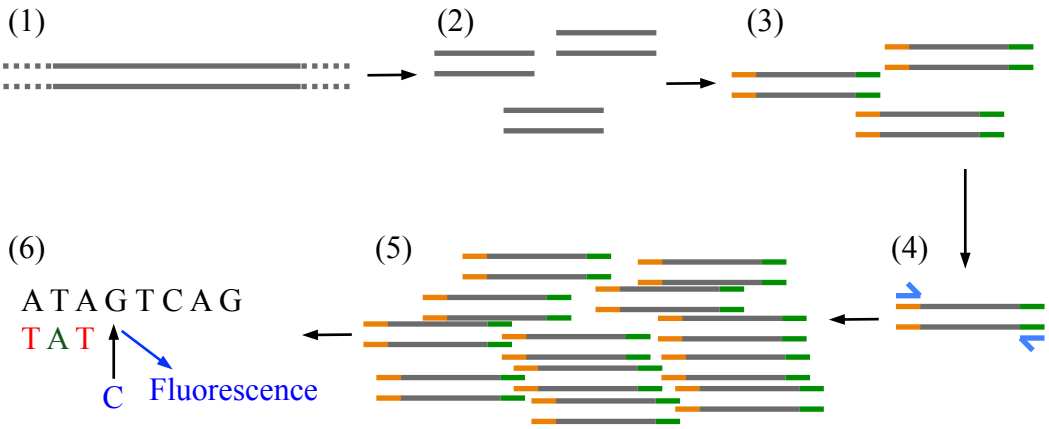


Figure 6.1: The preparation of (1) DNA template for sequencing involves (2) fragmentation, (3) the ligation of adapters and (4, 5) PCR amplification with primers recognising the adapter sequences. (6) Sequencing with the Illumina MiSeq platform consists in the sequential incorporation of nucleotides and emission of a fluorescent signal. Data analysis is then needed to determine the sequence of the DNA template.

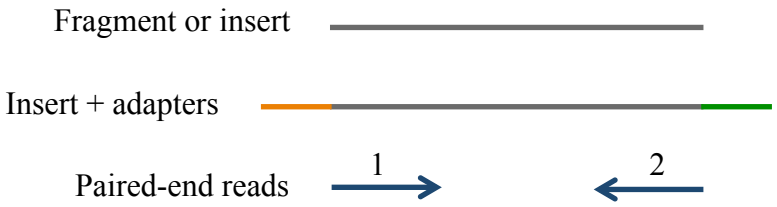


Figure 6.2: During library preparation from genomic DNA, DNA fragments, also called inserts, are produced. Adapter sequences (orange and green) are ligated to each extremity of the insert. Reads are produced through sequencing either from one end (single-end read) or both ends of the insert (paired-end reads).

The four phage genomes (SpT5, SpT152, SpT252 and SpT99/F3) were prepared for whole-genome sequencing with the Nextera XT DNA Library Preparation kit (Illumina). With this kit both DNA fragmentation and tagging are carried out simultaneously by a modified transposase (= tagmentation). The biological role of a transposase is to mediate the integration and excision of mobile genetic elements called transposons (Muñoz-López and García-Pérez, 2010). Importantly, transposases do not usually insert upstream of terminal bases (Dr Andrew Millard, personal communication). This detail is crucial for the interpretation of some of the results presented in this chapter.

Sequencing of the phage genomes was performed with the Illumina MiSeq platform, one of the most commonly used SGS platforms. It relies on the sequential incorporation of nucleotides and emission of a fluorescent signal to obtain nucleic acid sequence from the amplified library (Grada and Weinbrecht, 2013). The read length obtained with SGS is much shorter than with the Sanger sequencing method: from 100-150 bp, up to 250-300 bp nowadays.

Third-generation sequencing (TGS) methods are based on the direct inspection of single DNA molecules. These methods aim at overcoming some of the shortcomings of SGS methods, such as PCR amplification that can introduce errors in the template sequence as well as amplification bias (Schadt *et al.*, 2010). Most of these technologies are still in development and their advantages are expected to be speed, much longer read length and reduced costs.

Following sequencing with SGS, a huge amount of data is produced and must undergo several analysis steps. Raw sequence data first undergoes preprocessing to remove the adapter sequences and low-quality reads. The assembly of sequencing reads is carried out by mapping the data against a reference genome or by *de novo* assembly using algorithms that determine the best successive combination of sequencing reads based on the overlaps observed between reads (Baker, 2012).

Once whole genomes are obtained, genome annotation is performed to further study their characteristics. Gene prediction is the first step of a genome annotation pipeline. It is carried out with programmes such as Prodigal (Hyatt *et al.*, 2010) that base their prediction on the presence of start and stop codons. The predicted genes are then translated and their putative products are aligned to protein databases using fast sequence alignment tools like BLAST (Richardson and Watson, 2013). It is best to use curated databases such as SwissProt for this step.

Genome assembly and annotation of the phage genomes were performed by Dr Witold Kot and Dr Andrew Millard respectively (sections 2.17 and 2.18). The annotated genomes were then aligned with each other to compare their genetic organisation.

The four genomes were also studied in more details to determine if they had distinct cohesive ends or if they were circularly permuted without distinct genome ends. As described in section 1.4.2.a.iv, this characteristic is a consequence of the dsDNA phage packaging strategies. The genomes of phages with cohesive ends have distinct ends at identical locations in the sequence whereas the ends of circularly permuted genomes "move" along the sequence and contain terminal repeats. Based on these characteristics, it was attempted to predict whether the four phage genomes were circularly permuted or had cohesive ends through a bioinformatics approach. A PCR experiment was then performed to confirm (or not) the predictions.

A bioinformatics approach was also used to investigate further the characteristics of the atypical SpT99/F3 genome, and try and explain the unusual features of this phage described in section 3.4.2.

6.2. Study of the genetic organisation in the Warwick phages

To compare the four phage genomes in more details and study genome organisation, they were aligned using the programme Easyfig (Figure 6.3). Easyfig uses BLAST to create linear comparisons of multiple genomic regions ranging from single genes to whole prokaryote chromosomes (Sullivan *et al.*, 2011). Here, Easyfig produced an alignment using the GenBank files obtained after annotation of the assembled phage genomes. The annotation led to the identification of coding DNA sequences (CDSs) and the determination of the putative function of their product based on similarity with the SwissProt protein database (Table 6.1).

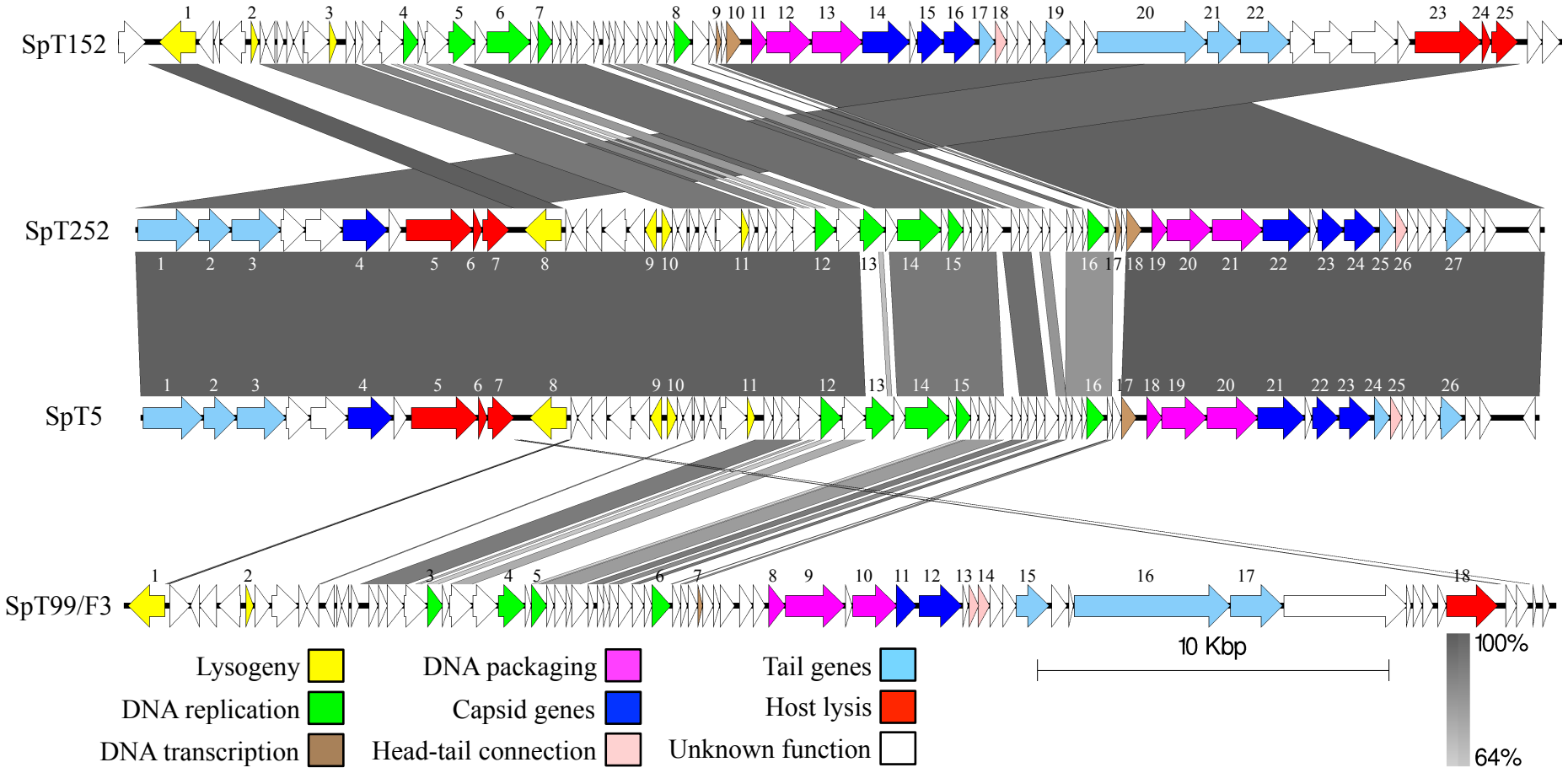


Figure 6.3: The four phages exhibited a level of similarity regarding genome organisation. CDS are shown as arrows and putative functions are indicated by colour coding. A BLAST similarity scale is shown at the bottom right. Details of putative functions are given in Table 6.1.

Table 6.1: Proteins of similar putative functions, when their function could be predicted, were encoded in the four phage genomes. Correspondence between CDSs and numbers are shown in Figure 6.3. A description of the proteins' function in the phage life cycle can be found in sections 1.4.2.a and 1.4.2.b. A search in the UniProt database described the phage Mu protein F as a putative capsid morphogenesis protein.

CDS number	Bacteriophages			
	SpT152	SpT252	SpT5	SpT99/F3
1	Integrase	Tail length tape-measure protein	Tail length tape-measure protein	Integrase
2	Helix-turn-helix protein	Tail protein	Tail protein	Helix-turn-helix protein
3	Excisionase	Tail endopeptidase	Tail endopeptidase	ssDNA binding protein
4	ssDNA binding protein	Capsid protein	Capsid protein	DNA replication protein
5	Replication initiation protein	Endolysin precursor	Endolysin precursor	Holliday junction resolvase
6	Helicase	Holin	Holin	dUTPase
7	Holliday junction resolvase	Cell wall hydrolase precursor	Cell wall hydrolase precursor	Transcriptional activator
8	dUTPase	Integrase	Integrase	Terminase, small subunit
9	Transcriptional activator	CI repressor	CI repressor	Terminase, large subunit
10	σ^{70} factor	Cro repressor	Cro repressor	Portal protein
11	Terminase, small subunit	Excisionase	Excisionase	Prohead protease
12	Terminase, large subunit	ssDNA binding protein	ssDNA binding protein	Capsid protein
13	Portal protein	Replication initiation protein	Replication initiation protein	Head-tail connector protein
14	Phage Mu protein F-like protein	Helicase	Helicase	Head-tail joining protein

CDS number	Bacteriophages			
	SpT152	SpT252	SpT5	SpT99/F3
15	Capsid protein	Holliday junction resolvase	Holliday junction resolvase	Tail protein
16	Capsid protein	dUTPase	dUTPase	Tail length tape-measure protein
17	Tail protein	Transcriptional activator	σ^{70} factor	Tail protein
18	Head-tail connector protein	σ^{70} factor	Terminase, small subunit	Endolysin
19	Tail protein	Terminase, small subunit	Terminase, large subunit	
20	Tail length tape-measure protein	Terminase, large subunit	Portal protein	
21	Tail protein	Portal protein	Phage Mu protein F-like protein	
22	Tail endopeptidase	Phage Mu protein F-like protein	Capsid protein	
23	Endolysin precursor	Capsid protein	Capsid protein	
24	Holin	Capsid protein	Tail protein	
25	Cell wall hydrolase precursor	Tail protein	Head-tail connector protein	
26		Head-tail connector protein	Tail protein	
27		Tail protein		

The whole-genome alignment produced with Easyfig concurred with the alignment obtained with Mauve (section 3.4.4.b). SpT252 and SpT5 were very similar to each other with only a few differences in the middle of the genomes. The most obvious difference was seen between CDSs 13 in SpT252 and SpT5, both were annotated as DNA replication initiation proteins. SpT152 showed a slightly lower level of similarity and SpT99/F3 exhibited very little similarity with the other phages. On average 67 CDSs were predicted for each genome and the putative function of their products was determined for around 36% of them (25% for SpT99/F3). Such a low proportion of annotated genes is not unusual when annotating phage genomes. Current databases are still largely incomplete when it comes to phage genes and proteins, and this led to a majority of CDSs to be annotated as "hypothetical protein".

In each genome, groups of CDSs with similar function or associated with the same molecular process were identified. Genes of the same functional group were usually situated next to each other in the genome and the position of each group of genes relative to the other ones was similar in all four genomes. Genes involved in lysogeny were followed by genes involved in DNA replication, then DNA transcription, DNA packaging, structural genes coding for capsid, head-tail joining and tail proteins, and host lysis.

Regarding genes involved in DNA transcription, it was interesting to see that a CDS coding for a σ^{70} factor was identified in three genomes out of four. The sigma factors of σ^{70} family are components of the bacterial RNA polymerase (RNAP) holoenzyme. They direct the core RNAP to specific promoter elements (Paget and Helmann, 2003). The presence of a CDS coding for a σ^{70} factor suggested that these phages do not encode their own RNAP, and redirect the host RNAP towards their genome by replacing the host σ^{70} factor, similarly to the *Bacillus subtilis* phage SPO1 that encodes a regulatory protein, gp28, that is homologous to σ factors from *E. coli* and *B. subtilis* (Costanzo and Pero, 1983, Gribskov and Burgess, 1986). The absence of a phage RNAP gene was not confirmed because the function of numerous CDSs remained unknown. However, the phage single subunit RNAP (ssRNAP) gene, found in the T7 phage and its relatives, is well conserved (Cermakian *et al.*, 1997) and should theoretically be easily identified by annotation programmes. It is therefore unlikely that the phages studied here encode their own RNAP.

In SpT152 and SpT99/F3, CDS 2 was annotated as helix-turn-helix protein. HTH motifs are typically found in DNA-binding proteins such as CI repressors involved in the maintenance of lysogeny (section 5.8). CDS 2 was situated between the integrase and the excisionase genes in SpT152. It was also found in a location similar to that of the SpT5 and SpT252 *c*I repressor genes in both SpT152 and SpT99/F3. It was therefore reasonable to propose that CDS 2 in SpT152 and SpT99/F3 coded for an analogue of CI repressors.

In SpT5 and SpT252, the genome organisation was similar to SpT152 and SpT99/F3 except that some groups of genes (structural and host lysis genes) seemed to have shifted from one end of the genomes to the other. This could be the result of genomic rearrangements or it may indicate that the genomes were circularly permuted. In the latter case the genome ends were chosen arbitrarily by the programme during the assembly process.

6.3. Were the four phage genomes circularly permuted or with cohesive ends?

6.3.1. Bioinformatic prediction based on read coverage across the genomes

Whether a genome is circularly permuted or has cohesive ends can be tested experimentally or it can be predicted it using a bioinformatic approach. The latter approach is possible when using sequencing data obtained after preparing DNA libraries with the Nextera XT DNA Library Preparation kit. As mentioned in section 6.1, this kit relies on transposase activity to fragment and tag DNA at the same time. Transposases do not usually insert upstream of terminal bases meaning that they are less likely to insert transposons within the distinct ends of cohesive genome termini. This results in a lower tagmentation rate in terminal regions and fewer sequencing inserts being produced. This affects the coverage of these regions after sequencing. The coverage describes the average number of reads that align to, or "cover", known references bases, e.g. an assembled genome. In the case of a genome with distinct ends, coverage should be much lower over cohesive termini than over the rest of the genome because fewer reads are produced. In the case of a circularly permuted genome, coverage should be more even over its entire length (Rihtman *et al.*, 2016). This property was used to try and predict *in silico* whether the phage genomes were circularly permuted or had cohesive ends.

To do the prediction, the method described in section 2.39 was used to produce diagrams illustrating read coverage across each phage genome (Figure 6.4).

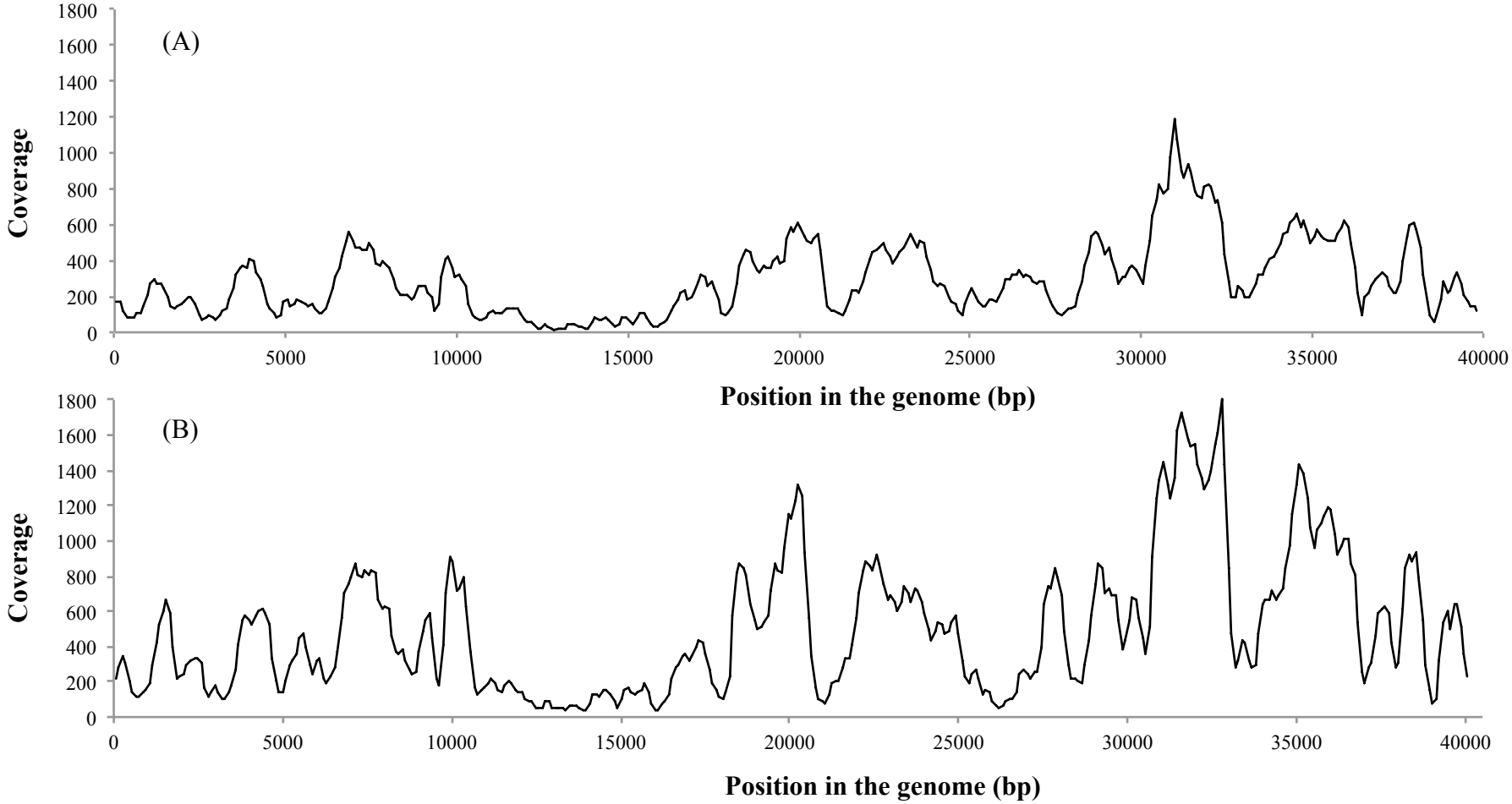
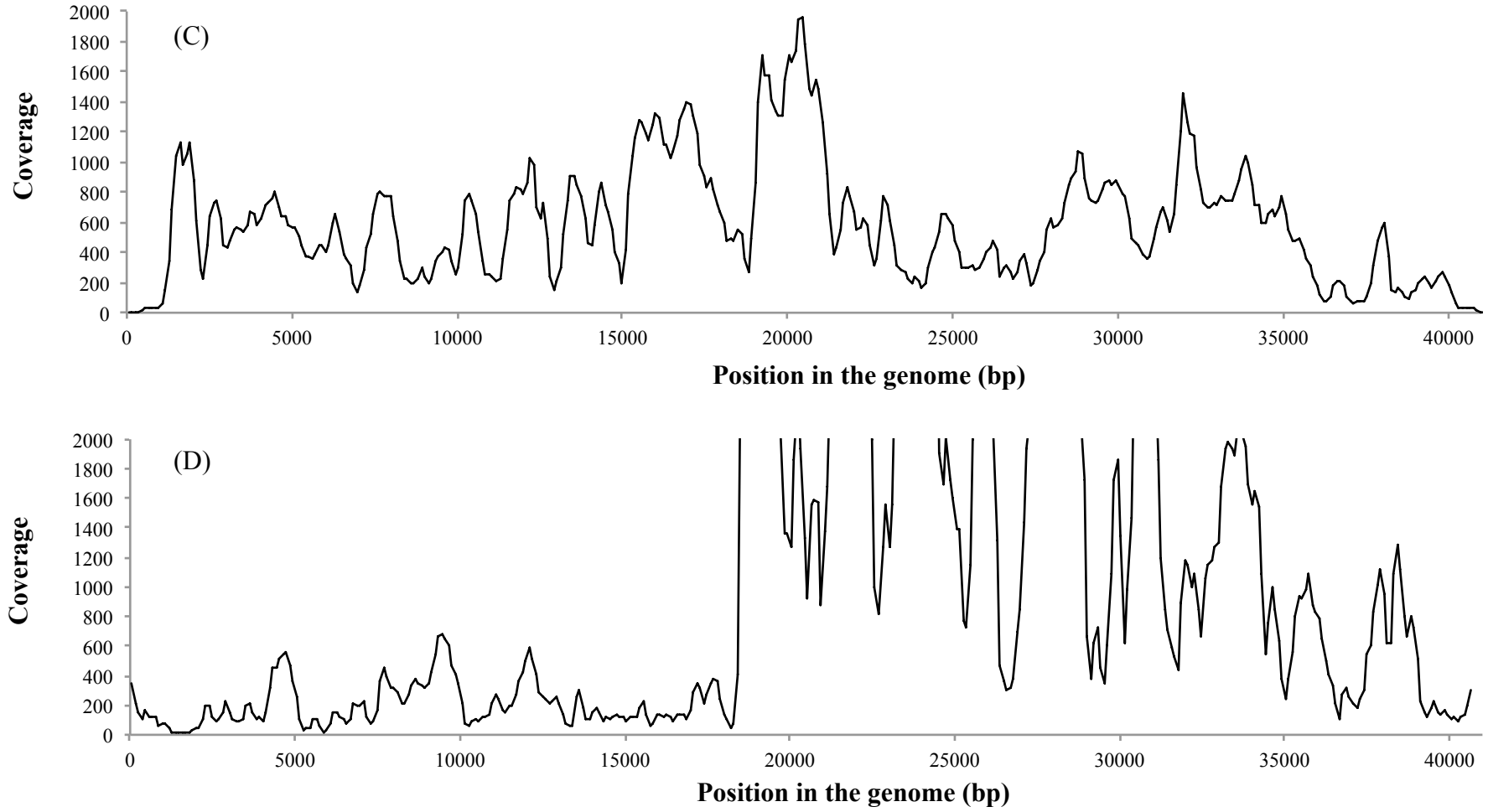


Figure 6.4 (C and D on next page): Read coverage was very irregular across the phage genomes. It clearly dropped towards the ends of the SpT152 genome. (A) SpT5, (B) SpT252, (C) SpT152 and (D) SpT99/F3 (coverage higher than 2000x not shown).


(C)
Coverage
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
5000
10000
15000
20000
25000
30000
35000
40000
Position in the genome (bp)
(D)
Coverage
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
5000
10000
15000
20000
25000
30000
35000
40000
Position in the genome (bp)

Coverage was very erratic across the four genomes. A flatter line would usually be expected, even though it is known that coverage can be biased depending on GC content and the chosen library preparation method (Rhodes *et al.*, 2014). Overall the average coverage was 600x. The definition of sufficient coverage depends on the application, but in general for phage genomes having coverage higher than 50x does not offer any particular advantage (Dr Andrew Millard, personal communication). The average coverage of the Warwick genomes was therefore very high. The highest coverage value (6000x) was seen with SpT99/F3. In fact, coverage across this genome was very unusual. Half of the genome had coverage below 600x and the other half had above 2000x coverage. This unusual pattern and its possible causes were further discussed in section 6.4.

SpT5 and SpT252 had coverage profiles similar to each other. This was to be expected because these phages were very similar to each other. One area of low coverage in comparison to the average coverage was observed between 13,000 and 14,000 bp in both genomes (down to 32x in SpT252 and 12x in SpT5). Sequence analysis of this region revealed that it had a GC content of 25%. The GC content of the whole genomes was 35%, which was similar to the expected GC content of their staphylococcal host (Rosypal *et al.*, 1966). The transposase used in the Nextera system was shown to have a preference for insert sites within A-T rich regions. This meant that regions with very low GC content (25% and below) were targeted by the transposase more often than other regions in the phage genomes and this led to some loss of signal and low coverage. The use of the Nextera XT DNA Library Preparation kit might therefore not be ideal for low GC genomes. It was however shown to be able to produce good quality DNA library for genomes down to 29% GC content (Lamble *et al.*, 2013).

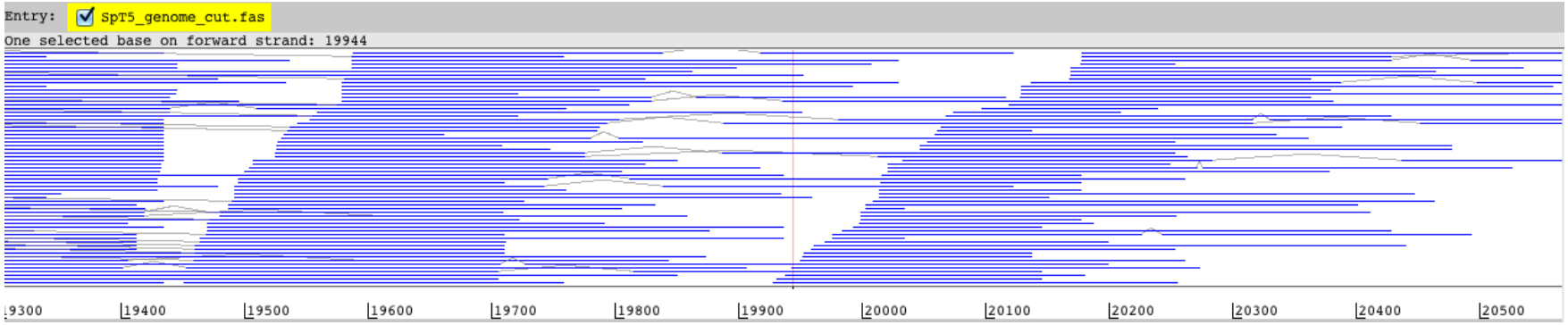
Coverage at the ends of the SpT152 genome dropped to a very low value (1x). This suggested that this genome had distinct cohesive ends. For SpT5, SpT252 and SpT99/F3, coverage value did not drop as drastically and remained around 200 to 300x. This indicated that these genomes might be circularly permuted.

6.3.2. Bioinformatics prediction based on read alignment across genome ends

Another way to predict whether genomes have cohesive ends or are circularly permuted is to determine if reads can align to both ends of a genome at the same time. If this is the case, this means that both arbitrarily chosen "ends" of the genome were sometimes connected when DNA was fragmented and the genome is probably circularly permuted. To show this, the four genomes were cut in half and both original ends were pasted together in a fasta file. The alignment of sequencing reads with the cut-and-pasted version of each genome was studied (section 2.40).

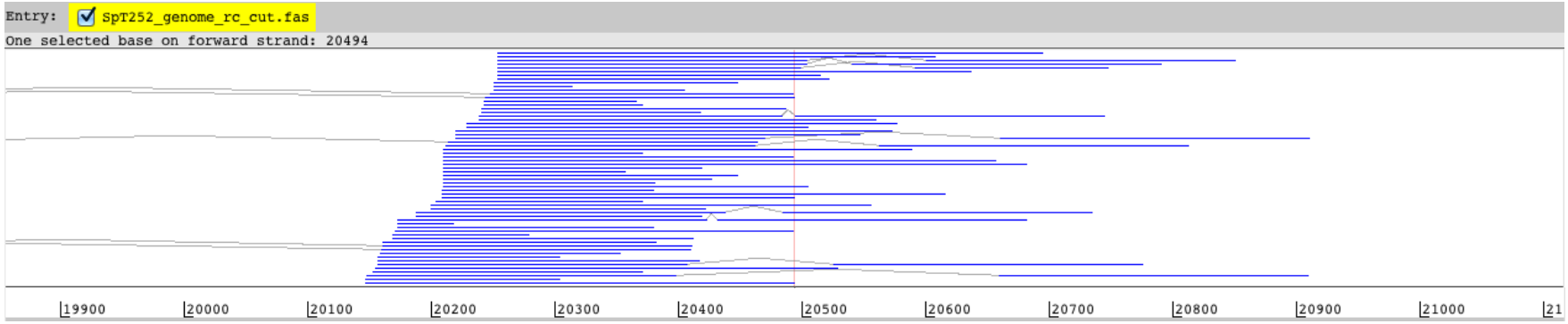
Numerous reads mapped or were paired across both "ends" of the SpT5, SpT252 and SpT99/F3 genomes suggesting once again that they were circularly permuted (Figure 6.5). These results concurred with the previous prediction based on read coverage. No reads mapped directly across the ends of SpT152 genome but three pairs of reads were paired across that region. This meant that on very few occasions inserts spanning over both ends of the genome were produced. The fact that the SpT152 genome had cohesive ends could therefore not be determined for certain with this bioinformatic approach.

(A)



Position in the genome (bp)

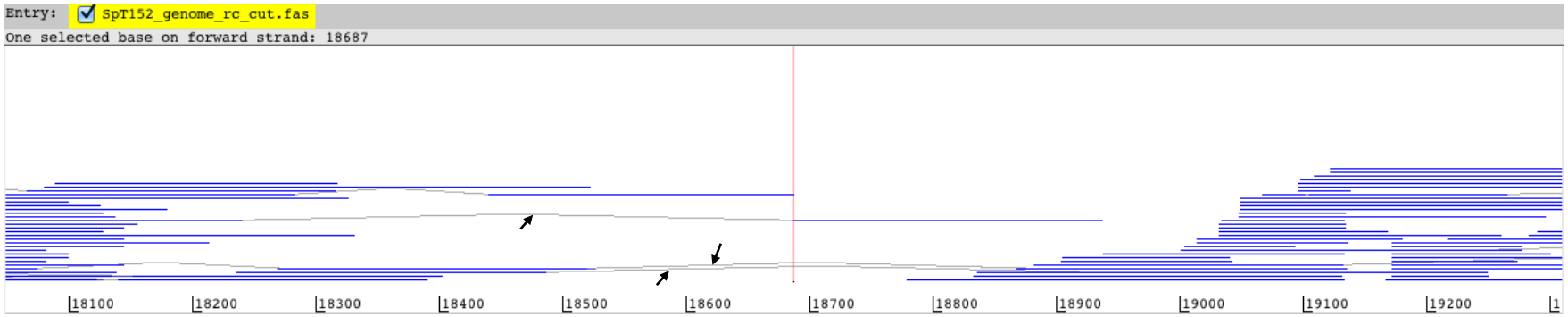
(B)



Position in the genome (bp)

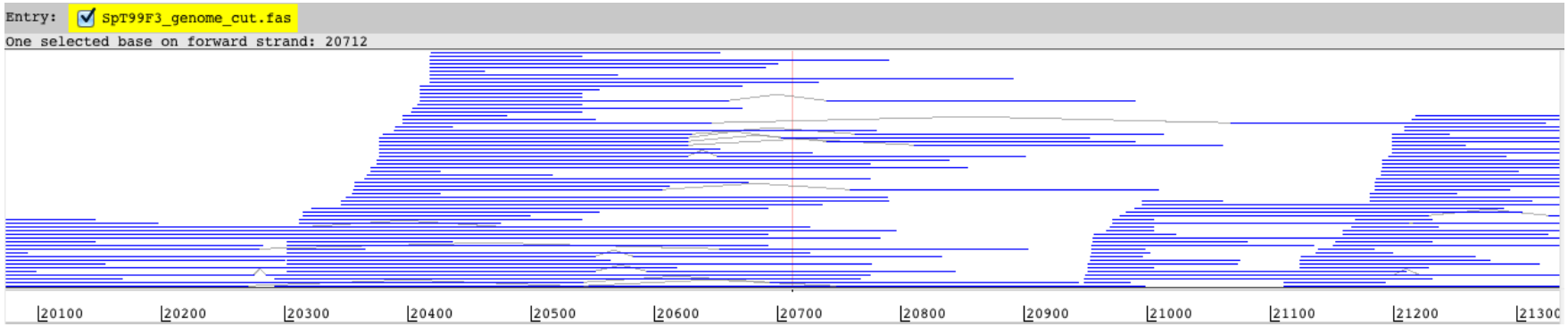
Figure 6.5 (C and D on next page): Sequencing reads (blue lines) either aligned or were paired (pairing shown with grey lines) across both ends of the (A) SpT5, (B) SpT252 and (D) SpT99/F3 cut-and-pasted genomes. The original first base of each genome is shown with a vertical red line. No reads mapped directly across the ends of the (C) SpT152 genome but three pairs of reads (shown with arrows) were paired a that region.

(C)



Position in the genome (bp)

(D)



Position in the genome (bp)

6.3.3. Confirmation of the bioinformatic predictions through PCR

To confirm the predictions from *in silico* analysis, PCR was performed with primers complementary to each theoretical end of the phage genomes (Figure 6.6).

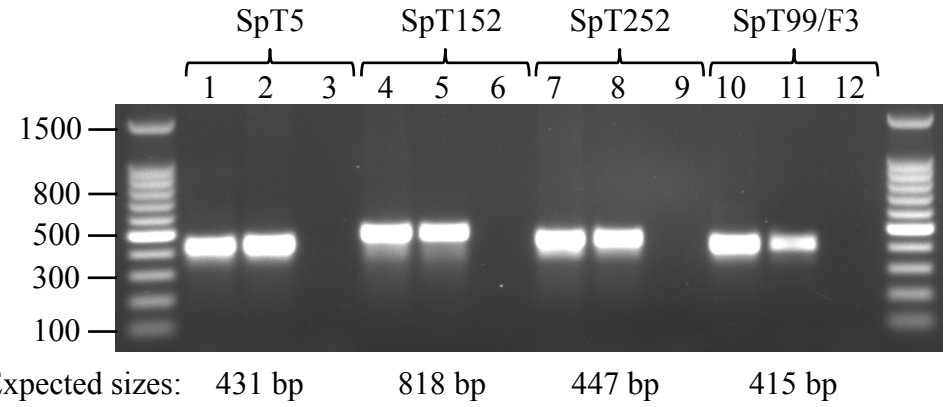


Figure 6.6: PCR was performed with primers complementary to each theoretical end of the phage genomes on phage DNA (1, 4, 7 and 10), on the corresponding lysogen's DNA (2, 5, 8 and 11) and water as a control (3, 6, 9 and 12). PCR amplification was seen with all sets of primers on both phage and lysogen's DNA. The size of the PCR product for SpT152 (~500 bp) was different from the expected size (818 bp).

PCR amplification was seen with all sets of primers on both phage and lysogen's DNA. For SpT5, SpT252 and SpT99/F3 this result confirmed the bioinformatics prediction; these genomes were most probably circularly permuted.

It was thought that SpT152 did not have a circularly permuted genome. This meant that no PCR amplification was expected on phage DNA because the genome ends should be defined and separate from each other in phage particles. On the other hand, PCR amplification was expected on the lysogen's DNA because the genomes of siphoviruses, including those with cohesive ends, undergo circularisation once they enter a host cell. Genomes are then cut open at a different site before integration into the host's genome (section 1.4.2.b.ii). PCR amplification should therefore be possible on lysogen's DNA because the ends observed in phage particles are joined together in the corresponding prophage.

PCR amplification was seen on both the phage and the lysogen's DNA indicating that SpT152 was likely to have a circularly permuted genome. The discrepancy

between the *in silico* prediction and the experimental result may be due to the fact that the GC content in the theoretical terminal regions was extremely low (19%) compared to the rest of the genome (35%). This probably explained why read coverage dropped so drastically in those regions, similarly to the regions with low coverage in the SpT5 and SpT252 genomes. The very low coverage in that region of the SpT152 genome may also have led to assembly errors and this would explain why there is a discrepancy between the expected size (818 bp) and the obtained size (~500 bp) of the PCR product.

To look for the presence of sequence repeats associated with circularly permuted genomes, alignments of the phage genomes with themselves were performed with Easyfig. The software identified sequence match between the ends of SpT152 suggesting once more that it may be circularly permuted (Figure 6.7). The poor quality of the sequence in these regions prevented the drawing of any definitive conclusions regarding terminal repeats. Such sequence matches were not found in SpT5, SpT252 and SpT99/F3. The presence of sequence repeats at the end of the SpT152 genome may constitute another possible explanation for the discrepancy between the expected size (818 bp) and the obtained size (~500 bp) of the PCR product. Primers were designed to be complementary to each theoretical end of the SpT152 genome and when calculating the expected size of the PCR product the length of the repeats was included (Figure 6.8, A). In reality, PCR amplification occurred on different genome permutations where sequence repeats were not present between both primer sites (Figure 6.8, B) leading to a shorter actual PCR product.

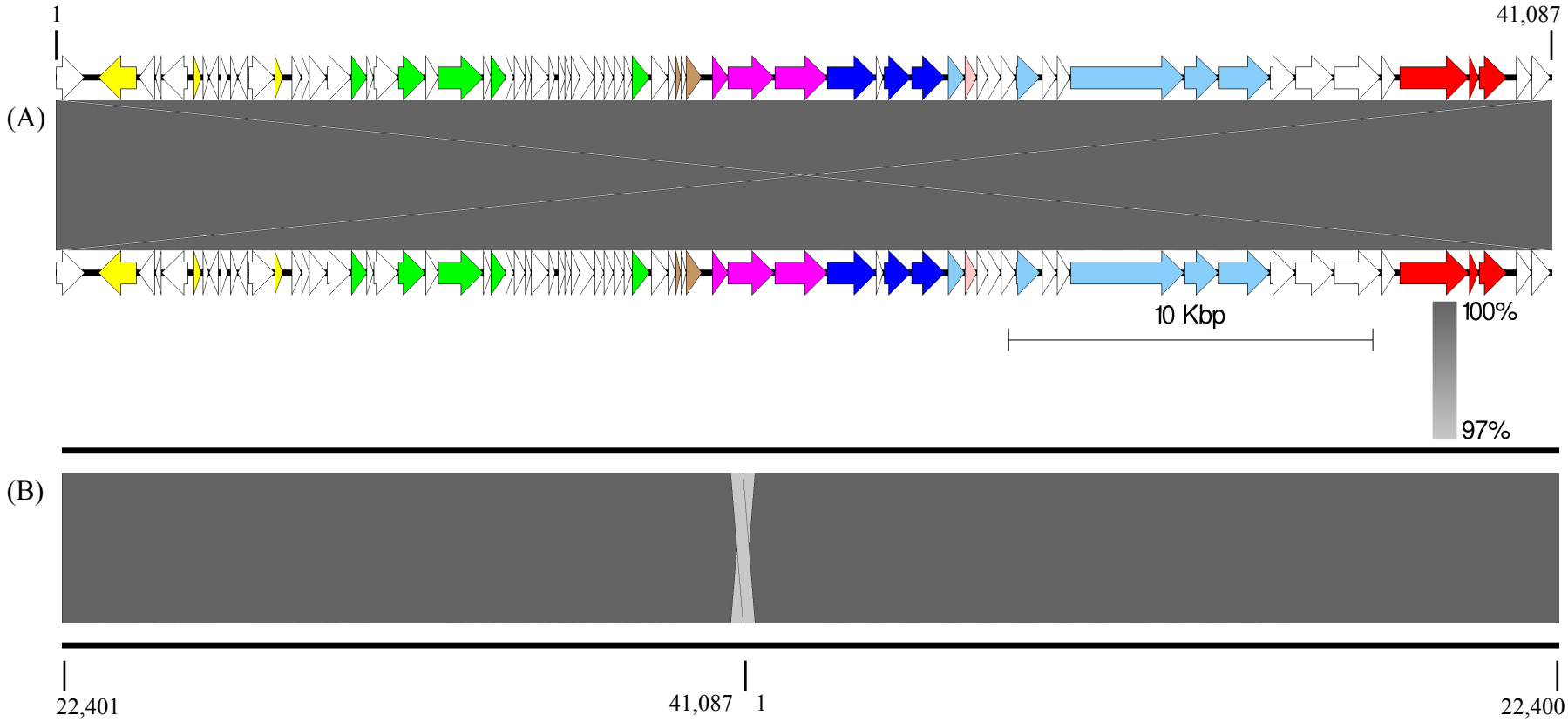


Figure 6.7: (A) Sequence matches were observed between both "ends" of the SpT152 genome when aligning it with itself using Easyfig. (B) These sequences matches were even more visible when aligning the cut-and-pasted version of SpT152 genome with itself (section 6.3.2). Raw fasta sequences were used for this, hence the absence of CDSs. The position in the genome is indicated in base pairs at the top and bottom of the figure. A BLAST similarity scale is shown in the middle.

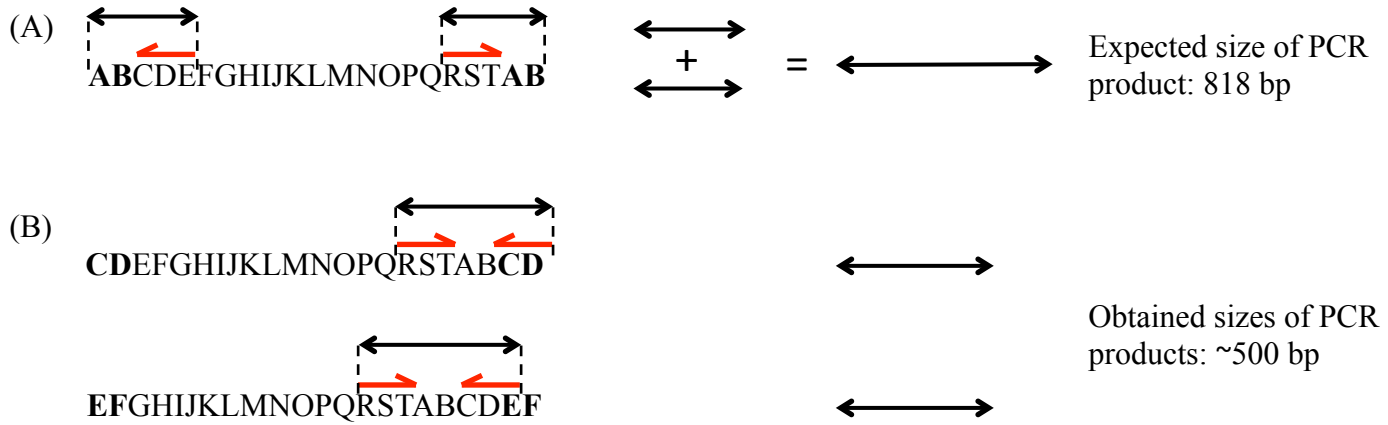


Figure 6.8: (A) Primers (in red) complementary to each theoretical end of the SpT152 genome (here represented with letters) were designed. When calculating the expected size of the PCR product (black arrow) the length of the terminal repeats (in bold) was included. (B) In reality, PCR amplification occurred on different genome permutations where sequence repeats were not present between both primer sites leading to a shorter PCR product.

6.4. Further study of the SpT99/F3 genome

SpT99/F3 stood out among the four phages because of its phenotypic and genotypic characteristics. It was able to form plaques on only a few strains out of the 72 available *S. pseudintermedius* strains. The lysate titres usually obtained with SpT99/F3 (10^8 PFU/mL) were lower than with the other phages (10^9 PFU/mL and higher). SpT99/F3 lysates produced through plate wash were systematically concentrated to reach a high titre. Amplification in liquid culture was never achieved. Most importantly, PFGE performed with SpT99/F3 genomic DNA revealed the presence of several bands when only one band would be expected (section 3.4.2).

One hypothesis to explain this result was the possible presence of a second phage (and maybe more) in the SpT99/F3 lysate. When electron microscopy (EM) was performed to observe this phage's morphology, two populations were seen according to Ian Hands-Portman, who helped with taking EM pictures. One type is depicted in section 3.4.5 and was of a similar size as SpT5, SpT152 and SpT252. The other population was much bigger and much more rare. Unfortunately, no picture of these giant phages was found in our records. This observation, even if it could not be verified, pointed towards the presence of at least two types of phages in SpT99/F3 lysate.

Another piece of evidence supporting the two-phage population hypothesis was the drastic change in read coverage across the SpT99/F3 genome (Figure 6.4, D). The fact that coverage was much higher over one half of the genome indicated that this half was overrepresented compared to the other one. One possible explanation for this was that the SpT99/F3 genome was a chimera of two genomes stuck together during the assembly process. The low coverage region (1 to 18,500 bp) would correspond to a phage present in low abundance and the high coverage region (18,500 to 40,750 bp) would correspond to a high-abundance phage. To prove that the SpT99/F3 genome was not a chimera created *in silico*, PCR was performed with primers recognising DNA sequences situated on either side of the region with the change in coverage (Figure 6.9, A). PCR amplification was seen on phage and lysogen's DNA indicating that within the population there were at least some individuals where the two regions were connected and were part of the same genome (Figure 6.9, B).

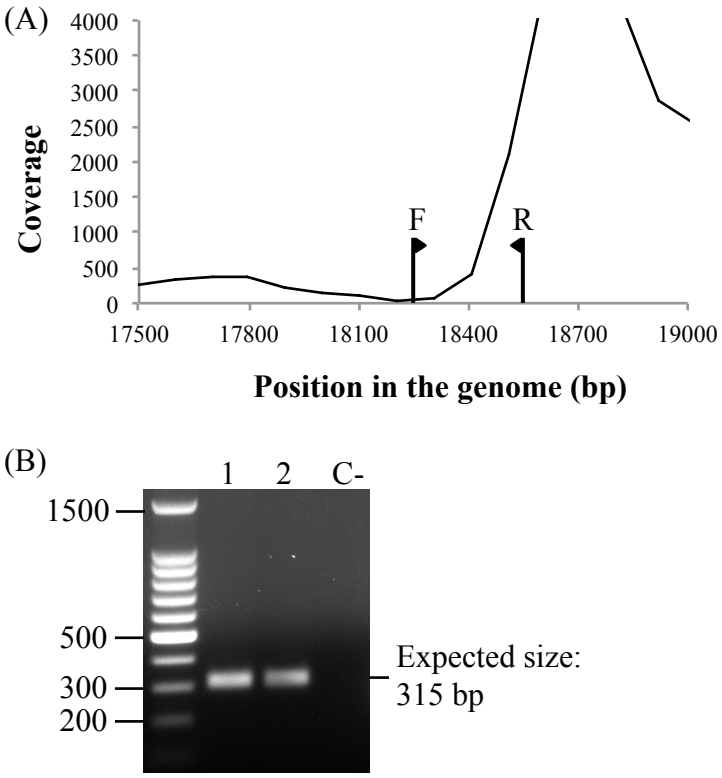


Figure 6.9: (A) Primers recognising DNA sequences on either side of the region with the change in coverage were used to show whether SpT99/F3 was a chimera of two genomes created during genome assembly. F = forward primer in region with 50x coverage. R = reverse primer in region with 3000x coverage. (B) PCR amplification was seen on SpT99/F3 DNA and DNA from its lysogen indicating that the two regions with difference in coverage were connected at least in some individuals within the population. 1: SpT99/F3, 2: S56F3, C-: water negative control.

Considering these results, another possibility would be that part of the phage population carried the entire genome and while another part of the population carried only half of it. In that case the high coverage over one half of the genome would be due to the added abundance of DNA from both types of phages. This situation would resemble the P2/P4 system. P2 is a temperate phage that infects *E. coli*. Its genome contains all the genes necessary for lytic growth and lysogeny. P4 is a satellite of the P2 phage. It can establish lysogeny in the absence or presence of P2 but it depends on the P2 helper genes for all the morphogenesis functions (Briani *et al.*, 2001). Its genome does not contain structural genes or genes required for cell lysis (Eriksson, 2005). There were two major differences between the P2/P4 system and the two potential SpT99/F3 genomes. Firstly, the high coverage region covered the second half of the SpT99/F3 region (from CDS 8, see Figure 6.3). This would mean that one

genome contained all the genes necessary for lytic and temperate lifestyles, similarly to the P2 phage, and the other genome, unlike P4, only contained structural genes and gene required for cell lysis. Secondly, P2 and P4, unlike the two potential SpT99/F3 genomes, have genomes that show little homology with each other (Deho and Ghisotti, 2006).

6.5. Conclusions

Following whole-genome sequencing with the Illumina MiSeq platform, the four genomes were assembled and annotated. The putative function of most of the identified CDSs remained unknown. To try and improve the original annotation, a second round of annotation was performed with Prokka using a reference database of all known viral proteins instead of SwissProt. This did not lead to any significant improvement. A lot of work remains to be done to enrich databases with well-characterised phage proteins. Nevertheless, it was possible to identify clusters of genes with similar function or involved in the same biological processes. This type of genome organisation (with succession of lysogeny – replication – packaging – head – tail – lysis genes) was observed in *Staphylococcus aureus* temperate phages (Xia and Wolz, 2014) as well as *Streptococcus* phages and dairy phages (Obregon *et al.*, 2003, Brüssow and Desiere, 2001). It is therefore probably a common type of genome organisation among temperate phages of the low GC Gram positive cocci.

The observation of the phages with electron microscopy revealed that they exhibited a typical *Siphoviridae* morphology (section 3.4.5). This family of phages is known to contain linear DNA (Ackermann, 2006) and regarding DNA packaging strategies, both cohesive genome ends and headful packaging (associated with circularly permuted genomes) are observed in temperate phages belonging to this family (Catalano *et al.*, 1995, Birdsell *et al.*, 1969, Marrero and Yasbin, 1986). Bioinformatic approaches based on read coverage and read mapping suggested that SpT5, SpT252 and SpT99/F3 had circularly permuted genomes and that SpT152 had a genome with cohesive ends. For the first three phages, PCR amplification across the “ends” of their genomes confirmed the bioinformatic predictions but no terminal repeats, characteristic of circularly permuted genomes, were found. For SpT152, PCR amplification occurred and putative terminal repeats were identified in the genome sequence refuting the *in silico* prediction.

The results presented in this chapter showed that the *in silico* predictions were not always reliable. This was probably due to the quality of the sequencing data especially for SpT152 where extremely low coverage led to assembly errors. The Nextera XT DNA Library Preparation kit is not well suited for low GC genomes and the *S. pseudintermedius* phage genomes have proven even more AT rich than expected based on the GC content of their host. It would therefore be beneficial to perform sequencing of these genomes with a different method and compare both results. The genome sequences obtained with the Nextera + MiSeq method were still accurate enough to allow the successful design of phage-specific primers (section 4.2) and sequence study in Chapter 5.

Phage SpT99/F3 exhibited unusual phenotypic and genotypic features. The analysis of the sequencing data seemed to support the idea that two or more phages were present in SpT99/F3 lysate. A small PCR test showed that the two halves of the assembled genome were connected at least in part of the population. It would be beneficial to sequence the genome of this phage again as well as observing it in electron microscopy one more time to check if the same results were obtained. Another experiment that could be performed is quantitative PCR with primers annealing to the region with change in coverage and primers annealing to the left and right of that region. A ratio of 1:1:1 would suggest that all three regions are present in equal quantities and are part of one single genome. If different ratios were observed, it would suggest that the assembled genome presented in this chapter is not representative of the entire population and some individuals carry only parts of that genome in their capsid.

Chapter 7 The ecology of *S. pseudintermedius* and its phages

7.1. Introduction

To successfully isolate phages, choosing the right source of phages is important and is based on the fact that phages and their host co-reside in the same type of environment. Co-residence was shown to occur between phages and hosts that live or survive in the environment such as cyanobacteria (Huang *et al.*, 2015) or bacterial species resulting from human or animal faecal contamination of wastewater (Wangkahad *et al.*, 2015, Yahya *et al.*, 2015). Water and soil are known to be environmental reservoirs of *Mycobacteria* (Makovcova *et al.*, 2014, Lecuona *et al.*, 2016, Mohanty *et al.*, 2016), some species of which can cause serious diseases in humans and animals. Mycobacteriophages were isolated from the same sources (Froman *et al.*, 1954, Carroll and Avio, 1975, Teng *et al.*, 2015). *Vibrio cholerae* and cholera phages can both be present in river waters and were shown to influence each other's population dynamics in a study in Bangladesh. Researchers found that seasonal outbreaks of cholera inversely correlated with the prevalence of cholera phages in rivers (Faruque *et al.*, 2005).

In the above examples, the isolated phages were often virulent but temperate phages infecting the same hosts exist as well (Pedulla *et al.*, 1996, Woods and Egan, 1974, Beilstein and Dreiseikelmann, 2008). For other bacterial hosts, the situation is sometimes different. The human pathogen *Clostridium difficile* that causes nosocomial antibiotic-associated diarrhoea was shown to carry diverse prophages in its genome (Shan *et al.*, 2012) and so far only temperate phages of this host have been isolated even though it can also be found in the environment (Zidaric *et al.*, 2010, Hargreaves *et al.*, 2013). It was hypothesised that the ability of *C. difficile* to undergo sporulation may select for lysogenic infections over lytic infections and this would explain why lytic phages of this bacterium have not been found (Hargreaves and Clokie, 2014). Phages infecting *Staphylococcus aureus*, an opportunistic pathogen that colonises the skin of humans and animals and causes nosocomial infections in clinical settings, are often temperate (Deghorain and Van Melderen, 2012). They were shown to contribute to pathogenesis and the spread of virulence genes in the *S. aureus* population (Blair and Carr, 1961a, Xia and Wolz, 2014, Moon *et al.*, 2015). Lytic phages infecting this bacterium have also been isolated such as

phage K (O'Flaherty *et al.*, 2004), SA11 (Kim and Myung, 2012) or phiIPLA35 and phiIPLA88, two phage that are lytic due to mutations in the lysogeny functions (Garcia *et al.*, 2009). The origin of phage K remains unclear (O'Flaherty *et al.*, 2005), SA11 was isolated from wastewater and the phiIPLA phages were found in milk. *S. aureus* usually resides on the skin so co-residence of staphylococcal phages and their host is less easy to justify in these types of samples. It is possible that the milk and water used in those experiments were contaminated with animal sources.

S. pseudintermedius, like *S. aureus*, is an opportunistic pathogen that resides on the skin of animals, dogs in particular. Several studies showed that the bacterium is present on the skin of healthy dogs (section 1.3.1). *S. pseudintermedius* has been identified as the main causative agent of skin infections. Consequently this type of infection is strongly associated with the presence of the pathogen on the skin: a study showed that 70% of dogs referred with bacterial pyoderma to a dermatology practice in Canada carried *S. pseudintermedius* on their skin (Beck *et al.*, 2012). This study also showed that microorganisms other than *S. pseudintermedius*, such as *S. aureus* (identified in 6.4% of dogs in that study), were present on the skin and could cause infections in dogs.

No information was available in the literature about phages of *S. pseudintermedius* and their ecology in the environment, and because of this a range of environmental samples was screened for the presence of these phages. Skin swabs were included in the screening based on the principle of co-residence of phages and their host on the skin of healthy dogs and dogs suffering from pyoderma. Dog faecal samples were screened too following the successful isolation of *S. pseudintermedius* phages from this type of samples in Denmark (section 3.2.1). The hypothesis that motivated the choice of these samples was that dogs might swallow *S. pseudintermedius*, and its phages if present, when licking the skin. If the bacterium and its phages survived the journey in the intestinal tract they would subsequently be found in faeces. A few water and soil samples were included in the screening in case *S. pseudintermedius* phages were found in the environment like some *S. aureus* phages. After screening more than 100 samples in total, the isolation of *S. pseudintermedius* phages from environmental samples remained unsuccessful (section 3.2.2).

A study was therefore undertaken to gain knowledge about the prevalence of *S. pseudintermedius* and its phages in the different types of samples described above. It was hoped that once a method of detection was developed it would help determine whether or not samples contained phages and/or their host, constituting a pre-screening tool to identify potential sources of phages and speed up the process of phage isolation. Until now the prevalence of *S. pseudintermedius* has only been studied on the skin. The proposed study would generate data about the prevalence of *S. pseudintermedius* in other types of samples. It could prove whether or not the bacterium was actually present in faeces and whether it could survive in the environment.

As described in the section 1.3.2, *S. pseudintermedius* is usually identified through biochemical tests (e.g. coagulase activity) and PCR amplification of the thermonuclease (*nuc*) gene following an initial cultivation step. However, to study the ecological reservoirs of a bacterial species, molecular culture-independent approaches are generally the most sensitive and specific assays. PCR-based methods were used in a variety of situations such as the detection of bacteria in plants (Kamle *et al.*, 2013), food (Strohmeier *et al.*, 2014, Wang *et al.*, 1997), human (McDowell *et al.*, 2001, Johnson *et al.*, 2000) and animal (Cheema *et al.*, 2007) samples. Viruses can be detected too (Allen *et al.*, 1999).

A PCR-based screening method was therefore developed to detect *S. pseudintermedius* and its phages. It consisted in whole-DNA extraction from dog samples and PCR amplification with primers specific for *S. pseudintermedius* or its phages. Several methods for DNA extraction were compared, and end-point PCR and quantitative PCR were tested. Indeed, qPCR assays can improve the sensitivity and the specificity of the method of detection as well as providing information about the load of a pathogen in a sample (Horváth *et al.*, 2013).

Quantitative PCR can be performed following two methods: SYBR Green or TaqMan®. The SYBR Green master mix contains a dye that becomes a hundred times more fluorescent when binding to dsDNA compared to its fluorescence in solution. The quantity of measured fluorescence is proportional to the quantity of dsDNA produced during the PCR reaction. The TaqMan® method involves the use of

a probe that binds to the region between both primer sites. The probe has a fluorescent dye at one end and a quenching dye at the other end so no fluorescence is observed when the probe is intact. During the PCR reaction the TaqMan® probe is degraded by the Taq polymerase 5' to 3' exonuclease activity and the fluorescent dye is released. The signal is proportional to the quantity of amplicon produced in the reaction. TaqMan® and SYBR Green methods have been compared previously in a range of applications and were found to be either equivalent in terms of sensitivity (Paudel *et al.*, 2011, Tajadini *et al.*, 2014) or the TaqMan® method proved to be superior (Matsenko *et al.*, 2008, Gunel *et al.*, 2011). In general, the use of a TaqMan® probe adds specificity to the assay and is therefore considered preferable, especially when working on complex samples such as faeces. The expected sensitivity of an assay for the detection of a pathogen ranges from 1000 to less than ten genome copies per gram of sample depending on the application (Leal *et al.*, 2014, Nagpal *et al.*, 2015). In this study the aim was to detect 100 genome copies per gram of sample (or per swab).

The temperate phage carriage of *S. pseudintermedius* strains was studied as well. Temperate phages were successfully isolated through mitomycin C exposure and co-culture in this project, prophages were identified in the genome of the E140 strain and other *S. pseudintermedius* strains (Moodley *et al.*, 2013, McCarthy *et al.*, 2015) supporting the idea that prophages might be widespread in the *S. pseudintermedius* population. To investigate this, a PCR-based screening was performed to look for the presence of prophages in *S. pseudintermedius* strains.

Finally the ecology of one particular phage, SpT5, was looked into in more detail to identify the bacterial strain of origin of this phage. It was not known where SpT5 came from because it was isolated through co-culture of seven bacterial strains together (section 3.3.1).

7.2. Detection of *S. pseudintermedius*

7.2.1. Development of an end-point PCR assay

7.2.1.a. Choice of primers

S. pseudintermedius and other *Staphylococcus* species are very closely related and primers targeting the *nuc* gene and discriminating between all of them through end-point PCR were developed previously (Sasaki *et al.*, 2010). They were tested on DNA from *S. pseudintermedius* (strain E140) and other *Staphylococcus* species to check their specificity (Figure 7.1). From then on, DNA and cells from the E140 *S. pseudintermedius* strain were used in the experiments. This strain was chosen because it was sequenced (Moodley *et al.*, 2013) and this provided useful information such as genome size for the design of experiments.

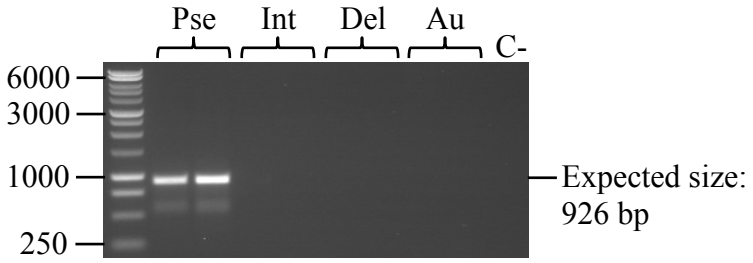


Figure 7.1: PCR amplification was seen on *S. pseudintermedius* (Pse) and not on *S. intermedius* (Int), *S. delphini* (Del) and *S. aureus* (Au) genomic DNA when using primers specific for *S. pseudintermedius*. C-: water negative control.

PCR amplification was seen only on *S. pseudintermedius* DNA. The high specificity of the primers was confirmed. The second fainter band visible on the gel was sequenced and it matched specifically *S. pseudintermedius* when aligned against the NCBI 'nr' database. It was decided to use these primers for the detection of *S. pseudintermedius* through end-point PCR.

7.2.1.b. Choice of DNA extraction methods

For DNA extraction from faeces, the FastDNA™ Spin Kit for Soil (MP Biomedicals) and the QIAamp® Fast DNA Stool Mini Kit (Qiagen) were compared (Figure 7.2). DNA was extracted in triplicates from two different samples with both kits. 10 µL of the resulting DNA was run on a gel to estimate the efficiency of extraction of both kits.

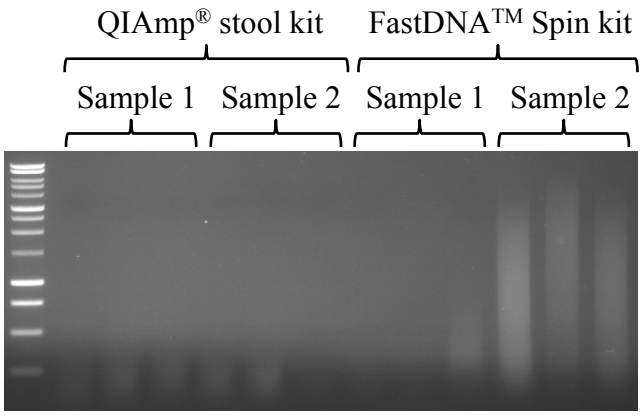


Figure 7.2: DNA extraction with the FastDNA™ Spin kit was successful with all three replicates of sample 2. Some DNA was also seen in one replicate of sample 1. No DNA was visible on the gel after extraction with the QIAmp® stool kit.

The results showed that the QIAmp® Fast DNA Stool Mini Kit was not efficient at extracting DNA from faeces. DNA was successfully extracted from one sample out of the two that were tested with the FastDNA™ Spin Kit. The extracted DNA appeared as a smear when run on an agarose gel. This meant that DNA was sheared during the process of extraction. However, there should still be pieces of DNA big enough for PCR amplification to be performed successfully. The FastDNA™ Spin Kit was already routinely used in the lab for the purpose of DNA extraction from faeces. It was therefore used for further development.

For DNA extraction from skin swabs, the Chemagic STAR kit for Nucleic Acid Isolation (PerkinElmer) and the NucleoSpin® Tissue kit (Macherey-Nagel) were tested. Swabs were initially sterile so the only way to compare the efficiency of both kits was to spike swabs with known numbers of *S. pseudintermedius* cells (E140 strain) and extract DNA. This was directly coupled with the determination of the

sensitivity of the PCR assay because the quantity of DNA extracted from spiked swabs was not high enough to be visible directly on a gel prior to amplification.

7.2.1.c. DNA extraction from spiked samples and PCR amplification

To determine how sensitive the end-point PCR assay was, faecal samples and swabs were spiked with known numbers of *S. pseudintermedius* cells (sections 2.41 and 2.42), DNA was extracted and PCR was performed. Faecal samples were spiked in triplicate with 10^8 cells and DNA was extracted with the FastDNA™ Spin kit. It was suspected that end-point PCR might not be sensitive enough for the detection of *S. pseudintermedius* in faeces. To obtain preliminary results regarding the sensitivity of the assay, samples were only spiked with a high number of cells.

Regarding swabs, the objectives of the experiment was to compare both DNA extraction kits as well as determining the sensitivity of the PCR assay. To do this, swabs were spiked with a wider range of cell numbers (low: 10^3, medium: 10^5 and high: 10^8 cells). DNA was subsequently extracted with either the Chemagic STAR kit or the NucleoSpin® Tissue kit. The efficiency of extraction was evaluated based on PCR amplification (Figure 7.3).

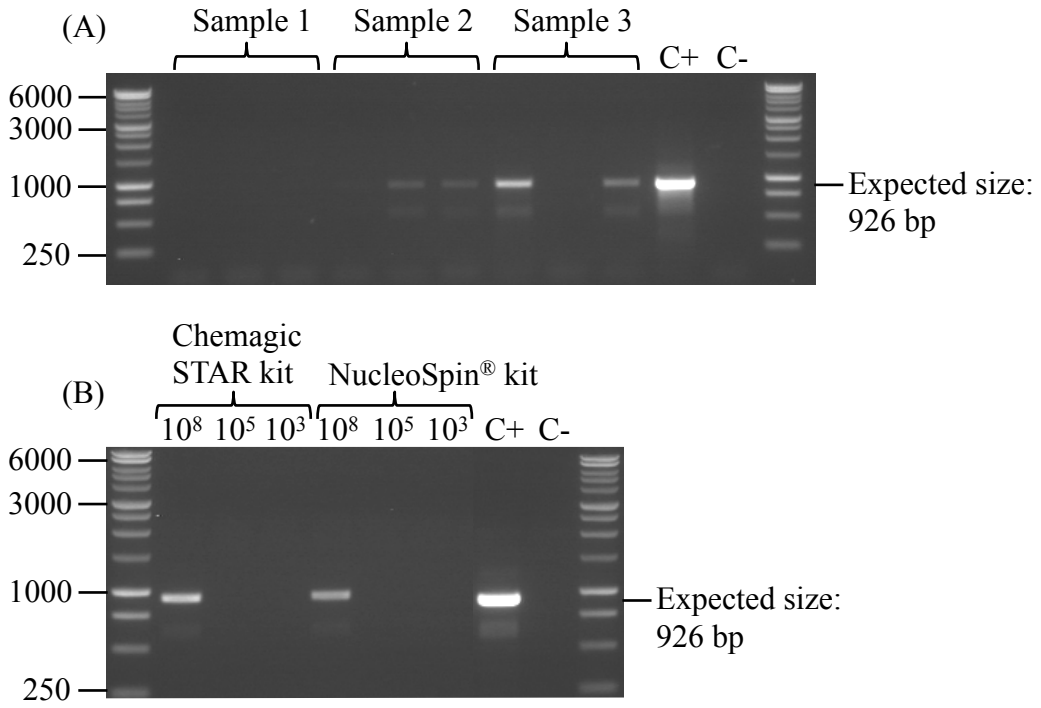


Figure 7.3: (A) PCR amplification was seen in only two out of three replicates for faecal samples 2 and 3, and no amplification was visible in sample 1 despite the high number of *S. pseudintermedius* cells added to the samples. (B) With both kits, PCR amplification was observed only on DNA extracted from swabs spiked with 10^8 *S. pseudintermedius* cells. C+: positive control. C-: water negative control.

PCR amplification was seen in only two out of the three replicates for faecal samples 2 and 3, and no amplification was seen in sample 1 despite the high number of *S. pseudintermedius* cells added to the samples. This suggested that the end-point PCR assay was not sensitive enough for the detection of *S. pseudintermedius* in faeces. Regarding swabs, the obtained results were the same for both kits: amplification was seen when spiking swabs with a number of cells and no amplification was observed at lower numbers of cells. The NucleoSpin® Tissue kit was chosen to perform subsequent DNA extraction from swabs because it was readily available in the lab.

The PCR assay was further optimised to try and improve its sensitivity. Parameters such as the BSA concentration and the annealing temperature were modified. The addition of dimethyl sulfoxide (DMSO) and the dilution of samples, particularly relevant for faecal samples that may contain PCR inhibitors, were tested as well. Loading more of the PCR reactions on agarose gels to make bands more visible was

tried too. In spite of all these tests, amplification was only observed with samples spiked with 10^8 cells. The end-point PCR assay was not sensitive enough. To overcome this problem, a qPCR assay for the detection of *S. pseudintermedius* was developed.

7.2.2. Development of a qPCR assay

When performing qPCR, it is usually recommended to have an amplicon size of 100 to 200 bp. The amplicon in the end-point PCR assay was 926 bp long. Primers specific for the *S. pseudintermedius nuc* gene with a 180 bp-long PCR product were therefore designed (section 2.46). It was not possible to design a TaqMan® probe specific for *S. pseudintermedius* because the target region between both primers was too similar in all SIG members. The qPCR assay was developed using the SYBR Green master mix (section 2.47). Optimisation was performed to improve the sensitivity of the assay on pure genomic DNA of *S. pseudintermedius*. A serial dilution from 100,000 to zero genome copies/µL was tested and the assay allowed the detection of 100,000 down to one genome copy/µL within 40 qPCR cycles when adding 10 µL of template DNA (Figure 7.4). The same serial dilutions were used as standard concentrations when performing further qPCR experiments (seven standards and one negative control).

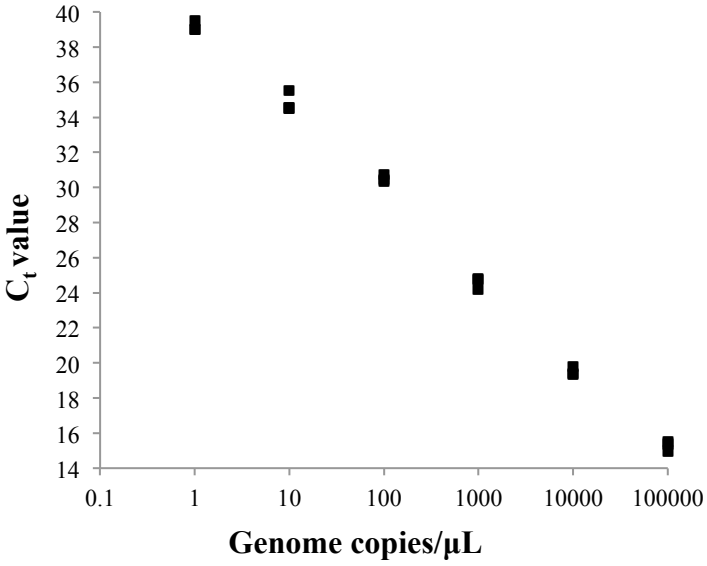


Figure 7.4: The detection of 100,000 down to one *S. pseudintermedius* genome copy/µL (tested in triplicate) was achieved within 40 PCR cycles. The same standard curve was used for subsequent qPCR experiments. Ct = threshold cycle.

To make sure that correct amplification was taking place, 10 µL of each standard qPCR reaction was loaded on a gel to check the size of the resulting amplicons (Figure 7.5, A and B). As expected the PCR product was 180 bp long for each reaction. No bands were seen with samples containing ten or one genome copy(ies)/µL because the DNA concentration was below the detection limit of the ethidium bromide-stained agarose gel. The qPCR machine was much more sensitive and could detect amplification.

In parallel of this experiment, the specificity of the qPCR assay was tested on *S. pseudintermedius* and other closely related species. 10^6 genome copies/µL were detected as expected and 180 bp-long amplicons were seen on a gel after PCR amplification on the three *S. pseudintermedius* strains that were tested (Figure 7.5, B, C and D). No amplification was detected and no bands were observed for the *S. intermedius*, *S. delphini* and *S. aureus* strains. This confirmed that the qPCR assay was specific for *S. pseudintermedius*.

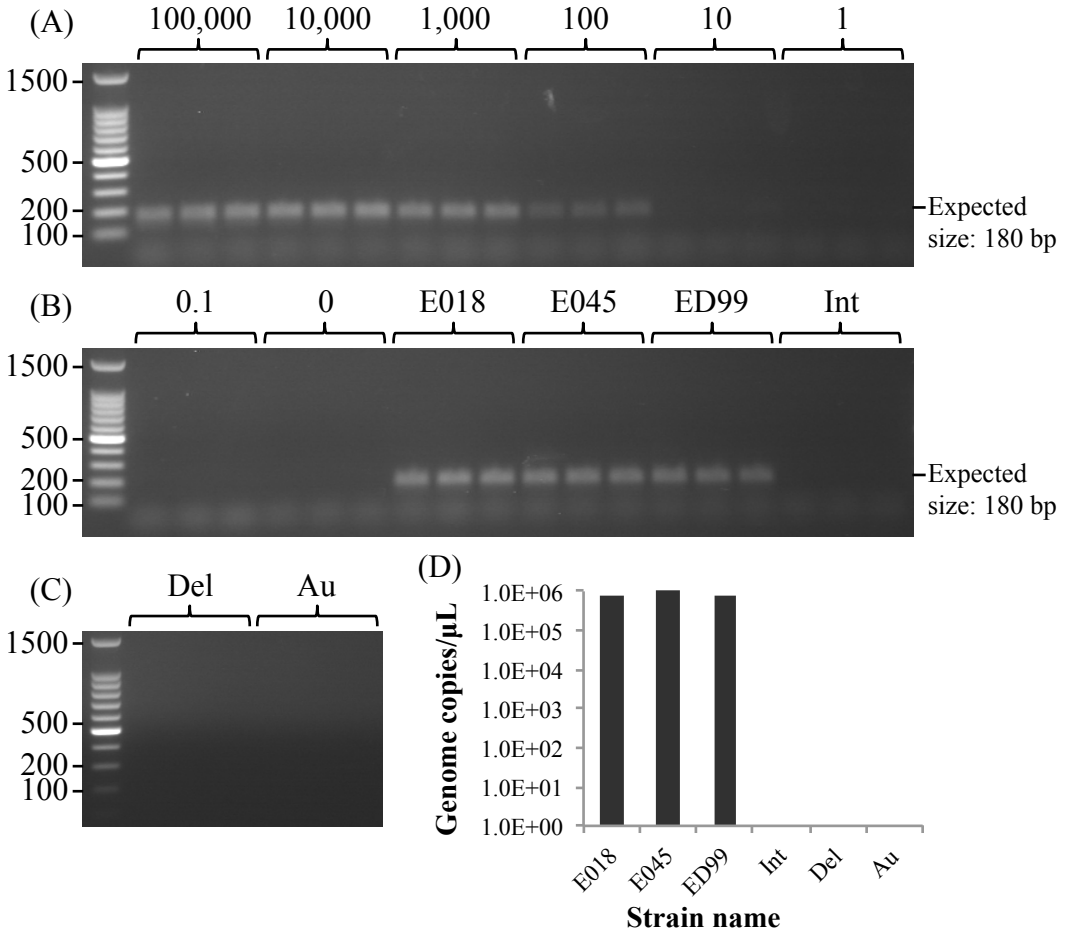


Figure 7.5: (A) and (B) A 180 bp-long PCR product was observed when loading 10 µL of each qPCR standard reactions, as expected. The quantity of DNA in samples containing ten and one genome copy(ies)/µL was below the detection limit of the agarose gel. (B) and (C) 180 bp-long amplicons were seen after PCR amplification on *S. pseudintermedius* strains (E018, E045 and ED99) but not on *S. intermedius* (Int), *S. delphini* (Del) and *S. aureus* (Au). (D) 10^6 genome copies/µL were detected in the *S. pseudintermedius* samples and no amplification was detected on the other *Staphylococcus* strains.

To determine the detection limit of the qPCR assay on faecal samples and swabs, these were spiked in triplicate with known numbers of *S. pseudintermedius* cells (from 10^8 to 10^2 cells). DNA extracted with the chosen methods and qPCR was performed. The number of genome copies per microliter was calculated with the ABI 7500 software and then expressed in either genome copies per gram of faeces or genome copies per swab (Figure 7.6).

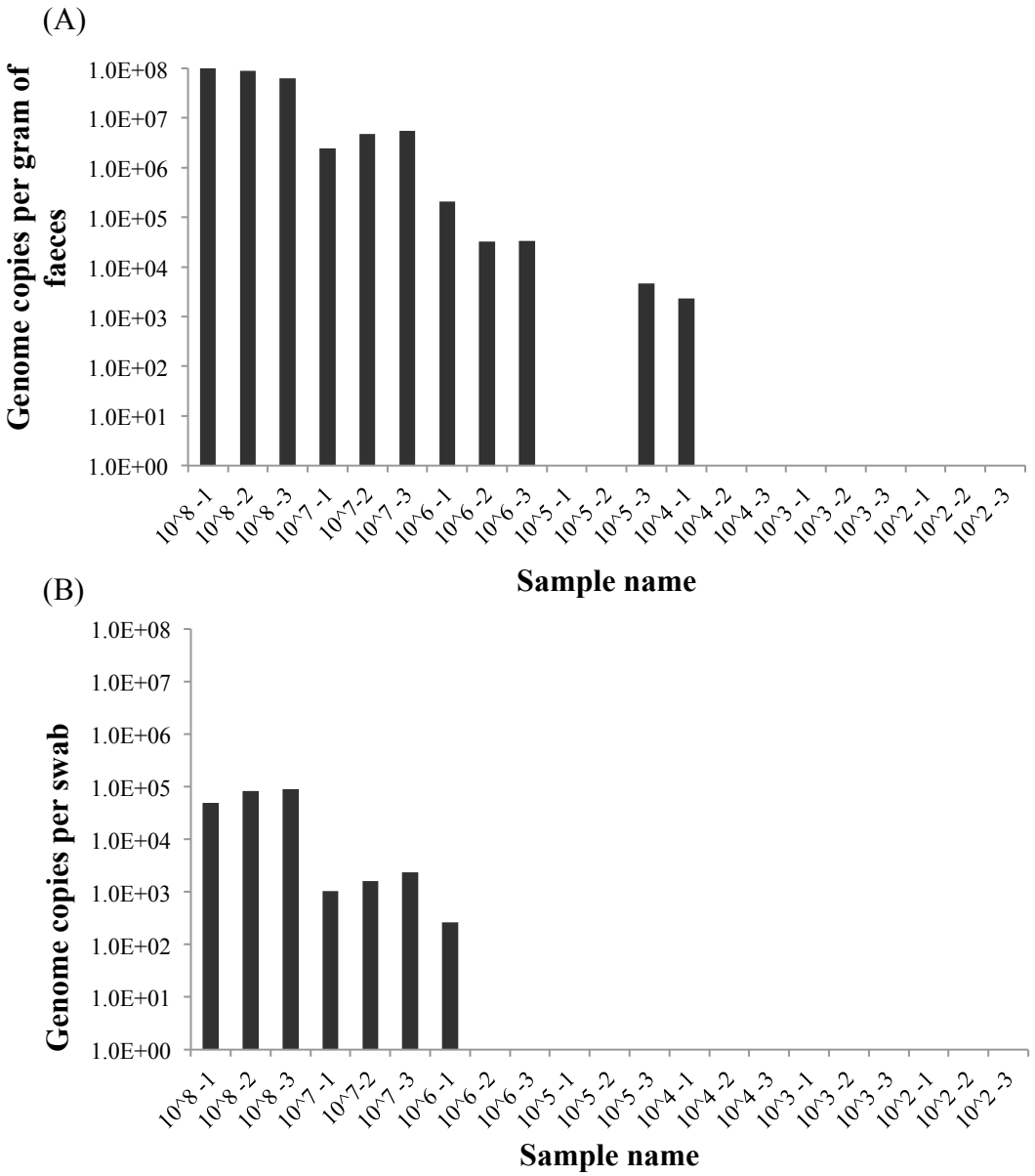


Figure 7.6: (A) For samples spiked with 10^8 and 10^7 cells per gram of faeces, the detected numbers of genome copies was the same as the numbers of cells added to the samples. When spiking faeces with 10^6 cells per gram of faeces and below, DNA recovery was less efficient (one to 1.5 log units lower than expected) or inexistent (no detection at all). (B) The detected numbers of genome copies per spiked swab were at least three log units lower than expected. Below 10^6 cells per swab, no amplification was detected.

Faecal samples were spiked with 10^8 to 10^2 cells per gram of faeces. After DNA extraction and qPCR it was possible to study the detection limit of the assay as well as the recovery of genome copies compared to the original number of cells added to the sample. For samples spiked with 10^8 and 10^7 cells per gram of faeces, DNA recovery was good because the detected numbers of genome copies were the same as

the number of cells added to the samples. When spiking with 10^6 cells per gram of faeces, DNA recovery was not as good because the detected numbers of genome copies were one to 1.5 log units lower than expected. Below 10^6 cells per gram of faeces, DNA recovery was poor or inexistent. When spiking with 10^5 and 10^4 cells per gram of faeces, DNA amplification was detected in only one out of the three replicates. Below 10^4 cells per gram of faeces, no DNA amplification was detected at all. This meant that the detection limit of the qPCR assay following DNA extraction from faecal samples with the FastDNA™ Spin kit was 10^6 cells per gram of faeces. The objective was to reach a detection limit of 10^2 cells per gram of faeces (section 7.1).

Regarding spiked swabs, DNA recovery was very poor in general. The detected numbers of genome copies were at least three log units lower than expected. Added to this, no amplification was detected at all for swabs spiked with less than 10^5 cells per swab. This strongly suggested that the DNA extraction method was not optimal.

7.3. Detection of *S. pseudintermedius* phages

7.3.1. Development of an end-point PCR assay

In parallel to the development of an assay for the detection of *S. pseudintermedius*, it was attempted to detect its phages as well. The integrase gene was very similar in all of the isolated phages except SpT99/F3 so it was chosen as an end-point PCR target (Figure 7.7).

Figure 7.7 (next page): The integrase gene was very similar in all of the isolated phages except SpT99/F3 as shown by an alignment of the integrase genes performed with the online tool Clustal Omega. T99F3 = SpT99/F3, L8 = SpL8, L10.5 = SpL10.5, L11 = SpL11, T152 = SpT152, T252 = SpT252 and T5 = SpT5.

```
T99F3      atgagtgttagaaaatacg------gtaataaatggtattatgattttggatacgaaggt 54
L8         atgccggtgtacaaagacgacaaaagcgataaatggtactttactgttagatacaaagat 60
L10.5      atgccggtgtacaaagacgacaaaagcgataaatggtactttactgttagatacaaagat 60
L11        atgccggtgtacaaagacgacaaaagcgataaatggtactttactgttagatacaaagat 60
T152       atgccggtgtacaaagacgacaaaagcgataaatggtactttactgttagatacaaagat 60
T252       atgccggtgtacaaagacgacaaaagcgataaatggtactttactgttagatacaaagat 60
T5         atgccggtgtacaaagacgacaaaagcgataaatggtactttactgttaggtacaaagat 60
           ***.  ** :..*** ***      * .********** *:*..* **.*.***.***.*

T99F3      a---------------aacgatataaaaagaaaggttttaaaaccaaacgtgaagccact 99
L8         atttacggcaataacaaaaggaaattaaaacgaggatttaagactaaacgtgaggctaaa 120
L10.5      atttacggcaataacaaaaggaaattaaaacgaggatttaagactaaacgtgaggctaaa 120
L11        atttacggcaataacaaaaggaaattaaaacgaggatttaagactaaacgtgaggctaaa 120
T152       atttacggcaataacaaaaggaaattaaaacgaggatttaagactaaacgtgaggctaaa 120
T252       atttacggcaataacaaaaggaaattaaaacgaggatttaagactaaacgtgaggctaaa 120
T5         atttacggcaataacaaaaggaaattaaaacgaggatttaagactaaacgtgaggctaaa 120
           *               **.*.:*:::***...***:*****.** ********.** *.:

T99F3      ------gaagctgaaacaatagctaaaaataaactaatgcaaggtataa-ttatcaacaa 152
L8         agcgcagaagctgaatttttaacagaa-gtcaacgaaggctatagtgattcgaacacata 179
L10.5      agcgcagaagctgaatttttaacagaa-gtcaacgaaggctatagtgattcgaacacata 179
L11        agcgcagaagctgaatttttaacagaa-gtcaacgaaggctatagtgattcgaacacata 179
T152       agcgcagaagctgaatttttaacagaa-gtcaacgaaggctatagtgattcgaacacata 179
T252       agcgcagaagctgaatttttaacagaa-gtcaacgaaggctatagtgattcgaacacata 179
T5         agcgcagaagctgaatttttaacagaa-gtcaacgaaggctatagtgattcgaacacata 179
                 *********: ::**.*:.** .*.*** ** **:* . : *:   *:**..:*

T99F3      taaaagttcttt-tatc--------gattattatgaacagtggatgga----agtcaaca 199
L8         tgaatatacattttatcattatttagacaatagtgacctgcggcctaaaactagaaaacg 239
L10.5      tgaatatacattttatcattatttagacaatagtgacctgcggcctaaaactagaaaacg 239
L11        tgaatatacattttatcattatttagacaatagtgacctgcggcctaaaactagaaaacg 239
T152       tgaatatacattttatcattatttagacaatagtgacctgcggcctaaaactagaaaacg 239
T252       tgaatatacattttatcattatttagacaatagtgacctgcggcctaaaactagaaaacg 239
T5         tgaatatacattttatcattatttagacaatagtgacctgcggcctaaaactagaaaacg 239
           *.**:.*:*:** ****        ** :**:.***.*:* **.  .*    **:.***.

T99F3      aaaagggcgtaattacagacaaagcttatcaaacatacgttaatgctataaaacaattca 259
L8         gaaagagaatgaatacaaac--tgcatataaaaccgaagtttgggca--------catca 289
L10.5      gaaagagaatgaatacaaac--tgcatataaaaccgaagtttgggca--------catca 289
L11        gaaagagaatgaatacaaac--tgcatataaaaccgaagtttgggca--------catca 289
T152       gaaagagaatgaatacaaac--tgcatataaaaccgaagtttgggca--------catca 289
T252       gaaagagaatgaatacaaac--tgcatataaaaccgaagtttgggca--------catca 289
T5         gaaagagaatgaatacaaac--tgcatataaaaccgaagtttgggca--------catca 289
           .****.*..*.*:****.**  :**:***.****. *.***:. **:        .:***

T99F3      agaa-------atttctctatactgaaaacctagacga----t-attac-tttgaataat 306
L8         agatgaacaaaattactcagcaac-aatgccaagagttcagaaagtatcttatggataat 348
L10.5      agatgaacaaaattactcagcaac-aatgccaagagttcagaaagtatcttatggataat 348
L11        agatgaacaaaattactcagcaac-aatgccaagagttcagaaagtatcttatggataat 348
T152       agatgaacaaaattactcagcaac-aatgccaagagttcagaaagtatcttatggataat 348
T252       agatgaacaaaattactcagcaac-aatgccaagagttcagaaagtatcttatggataat 348
T5         agatgaacaaaattactcagcaac-aatgccaagagttcagaaagtatcttatggataat 348
           ***:       ***:***:. *.  **:.**:***  :    : .*::* *:**.*****

T99F3      ttcacaacaatttt---ttatcgaaaattcttaaaatggtatggtgataatcatgcgaca 363
L8         atcaattcttttaacagtgcgcgtac------aatttggtcagggtttaaa---gtg--g 397
L10.5      atcaattcttttaacagtgcgcgtac------aatttggtcagggtttaaa---gtg--g 397
L11        atcaattcttttaacagtgcgcgtac------aatttggtcagggtttaaa---gtg--g 397
T152       atcaattcttttaacagtgcgcgtac------aatttggtcagggtttaaa---gtg--g 397
T252       atcaattcttttaacagtgcgcgtac------aatttggtcagggtttaaa---gtg--g 397
T5         atcaattcttttaacagcgcgcgtac------aatttggtcagggtttaaa---gtg--g 397
           :***.::*::**::     . **:*.      **::****.:**  :***:   * *  .
```

```
T99F3      gaatcagttaaaaaaatacacaattgtcttaa-agcagcgctatccgacgctatgcaaga 422
L8         ttatcaat------tatgctaaaaagtattttggattgcgc--g---------------- 433
L10.5      ttatcaat------tatgctaaaaagtattttggattgcgc--g---------------- 433
L11        ttatcaat------tatgctaaaaagtattttggattgcgc--g---------------- 433
T152       ttatcaat------tatgctaaaaagtattttggattgcgc--g---------------- 433
T252       ttatcaat------tatgctaaaaagtattttggattgcgc--g---------------- 433
T5         ttatcaat------tatgctaaaaagtattttggattgcgc--a---------------- 433
            :****.*      :**.*:.**::**.**:: .. :****

T99F3      agggcttatttataaagaccctacttataaa------gctattgtaaaagggaaaaagcc 476
L8         --------------tcgacccaactatatcaattaaacctatt---ccacgtactaagcc 476
L10.5      --------------tcgacccaactatatcaattaaacctatt---ccacgtactaagcc 476
L11        --------------tcgacccaactatatcaattaaacctatt---ccacgtactaagcc 476
T152       --------------tcgacccaactatatcaattaaacctatt---ccacgtactaagcc 476
T252       --------------tcgacccaactatatcaattaaacctatt---ccacgtactaagcc 476
T5         --------------tcgacccaactatatcaattaaacctatc---ccacgtactaagcc 476
                        :.*****:***::::.*       ****    ..* * *.:*****

T99F3      tag-ccagccagaagaagacaaatttatgagtatagcagattataaaaaattgaaaaatt 535
L8         taaaccaa----------actttatgatgagagaa---gaatttgaag-atcgtat--aa 520
L10.5      taaaccaa----------actttatgatgagagaa---gaatttgaag-atcgtat--aa 520
L11        taaaccaa----------actttatgatgagagaa---gaatttgaag-atcgtat--aa 520
T152       taaaccaa----------actttatgatgagagaa---gaatttgaag-atcgtat--aa 520
T252       taaaccaa----------actttatgatgagagaa---gaatttgaag-atcgtat--aa 520
T5         taaaccaa----------actttatgatgagagaa---gaatttgaag-atcgtgt--aa 520
           **. ***.          **::::* *****:.:*   **:*:*.**. ** *:.:  ::

T99F3      atgtggcaaatgtccctactcaatcatatttgtttatctacattttagt-tataacagga 594
L8         aagaagtagaagaacaaga-ttatcggga---actatttactttgatgttttatacaggt 576
L10.5      aagaagtagaagaacaaga-ttatcggga---actatttactttgatgttttatacaggt 576
L11        aagaagtagaagaacaaga-ttatcggga---actatttactttgatgttttatacaggt 576
T152       aagaagtagaagaacaaga-ttatcggga---actatttactttgatgttttatacaggt 576
T252       aagaagtagaagaacaaga-ttatcggga---actatttactttgatgttttatacaggt 576
T5         aagaagtagaagaacaaga-ttatcggga---actatttactttgatgttttatacaggt 576
           *:*:.* *.*:*:.*.:..  :***. .:   : *** ***:** ::** *:::*****:

T99F3      gctcgttttggtgaagttcaaaaat---taacaactgatgactta------------g-- 637
L8         ttaagagtcggcgaagcgatggctttagtttggacagactacaataaatataaaaaagaa 636
L10.5      ttaagagtcggcgaagcgatggctttagtttggacagactacaataaatataaaaaagaa 636
L11        ttaagagtcggcgaagcgatggctttagtttggacagactacaataaatataaaaaagaa 636
T152       ttaagagtcggcgaagcgatggctttagtttggacagactacaataaatataaaaaagaa 636
T252       ttaagagtcggcgaagcgatggctttagtttggacagactacaataaatataaaaaagaa 636
T5         ttaagagtcggcgaagcgatggctttagtttggacagactacaataaatataaaaaagaa 636
             :.*: * ** ****  .:...:*   *:: .**:**  **:::           *

T99F3      -atttcattaataacacga------------ttcatttaagag------gtacaaagaca 678
L8         atatccatcaataaaacgatggacatctctaatcggtcaatataccctcggcctaaaaca 696
L10.5      atatccatcaataaaacgatggacatctctaatcggtcaatataccctcggcctaaaaca 696
L11        atatccatcaataaaacgatggacatctctaatcggtcaatataccctcggcctaaaaca 696
T152       atatccatcaataaaacgatggacatctctaatcggtcaatataccctcggcctaaaaca 696
T252       atatccatcaataaaacgatggacatctctaatcggtcaatataccctcggcctaaaaca 696
T5         atatccatcaataaaacgatggacatctctaatcggtcaatataccctcggcctaaaaca 696
            ::* *** *****.****           :**. * ** *       * .*:**.***

T99F3      gtaacatcagatcgtatagtcgatgtcccgagtgaagacatgagggttttaaaaagaaca 738
L8         gatagttctgaagatatagtacctttacctaaatttatcaat-gaaatgttatctgaacg 755
L10.5      gatagttctgaagatatagtacctttacctaaatttatcaat-gaaatgttatctgaacg 755
L11        gatagttctgaagatatagtacctttacctaaatttatcaat-gaaatgttatctgaacg 755
T152       gatagttctgaagatatagtacctttacctaaatttatcaat-gaaatgttatctgaacg 755
T252       gatagttctgaagatatagtacctttacctaaatttatcaat-gaaatgttatctgaacg 755
T5         gatagttctgaagatatagtacctttacctaaatttatcaat-gaaatgttatctgaacg 755
           *::* :**:**: .******. .* *.** *.: ::.:**:  *..:* *:*:.:****.
```

```
T99F3      atgac----tgaaatgccaattaacat-------ga---a-tagacaatt--gtttaata 781
L8         ctaccaacgtgaaaaacagatgaacaaatattttgatgaacaaaactattttatatttgg 815
L10.5      ctaccaacgtgaaaaacagatgaacaaatattttgatgaacaaaactattttatatttgg 815
L11        ctaccaacgtgaaaaacagatgaacaaatattttgatgaacaaaactattttatatttgg 815
T152       ctaccaacgtgaaaaacagatgaacaaatattttgatgaacaaaactattttatatttgg 815
T252       ctaccaacgtgaaaaacagatgaacaaatattttgatgaacgaaactattttatatttgg 815
T5         ctaccaacgtgaaaaacagatgaacaaatattttgatgaacaaaactattttatatttgg 815
           .*..*    *****:.*..** ****:       **   *  *.**:***  .*:*:: .

T99F3      caggctattctctaataacaaacaatgcagtaacaaaaatacttcaaaaattctgtttag 841
L8         cggacttgcccctaaa--cattatagccatgttcataaa---------------------- 852
L10.5      cggacttgcccctaaa--cattatagccatgttcataaa---------------------- 852
L11        cggacttgcccctaaa--cattatagccatgttcataaa---------------------- 852
T152       cggacttgcccctaaa--cattatagccatgttcataaa---------------------- 852
T252       cggacttgcccctaaa--cattatagccatgttcataaa---------------------- 852
T5         cggacttgcccctaaa--cattatagccatgttcataaa---------------------- 852
           *.*.**:  * ****:  **::.:*  **  ::**:***

T99F3      aaaataaattaggaaatttc-------actctacatgcaataagacacacacattgctct 894
L8         aaatttaacaaggcttttcctaattatagcatacatgccctaagacattcatatgcatct 912
L10.5      aaatttaacaaggcttttcctaattatagcatacatgccctaagacattcatatgcatct 912
L11        aaatttaacaaggcttttcctaattatagcatacatgccctaagacattcatatgcatct 912
T152       aaatttaacaaggcttttcctaattatagcatacatgccctaagacattcatatgcatct 912
T252       aaatttaacaaggcttttcctaattatagcatacatgccctaagacattcatatgcatct 912
T5         aaatttaacaaggcttttcctaattatagcatacatgccctaagacattcatatgcatct 912
           ***:*:** :***.::** *       *  .*******..******* :** **  .***

T99F3      tatctattgcatggcggtgtatcaatttactatat---atctaaaaggcttggtcacgct 951
L8         tacctagcaaataacggtgtagatatattcgtactacaatcattaatgc---gtcatgct 969
L10.5      tacctagcaaataacggtgtagatatattcgtactacaatcattaatgc---gtcatgct 969
L11        tacctagcaaataacggtgtagatatattcgtactacaatcattaatgc---gtcatgct 969
T152       tacctagcaaataacggtgtagatatattcgtactacaatcattaatgc---gtcatgct 969
T252       tacctagcaaataacggtgtagatatattcgtactacaatcattaatgc---gtcatgct 969
T5         tacctagcaaataacggtgtagatatattcgtactacaatcattaatgc---gtcatgct 969
           ** ***  ..**..******* .:**:*:* ::.*   ***:::** **   **** ***

T99F3      aatat---caaaacaacgttagaagtatattctcatctgttagaagagactcaggtcgaa 1008
L8         caaataaccgaaacaatggg-tacgtatagtc--attt------atatactcagaaa--- 1017
L10.5      caaataaccgaaacaatggg-tacgtatagtc--attt------atatactcagaaa--- 1017
L11        caaataaccgaaacaatggg-tacgtatagtc--attt------atatactcagaaa--- 1017
T152       caaataaccgaaacaatggg-tacgtatagtc--attt------atatactcagaaa--- 1017
T252       caaataaccgaaacaatggg-tacgtatagtc--attt------atatactcagaaa--- 1017
T5         caaataaccgaaacaatggg-tacgtatagtc--attt------atatactcagaaa--- 1017
           .*:**   *.****** *    *.***** **  ** *      * * ******.:.

T99F3      gaaaaacaaaaaactattaacttgatt-aaaagtatgtaa     1047
L8         ---aaacatgatgccatagccatttttgacgagtaa----     1050
L10.5      ---aaacatgatgccatagccatttttgacgagtaa----     1050
L11        ---aaacatgatgccatagccatttttgacgagtaa----     1050
T152       ---aaacatgatgccatagccatttttgacgagtaa----     1050
T252       ---aaacatgatgccatagccatttttgacgagtaa----     1050
T5         ---aaacatgatgccatagccatttttgacgagtaa----     1050
              *****:.*:.* **:..*:* :** *..****:
```

The integrase primers were tested on phage and bacterial DNA (Figure 7.8).

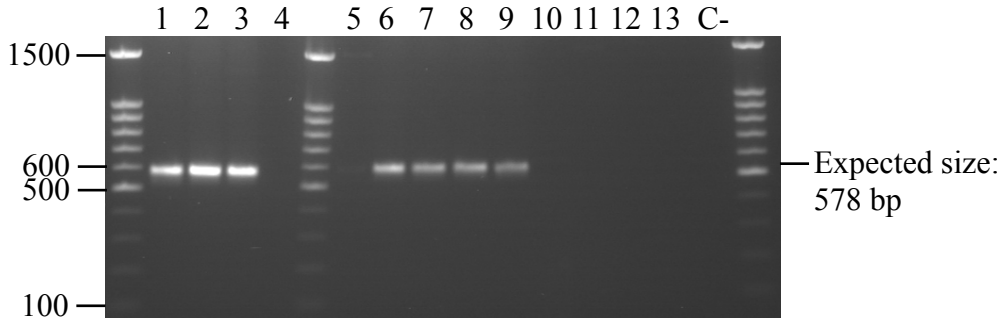


Figure 7.8: Amplification was seen on phage and bacterial DNA when using primers targeting the phage integrase gene. Amplification of bacterial DNA indicated the presence of prophages in the tested bacterial strains. 1: SpT5, 2: SpT152, 3: SpT252, 4: SpT99/F3, 5: E045, 6: E045 lys SpT5, 7: E140, 8: AB252, 9: JZ152, 10: E139, 11: ED99, 12: S56F3, 13: E018, C-: water negative control.

Amplification was seen on phage DNA but also on bacterial DNA indicating the presence of prophages. The integrase gene is essential for establishing lysogeny and since only genomes of temperate phages were available to design primers, it was difficult to target genes that were not present in prophages. There was no amplification in ED99 supporting the fact that it is prophage-free (Ben Zakour *et al.*, 2011). As expected, no amplification was visible for SpT99/F3. This confirmed that a level of variability existed among phages and the set of primers may not capture the whole diversity of *S. pseudintermedius* phages.

The development of an end-point PCR assay for the detection of phages in swabs and faeces was not carried out because of all the issues encountered during the development of a similar method for the detection of *S. pseudintermedius*. Time was lacking for extensive optimisation. Instead, PCR screening was performed with the already available primers to obtain information about the prevalence of prophages within *S. pseudintermedius* strains.

7.3.2. Colony PCR for the detection of prophages

7.3.2.a. Phage-specific PCR to look for prophages similar to the four selected phages (SpT5, SpT52, SpT152 and SpT99/F3)

The host range of bacteriophages can vary greatly. Some phages can infect several bacterial genera (Bielke *et al.*, 2007, Meaden and Koskella, 2013, Weigele *et al.*, 2007), often they are limited to one species of bacteria and some phages can only infect a few strains within one species. In the case of the *S. pseudintermedius* phages isolated in this project the host range was very narrow, restricted to less than half (in the case of SpT99/F3 less than a quarter) of the 72 strains that were tested in total (Table 3.6). Bacterial resistance to phage infection is achieved by using restriction-modification systems, by using CRISPR-Cas systems or by not expressing the phage receptor. The presence of a prophage in the chromosome also confers resistance to phage infection to lysogens through homo/heteroimmunity (section 1.4.2.b.v).

To test whether lysogeny was widespread in *S. pseudintermedius* and could explain the narrow host range of the tested phages, primers specific for the Warwick phages (sections 2.37 and 4.2) were used to screen *S. pseudintermedius* strains for the presence of prophages in their genome. For each phage, 28 strains (MRSP and MSSP, not necessarily the same ones for each phage depending on their host range) that did not support phage growth were selected and screened through colony PCR with the corresponding Warwick phage-specific primers (Table 7.1). A known lysogen and the strain used to amplify each phage in the lab were included in the screening as positive and negative controls respectively.

Table 7.1: PCR screening of *S. pseudintermedius* strains that did not support phage growth with Warwick phage-specific primers revealed that only a few strains contained a similar prophage in their genome.

Phage name	Number of PCR-positive *S. pseudintermedius* strains / total number of tested strains
SpT5	2 / 28
SpT252	3 / 28
SpT152	1 / 28
SpT99/F3	1 / 28

PCR screening revealed that for each phage only a few strains (up to three out of 28) contained a similar prophage in their genome. The absence of phage growth on the bacterial strains used in this experiment was probably not due to the presence of prophages in most cases. Other mechanisms such as restriction-modification systems may explain the narrow host range of the Warwick phages.

7.3.2.b. PCR screening to look for other prophages

To extend the search for prophages, the primers targeting the integrase gene were used to perform colony PCR on all the bacterial strains used in the previous Warwick phage-specific test. Again, known lysogens and amplification strains were included in the experiment as positive and negative controls respectively. Results obtained with phage-specific PCR were included (positive if amplification was observed for at least one phage-specific set of primers) and based on these results alone the theoretical presence of an integrase gene was deduced. The actual results of the integrase PCR screening were then compared to the theoretical ones (Table 7.2).

Table 7.2: The comparison of theoretical and obtained results for integrase gene PCR showed a lack of correspondence for some of the 40 *S. pseudintermedius* strains that were tested. Second column: results obtained with phage-specific PCR, (+) = strain was positive for at least one phage, (-) = no PCR amplification for any phage. Third column: theoretical results for integrase gene PCR, based on phage-specific PCR results, (+) = PCR amplification should be observed, (-) = no PCR amplification should be seen. Fourth column: obtained results for integrase gene PCR, (+) = PCR amplification, (-) = no amplification.

S. pseudintermedius strains	Result for phage-specific PCR	Theoretical result for integrase gene	Obtained result for integrase gene
E029	-	-	+
E046	+	+	+
E052	+	+	+
E075	+	+	+
E086	-	-	+
E140	-	-	+
E018	-	-	-
E020	-	-	-
E022	-	-	-
E025	-	-	-
E122	-	-	-
E125	+	+	-

S. pseudintermedius strains		Result for phage-specific PCR	Theoretical result for integrase gene	Obtained result for integrase gene
E135		-	-	-
E069		-	-	-
AB178		-	-	-
AB255		-	-	-
AB316		-	-	-
08BKT		-	-	-
HK14		-	-	-
Y1		-	-	-
S56C3		-	-	-
S56D2		+	+	-
S56H7		-	-	-
S57E7		-	-	-
S60D7		-	-	+
S61H5		-	-	+
S61I9		-	-	+
S62A2		-	-	-
S63G7		-	-	-
S66E5		-	-	-
S76I4		-	-	-
JZ22		-	-	-
JZ31		-	-	-
JZ133		-	-	-
JZ151		-	-	-
JZ170		-	-	-
JZ208		-	-	-
JZ220		-	-	-
AB561		-	-	-
AB564		-	-	-
Amplification strains	E045	-	-	-
	E139	-	-	-
	ED99	-	-	-
Lysogens	E045 lys SpT5	+	+	+
	AB252	+	+	+
	JZ152	+	+	+
	S56F3	+	-	-

Theoretical and actual results were consistent for E046, E052 and E075 suggesting the presence of functional prophages in these strains. E125 and S56D2 were positive for Warwick phage-specific PCR but negative for integrase gene, indicating the presence of possibly defective prophages in their genome. E029, E086, E140, S60D7, S61H5 and S61I9 were negative for Warwick phage-specific PCR and positive for integrase gene. This suggested that they contained prophages different from the four Warwick phages. Regarding the controls, the amplification strains were negative for Warwick phage-specific PCR and integrase gene as expected. The lysogens were positive for both Warwick phage-specific PCR and integrase gene as expected, except S56F3. Primers were designed to target the integrase gene present in all the isolated phages (Warwick and Danish) except SpT99/F3. This phage had an integrase gene in its genome that was identified later. This explained the absence of PCR amplification in its lysogen (S56F3). No other strains were only positive for SpT99/F3.

Such PCR-based experiments have limitations. First, PCR inhibition can occur when performing colony PCR leading to false negatives. The fact that amplification was systematically seen in the positive controls suggested that this did not happen. Secondly, false positives can occur too, especially when the region to amplify is short as it is the case with the SpT5 PCR product (125 bp). Thirdly, screening through PCR for a single gene gives no indication of whether or not this gene is part of a functional prophage. The integrase genes identified in *S. pseudintermedius* strains may be the remnants of degenerated prophages. Finally, targeting only one gene may not capture the whole diversity of *S. pseudintermedius* phages. For example, phages similar to SpT99/F3 were not targeted in this study. Still, this simple experiment gave an idea of how widespread phages other than SpT99/F3-like phages were. An integrase gene was detected in 11 out of 45 strains (E045 lys SpT5 was not included as it was isolated specifically for this project – see section 4.2. ED99 was not included either because it was a known prophage-free strain). This indicated that prophages similar to temperate phages isolated in this project were not very common.

7.3.2.c. Looking for the strain of origin of phage SpT5

SpT5 was isolated through co-culture in Denmark with a mix of seven *S. pseudintermedius* strains so the strain of origin (or lysogen) of this phage was not known at the time of isolation (section 3.3.1). To overcome this problem, a lysogen of this phage was isolated during the project. Primers specific for the SpT5 genome were designed and used to confirm the state of lysogeny of E045 lys SpT5 through colony PCR (section 4.2). The same primers were then used to screen the seven strains present in the mix from which SpT5 originated to try and identify the strain of origin of this phage (Figure 7.9).

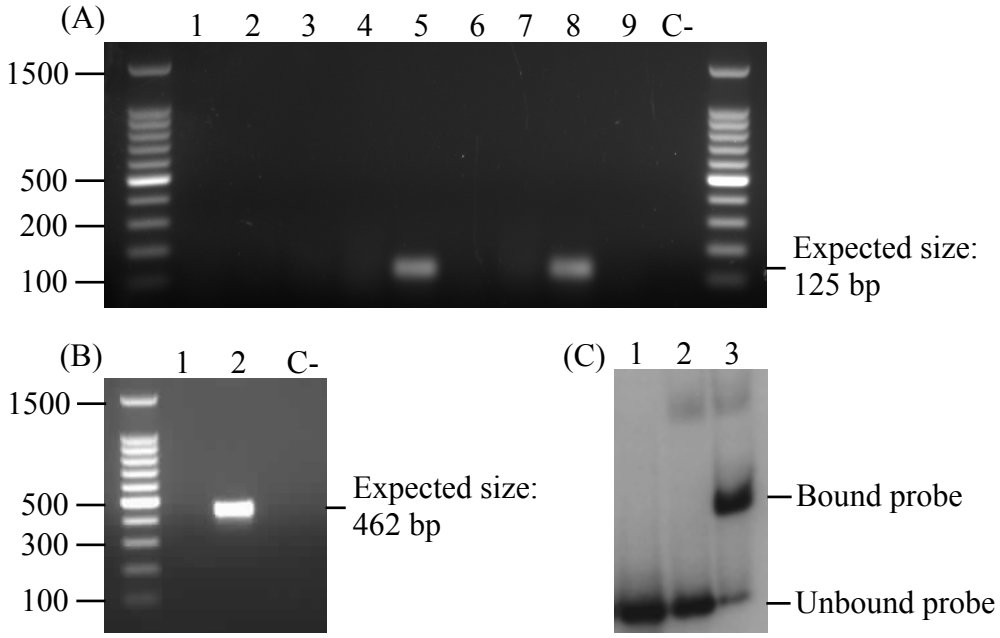


Figure 7.9: (A) PCR amplification with SpT5-specific primers was seen on *S. pseudintermedius* strains E133 and E045 lys SpT5 (positive control). 1: E018, 2: E022, 3: E025, 4: E122, 5: E133, 6: E140, 7: S61H5, 8: E045 lys SpT5, 9: E045 (negative control) and C-: water negative control. (B) PCR amplification with primers targeting the SpT5 immunity region was not seen on E133. 1: E133, 2: SpT5 and C-: water negative control. (C) No shift was observed when exposing the SpT5_op DNA probe to E133 cell lysate. 1: free DNA, 2: DNA + E133 and 3: DNA + E045 lys SpT5.

When performing colony PCR on the seven strains present in the co-culture mix, amplification was seen on E133 (Figure 7.9, A). This was surprising because SpT5 was able to form plaques on E133 (Table 3.6) and a phage cannot normally grow on its own lysogen. To confirm whether or not E133 was an SpT5 lysogen, primers targeting the phage immunity region were designed and PCR was performed on

genomic DNA. In this case no amplification was seen on E133 indicating that it was not lysogenized with SpT5 (Figure 7.9, B). This result was confirmed through gel shift assay. When the SpT5_op DNA probe was exposed to E133 cell lysate, no shift was observed (Figure 7.9, C). The CI repressor was therefore absent from the cell lysate and E133 was not a lysogen of SpT5. The strain of origin of this phage could not be identified. SpT5 is very similar to the Danish phages and it was isolated in Denmark in the same lab where the Danish phages were themselves isolated and handled. It is therefore possible that SpT5 came from the laboratory environment rather than one of the tested bacterial strains.

7.4. Conclusions

Following the unsuccessful attempts at isolating *S. pseudintermedius* phages from environmental samples, a study of the prevalence of the pathogen and its phages in these samples was undertaken. Samples were chosen for phage isolation because it was proposed that *S. pseudintermedius* was present in them and that phages would co-reside with their host. However, there was no proof that the bacterium or its phages were present in the samples screened in this project. Developing a method for the detection of *S. pseudintermedius* and its phages would therefore provide a pre-screening tool to identify potential sources of phages and speed up the process of phage isolation. The study would also generate data about the ecology of the bacterium in a more general context than just the skin of dogs.

The development of an end-point PCR assay for the detection of *S. pseudintermedius* was attempted using primers that were already available in the literature. Amplification was seen only when samples were spiked with 10^8 cells per swabs or per gram of faeces. The assay was not sensitive enough. To overcome this problem, a qPCR assay was developed. When tested on pure *S. pseudintermedius* DNA, the qPCR assay could detect a concentration as low as 10 genome copies/µL but when spiking faecal samples or swabs DNA recovery was generally poor and signal was not detected below 10^5 cells per swab or per gram of faeces.

Further optimisation for the detection of *S. pseudintermedius* and its phages was not carried out because of a lack of time and a number of issues associated with sample collection. Indeed, while the method of detection was developed, contact was taken

with several veterinary practices to start collecting samples (skin swabs and faeces) to make sure they would be available without delay once the assay was optimised. Sample collection was slow because dermatology cases presented only sporadically in regular vet practices and veterinarians sometimes forgot to collect samples.

There was also concern over the possibility to access truly positive samples from confirmed *S. pseudintermedius* pyoderma. Confirming the presence of the pathogen in skin lesion is time-consuming and was not done systematically. A certain number of samples were collected from putative pyoderma. The lack of known positive samples meant that it was not possible to know the load of pathogen that could be expected in a given sample. The arbitrary detection limit of 100 genome copies per gram of samples may not be low enough. Enrichment could be performed to amplify the pathogen before detection and in that case the original load of bacteria could not be determined.

Other issues were related to the use of swabs for sample collection. Ideally, the load of bacteria should be expressed in genome copies per gram of sample. However, with swabs it is difficult to know how much sample was collected. In a small experiment, swabs were weighed before and after swabbing. It was found that they did not gain weight and might even lose weight if swab material was left on the skin. The detection of *S. pseudintermedius* and its phages in those samples would therefore be limited to information about presence or absence without information about the load. Another problem was the impossibility to do true replicates of each swab. When a study is based on environmental thus variable samples, replicates are essential (King *et al.*, 2015). When swabbing, the microbiome at the surface of the skin was disturbed and swabbing a second time would not represent the same microbiome. To overcome this problem, samples could be collected from different areas on the body of one dog and these would count as replicates.

Regarding the detection of *S. pseudintermedius* phages, the study was focused on the ecology of temperate phages instead of looking for phages in dog samples. A colony-PCR screening performed with Warwick phage-specific primers or primers targeting the integrase gene revealed that 11 strains out of the 45 (24%) that were tested contained prophage genes. This suggested that lysogeny was not widespread in the *S.*

pseudintermedius population. In comparison, studies on lysogeny in other *Staphylococci* including *S. aureus* showed the presence of temperate phages in up to 80% of the tested strains (Rountree, 1949, Blair and Carr, 1961b, Matsuzaki *et al.*, 2003). The fact that lysogeny was not very common in *S. pseudintermedius* suggested that the narrow host range of the Warwick phages was not due to homo/heteroimmunity in most cases. In another study about prophage carriage in *S. aureus*, eight different families of bacteriophages were defined based on their integrase gene alone (McCarthy *et al.*, 2012). It was possible that *S. pseudintermedius* strains contained other prophages that harboured different integrase genes that were not targeted by the designed primers.

Regarding the ecology of phage SpT5, the same colony-PCR approach was used to identify its strain of origin. A few experiments showed that SpT5 could not be traced back to one of the strains used in the co-culture experiment that led to its isolation. It might have come from the laboratory environment where other *S. pseudintermedius* phages were previously handled.

Chapter 8 Summary and discussion

8.1. Summary of the results presented in this thesis

8.1.1. Isolation of *S. pseudintermedius* phages and Vir mutants

The isolation of phages infecting *S. pseudintermedius* was attempted from a variety of samples (wastewater, dog faeces and skin swabs). The objective was to find lytic phages adapted for phage therapy but this proved unsuccessful. The isolation of temperate phages through co-culture and mitomycin C induction yielded almost twenty phages that were characterised genetically and phenotypically. Four phage candidates, SpT5, SpT152, SpT252 and SpT99/F3, were kept for further tests. Random mutagenesis and multiple passaging approaches were used to produce Vir mutants from these four phages. When this remained unsuccessful, site-directed mutagenesis was considered as the next best option for the production of Vir mutants. For this purpose, an operator was found in the SpT5 genome and mutations leading to absence of CI repressor binding were identified. These mutations should lead to a virulent phenotype when introduced in the SpT5 genome.

8.1.2. Study of the biology of *S. pseudintermedius* phages

The study of the predicted tertiary structure of the SpT5 CI repressor led to the identification of a helix-turn-helix domain typical of DNA-binding proteins. Bioinformatics analyses of the four phage genomes revealed that they exhibited a genetic organisation similar to other phages infecting Gram-positive cocci with clusters of genes involved in the same biological processes (e.g. virion assembly, host lysis, lysogeny). It was also suggested that they have circularly permuted genome corresponding to a headful packaging system and that SpT99/F3 lysate may contain more than one type of phages. A study of the ecology of *S. pseudintermedius* and its phages was initiated and later discontinued because of problems encountered during method development. The genome of *S. pseudintermedius* strains was screened for prophages through a PCR-based approach. The experiment indicated the presence of prophage genes in one fourth of the tested strains (11 out of 45 strains tested in total).

8.2. Suggestions for future work

8.2.1. Further study of the biology of *S. pseudintermedius* phages

A few suggestions were made in the previous chapters to pursue the work on the biology of *S. pseudintermedius* phages: DNase I footprinting to identify other operators in the SpT5 genome (section 5.9), crystallisation of the SpT5 CI repressor to explore its structure (section 5.9) and the use of antibiotics different from mitomycin C to maybe induce different families of phages from *S. pseudintermedius* lysogens (section 3.5). It was also suggested to perform electron microscopy observation and genome sequencing on phage SpT99/F3 along with qPCR on several regions of its genome to try and understand its unusual characteristics (section 6.5).

8.2.2. Further steps towards phage therapy

8.2.2.a. Ensuring the absence of harmful genes in future therapeutic phages

Regarding the development of phage therapy, one important step is to check the genome of phage candidates for the presence of undesirable genes such as antibiotic resistance genes or toxin genes (Skurnik and Strauch, 2006). Temperate phages are more likely to contain such genes. Shiga toxin phages infecting *E. coli* are one example of temperate phages that can convert bacteria into dangerous toxin-producing pathogens (Brussow *et al.*, 2004). The genome annotation performed with Prokka (an online programme that predicts the function of genes and uses the manually curated SwissProt database as a reference, see section 6.2) on the four selected phage genomes did not identify such genes, however a lot of coding sequences remained of unknown function (Figure 6.3). The use of specially designed programmes such as ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) or Island Viewer (http://www.pathogenomics.sfu.ca/islandviewer2/resources.php) may help find harmful genes if they are present.

8.2.2.b. Site-directed mutagenesis for the production of Vir mutants

The next step is the introduction of the point mutations identified through gel shift assay into the genome of SpT5 through one of the approaches described in section 5.9: the transformation of SpT5 lysogens with mutated ssDNA of the operator region or the transfection of non-lysogens with the phage genome and the same mutated ssDNA at the same time. Even though phages are known to evolve quickly, it is not expected that they would readily revert to a temperate phenotype because there

should be no selective pressure for them to do so. Added to this, in the event of a Vir mutant reverting to lysogeny over the course of a treatment, it is expected that the other non-revertant phages administered at the same time would still be capable of killing the newly lysogenized host.

It is also useful to test the host range of the Vir mutant because it is likely to change compared to the parent phage. It is hoped that the host range will be broader because Vir mutants can infect lysogens. However, the prophage screening study (section 7.3.2.b) suggested that lysogeny is not hugely widespread in the *S. pseudintermedius* population (11 out 45 strains tested positive for the presence of prophage-associated genes). Overcoming homoimmunity may therefore not be enough to significantly broaden the spectrum of the phage. One possibility would be to further mutagenize the Vir mutants to obtain host range mutants that could be combined in a cocktail. A similar experiment was successfully performed on an *S. aureus* Vir mutant phage (Rapson, 2002).

8.2.2.c. *In vitro* testing

Once a phage or phage cocktail with satisfactory host range is obtained, its efficacy should be tested *in vitro* against a range of *S. pseudintermedius* strains (ST68 and ST71 strains in particular). Some studies showed that the *in vitro* virulence of a phage does not always correlate with its effect *in vivo* because the environment is much more complex and the bacterial state, including expression of the phage receptor, may be different (Kropinski, 2006, Tsonos *et al.*, 2013, Henry *et al.*, 2013). It is therefore recommended to move on to *in vivo* studies whenever possible to evaluate the efficacy of phage therapy against pyoderma in more realistic conditions.

8.2.2.d. *In vivo* testing

8.2.2.d.i. Animal model and purification of the phage preparation

A mouse model of *S. pseudintermedius* skin infection was developed at the Statens Serum Institute in Denmark and could be used for the *in vivo* studies (Prof. Luca Guardabassi, personal communication). It is important to ensure the purity of the phage preparation before it is applied on animal skin. Indeed, crude phage lysates may contain toxins, whole live cells or cell membrane components that could be dangerous or could trigger inflammatory reactions. They have to be removed before

in vivo application of the phage product. The traditional phage purification method involving precipitation with polyethylene glycol (PEG) followed by separation on a cesium chloride (CsCl) gradient can yield high concentration of phage with a satisfactory level of purity (Merril *et al.*, 1996). However, it is time-consuming and it cannot be easily scaled-up for the production of bigger quantities of culture required for commercial applications. Other methods have been proposed to purify phages. These involve a succession of centrifugation and filtration steps, along with chromatographic procedures, which aim is to produce a phage suspension with as few non-phage contaminants as possible (Gill and Hyman, 2010). Alternatively, some advocate a minimalist approach to purification where the aim is merely to remove living bacteria and toxins through filtration and chromatography. Phages are then left in a mixture of non-toxic biomolecules and media components (Merabishvili *et al.*, 2009). In any case, the first step after successful production of a "pure" phage product is to assess its potential adverse effects, e.g. inflammation, by applying it on non-infected skin. Once the safety of the product is confirmed, its ability to clear an infection can be tested.

8.2.2.d.ii. How to administer phages

In a study on experimental phage therapy of burn wounds, a phage suspension was sprayed onto the area to treat. The authors reported the tendency of the liquid to run off the wound and recommended the use of a suitable carrier such as a phage-containing gel or a phage-impregnated membrane similar to PhagoBioDerm (section 1.2.2) (Rose *et al.*, 2014). Both approaches could be tested for the delivery of phages onto pyoderma lesions. The dose of phages needed for clearing the infection has to be determined. Due to the ability of phages to multiply *in situ* as long as the host is present, a low initial dose may be sufficient. This so-called "active" phage therapy relies on the self-amplifying and self-limiting (as amplification stops when the host is not present anymore) properties of phages to reach the appropriate dose (Loc-Carrillo and Abedon, 2011). These properties also offer the possibility for single-dose treatment, or at least less frequent applications, which would make the treatment of pyoderma more convenient and easier to comply with.

8.3. Phage therapy for the treatment of pyoderma: issues and options

8.3.1. Using temperate phages in phage therapy: is it safe?

The results obtained during this PhD project showed that developing phage therapy for a particular indication is not always a straightforward process. The fact that only temperate phages were found was an important hurdle. The possibility to use virulent mutants of these phages was explored and discussed in this thesis. There is concern, expressed by some members of the phage community, regarding the ability of modified temperate phages to recombine with prophages present in the hosts they infect. Given that it was suggested that temperate phages play a major role in the transfer of genetic material between *S. pseudintermedius* strains (McCarthy *et al.*, 2015, Couto *et al.*, 2016), this could lead to the transduction of potentially harmful genes from one host to another. However, if transductants are produced they should still be killed by the Vir mutant phage that infected them, given their strictly lytic nature (Abedon *et al.*, 2011). This, along with checking the absence of harmful genes in the genome of the Vir mutant, should ensure the safety of the treatment.

Engineered temperate phages can also be used therapeutically as delivery systems for genes encoding bactericidal proteins (Westwater *et al.*, 2003) or genes conferring sensitivity to antibiotics to combat resistant pathogens (Yosef *et al.*, 2014). Another alternative is to use phage enzymes, such as the endolysin, instead of whole phages. Studies showed that some endolysins applied externally are able to degrade the cell wall of Gram-positive bacteria *in vitro* and *in vivo* (Fischetti, 2010). Both options could also be considered for the treatment of pyoderma. However, these approaches lose the self-amplifying and self-limiting properties of using intact phages, which are important advantages of phage therapy (section 8.2.2.d.ii).

8.3.1.a. Limited knowledge about the exact pathogenesis of pyoderma

S. pseudintermedius has been identified as the main causative agent of bacterial pyoderma. It is known is that this bacterium is found on the skin of healthy dogs, that it is also found in pyoderma lesions and that it expresses virulence factors favouring adherence to and invasion of skin cells (Pietrocola *et al.*, 2015). However, the exact pathogenesis of pyoderma is not known. Other bacteria, e.g. *E. coli*, *Proteus* spp. and *Pseudomonas* spp. (Ihrke, 1987, Dowling, 1996), may be involved and this may

influence the outcome of phage therapy that would target only *S. pseudintermedius*, hence the importance to know as much as possible about the etiology of the disease. One solution would be to use phages topically along with a systemic application of antibiotics that have a broader spectrum of action. The synergistic effect of phages and antibiotics to kill bacteria was demonstrated *in vitro* and *in vivo* on *S. aureus* (Kirby, 2012, Chhibber *et al.*, 2013), and could be useful in the case of pyoderma.

8.3.2. Using *S. pseudintermedius* phages for prophylaxis

In human medicine, the contamination of the hospital environment is known to play a role in the transmission of pathogens such as MRSA (Mitchell *et al.*, 2014). The decontamination of the environment is essential to reduce the incidence of MRSA nosocomial infections. Added to this, there is a relation between carriage of *S. aureus,* usually in the nares, in human patients and the occurrence of surgical site infections (Levy *et al.*, 2013). To reduce the incidence of *S. aureus* infections, the nasal decolonisation of patients is recommended (Coates *et al.*, 2009). A study showed that phages have the potential to decontaminate fomites (Jensen *et al.*, 2015) and a review suggested that they could be used to prevent MRSA infections through nasal decolonisation (Mann, 2008).

The prophylactic use of *S. pseudintermedius* phages could therefore be considered for the decontamination of the skin of dogs before surgery or to limit transmission through direct contact with other animals (e.g. during an overnight stay at the practice). The decontamination of surfaces at veterinary practices with phages may be possible as well, however there is a lack of study to show that *S. pseudintermedius* survives in the environment. Only one study was conducted recently to determine the presence of *S. pseudintermedius* other than on the skin of dogs. It showed that *S. pseudintermedius* was occasionally found on clothing worn by veterinary personnel (Singh *et al.*, 2013).

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Appendix A

A.1. How to determine the sequencing read coverage across phage genomes (on Macintosh computer)

The installation of BWA-MEM, Samtools, Qualimap and their corresponding binary files beforehand is required.

Go to Terminal and type (words in italic must be replaced by relevant folder and file names):

cd *WorkingFolder (with .fas and .fastq files)*

bwa index –a bwtsw *SpTX_genome*.fas

bwa mem –t 1 *SpTX_genome*.fas *SpTX_R1*.fastq *SpTX_R2*.fastq > *SpTX_aln*.sam

samtools view –b –S *SpTX_aln*.sam > *SpTX_aln*.bam

samtools sort –m 1000000000 *SpTX_aln*.bam *sorted_SpTX_aln*

cd *QualimapFolder*

./qualimap

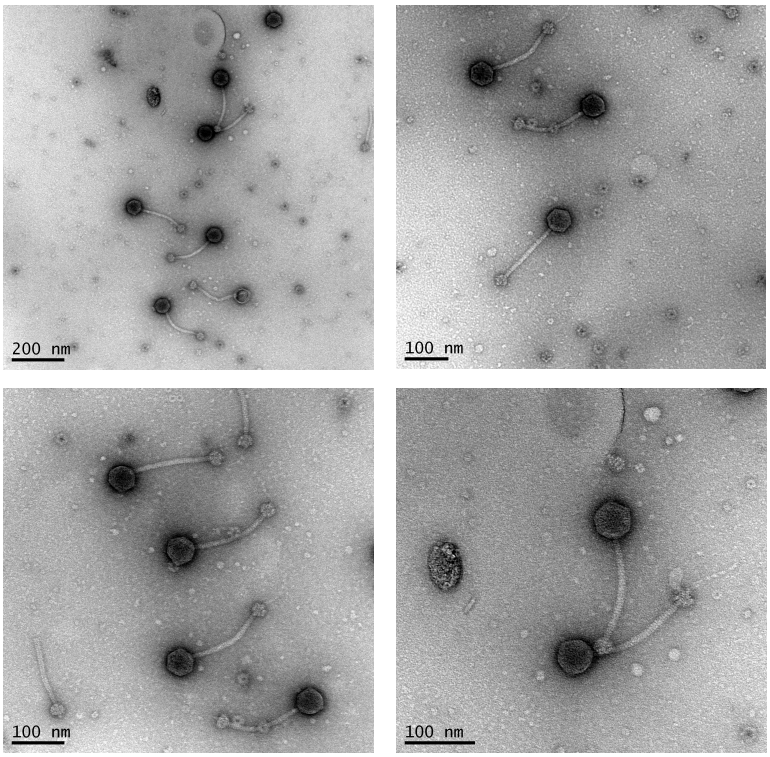
and open *sorted_SpTX_aln*.bam

Open the coverage across genome plot, right click > Export plot data. This creates a .txt file. Copy data from the .txt file into an Excel file to produce diagrams of coverage across genome.

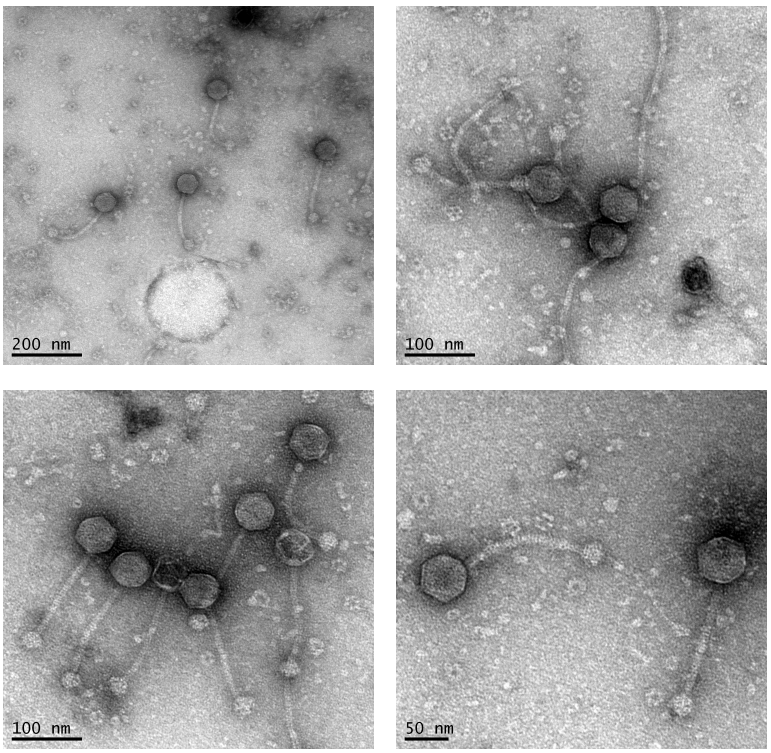
Appendix B

B.1. Additional electron microscopy pictures of the four Warwick phages

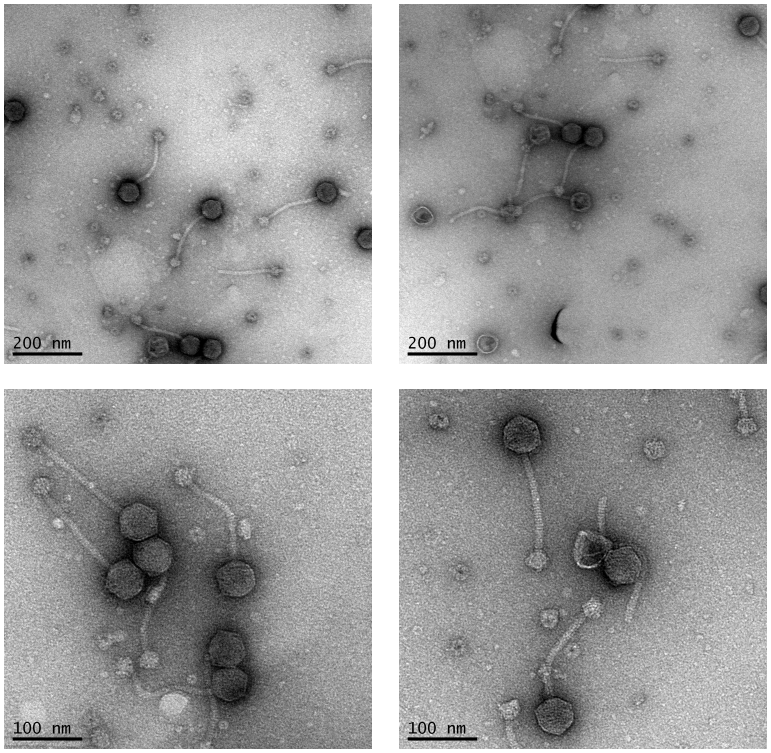
SpT5



SpT152



SpT252



SpT99/F3